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(52) Abstract: Methods and compositions for reducing the incidence of C. jejuni bacteria infections, in poultry and in humans asxi other animals are formulated to include C. jejuni antigens, and particularly CadF, FlpA and FkA. The antigens may be provided in the form of polypeptides or by hosts that produce the antigens. Fiferonectin binding proteins of C. jejuni may also be used to deliver substances of interest to humans and other animals.

(54) Title: ANTIGEN COMPOSITIONS AND METHODS OF INHIBITING CAMPYLOBACTER JEJUNI BACTERIAL INFECTION AND USES OF THE ANTIGEN COMPOSITIONS


(72) Inventors; and
KONNEL, Michael [US/US]; 305 SE South St., Pullman, WA 99163 (US).


(75) Inventors/Applicants (for US only): KONNEL, Michael [US/US]; 305 SE South St., Pullman, WA 99163 (US).

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ANTIGEN COMPOSITIONS AND METHODS OF INHIBITING
CAMPYLOBACTER JEJUNI BACTERIAL INFECTION AND USES OF THE
ANTIGEN COMPOSITIONS

The present disclosure relates to reducing the incidence of C jejuni bacteria infections in poultry and in humans, and more particularly to novel antigenic compositions, vaccines, and methods for generating an immune response against C jejuni bacteria in an animal.

BACKGROUND

The following includes information that may be useful in understanding the present inventions. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently described or claimed inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

The bacteria! genus Campylobacter are gram-negative, spiral-shaped motile pathogenic-bacteria that include numerous species associated with many animals, both domestic and wild, commonly found in chickens, turkeys, cattle, sheep, horses and rodents. The bacteria can infect the host animal through various routes of transmission, including by food, water, and through contact with other animals. Contamination of meat in a slaughterhouse is also prevalent. Humans are frequently infected with Campylobacter jejuni by handling or consuming poultry products. Campylobacter infections in humans also surpass the number of Salmonella infections (Walker et al., Microbiological reviews, 30; 81-94(1986)). Campylobacter jejuni is the most common Campylobacter species isolated in association with human diarrhea. Campylobacter jejuni is responsible for approximately 2 to 3 million cases of foodborne illness per year in the U.S. with an estimated cost of treatment and loss of productivity of $8 billion annually (Suzby, J. C. et ai (1991) Infect. Dis. 176 Stf Pl 2:S192-1 97). Diarrhea caused by Campylobacter jejuni typically manifests for about 2-7 days and is self-limiting, but the infection in young children, elderly and immunocompromised individuals often requires antibiotic treatment. Campylobacter can cause enteric infections in humans, and are occasionally the cause of more severe diseases like meningitis, neurologic complications, appendicitis, urinary tract infection, and spontaneous abortions (Blaser et al., New Engl. L Med, 305: 1444-1452 (1981), Butzter et ai, Clinics in Gastroenterol., S: 737-765 (1979), Schwerer et al., J. Endotox. Res, 2: 395-403 (5995) and Sailoway et al., Infect Imrnoun. 64: 2945-2949 (1996). Campylobacter jejuni infections are also associated with Guillain-Barre syndrome (Atlas, B. M. (2003), Clin. Meet Dis. 32:1201-1206).
Given the seriousness of the problems associated with this infectious agent, novel compositions and methods for inhibiting and preventing *Campylobacter jejuni* infections in birds, poultry, and in humans are very much needed. The inventions described herein satisfy this need.

**BRIEF SUMMARY**

An embodiment of the invention relates to *C. jejuni* antigens which can be used to elicit an immune response against *C. jejuni* bacteria in an animal. In some embodiments, the immune response is a protective immune response, and prevents (or substantially decreases) the ability of *C. jejuni* bacteria to colonize or establish an infection in an animal to whom the antigens have been administered. While animals of any age can benefit from administration of the antigens, young (juvenile), especially newborn animals, are frequently targeted for vaccination.

The CadF, FlpA and FshA antigens have been identified as particularly effective antigens. Without being bound by theory, it is believed that these antigens, which are at least in part exposed on the surface of the *C. jejuni* bacteria, play a role in and may be necessary for mediating the attachment of *C. jejuni* bacteria to, and hence infection of, animal cells. Administration of these antigens to an animal (either the entire protein, or immunotopically effective portions thereof) results in the production of antibodies to these antigens by the animal. When the animal is subsequently exposed to *C. jejuni*, the antibodies bind to these antigenic proteins on the bacteria’ surface and prevent or block the bacteria from binding to and infecting the eelii.

While CadF, FlpA and FiaA are particularly effective antigens for use against *C. jejuni* infection, either alone or in combination, the use of other antigens to which *C. jejuni* antibody responses are prevalent is also contemplated, including one or more of *C. jejuni* proteins: porins, CmeA, CmeC, CjaA, CjaC, CJS1 176.0126 or a CJSI 176.0126 homolog, CJS1 176.0128 or a CJSI 176.0128 homolog, CJS1 176.0164 or a CJSI 176.0164 homolog, CJS1 176.0164 or a CJSI 176.0164 homolog, CJS1 176.0586 or a CJSI 176.0586 homolog, CJS1 176.185 or a CJSI 176.185 homolog, CJS1 176.1295 or a CJSI 176.295 homolog, CJS1 176.525 or a CJSI 176.525 homolog, FlaB, FlgE2, FlgF2, PldB3, PorA, MapA, and SdhB, or one or more antigenic fragments of any of these.

FlpA, hi some embodiments, may be used to provide antigens, pharmaceuticals, drugs (e.g. anticancer drugs), toxins*, etc, to human cells based on its binding to human eelik and ihroBectin. Other antigens of *C. jejuni* may be used similarly, e.g. in the form of a chimera or fusion product with the substance of interest.
The invention provides a method for preventing or treating *Campylobacter jejuni* colonization in an animal. The method comprises the step of providing to the animal 1) one or more *C. jejuni* polypeptides; or 2) a host genetically engineered to contain and express nucleic acid sequences encoding one or more *C. jejuni* polypeptides.

The invention also provides a host genetically engineered to contain and express nucleic acid sequences encoding one or more *C. jejuni* polypeptides.

Further embodiments of the invention provide a modified bacterial S-layer protein having an internal insertion of at least one heterologous polypeptide from a *C. Jejuni* bacterium.

The invention also provides an antigenic composition for generating an immune response to *C. jejuni* in an animal. The antigenic composition comprises 1) at least a first polypeptide selected from a group consisting of *C. jejuni* CadF, FlaA, F1pA, and antigenic fragments thereof; and 2) at least a second *C. jejuni* polypeptide that is different from said first polypeptide.

The invention further provides a method for delivering a substance of interest to a subject. The method comprises the steps of: 1) providing the subject with a fusion product containing a fibronectin binding protein of *C. jejuni* and a substance of interest, wherein said fusion product contains both heterologous polypeptide and said substance of interest are associated with one another; and 2) permitting said fusion product to bind fibronectin in cells or tissues of said subject.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1A and B shows the reactivity of the *C. jejuni* S3B-SPF sera against *C. jejuni* outer membrane proteins extracted from A, homologous (S3B) and B, heterologous (81-176) strains. Numbers on the x-axis indicate the identification numbers of the serum samples. Vertical bars represent the arithmetic mean and the error bars represent the standard deviation for triplicate samples. The horizontal lines represent the negative cutoff value determined from nine sera collected from control chickens not colonized with *C. jejuni*.

Figure 2. Data representing the presence (X = strong reactivity, O = weak reactivity) or absence (blank) of bands that reacted with the maternal antibodies as determined from immunoblots of outer membrane protein (OMP) extracts of the *C. jejuni* homologous (SSB) and heterologous (81-176) strains probed with the *C. jejuni* S3B-SPF sera, represented in tabular form.

Figure 3 shows *C. jejuni* S38-SPF pooled sera contain antibodies that reduce the motility of *C. jejuni* S3B, but not of *C. jejuni* 81-176. Panels. A, Motility assays performed with the *C. jejuni* S3B

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strain; and B, Motility assays performed with the C jejuni 81-176 strain. The horizontal lines (white) indicate die diameter of the spots (i.e., torn the center to the edge of the bacterial zone). Figure 4 illustrates C. jejuni 81-176 outer membrane proteins identified by tC/MALOI/ TOF-TOF.

Figure 5 depicts predicted "best-fit" immunogenic membrane-associated C jejuni proteins identified by nano-LC/MS/MS.

Figure 6 shows the number of C. jejuni bound to chicken LMM hepatocellular carcinoma epithelial cells. Each bar represents the mean ± standard deviation of C. jejuni bound to the LMM cells per well of a 24-well plate. The asterisk (*) indicates a statistically significant difference (P < 0.05) between the C. jejuni F3801 wild-type isolate and an isogenic mutant, as determined using Student's t-test.

Figure 7 shows CadF, PEB 1, and Cj1279c contribute to C. jejuni colonization of broiler chickens. The ‘N’ indicates the number of chickens in the group of 10 that were colonized with C Jejuni (limit of detection K 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.

Figure 8 shows that Cj1279c (FtA) encodes an adhesin. Each bar represents the mean ± the standard deviation for the number of C. jejuni bound to the LMM cells in each well of a 24-well plate. The asterisk (*) indicates a statistically significant difference (P < 0.05) between the C. jejuni F3801 wild-type isolate and an isogenic mutant, as determined using Student's t-test.

Figure 9, Salient features of the FlpA gene and its deduced amino acid sequence in the C jejuni NCTC 11168 strain. YheβpA gene in the C. jejuni F3801 strain is the second gene in a polyeistrottie opetxm containing a total of 4 genes. The FlpA gene in NCTC H 168 is 1236 nucleotides. Examination of the FlpA deduced amino acid sequence revealed few residues differed in the NCTC 11168, RM 122J, 81-176, 81L16, and F380U strains (arrowheads), three fibronecttn type 3 domains (Vn3, residues 40-132, 135-227, and 220-407), and a prokaryotic membrane lipoprotein lipid attachment site (arrow). 'v' = residues that vary in one or more of the four Cjefu 3i sequences strains listed above (SIIQ ID NO; 50); ‘b’ = fibronecttn, type 11 domain (SSSF49265, residues 40 to 132, 135 to 227, 220 to 407); ‘e’ = prokayotin membrane lipoprotein attachment site.

Figure IA and B, Detection of FlpA in C jejuni whole cell lysate (VVCL) and outer membrane protein (OMP) extracts prepared from the C. jejuni F38011 wild-type strain and βp A isogenic mutant as judged by immibioblot analysis with a FtpA-specific serum. WCL and:
OMF extracts were resolved by SDS-PAGE (12.5% polyacrylamide) and immunoblot analysis as outlined in "Materials and Methods." The blot on the left side of the panel (A) shows a blot of C. jejuni WCLs probed with the FlpA-specific serum and the blot on the right side of the panel (B) shows a blot of C jejuni OMPs probed with the HpA-specific serum. Lanes: 1, C jejuni F3B01 wild-type strain; 2, C. jejuni flpA mutant; and 3, C. jejuni flpA (JlpA") complemented strain. The position of the FlpA 46 kDa protein is highlighted (arrow). The positions of the molecular mass standards are indicated on the left (in kDa).

Figure 11. The FlpA protein contains surface exposed domains. Indirect immunofluorescence microscopy was performed with the C. jejuni F3S01 1 wild-type strain (Panels A-C) and the FlpA isogenic mutant (Panels D-F) as outlined in "Materials and Methods," C jejuni were incubated with the rabbit FlpA-specific serum (Panels A-F) followed by incubation with a Cy2-conjugated goat anti-rabbit secondary antibody. All bacteria were visualized by staining with 4',6-diamidino-2-phenylindole (DAFI). The specimens were visualised using a Nikon Eclipse TE2000 inverted epifluorescence microscope. The C. jejuni wild-type strain and the flpA mutant incubated with the rabbit u-c. jejuni whole-cell serum were readily observed (not shown), whereas only the C jejuni wild-type strain incubated with the rabbit FlpA-specific serum was stained.

Figure 12. FlpA promotes the binding of C. jejuni to human epithelial cells. In vitro adherence-assays were performed with human TNT 407 epithelial cells and the C jejuni F3S01 1 wild-type strain, cadF isogenic mutant, and flpA (JlpA") complemented strain as outlined in "Materials and Methods." The C. jejuni cadF mutant was included as a negative control, as it is well documented that CadF is an adhesin. Values represent the mean ± standard deviation of viable bacteria bound to INT 407 cells per well of a 24-wel tissue culture tray. The asterisks (*) indicates that the number of bacteria bound to the FNT 407 cells was statistically different (P < 0.01) from that of the C jejuni wild-type strain as judged by the Student's t-test.

Figure 13, inhibition of C. jejuni binding to human INT 407 epithelial cells with FipA-specific serum. The bacteria were incubated with indicated dilutions of the FlpA-specific serum or pre-bleed serum for 30 min prior to inoculation of the FNT 407 cells. Adherence assays were performed as outlined in "Materials and Methods," Values represent the mean ± standard deviation of viable bacteria bound to INT 407 cells per well of a 24-well tissue culture tray. The asterisks (*) indicates that the number of bacteria bound to the TNT 407 cells was statistically different (P < 0.01) from that of the C jejuni wild-type strain as judged by the Student's t-test.
Figure 14. FIpA binding to fibronectin is saturable. Fibronectins-coated wells were incubated with 2-fold serial dilutions of FIpA-GST and CadF-GS1 proteins, and bound proteins detected as outlined in "Materials and Methods." The C. jejuni CadF protein was included as a positive control. The samples were tested in triplicate, and each data point contains the mean ± standard deviation of a representative experiment.

Figure 16A-C. Schematic of Fn and Fn fragments used in this study: A, fitil-tengih fibronecetin (Fn); B, the Fn N-troinai domain (NTD); C, the gelatin-binding domain (GBD). The KTD and GBD are produced by digestion of Fn with thermolysin.

Figure 17A-C. Primary structure of the FIpA protein, FIpA domains, and synthetic peptides: A, full-length FIpA 'S' marks the signal peptide, B, GST fusion proteins of the three FIpA FN3-like repeats, C, amino acid sequence of the FIpA domain 2 (FIpA-D2) and the seven synthetic peptides F1-P7. Amino acid sequence predicted β-sheet secondary structure arc indicated with by <-> and gray boxes.

Figure 18A and B. A, Adherence of FIpA and the three FSpA FN3-like domains (DK 02, and D3) to Fii-coated wells. EL-ISAs were performed with wells coated overnight with 1 ug of Fn. Serial dilutions of the GST fusion proteins (FIpA full-length, FIpA-DL, FIpA-D2, and FIpA-D3) were added to each well. The amount of FIpA protein bound was detected with a primary antibody against GST and a secondary HRP conjugate. All samples were performed in triplicate as described in the Material and Methods. B, Adherence of Fn to wells coated with FSpA and the three FIpA FN3-like domains. For ELISA experiments wells were coated overnight with FIpA full-length. FIpA-DL, FIpA-D2, and FIpA-.03. Serial dilutions of FIpA were added to each well. The amount of FIpA protein bound was detected with a primary antibody against Fn and a secondary liU* conjugate.
Figure 19. Adherence of FB for well coated with FlpA peptides P1-P5. To determine which peptides bound Pn RJSAs plates were coated overnight with the five FlpA peptides. Fn was incubated in the wells, and detected as previously.

Figure 20. FlpA P7 (Nl 47-SI 66) contains the FlpA Fn-binding domain. ELISAs were performed as previously. Microliter plates were coated with the FlpA peptides and the amount of FlpA bound was determined spectrophotometrically.

Figure tl. ClustalW sequence alignment. The amino acid sequence of FlpA-D2 (aa 135-224) was compared to the sequences of the FN3 domains from Fn. FlpA-D2 was most similar to FN3 sharing 22.9% sequence identity, 15.7% conserved substitutions, and 21.7% serai-conserved substitutions.

Figure 22-A-B. FipA binds a site within the gelatin-binding domain (GBD) of Fn. ELISAs were performed to determine if FlpA bound the NTD or GBD of Fn. Microliter plates were coated with Yn, GBD, NTD, or ovalbumin (negative control) overnight. Serial dilutions of FlpA full-length, B, FlpA-D1, C, FlpA-02, and t>. FlpA-D3 were added to the wells and the amount of FlpA proteins bound was determined as previously.

Figure 23 illustrates the colonisation of broiler chickens by C jejuni. Broiler chicks were administrated Lactobacillus by oral gavage (−10⁸ CFU) at day one of hatch and 4 days post-hatching. Chicks receiving C jejuni challenge were administrated C. jejuni F3801 l by oral gavage (−10⁸ CFU) at day 14 post-hatching. Half of the chickens were euthanized and necropsied at (A) Day 7 post-challenge and the remaining chickens at (B) Day 14 post-challenge. A cecum was dissected from each chicken, weighed, diluted in an equal volume of MM broth, and thoroughly stomached. Samples were serially diluted and plated onto Campy Cefex agar for enumeration.

Figure 24.4 and B shows anti-C. jejuni serum antibodies. Levels of mitx-Cjejuni serum antibodies in broiler chickens were determined at (A) Day 7 and (B) Day 14 post-challenge by ELISA as outlined in Materials and Methods. Microliter plates were coated with C. jejuni whole cell lysates, and incubated with sera from the chickens. Chicken sample identification numbers and treatment groups are indicated on the X-axis. Antibody level for each specimen is indicated by white bars. Microbial counts for C. Jejuni (gray) and Lactobacillus (black) are shown as CFU/gram cecal contents.

Figure 25A mut B shows the anti-alpha toxin serum antibodies. Levels of anti-Clostridium perfringens alpha-toxin serum antibodies in broiler chickens were determined at (A) Day 7 and (B) Day 14 post-challenge by ELISA as outlined in Materials and Methods. Chicken sample
identification numbers and treatment groups are indicated on the X-axis. Antibody level for each specimen is indicated by white bars. Microbial counts for C. jejuni (gray) and Lactobacillus (black) are shown as CFU/gram cecal contents.

Figure 26 shows the reactivity of the antibodies in a serum collected from a broiler chicken orally inoculated with C. jejuni. The blood was collected from the chicken at 15 days post-inoculation with C. jejuni S1116. Lanes: 1, C. jejuni S1116 whole cell lysate (wcl); 2, C. jejuni S3116 outer membrane protein (omop) preparation; and 3, C. jejuni S1116 wcl. The blot shown in the right panel was incubated with a 1:50 dilution of the chicken serum. The arrows highlight the known proteins (62 kDa = FlmA, 37 kDa = CadF), thus far found to react with every sera tested in 10). The bullets indicate proteins whose identity will be determined by mass spectroscopy.

Figure 27 shows an analysis of the C. jejuni FlmA filament protein. The deduced amino acid sequences from 26 FlmA proteins were aligned to determine the conserved regions. Shown with shading are the residues/regions with greater than 99% and 50% amino acid identity, respectively. The black lines indicate regions of increased variability (i.e., non-conservation, insertion, or deletion). The 30-mer indicated below the color graphic (SEQ ID NO; 3) represents a region where 28 of 30 residues are greater than 90% conserved. Relative to the regions downstream of residue 220, the r1-220 region represents a good target for incorporation into a Lactobacillus S-layer protein.

Figure 28 shows a hydropathy profile of the L. helveticus SlpA protein. Shown cm the Y-axis is the hydropathy in Keai/nuol, calculated for 19 residue windows centered at the residue numbers listed on the X-axis.

Figure 29 shows the incorporation of the CadF Fn-BD into the I. hthvics S layer protein. Panel A is a CBB-R25G stained gel! Panel B is a blot probed with goat k-CadP serum. Lanes: 1, S-layer protein extracted from a wild-type strain; 2, transfotmarst with a wild-type slpA allele; 3 and 4, independent transformants with the CadF Fn-BD (!2nier) in site 1 of the slpA gene. The S-layer with the CadF Fn-BD is indicated (arrow).

Figure 30 shows the experimental design to compare the number of Campylobacter colonizing chickens inoculated with a Lactobacillus vaccine sixain versus non-vaccinated chickens. There were 20 birds in each experimental group.

Figure 3! illustrates the competitive exclusion of C. jejuni colonization of chickens wsh recombinant Causwacter creseecicus, N = the number of birds (of 10) that have detectable
numbers of *C. jejuni* in their cees two weeks post-inoculation. *C. cresciens* CadF/FlaA/PorA is the vaccine strain.

**Figure** 32 illustrates a *Caulobacter cresciens* CadF/FlaA2/PorA vaccine strain that inhibits colonization of chickens and stimulates a specific antibody response against a CadF peptide (amino acids 128443).

**Figure** 33A-C. Comparison of FlaA sequences from 8 different *C. jejuni* strains.

**DETAILED DESCRIPTION**

An embodiment of the invention provides immunogenic *C. jejuni* antigens that are immunogenic and which can be used to elicit an immune response against *C. jejuni* in an animal to whom the antigens are administered. Particular antigens of interest include CadF, FlaA and FlpA, as well as others described herein, e.g., in Table 1.

Results presented herein shows for the first time that CadF, a well-characterized 3? kDa fibroactin binding protein, is immunogenic in chickens. Noteworthy is that the CadF protein, which is necessary for *C. jejuni* colonisation of chickens (Zitprin, R., et al. (1999), Avian Dis. 43:586-589), was detected in both the *C. jejuni* S3B and 81-176 strains. A preferred epitope of CadF identified and provided herein includes the 30-amino acid sequence HYGAGVKFRLDSL ALRLETRDQ1NFnHAN (residues 527-156, SEQ ID NO: J) and fragments thereof that are capable of producing a desired immunological response, for example, the sequence FRLS (SEQ ID NO: 2).

FlpA antigen, or immunogenic portions thereof, may also be used in the compositions and methods of the invention. In one embodiment, the immunogenic portion of FlpA is and residues 141-170 of FlpA: **FVQAVTNIPLNKILWPRHPDFRVDYTIE** (SEQ ID NO: 3).

FlaA antigen, or immunogenic portions thereof, may also be used in the compositions and methods of the invention. In one embodiment the immunogenic portion of FlaA is residues 278-307 of FlaA: **IANVKDTTGV** ASIA**NGQ**VLTSA**DGRG** 11 (SEQ ID NO: 4).

**Definitions**

Terms that are not defined below or elsewhere in the specification shall have their art-recongnized meaning.

Amino acids used in compounds provided herein (e.g., peptides and proteins) can be genetically encoded amino acids, naturally occurring non-genetically encoded amino acids, or synthetic amino acids. Both L- and D-enantiomers of any of the above can be utilized in the compounds. The following abbreviations may be used herein for the following genetically encoded amino acids (and residues thereof): alanine (Ala, A); arginine (Arg, R); asparagine
(Asp, N); aspartic acid (Asp, D); cysteine (Cys, C); glycine (Gly, G); glutamic acid (Glu, E); glutamine (Gln, Q); histidine (His, H); isoleucine (ILE, I); leucine (Leu, L); lysine (Lys, K); methionine (Met, M); phenylalanine (Phe, F); proline (Pro, P); serine (Ser, S); threonine (Thr, T); tryptophan (Trp, W); tyrosine (Tyr, Y); and valine (Val, Y).

The invention provides nucleic acid sequences (which may be genes) which encode the C-globins? antigens described herein, as well as nucleotide sequences that are variants of or homologous to those sequences. Such polynucleotides typically have at least about 70% homology, and may exhibit at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% homology with the relevant sequence, for example over a region of at least about 15, 20, 30, 40, 50, or 100 or more contiguous nucleotides. As used herein, the terms "homology and homologies" are also used to refer to corresponding polynucleotides or proteins from genetically related but not identical organisms (e.g., different strains of bacteria as identified by their strain numbers). These polynucleotides and polypeptides may be identical, or they may, for example, have sequence variations.

Homologous or variant sequences typically differ from the sequence disclosed herein by at least (or by no more than) about 1, 2, 5, 10, 15, 20 or more mutations (which may be substitutions, deletions or insertions). These mutations may be measured across any of the regions mentioned above in relation to calculating homology. The homologous sequence typically hybridizes selectively to the original sequence at a level significantly above background. Selective hybridisation is typically achieved using conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50 °C to about 60 °C).

Levels or degrees of homology may be calculated based on any method in the art. For example the IAVGC Package provides the BESITO program, which can be used to calculate homology (Devereux et al., Nucleic Acids Research 52, p357-395 (1984)). The PILIKJP and BI-AST algorithms can be used to calculate homology or align sequences, for example as described in Altschul S. F.; J Mol Evol 36: 290-300 (1993); Altschul S. F. et al\ J Mol Biol 215: 403-10 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

The invention also includes nucleic acid sequences that are complementary to the sequences disclosed herein. Complementary sequences may be DNA, RNA or hybrids thereof. The term "complementary" generally refers to the natural binding of polynucleotides by base pairing, and may be complete or partial "Hybridizable" and "complementary" are terms that are used to indicate a sufficient degree of complementarity such that binding, preferably stable
binding sufficient to carry out an intended action, for example, occurs between the DNA or RNA target and the polynucleotide.

The term "stringent conditions" refers to conditions that permit hybridization between polynucleotides, as is understood by those of skill in the art. For example, stringent salt concentration will ordinarily be less than about 750 nM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and most preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Stringent temperature conditions will ordinarily include temperatures of at least about 30°C, more preferably of at least about 37°C, and most preferably of at least about 42°C, Berger and Kimmef, Methods In F.nxymoiology, VoS. 152: Guide To Molecular Cloning Techniques, SaB Diego (1987); Academic Press, Inc. and Sambrook ef al, Molecular Cloning (1989); A Laboratory Manual, 2nd Ed., VoIs 1-3, Cold Spring Harbor Laboratory).

In general, the terms "protein", "polypeptide" and "peptide" refer to any polymer of two or more individual amino acids (whether or not naturally occurring) linked via peptide bonds, "protein ", "polypeptide" and "peptide" may be used interchangeably herein. Similarly, protein fragments, analogs, derivatives, and variants are encompassed by these terms. The term "fragment" or "portion" of a protein refers to a polypeptide comprising fewer than all of the amino acid residues of the protein. A "domain" of a protein is also a fragment, and comprises the amino acid residues of the protein often required to confer activity, function, or structure.

In some embodiments, polypeptides provided herein have conservative amino acid substitutions relative to a reference amino acid sequence. The present invention encompasses C. jejuni proteins (polypeptide, peptides) as described herein, as well as amino acid sequences variants having about at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% or more sequence identity with the disclosed amino acid sequence. More typically, such variant sequences have between 1 and 5 (e.g., 1, 2, 3, 4, or 5) conservative amino acid substitutions relative to the amino acid sequences that are explicitly disclosed herein. An antigenic fragment (or epitope, antigenic determinant, etc.) includes any part of a polypeptide as long as it is capable of eliciting a desired immune response.

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A common aspect of the inventions relates to methods and compositions for reducing the number of C jejuni that colonise animals by promoting an immune response to C jejuni in animals. In some embodiments, the animals are birds such as chickens. This directly addresses human infections resulting from exposure of C jejuni through poultry because a decrease in the number of C jejuni exposed chickens is expected to result in lower exposure of humans to C
This problem was approached by first looking at chicken maternal antibodies. In chickens, the levels of antibodies against \textit{C. jejuni} \textit{mt} vary considerably through the lifecycle of the chicken. At the time of a chicks hatching, maternal antibodies against \textit{C. jejuni} axe at high levels. These maternal antibodies transferred from the mother to the chick remain high in the chick for 3-4 days, at which point they gradually decrease until they become undetectable at about 2-3 weeks of age. Importantly, the colonization of chicks by \textit{C. jejuni} coincides with the decrease in these maternal antibodies. The mechanisms by which maternal antibodies protect clucks against \textit{C. jejuni} infections are not clear. They may interfere with bacterial motility, promote clearance by agglutination, block ion/nutrient transport, decrease viability through complement-mediated killing, and/or block the interaction between bacterial adhesions and host cell intentional receptors. Stem, NJ, (1990), Avian Dis. 34:595-601. Antibodies against the bacteria start being generated after the chick has been colonised with \textit{C. jejuni}. These antibodies may not be sufficient to clear an existing colonization, but once the chick’s own antibody production sets in, a decrease in the number of \textit{C. jejuni} organisms can be observed. Shoaf-Sweeney, KT, \textit{et al} (2008), Appl Environ, Microbiol 74(22) 6867-75.

Antigenic proteins recognized by chicken maternal antibodies believed to be useful lot the design and preparation of vaccines are identified and described herein. The studies described in Example 1 identified \textit{C. jejuni} membrane-associated proteins recognized by maternal antibodies, as the antibodies passed from bens to chicks are partially protective against \textit{Campylobacter} colonization of chicks. The proteins identified were further characterized to evaluate their efficacy as \textit{C. jejuni} vaccine candidates. While a total of 60 proteins were identified in the OMP extracts from \textit{C. jejuni} 81-176, fewer proteins (\textit{i.e.}, ~20) were identified that reacted with the antibodies in the Cj S3B-SPF sera. The identified proteins include CadF, CmcA, CmcC, CjaA, CjaC, CJJ81 176 0126, CJJ8H76 0126, CJJ8 \pi 76 0164, CXISi 176 0586 CJI8 U76 H85, CJI81 17&J295, CJJB1 176J525, FlAa, FlAB, FlgF, FlpA, PEB2, PEB3, PorA, MapA, and SdhB (see Table 1).

The Cj S3B-SPF sera contained antibodies that reacted with the flagellar hook protein (FlgE2) and the flagellar FlaA and FlAB filament proteins in \textit{C. jejuni} S3B and 81-176. FlgE2 has a molecular mass of 89.4 kPa and is required for motility, flagellar assembly, and protein secretion in \textit{C. jejuni} mendixs on, D. R., and V. J. DiRita (2003). MoL Miciobiol. 50:687-702; KoBkel, M. E. \textit{et al} (2004), \textit{J. Bacteriol} 186: 3296-3303. Although the predicted molecular mass of these proteins is around 59 kDa, glycosylation has been shown to alter the mass of the proteins by up to 10% depending on the level of modification (Thibault \textit{P. et al} (2001), \textit{J. Biol.}}
C jejuni. A band between 65 and 63 kDa was observed as judged by immunoblot analysis with an anti-C. jejuni flagelliti specific serum. It is possible that this band represents glycosylated forms of the FlaA or FlaB proteins, whereas the proteins with apparent molecular masses of less than 60 kDa represent degradation products.

The C jejuni S3B-SPF sera contained antibodies that reacted against C jejuni strain-specific proteins as well as proteins common amongst C jejuni strains. For example, a 40 kDa immunoreactive protein, CmeA (band 9), was recognized in the OMP extracts of the C jejuni S3B and 81-176 strains by all of the C jejuni S3B-SPF sera, whereas a 54 kDa protein, presumably CmeC, was primarily recognised in the OMP extracts of the C jejuni S3B strain. Together the CmeA, B, and C proteins comprise a resistance-nodulation-diversity (RND) efflux pump that is involved in resistance to a broad range of antimicrobials and bile salts (Lin, J., L. O. Michel, and Q. Zhang. (2002), Antimicrob. Agents Chemother. 46:2124-2131; Lin, J. e ai. (2003), Infect. Immun. 71:4250-4259). CmeB is the inner membrane efflux transporter, whereas CmeA is localized in the periplasmic space, and CmeC forms an outer membrane channel. CmeABC is widely distributed in C jejuni isolates (Un J., L. O, Michel, and Q. Zhang. (2002), Antimicrob. Agents Chemother, 46:2124-2131), and comparison of the deduced amino acid of each protein from four C jejuni strains (NCTC 1168, RM 1221, 81116, and 81-176) revealed that sequence of each protein was well conserved (> 98% similarity) amongst these strains.

Two outer membrane substrate-binding proteins involved in amino acid transport, CjsA (band 12) and CjaC (band B), were identified. CjaA has been characterized as an extracytoplasmic solute receptor in a putative ATP-binding cassette-type cysteine transporter (MitHer, A. et ai. (2005), Mol Microbiol. 57:143-155), while CjaC has been shown to be required for histidine transport (Garvis, S. G., Q. J. Puxcm, and M. E. Konke! (1996), Infect. Immun. 64:3537-3543). It is possible that amino acid transport system proteins may serve as good vaccine components because C jejuni is asaccharolytic and relies on exogenous sources of amino acids for energy production. The C jejuni S3B-SPF sera was found to contain antibodies that reacted against the CjaA and CjaC proteins in the C jejuni S3B strain, but not in the 81-176 strain. Pawelec et al (2000), FEMS Microbiol. Let. 185:43-49 demonstrated genetic diversity in both cjaA and C jejuni isolates, with as much as 16% variation noted at the nucleotide level. The relevant antigenic surfaces of CjaA and CjaC are cross-reactive with each other. Thus, slight variations in the amino acid sequences may account for the reduced or absent antibody response to CjaA and CjaC in the C jejuni 81-176 strain.
The results presented in Example 1 shows for the first rime that CadF, a well-characterized 37 kDa fibronectin binding protein, is immunogenic in chickens. Noteworthy is that the CadF protein, which is necessary for C. jejuni colonization of chickens (Ztprin, R. L., et ai (1999), Avian Dis. 43:586-589), was detected in both the C. jejuni S3B and 81-176 strains. A preferred epitope of CadF identified and provided herein includes the 30-amino acid sequence HYJAGVKFR1SDSRAELETR01NFMHAN (residues 127-156, SEQ ID NO:1) and fragments thereof that are capable of producing a desired immunological response. Such fragments or portions of this sequence include, for example, fragments comprising at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 contiguous amino acids capable of producing a desired immunological response. hi one embodiment, the fragment comprises at least 4 contiguous amino acids, for example, the sequence FRLS (SEQ ID NO:2). One desired immunological response of an antibody that binds to a CadF epitope is inhibiting CadF binding to fibronectin. Another desired immunological response is to sufficiently inhibit Campylobacter colonization of chickens, which may be caused by inhibiting CadF binding to tibotteelin.

Several C jejuni OMPs were also found to be immunogenic. These proteins included PEB3, MapA, and CJSI 1168. The function of FEB3 is not known (Pei, Z. H., et al. (1991), j. Biol. Cheni. 266:16363-16369). MapA is an outer membrane lipoprotein that has been used as an identification tool to distinguish between C jejuni and C. coli (Stucki, U. et al. (1995), j. Clin. Microbiol. 33:855-859), and to detect and diagnose individuals with C. jejuni infection (Campbell, L.-K. et al (2006), Mod. Pathol. 19:1042-1046). CJSI 1168 has been identified as a hypothetical outer membrane protein based on its amino acid sequence. C. jejuni antigens utilised in various embodiments of the invention are summarised in Table 1.

Table 1. Listing of C jejuni antigens and corresponding Genbank Accession numbers.

<table>
<thead>
<tr>
<th>NCTC1168</th>
<th>81-176</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene/locus</td>
<td>Protein</td>
</tr>
<tr>
<td>cadF/CJJ1478c</td>
<td>CadF</td>
</tr>
<tr>
<td>cmeA/CJ0367c</td>
<td>CmeA</td>
</tr>
<tr>
<td>SEQ ID NO: 9</td>
<td>SEQ ID NO: 10</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
</tr>
<tr>
<td>cmeC/Cj0363c</td>
<td>CmeC</td>
</tr>
<tr>
<td>SEQ ID NO: 13</td>
<td>SEQ ID NO: 14</td>
</tr>
<tr>
<td>cjaA/Cj0982c</td>
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<td>SEQ ID NO: 17</td>
<td>SEQ ID NO: 18</td>
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<tr>
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<td>SEQ ID NO: 21</td>
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<td>SEQ ID NO: 30</td>
</tr>
<tr>
<td>Cj0561c</td>
<td>Cj0561c</td>
</tr>
<tr>
<td>SEQ ID NO: 33</td>
<td>SEQ ID NO: 34</td>
</tr>
<tr>
<td>omp50/Cj1170c</td>
<td>Omp50</td>
</tr>
<tr>
<td>SEQ ID NO: 41</td>
<td>SEQ ID NO: 42</td>
</tr>
<tr>
<td>flipA/Cj1279c</td>
<td>FlipA</td>
</tr>
<tr>
<td>SEQ ID NO: 45</td>
<td>SEQ ID NO: 46</td>
</tr>
<tr>
<td>Cj1540</td>
<td>Cj1540</td>
</tr>
<tr>
<td>SEQ ID NO: 49</td>
<td>SEQ ID NO: 50</td>
</tr>
<tr>
<td>flaA/Cj1339c</td>
<td>FlaA</td>
</tr>
<tr>
<td>SEQ ID NO: 53</td>
<td>SEQ ID NO: 54</td>
</tr>
<tr>
<td>flaB/Cj1338c</td>
<td>FlaB</td>
</tr>
<tr>
<td>SEQ ID NO: 57</td>
<td>SEQ ID NO: 58</td>
</tr>
<tr>
<td>flgE*/cj1729c</td>
<td>FlgE**</td>
</tr>
<tr>
<td>SEQ ID NO: 61</td>
<td>SEQ ID NO: 62</td>
</tr>
</tbody>
</table>

15

SUBSTITUTE SHEET (RULE 26)
**GeπBaak Nceti e Sequence Gene ID #**

**FlgE, aka FlgH2,**

Another aspect of the invention is the identification and characterisation of bacterial adhesin proteins, and more particularly to identify and characterize adhesin proteins of *C. jejuni* useful for making vaccines against *C. jejuni*. Bacterial adherence to host epithelial cells is believed to be critical for chicken colonization, as cell attachment may prevent clearance of the bacteria via host mediated mechanical force. Studies were performed to assess the conservation of putative *C. jejuni* adhesin-encoding genes *cadF*, *atpA*, *βpA*, *pehIA*, *porA*, *Q1279c* (*βpΛ*), and *Q1349c* and additionally to identify the contribution of the corresponding proteins in *C. jejuni* host cell interactions. The results presented in Example 2 show that the *cadF*, *jlpΛ*, *porA*, *pehIA*, *flpA*, and *CjI349c* genes were conserved amongst the isolates, whereas the presence of the *capA* gene was variable. The results further showed that the *C. jejuni* CadF, CapA, FipA, and CjI349c 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. Included in these finding is the first novel showing that FlpA promotes the binding of *C. jejuni* to host cells and plays a role in *C. jejuni* colonization of chickens.
Experiments were 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, four of which had not been identified previously. The clonal complexes identified amidst the C. jejuni livestock (*i.e.*, bovine, porcine and ovine) isolates included two complexes, CC42 and CXXsI, that were determined in previous studies to be associated significantly with bovine and ovine (Folks, F. M., *et al.* (2003), Microbiol. 69:7409-7413; Kwan, P. S., *et al.* (2008), Microbiol. 74:5130-5138). Furthermore, the eleven clonal complexes identified in the 41 poultry isolates included several poultry-associated complexes (*i.e.*, CC45, CC257 and CC354; (Dingle, K. E., *et al*., (2002) Emerg. Infect. Dis. 8:949-955). 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 discussed above, genetic analysis of the adhesin profiles amongst the strains via dot blot assays demonstrated conservation of the C. jejuni *cadF*, *lipA*, *porA*, *peblA*, *jipA*, and *Qj1349c* 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*, *JipA*, *PEBS*, *Cj1279c*, and *Cj1349* are all greater than 95% identical between C. jejuni strains, and CapA is greater than 85% identical between C. jejuni strains. These studies further indicate that the *dipA* 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.

This is the first time the functional role of the C. *jejuni* proteins examined 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 *JipA*- and *PEBS* did not appear to play a role in cell adherence. For example, it was found that insertional mutagenesis of *jipA* did not result in a reduction in binding to chicken LMH cells. In agreement with the results from the *in vitro* binding assays, the *jipA* mutant was able to colonize broiler chickens at a level comparable with that of a wild-type isolate.

While it was found that a C. *jejwu* peblA mutant bound to chicken IMH cells at a level comparable to that of a wild-type isolate, the mutant did not colonize broiler chickens. The *in vitro* data indicates that *PEBS* does not appear to act as an adhesin but rather plays a critical role in aspartate and ghitamaie transport. The CapA protein was identified as a putative
autotransporter based on in silico analysis. Ashgar et al. (2007, L Bacterid. 189:1856-1865) reported that a capA knockout failed to colonize and persist in Rhode Island Red chickens. Studies described herein showed that the capA gene was not conserved amongst C. jejuni isolates. Indeed, 15 of the C. jejuni poultry isolates utilized in this study lacked the capA gene. It was also found that the C. jejuni cvpA 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 (his data and that of Ashgar et al) is not known. However, based on these results, it is concluded that the CapA protein is an adhesion protein that 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 (Koonci, M. E. et al. (2005), Mol Microbiol. 57:1022-1035; Kocikel, M. E. et al (1997), Mol. Microbiol. 24:953-963; Kocikel, M. E. et al. 1999, J. Clin. Microbiol. 37:530-517; MonteviJe, M. R., and M. E. Konkel (2002), Infect, VIIro. 70:6665-6671). The results presented here show that the C. jejuni cadF mutant demonstrated a 41% reduction in binding to chicken LMK cells and was unable to efficiently colonize broiler chickens. Since the Fn-binding protein CadF is critical to C. jejuni host cell adherence, it is hypothesized that FlpA and Cj1349c may play a role in host-cell attachment. Cj1349c has been annotated as a putative Fbn-fibrinogen-binding protein. The Cj1349c mutant demonstrated a 14% reduction in binding to chicken LMH cells (P < 0.05). However, reduced colonization of broiler chicks was not observed 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 Vn type III domains. Interestingly, the jlpA 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 Cj13278c. The Cj13278c mutant did not show a significant reduction in binding to chicken LMH cells relative to the mid-type isolate. These data suggest that FlpA is a novel C. jejuni adhesin involved in C. jejuni-hos P cell adherence and chicken colonization.

In summary, the ausIF, jlpA, pebiA, porA, βpa, and Cj1349c genes are conserved amongst C. jejuni isolates, whereas the presence of the capA Sm and h variable. CadF, CapA, FlpA, and Cj1349c proteins facilitate C. jejuni adherence to chicken LMH cells, which is consistent with the hypothesis that more than one protein contributes to the binding of C. jejuni
to host epithelial cells. The results indicate 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 Cj i349c proteins are not essential for *C. jejuni* to colonize chickens, but the
possibility that they contribute to the process cannot be ruled out. Additionally, the PEFi
proteins are required for *C. jejuni* to colonize chickens. This finding is likely due to the fact that
they are involved in amino acid transport required for viability within the host. These results have led to the determination that FlpA (Cj 1279c) is a novel *C. jejuni* adhesin.

In Example 3, the binding properties of FlpA were further characterized and FlpA was
determined to be a member of the Microbial Surface Components Recognizing Adhesive
Matrix Molecules (MSCRAMMs) family. Experimental evidence showed that *C. jejuni* FlpA is
exposed, promotes the attachment of *C. jejuni* to host epithelial cells, and has
fibroectin (Fn) binding activity. The identification of FlpA as a second MSCRAMM in *C.
jejuni* highlights the importance of Fn binding in host colonization and disease.

In addition, in Example 4, the specific sites of FlpA and Fn adherence were determined.
RLJSAs were recombinant proteins encoding each of the three FlpA domains demonstrated that
RPA-D2 contained the Fn-binding domain. Using an array of synthetic peptides spanning the
FlpA-D2 amino acid sequence, seven amino acids PHPDFRV (SEQ ID NO: SI) were
identified within FlpA-02 with maximal Fn-binding activity. Since FN? repeats are involved
in intramolecular interactions with the N-terminus of Fn, the ability FlpA to bind two
the peptidolytic fragments generated form the N-terminus of Fn - the 30 kDa N-terminal domain
(NTD) and the gelatin-binding domain (CrBD) were determined. FlpA bound the Fn gelatin-
binding domain (GBD), but not the NTO. Furthermore, the amounts of FlpA bound to the OBO
and full-length Fn were similar, indicating the GBD is the primary site of FlpA adherence to Fn.
Collectively, these data demonstrated that residues PHPDFRV within FlpA-D2 mediate
adherence to the GBD of Fn.

Vaccine compositions

The invention provides vaccine or antigenic (immunogenic) compositions which
comprise one or more *C. jejuni* antigens (e.g. proteins or polypeptides), or antigenic fragments
thereof. The antigens may be chemically synthesized, prepared by recombinant technology (e.g.
expressed by transgenic an organism that is genetically engineered to contain and express nucleic
acids encoding the amino acid sequence), or isolated from cultures of *Z. jejuni* bacteria. Preferred antigens
are listed, for example, in Table 1. The SEQ ID NOS: for each of the sequences in Table 1
(both the amino acid sequence and the nucleotide sequence encoding the amino acid sequence)
are provided in the Sequence Listing filed concurrently herewith. In some embodiments, the
antigens are one or more of CadF, FlpA and FlmA. or one or more antigenic fragments thereof. The antigenic fragments include but are not limited to 30-mer antigenic epitopes as follows: residues 127-156 of CadF: HYGAGVFKRLSDSLRLLETROOINFQRAN (SEQ ID NO: 1) or the 4-mer identified within this sequence, FRLS (SEQ ID NO:2); residues 141-170 of FlpA: FVQAVTNLPNRJKLWRPHDPFRVDSYUE (SEQ ID NO: 3) and residues 278-307 of FlmA: INAVKDTTGVASIDANGQLVLTSAADGRGI (SEQ ID NO: 4). Other antigenic fragments of these sequences may also be used, include, for example, fragments comprising at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 contiguous amino acids capable of producing a desired immunological response. Sequence variants of these proteins and antigenic fragments, as described herein, may also be utilized in the compositions. The antigens may be present in a single polypeptide construct which contains multiple copies of the same antigen, or copies of different antigens, i.e., a chimeric construct. For example, a chimeric polypeptide may include at least two of CaoT, FlpA and FlmA, or immunogenic fragments thereof, e.g., one or more copies of at least two of the three 30mers described above, and/or various other shorter sequences (e.g., FRLS (SEQ ID NO:2)). Chimeras may also include various spacer or linker sequences between the antigens in the construct.

In some embodiments, the C. jejuni proteinaceous antigens per se are present in a composition that is used to vaccinate an animal. However, the invention also encompasses hosts or vectors comprising nucleic acid sequence which encode the antigens, and in some embodiments, the compositions include such hosts. For example, various bacterial and viral hosts may be genetically engineered to contain and express sequences encoding the antigens, and then the genetically modified bacteria or viruses may be used to infect the animal and to express the antigens within the animal. Generally, the C. jejuni antigens are heterologous with respect to the host, i.e. the C. jejuni antigens are not naturally (in nature) found in the host organism. The host organisms may be attenuated so that they themselves do not cause any disease symptoms. Examples of suitable bacterial vectors include but are not limited to various plasmids that replicate in Lactobacillus (discussed in detail below), such as pAS3 and pLBS-GFP-EmR ([Bhowmik T, et al. (1993) J Bacteriol. 175:6341-4; Mota RM, et al. (2006) BMC Biotechnol. 5:62]). Such vectors also generally include one or more promoters to insure active transcription of the genes encoding the antigens, and may also include various enhancer sequences, stop signals, etc., as appropriate to achieve adequate expression of the sequences.

In one embodiment of the invention, the antigens of the invention are expressed in a bacteria! host as a chimera which also includes an export protein that causes the antigens to be transported to and/or presented on the surface of the host bacterium. The antigens are thus
readily accessible to the immune system of a vaccine recipient, and the elicitation of an immune response is encouraged or facilitated, in one exemplary embodiment the export sequence is the export signal and cell wall anchor sequence of the Lactobacillus S-protein. (i.e. residues 1 to 32 of the UiSA S-layer protein from _L. crispatus_ strain MH315 [complete _lhsA_ S-layer protein amino acid and encoding nucleotide sequences are provided in SEQ ID NOS. 166 and 169, respectively], residues 320 to 422 of the LbsB S-layer protein from _L. crispatus_ strain M10 [complete LbsB S-layer protein amino acid and encoding nucleotide sequences are provided in SEQ ID NOS. 170 and 171, respectively]). Other export and anchoring sequences may be used in a similar manner, examples of which include, but are not limited to _L. acidophilus_ strain NCFM SipA (the amino acid and encoding nucleotide sequences of which are provided in SEQ ID NOS: 172 and 173, respectively), and SlpB (the amino acid and encoding nucleotide sequences of which are provided in SEQ ID NOS: 174 and 175, respectively), and the _I. helveticum_ strain CRNZ32 SipA ([the amino acid and encoding nucleotide sequences of which are provided in SRQ ID NOS: \( \text{\textbackslash n} \) 76 and 177, respectively).

The antigens or hosts which produce the antigens are generally provided to a vaccine recipient in a suitable physiological compatible carrier, examples of which include but are not limited to normal saline solutions (e.g. buffered at pH 7.0-8.0) and water (see, for example, *Remington’s Pharmaceutical Sciences*, Mack Publishing Co., Airl. G. 

In order to facilitate delivery of the antigens, the formulation may include, as optional ingredients, other substances known in the art, e.g. diluents, solubilising or emulsifying agents, salts, buffering agents, excipients, penetration enhancers, surfactants, antioxidants, stabilizers, preservatives, wetting agents, lipids, chelating agents, etc., so long as they do not interfere with the biological activities of the components of die compositions provided herein. The formulations are generally sterile, except that host organisms may be present. A preferred carrier for vaccines provided herein, especially bacterial vaccines, is water.

**Methods of Vaccination**

Another aspect of the invention is directed to methods of treating or preventing a _C. jejuni_ bacteria colony formation in an animal by administering a therapeutically effective amount of the antigens described herein. Also, the _C. jejuni_ antigens could be used in combination with other antigens (e.g. human Infect&nts such as HIV, malaria, tuberculosis, etc.), e.g. in the form of a chimera or fission product. A ‘therapeutically effective amount’ is the amount of the antigen that will elicit a desired response, for example, an immune response in the vaccinated animal.
One desired immunological response may be the generation of antibodies that bind to the antigens described herein, when located in a live, potentially infective *C. jejuni* to which a vaccinated animal is exposed after vaccination, e.g. by binding to a CadF epitope and inhibiting CadF binding to fibronectin. Another desired immunological response is to sufficiently inhibit *Campylobacter* colonization of an animal, which may be caused by inhibiting CadF binding to fibronectin.

In some embodiments, the animal is a bird, but this need not always be the case. Other species also harbor *C. jejuni* and may be vaccinated as described herein, including but not limited to domestic pets, rodents, various wild animals, and especially animals that are used as food, e.g. cattle (especially calves), pigs, etc. The vaccine may be administered to birds, and these may be of any type (e.g. domesticated, wild, in protected areas such as zoos and wildlife preserves, etc.). However, the vaccine will be especially useful for inoculating birds that are raised for food, e.g. chickens, turkeys, geese, ducks, ostriches, etc., or those that are raised in proximity to humans (e.g. pels such as parrots, canaries, etc., or prize hens, roosters, etc).

In some embodiments, the vaccine is administered to young or juvenile animals, or even to animals that are very young, e.g. newborn. For example, when the animal is a bird, administration may be to a bird that is less than one month old, e.g. to a bird that is between one day and three weeks in age, or to a bird that is between one day and two weeks in age. In some embodiments, the vaccine is administered within the window of time between when maternal antibodies are still active in the animal, and the time when maternal antibodies wane. The vaccine formulation may be administered only once, or may be repeatedly administered, e.g. for up to about 2, 3, 4, or 5 days or more.

The vaccine and immunogenic formulations provided herein are generally intended for use in immunizing birds, especially chickens, although this need not always be the case. Typical routes of administration include but are not limited to addition of the antigens and/or of hosts which produce the antigens to substances which are ingested by the animal, usually water, although addition to other food sources are not excluded. In other embodiments, the antigens and/or hosts which produce the antigens are administered as an aerosol, e.g. a virus that is genetically engineered to contain and express the antigens may be introduced into the environment of the animal, chicken e.g. as an aerosol, and enter via inhalation into the respiratory tract, in still other embodiments, and especially when individual domesticated animals (e.g. pete) are concerned, administration may be via injection, e.g. intramuscular, subcutaneously, etc.
When vaccines are given orally via a water system, it is preferred that sanitizers are
absent when vaccines are being given. To neutralize the effects of chlorine and certain elements
in the domestic water supply, a neutralizing agent such as skimmed milk may be added to the
water (a rate of 500 ml of skimmed milk to 10 liters of water is suitable). For example,
skimmed milk can be added about thirty minutes before the vaccine, in order to achieve a good
vaccine administration, it is preferable to administer the vaccine such that all the animals drink
the vaccine medicated water within 1-2 hours. This is preferred because after this time period
the viability of a live vaccine can rapidly decline. In broilers (aka fryers) or birds less than 4
weeks of age, this may be achieved by withholding water for about 30 minutes before giving
the vaccine to make the birds thirsty. In birds over 4 week of age, the water may be withheld
from about 2 to 8 hours or overnight (about 6, 7, 8, 9, 10, 12, or more hours). For younger
birds, water may be withheld for about 20 minutes, 45 minutes, one hour, 90 minutes, or up to a
couple of hours. As birds drink when feeding, vaccination should be timed to coincide with
food being present in the feed (racks. The water can be provided at a time when birds are likely
to be drinking, such as morning time for broilers. Lighting can be used to influence the birds
drinking, where turning the lights down is used to reduce drinking and turning the lights on is
used to stimulate the birds to go to feeders and drinkers. Following vaccination clean water
should be again made available to the birds.

The dose of antigen that is administered may vary from situation to situation, depending
on, for example, the type of animal being vaccinated, the age or breed, the particular antigen
that is used, the form of the antigen (e.g. antigen alone, or a host that manufactures the
antigens), etc. The data obtained from the cell culture assays and animal studies can be used in
formulating a range of dosages for use. Guidance as to particular dosages and methods of
delivery is provided in the literature and generally available to practitioners; in the art, in
general, dosage is sufficient to achieve, in the animal, a level of antigen that is from about 0.01
mg/kg to about 100 mg per kg of body weight. If a bacterial or viral host is administered,
sufficient amounts to allow the host to colonize the gastrointestinal (GI) tract of the host, and/or
to produce sufficient antigen to cause a significant immune response in the vaccine recipient are
administered. A suitable amount will usually be at least about $10^5$ cfu, and preferably about $10^6$
cfus or more per dose. Larger dosages such as about $10^7$, $10^8$, $10^9$ and up to about $10^{10}$ cfu may
be used. This allows a sufficient amount of bacteria or virus to pass into the intestine, if
required. The above amounts of bacteria or virus may correspond, for instance, about 10^9 to
about $10^8$ cfu per kg of body weight of the animal. The concentration of bacteria or viruses in the
vaccine are typically at least about $1 \times 10^6$ ml, such as at least about $5 \times 10^5$ CrVml, $1 \times 10^8$ ZmI, $1 \times 10^5$ ml, or $1 \times 10^6$ ZmI.

Several vaccines for use in preventing diseases in poultry have been reported (See, e.g., U.S. Pat. Nos. 5,294,441, 5,389,368, 5,468,485, 5,387,744, and 6,866,547, the complete contents of each of which are hereby incorporated by reference), and the information contained therein may be useful with respect to vaccine compositions and method of administration.

**Probiotic Bacteria**


In another aspect of the invention, probiotic organisms were evaluated to explore whether certain species have a novel and beneficial effect on the colonization of *C. Jejuni* in chickens. The effect of administration of four probiotic *lactobacilli* on the colonisation of *C. jejuni* in chickens was evaluated (Example 5). The four probiotic *lactobacilli* examined were *Lactobacillus strains* (*L. acidophilus* NCFM, *Lactobacillus cnsipaus* CCM 58M), *Lactobacillus gallinam* ALCC 33199 and *Lactobacillus helveUcus* CNRZ32). The strain of *C. jejuni* utilized in these studies was F380I2, because it was found to be most sensitive to inhibition by lactobaciUm.

In order to determine the effect of administration of probiotic *lactobaciUm* on the chicken immune system, serum antibodies for *C. jejuni* (Figure 12) were determined at 21 and 28 days post-hatching. Anti- *C. jejuni* antibodies were not detected in birds belonging to groups that were not challenged with *C. jejuni* or in birds not found to be colonised with *C. jejuni*. Antibodies were detected in sera of colonized chickens at 2 weeks post inoculation with *C. jejuni*. Antibody production against *C. jejuni* did not clear the organism from the cecum. The results from Example 5 indicate that it is likely that immune modulation with probiotic *Lactobacillus* strains did not affect the ability of *C. jejuni* to colonize the gastrointestinal tract of chickens.

The ability of probiotic *lactobacilli* to inhibit the growth of *C. jejuni in vitro* and the effect of the administration of these probiotic on *C. jejuni* colonisation of chickens were
further evaluated. The results unexpectedly showed that birds receiving *L. crispatus KM 5S10* exhibited a low rate of colonisation by *C. jejuni*. Additionally, birds receiving *L. crispatus* had a high rate of recovery of lactobacilli from the cecum of chickens. Strain typing by PCR confirmed that lactobacilli recovered from the cecum of chickens are in fact, *L. crispatus*. Additionally, several isolates from chickens not receiving *L. acidophilus, L. gahinarow*, and *L. helveicus* were also positively identified as *L. crispatus*. *L. crispatus* is commonly isolated from chickens and has been identified as a predominant *Lactobacillus* species in the alimentary tract of chickens (Abbas Hilmi, H. T. *et al.* (2007), Appl Eimron Microbiol 73:7867-73). The fact that *L. crispatus* is able to remain in the alimentary tract of chickens for prolonged periods of time likely enhances the probiotic ability of this species. Moreover, when this fact is considered in view of the date presented that *L. crispatus* exhibited a low rate of colonisation by *C. jejuni*, provides evidence that this species might be a good candidate for the development of a recombinant bacterial vaccine.

The four *Lactobacillus* species evaluated are known to have genes encoding S-layer proteins (Avali-Jaaskelame *et al.*, S., and A. PaJva. (2005), FEMS Microbiol Rev 29:51-29). S-layer protein has been shown to be involved in adherence to host tissues (Doig, P., L. Emody, and T. J. Trust (1992), J Biol Cheni 267:43-9) and, in particular, the S-layer of *I. acidophilus* isolated from fowl has been shown to be involved in interaction with avian miestinaS epithelial cells (Schneitz, C. L. Nuotrho, and K, Lounatma (1993), I Appl Bacterid 74:290-4). The S-layer protein of *I. crispatus* JCM 5810, the strain evaluated in Example 5, was shown to be responsible for the strain's ability to adhere to collagen containing regions in the chicken colon. The S-layer protein from a different *L. crispatus* strain, ZJO01, was shown to inhibit adhesion of *Salmonella typhimuHum* and *E. coli* to HeLa cells. Thus, it is likely that the S-layer protein of *L. crispatus* is important for colonization of the chicken gastrointestinal tract and for inhibition of *C. jejuni* colonization.

Several mechanisms for competitive exclusion have been considered, including the saturation and obstruction of attachment sites for the pathogen by native and probiotic flora, competition for essential nutrients limiting the ability of the pathogen to grow, production of antagonistic molecules including organic acids, hydrogen peroxide and bacterioeins, and modulation of immune responses. Lactobacilli produce a number of anu-microbia products including bacterioeios, organic acids, and hydrogen peroxide (Barefoot, S. F., and C. G. Nettles (1993), J Dairy Sci 76:2366-79), which may inhibit *C. jejuni* and other pathogens in vitro. Lactobacillus saivarus NRRI. B-30514 has previously been identified as having anti-*C. jejuni* activity (Stem, N. J. *et al.* (2006), Antimicrob Agents Chemoth 50:31 11-6). Its baderioein,
OR-7, has been shown to reduce colonization of C. jejuni in chickens when administered in feed. Heat treatment of supernatants and trypsin and proteinase K treatment of supernatant* and agar plates did not effect inhibition of C. jejuni by the Lactobacillus strains evaluated, suggesting inhibition was not due to the production of a bacteriocin. These data strongly suggest that the combination of organic acids and hydrogen peroxide produced by the kctobaeilli are responsible for inhibiting C. jejuni in vitro.

Lactobacillus Bacterial Vaccines and Other Host-based Vaccines

Another aspect of the invention involves the administration of a Lactobacillus strain to chickens that has probiotic as well as vaccine properties. While not wishing to be bound by theory, it is believed that a Lactobacillus strain that displays C. jejuni epitopes stimulates production of C. jejuni-specific IgA antibodies, resulting in a reduction in C. jejuni colonisation. For example 5, three species of Lactobacillus (i.e., L. acidophilus, L. crispatus, and L. helveticus) were tested for probiotic properties in order to select one to develop as a vaccine. Some species of Lactobacillus synthesize an surface (S)-layer protein (Avali-Jaaskelaraen, S., and A. Palva (2005) FEMS Microbiol. Rev. 29:51 1-529, Boot, H. I. et al. (1996), Microbiology, 142 (Pt 9):2375-2384). These three species were chosen because they normally colonize the ilea of chickens and synthesize a surface (S)-layer protein. Moreover, the S-layer protein, which coats the surface of the bacterium, can tolerate incorporation of foreign epitopes (Ashgar, S. S. et al. (2007), J. Bacteriol. 189:1 856-1865). Generally, the S-layer is composed of one to three proteins ranging from 40 to 200 kDa, and comprises 10-15% of the total cellular protein. The S-layer of L. acidophilus' ATCC 4365 is encoded by two genes slpA and slpB, which are located in opposite orientation from one another and separated by a 3 kb DNA-region, L. helveticus JCM SSH) contains two S-layer encoding genes, cbsA and cbsB, but only the cbsA gene is expressed. L. helveticus contains one S-layer gene, termed slpA. The genetic techniques for Lactobacillus species are advanced (Mota, R. M. et al (2006), BMC Biotechnol. 6:2), and it is feasible to insert relatively large genetic segments within the S-layer gene, thereby achieving secretion, cell surface attachment, and high-density presentation of foreign epitopes (Avali-Jaaskeiainen, S., and A. Palva (2005) FEMS Microbiol. Rev. 29:51 1-529).

Colonization of the ileum by Lactobacillus is desirable, as this section of the digestive tract contains a high number of Peyer's patches that are involved in antigen sampling and antibody production (Vaughn, L. E. et al (2006), Avian Pis, 50:298-302). A mucosal immune response against C. jejuni results in antibodies that bind to the surface of bacteria and prevent or inhibit it from colonizing the digestive tract. To ensure that C. jejuni proteins that have the
potential to generate neutralizing antibodies are incorporated into the S-layer, the specific C
Jejuni proteins and the regions within (hose proteins against which poultry normally generate
antibodies are identified.

C jejuni genes could also be combined with various viral hosts or vectors, (e.g. adenovirus, baelovieuvs, herpes virus, pox virus vectors, etc.) or other bacterial hosts or vectors (e.g. Escherichia coii). Such hosts may be attenuated.

**Recombinantly engineered S-layer protein**

Other embodiments of this invention are directed to engineered recombinant S-layer
proteins that have one or more antigenic C jejuni antigenic sequences inserted into the S-layer
protein. A particular S-layer protein can be engineered to have, for example, one, two, three,
four, five or more antigenic polypeptide sequences inserted into the S-protei,, One
embodiment has polypeptide sequences from each of the Cadi\(^7\), FlA, and RpA proteins
inserted into the S-layer protein. The polypeptide may be inserted at a location where the
polypeptide is exposed at the surface of a bacterial cell when the surface layer protetin is
expressed. When expressed at the surface, the heterologous polypeptide can more readily
interact with another moiety, for example in a ligand/receptor with another polypeptide (e.g., an antibody). Alternatively, the heterologous peptide may be inserted such that it is not expressed
at the surface of the S-protein such as proximal to a cell wall anchor or binding domain.

Two main domains have been identified for the S-layer protein of *Lactobacillus acidophilus* (see Pouwc\(\acute{e}\), PH et al., (1998), Int J Food Microbiol41; 155-167; Seegers, JF (2002) 20:508-15; U.S. patent application serial number 10/500,307, published as
US20050233408, the complete contents of which is herein incorporated by reference). The N-
terminal region constitutes about two thirds of the molecule, and it is involved in crystallisation
and assembly. This region makes up amino acids 1 to 290 and forms the S-layer above the cell
wall. The second main domain consists of amino acids 290 to 412. This portion of the protein
is buried in the S-layer and it constitutes the cell wall anchor. The N-terminal region of the
*Lactobacillus acidophilus* S-layer protein can subdivided into three portions; residues 1 to
about 114, residues from about 115 to about 155 or so, and residues from about 160 to 290.
The region comprising amino acid residues from about 115 to about 155 or 160 appears to be
loop region that is exposed at the bacterial surface. This region is a preferred site for insertion
of heterologous polypeptides, including insertion at a position from 100 to 160, such as from
110 to 150, 110 to 140, preferably from 120 to 140, 120 to 130, or at about position 125. A
different amino acid residue numbering scheme will apply to alternative species of
*Lactobacillus*. 

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Antibodies

The present invention also provides antibodies that are specifically immunoreactive with the proteins described herein. Accordingly, the antibodies of the invention will specifically recognize and bind polypeptides that have an amino acid sequence identical, or substantially identical to the amino acid sequence disclosed herein, or an immunogenic fragment thereof. The antibodies of the invention usually exhibit a specific binding affinity of at least about $10^7$, $10^8$, $10^9$, or $10^{10}$ M$^{-1}$. Antibodies may be polyclonal or monoclonal and can be made and purified by a variety of means well known to those of skill in the art. See, for example, Coligan, Current Protocols in Immunology, Wiley/Greene, NY (1991); Stites et al. (eds.) BASIC AND Clinical Immunology (7th ed.) Latige Medical Publications, Los Altos, CaHf., and references cited therein ("Stites"); Coding, Monoclonal antibodies: Principles and Practice (2d ed.) Academic Press, New York, N.Y. (1986); Kohter and Mustek, 1975, Nature 256: 495-97; and Hariow and Lane. The antibodies of the invention may be of any isotype, for example, IgM, IgD, IgG, IgA, and IgE, with IgG, IgA and IgM most referred. Some monoclonal antibodies of the present invention are humanized, human or chimeric, and may be multifunctional. See, for example, Queen, et al., Proc. Natl Acad. Sci. USA 86: 10029 (1989); ILS. Pat. Nos. 5,563,762; 5,693,761; 5,585,089 and 5,530,101. Useful antibodies can also be produced using phage display technology (see, for example. Dower et al., WO 91/1722; and McCaffrey et al., WO 92/01047). Single chain antibodies can be produced using methods well known in the art (see, for example, Colcher et al., Ann. NY Acad. Sci. 880: 263-80 (1999); Reiter, Clin. Cancer Res. 2: 245-52 (1996); U.S. Pat. Nos. 4,946,778; 3,260,203; 5,455,030; 5,518,889; and 5,534,621). The antibodies of the invention have a variety of uses, for example for example, isolation or detection of polypeptides, inhibition of activity, etc.

Other uses of C. Jejuni antigens

Those of skill in the art will recognize that the antigens described herein can be used in a variety of other applications, including but not limited to; diagnostics, research reagents (e.g. for investigations! purposes), etc. All such uses are intended to be etibompassed by the invention.

Various aspect* of the invention will now be described with reference to the following experimental section that will be understood to be provided by way of illustration only and not to constitute a limitation on the scope of the invention.
EXAMPLE 1 Maternal antibodies of *Campylobacter jejuni* proteins recognized by chicken sera

In a previous study, chicks with maternal antibodies generated against the S3B strain of *C. jejuni* provided protection against *Campylobacter* colonization (Sahin *et al.*, 2003. AEM69:S372). Serum samples, collectively referred to as the CJ S38-SPF sera, were obtained from the previous study. These sera were determined to contain maternal antibodies that reacted against *C. jejuni* whole cell lysates as judged by ELISA. The antigens recognized by the CJ S3B-SPF antibodies were identified by sramunoblot analysis, coupled with mass spectrometry, of *C. jejuni* outer membrane protein extracts. This approach led to the identification of *C. jejuni* proteins recognized by the maternal antibodies, including the flagElln proteins and CadF adhesion. In vitro assays revealed that the CJ S3B-SPF sera retarded the motility of the *C. jejuni* S3B homologous strain, but did not retard the motility of a heterologous strain of *C. jejuni* (S1-176). Collectively, this Example provides a list of *C. jejum* proteins against which protective antibodies are generated in hens and passed to chicks.

**MATERIALS AND METHODS**

Bacterial cultures of chicken sera. The *Campylobacter jejuni* S3B strain was isolated from a chicken. The *C. jejuni* 81-176 strain was isolated from an individual with diarrhea containing blood and leukocytes (Koriatk J. A., *et al.*, 1985. Infect, *Ok*. 152:592-596). *C. jejuni* S3B and 81-176 were cultured on Mueller Kinton (MH) agar plates containing 5% citrate buffered bovine blood (MH-blood) under microaerobic conditions (5% O2, 10% CO2, 85% N2) at 37°C. The bacteria were subcultured to a fresh MH-blood plate every 48 h.

The generation of the sera is described in detail elsewhere (Sabin, O. *et al.*, 2003. Appl. Environ. Microbiol. 69:5372-5379). Briefly, specific pathogen-free (SPF) from Write Leghorn chickens were obtained from a supplier and hatched in isolation. The chickens were examined for Ith absence of *C. jejuni* colonization by cloaca swabs, bred at 22 weeks of age, and after an additional 2 weeks were inoculated with the *C. jejum* S3B strain. Fertilized eggs were collected from the inoculated hens and hatched in isolation. In total, blood was collected from nine SPF White Leghorn chickens at 2 days of age. The serum was harvested from each blood sample and stored at -20°C. 25 to 100 ml of each serum sample were obtained. Throughout this paper those serum samples are referred to as the CJ S3B-SPF sera.

Preparation of *C. jejuni* outer membrane proteins. Outer membrane proteins (OMPs) were prepared using N-Meuroyl-sarcosine as previously described by de MSIQ and Pechere (de MeIo, M. A., and J. C. Pechere, 1990, Infect, Immunun, 58:1749-1756) with slight modifications.
Briefly, bacteria were grown overnight in M H broth with a h a g a t ? C under microaerobic conditions. The bactetial cell suspensions were sonicated five times (30 s each) with a 30 s cooling period on ice between each pulse with a Branson Sonifier Cell Disrupter (model 250; Branson Sonic Power Co., Danbury, CT). Cell debris was removed by centrifugation at 6,000 x g for iθ mìn. The crude membranes were obtained by cectrifugation at 100,000 x g at 4°C for 2 h. The resulting pellets were suspended in H) mM Tris (pH 7.5), and the protein concentration of each sample was determined using the bkinchonink acid (BCA) assay as outlined in the manufacturer’s instnserkms (Pierce-, Rockford, IL). N-iauroyl-saresme (Sigma) was added to the crude extracts at a protein to detergent ratio of 1:4 total (w/w). The samples were incubated at room temperature with gentle rocking for 30 mìn. The OMPs were obtained by centrifugation at 100,000 x g at 15°C for 2 h. The pellets were washed with 50 mM Tris (pH 7.5), suspended in the same buffer, and stored at -20°C. The protein concentration of the OMP extracts was determined by bicinchonink acid BCA assay.

Enzyme-linked iBitrøøsorba πf assays. EIISAs were performed to determine the level of C. jejuni-specific IgGs antibodies the Cj S3B-SPF sera, Mictotiter plates (Corning Incorporated, Cornmg, NY) were coated with 100 pi of ovalbumin (negative control), C jejuni S3B whole cell lysates, or S1-176 whole cell lysates diluted to H) µg/ml in coating buffer (50 mM Na₂CO₃, Sl mM NaHCO₃, pH 9.6). After incubation at 4°C for 18 h, the coated plates were incubated with 0.5 % {w/v} bovine serum albumin (BSA; Sigma) in phosphate buffer (PBS; 0.14 M NaCl, 5 mM Na₂HP0₄, 2H₂O, 1.5 mM KII₃PO₄, 19 r»M KCl pH 7.4) at room temperature for 2 h to reduce the non-specific binding of antibodies. The Cj S3B-SPF serum samples were diluted 1:200 in PBS containing 0.5 % BSA. 100 ul of each sample was added to wells in triplicate, and incubated for 2 h at room temperature. The plates were rinsed 3 times with wash buffer [(0.15 M NaCl, Ql % (v/v) Tween* 20, pH 7.4)j and 100 l of rabbit anti-chicken IgG conjugated to peroixidase (1:1000; Sigma) diluted * PBS containing 0.5 % (w/v) BSA and 0.1 % (v/v) Tween 20 was added to the wells. Alter 1 h of incubation at room temperature, the plates were rinsed 2 times with wash buffer and 2 times with FBS. Tetrametiøyhenzidine (TMB) substrate (Pieree-Hndoge π) was added to the wells mid the reaction was stopped with 0.1 8 N H2SO4 after 10 min of development. Absorbances {A_490} were determined at 490 nm with an ELx808 Ulira Micropkt ε Reader (BioTek Instruments, iuc S Winoo&ki, VT). The absorbances obtained using the chicken sera incubated with ovalbumin were subtracted from the appropriate sera sample
values to remove background signal. Student's M test was performed on \( A_{d/g} \) values to
determine statistical significance between sample groups (\( P < 0.005 \)). Nine sera were collected
from chickens not colonized with \( C. \) jejuni and used to calculate the negative cutoff using
Student's t distribution. Absorbance values greater than the negative cutoff value were
considered positive for \( C. \) jejuni-specific antibodies (8).

SBS-PAGE and Smuttioblot analysis. Bacterial OMPs (0.5 \( \mu \)g/\( \mu \)l) were solubilized in
single-strength electrophoresis sample buffer and boiled for 5 min. Proteins were separated by
SDS-polyacrylamide gel electrophoresis (PAGE) using 12.5% polyacrylamide minigels as
proteins were either stained with Coomassie brilliant blue R250 (CBB-R2S0) or transferred to
polyvinylidene fluoride (PVDF) membranes (Timnohicm P; Millipore Corporation, Bedford,
MA). Membranes were blocked in PBS containing 0.05 \( \% \) (v/v) Tween 20 (PBS-T) with 20 \%
fetal bovine serum and incubated with the \( C. \) jejuni S3B-SPF sera (1:200 dilution) overnight at 4°C.
Subsequently, biota were washed 3 times with PBS-T. Bound antibodies were detected with
rabbit anti-chicken peroxidase-cmjjugated IgG (1:1000 dilution, Sigma). CadF was detected
using a goat anti-CadF specific serum (#463) coupled with a rabbit anti-goat peroxidase-
conjugated IgG (1:1000, Sigma). The FlaA and FlaB flagellin proteins were detected using a
rabbit antisrifl-\( C. \) jejuni flagellin specific serum with goat anti-rabbit peroxidase-conjugated IgG
(1:4000, Sigma). The blots were washed 3 times with PBS-T and developed with 4-chloro-1-
naphthol peroxidase chromogenic substrate (ThepnoN Scientific, Roekford, IL) as ouUmed by
the manufacturer.

LC/MAUDI/TOF-TOF and data analysis. OMP extracts were trichloroacetic acid (TCA)
precipitated and washed 3 times with acetone. The dried pellets were resuspended in 25 \( \mu \)l 8 M
urea, 100 \( \mu \)M NH\(_4\)HCO\(_3\), ami \( \mathrm{th}s \) pH was adjusted to 7.5-8.0 with NH\(_4\)HCO\(_3\). Proteins were
reduced with DTT at a final concentration of 5 \( \mathrm{mM} \) at 37°C for 30 min and then alkylated \( \mathrm{with} \)
ioodoactemide at a final concentration of 25 \( \mathrm{mM} \) at 37°C for 30 min in the dark. The solution
was diluted 4 times with 100 \( \mu \)M NH\(_4\)HCO\(_3\) and 1 \( \mu \)g trypsin (Sequence grade. Fro-mega,
Madison, WT) was added for overnight digestion at 37X. The digest solution was concentrated
with speed vacuum to a final volume of 20-30 \( \mu \)l. The LC MALDI plate was prepared using
the Tempo™LC MALDI system (Applied Biosystems, Foster City, CA). 5 \( \mu \)l digest solution
was loaded onto the analytical column (Chromolith® CapROD RP-18e, 100 \( \mu \)m x 150 mm,
Merck KGaA, Darmstadt, Germany) by the autosampler and separated at a flow rate of 2
\( \mu \)l/min using the following gradient: 5 \% B for 0-2 min, 5-20 \% B for 2-25 min, 20-60 \% A for

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25-50 min, 95 % B for 50-60 min, and 0 % B for 60-70 rain. Mobile phase A was 0.1 % trifluoroacetate acid (TFA) in 2 % acetonitrile and mobile phase B was 95 % TFA in 95 % acetonitrile. 5 mg/ml MALDI matrix, α-eyario-4-hydxyeinnamk acid (CHCA), was prepared in the solution of 50 % acetonitrile, 0.1 % TFA, and 5 mM ammonium monophosphate and delivered at a flow rate of 2 μl/min. The LC effluent and matrix solution were mixed via an Upchurk T connector and the mixtures were then spotted on a blank MALDI plate (123 X 81 mm) every 4 s during the 50 min LC gradient. The MS and MS/MS spectra were acquired with a 4800 MALDI/TOF-TOF mass spectrometer (Applied Biosystems, Foster City, CA). 1000 laser shots were used for each reflector MS spectrum and 2500 laser shots were collected for each MS/MS spectrum. The precursor peaks with S/N > 40 were selected for MSMS experiment and the 25 strongest precursors were allowed for MSMS per spot with the weakest precursor submitted first. Peaks with S/N > 10 were extracted and searched against the c. jejuni 81-176 database (CJJS 1176 downloaded from NCBI, 1758 ORFs) using ProteinPilot™ software (version 2.0.1, revision 67476, Applied Biosystems, Foster City, CA). Search parameters were set as follows; enzyme, trypsin; Cys alkylarion, iodoacetamide; Special factor, urea denaturation; Species, none; and ID focus, biological modification. The protein confidence threshold cutoff for this report is ProtScore 2.0 (unused) with at least one peptide with 95%, confidence. See the supplemental data for the complete ProteinPilot results. Protein subcellular localisation was determined by PSORTb (http://www.psort.org/psortb/).

Nano-IX/MS/MS and data analysis. Nano-LC/MS/MS was performed as described previously (Tang, X., et al. 2005, Anal. Chem. 77:31 1-31 8), and used to identify the reactive bands as determined by iTRAQ labelling with the Cj S3B-SPF sera. Briefly, bands representing immunoreactive proteins were excised from SDS - 12.5 % polyacrylamide gels that had been stained with CBB-R250. Atler band excision, each gel piece was (Sustained with a solution containing 50 % methanol and 5 % acetic acid. The disulfide bonds within the proteins were dissociated within the gel by peiformic acid oxidation. The gel was dried, and the proteins were digested with trypsin overnight at 37°C. Nano-LC/MS/MS analysis was done using a electrospray-km trap (Esquire- HCT, Brisker Daikonics, Billerica, MA) mass spectrometer coupled with a nano-HFIA. The resulting data were used to perform searches against the C. jejuni 81-176 genome database using the program MASCOT licensed in house (Version 2.1.0, MatrixScience Ltd, London). Protein hits with probability-based Mowse scores exceeding their thresholds (p < 0.05) were automatically reported. The protein hits were further filtered using more stringent MândPIT scoring and an ions score cutoff of 0.05, which removed all the peptides with expect value (E) > 0.05.
Motility assay. To evaluate the function attributes of the anti-Campylobacter maternal antibodies, motility assays were performed as described previously with slight modifications. (Koncicel, M. E., et al., 2004, J. Bacterid. 186:3296-3303). C. jejuni S3B and 81-176 strains were grown for 24 h on MH-bioceplates, harvested by centrifugation at 6,000 g, and suspended to an OD₅₅₀ ≈ 0.18 in Minimal Essential Medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Bacterial suspensions were then diluted 1:100 in the same media that contained either sera from C. jejuni iminoculated chickens, pooled Cj S3B-SPF sera, or heat-inactivated Cj S3B-SPF pooled sera. Complement was inactivated through heat treatment at 56°C for 30 min. The bacterial suspensions were mixed, and 10 μl aliquots were spotted onto the surface of semisolid MH medium with 0.4 % agar. Motility plates were incubated for 48 h at 3TC under microaerobie conditions.

RESULTS

Chicks hatched from hens colonized wills Campylobacter possess anti-C jejuni maternal antibodies. Nine serum samples (designated 12 K 123, 129, 132, 135, 139, 140, 144, and 147) generated in a previous study were obtained and termed the Cj S3B-SPF sera. These sera were collected from 2-day old SPF while Leghorn chicks hatched from hens inoculated with the C. jejuni S.1B strain. To determine the level of C. jejuni-specific IgG maternal antibodies in each serum, ELISA were performed with wells coated with whole cell lysates (WCL) prepared from C. jejuni homologous (S3B) and heterologous (81-176) strains (Figure 1). Non-specific antibody reactivity was determined by calculating the negative cutoff value of antibody reactivity for the sera harvested from nine chickens not colonized with C. jejuni (control sera). The reactivity of the control sera against the WCL of the C. jejuni S3B strain was less than (P < 0.005) that obtained for the WCL of the C. jejuni 81-176 strain (Figure 1 horizontal lines).

Each of the Cj S3B-SPF serum samples contained antibodies that reacted specifically against the WCL of the C. jejuni S3B and 81-176 strains as judged by EUSA. However, an increase was observed in the reactivity of the Cj S3B-SPF sera against the WCL of the S3B homologous strain (mean A₅₅₀ 0.665) when compared to WCLs from prepared from the 81-176 heterologous strain (mean A₅₅₀ 0.463) (P < 0.005). The differences in reactivity with the VVCLs suggested that the Cj S3B-SPF sera either contained antibodies that react with antigens unique to the C. jejuni S3B strain or that variations in the amino acid composition of strain specific antigens occur that contribute to the increase in reactivity of the sera against a specific strain. The increase in the reactivity of the Cj S3B-SPF sera against the WCLs from the C. jejuni S3S
strain versus the Sl-) 7 6 straits may partially explain a n observed delay in onset of colonization
with the C jejuni S3B challenged chicks and reduced rate of horizontal spread among the flock.

Identification of outer membrane proteins (OMFs). LC/MALDI/TOF-TQF was performed
with the total OMP extracts prepared from the C jejuni 81-176 strain, for which the genome
has been sequenced, to ensure that the composition of the preparations was predominantly outer
membrane protein and not cytoplasmic proteins (Figure 5). The ProteioPilot iM software was
employed as the search engine for protein identification using LC/MALDI/TUF–TQF data.
Since the unused ProtScore is a measurement of all the peptide evidence for a protein that is not
explained by a higher ranking protein and is a true indicator of protein evidence, we set the
anised score at 2.0 as the threshold cutoff for protein identification with at least one peptide
with 99% confidence. With these criteria, 60 proteins were identified with 2944 MS/MS spectra searching against the C jejuni 81-176 database (total 1758 ORFs) (Figure 5). Of the 60
proteins identified, approximately 32% were localized in the cytoplasm as determined by
PSORTb. Additional analysis of the proteins contained within the OMP extracts revealed that
18% were categorised as unknown subcellular location and 50% were identified as extracellular, outer membrane, periplasmic, inner membrane proteins, or designated as
unknown subcellular location with a signal peptide.

Reactivity of the CJ S3B-SFF sera against the OMPs of homologous and heterologous C.
jejuni strains. To determine the reactivity of the antibodies contained within the Cj S3B-SFF
sera. OMP extracts were separated by SDS-PAGH, transferred to PVDF membranes, and
unknown analysis was performed with the S3B-SFF sera. The Cj S3B-SFF sera produced
repeatable banding profiles for the OMP extracts from both the C jejuni S3B homologous
strain and 81-176 heterologous strain as judged by immunoblot analysis (Fig. 2A). The reactive
bands in the OMP extracts ranged from 16 to 90 kDa. The representative banding profiles
generated against the C jejuni S3B and 81-176 strains were similar, but some bands were
unique to a particular strain.

Inspection of blots revealed that the S3B-SFF sera contained antibodies that reacted
against strain-specific proteins and against proteins shared amongst the C jejuni S3B and 81-
176 strains. Proteins with apparent molecular masses of 83, 65, 60, 56, 54, 42, 37, 26, and
20 kDa (bands 1-6, 9, 11, 14, and 16, respectively, see Figure 2) were cross-reactive with both
the C jejuni S3B homologous and 81-176 heterologous strains. Immunoreactive proteins
specific to C jejuni S3B were observed at approximately 32, 28, 16 kDa (bands 12, 13, and 17,
spectively) (Figure 2) immunoreactive proteins unique to C jejuni 81-176 were observed at
approximately 50, 45, 40 and 23 kDa (bands 7, 8, 10 and 15, respectively) (Figure 2). These
results indicated that the chicks possessed both maternal antibodies that reacted against the particular C. jejuni strain with which the hens were colonized aacJ maternal antibodies that reacted with proteins shared amongst C. jejuni strains.

Immunoblots were performed to determine if the Cj S3B-SPF sera contained antibodies reactive against CadF protein. A reactive band (band 1t), corresponding to a protein with a Mr of 3? kDa, was observed in the OMP extracts from the C jejuni 53B and 81-176 strains using each of the nine Cj S3B-SPF sera (Figure 2). Fine bands observed at 37 kDa with the Oj S3B-SPF sera had the same relative migration as the CadF protein detected using a goat anti-CadF specific serum (Figure T).

Identification of the bands recognized by the Cj S3B-SPF sera, Nano-LC/MS/MS was used to identify the C jejuni membrane-associated antigens recognized by the Cj SSB-SPF sera. The OMP extracts from the C jejuni S3B and 81-176 strains were separated by SDS-PAGE, and either stained with CRB-R25O or transferred to PVDF membrane. The blot was incubated with a representative C.j 0 -ml S3B-speii te serum to identity the reactive proteins. Seventeen reactive bands were identified; fourteen of the seventeen bands were subjected to nano-LCVMS’MS.

Bands 1, 2, 4-10, and 12-16 were excised individually from the gel and subjected to trypsin digestion followed by nano-LC/MS/MS. Careful attention was paid to excise those protein bands that were in perfect alignment with the reactive bands in the corresponding immunoblot. The proteins identified are listed in Figure 4. The predicted "best fit" protein matches were OMFs with significant MASCOT scores and had a molecular weight corresponding to the migration of the protein in a SDS - 12.5 % polyacrylamide gel. Confidence in protein matches was established using MudPIT scoring and an ion score cutoff of θ.05.

Bands 3 and 11 were identified via tmminiotkit analysis using protein specific sera, and band 1? was identified based on its apparent molecular mass. The 65 kDa protein (band 3) was identified as flagellum using an anti-C jejuni flagellin serum and the 3? kDa protein (band 1t) was identified as CadF using an anti-C jejuni CadF serum. It is likely that the 16 kDa immunoreactive band (band 1?), which was detected only in the OMP extracts prepared from the r jejuni S3B strain, is Upooaigosacoharide (LOS) (Stem, N. J., and S. Pretønk (2006), J. Food Prot. 69:1034-1039).

The Cj S3B-SFF bands unique to C Jejuni 81-176 are flagellinu. A number of the bands were found to contain peptides that matched the. FlaA or FlaB sequence as judged by narro-LC/MSZMS (Figure 4). This finding raised the possibility that a particular band may have been
immunoreactive because of the presence of flagellin protein sobs.mi.t8. To determine whether
the reactivity of these bands was due to flagellin subunits or another protein distinct from
flagellin, C. jejuni S3B and 81-176 OMF extracts were probed with the aiU-C jejuni flagdlm
serum. As expected, the 65 kDa protein (bands 3) reacted with the mti-C. jejuni flagdlm
serum. In addition, proteins of 50 kDa (band 7), 45 kDa (band 8), 40 kDa (band 10) and 23 kDa (band
.15) were detected in the OMP extracts from C. jejuni strain 81476 but not in C. jejuni S3B
strain. The immunoreactive bands of 50 kDa, 45 kDa, 40 kDa, and 23 kDa were the only
proteins unique to the C. jejuni S1-176 OMP extracts (i.e., not detected in the C. jejuni S3B
OMP extracts). The bands of 50 kDa, 45 kDa, 40 kDa, and 23 kDa in the C. jejuni 81-176
OMP extracts were determined to be FlaA or FlaB by nano-LC/MS/MS.

**C. jejuni S3B-specific antibodies inhibit the motility of the homologous strata, but not the**
**heterologous strain.** Motility assays were performed with the Cj S3B-SPP sera and both the
C. jejuni S3B (Figure 3A) and 81-176 strains (Figure 3B). In contrast with the C. jejuni 81-176
strain, only the C. jejuni S3B strain showed a reduction in motility when compared with the
same strain with control sera harvested from birds not colonized with C. jejuni. This
observation was true for both the Cj S3B-3PF heat-inactivated sera, as well as the Cj S3B-3PF
untreated sera, demonstrating that the reduction in motility is due to antibodies binding to the
bacteria, and not due to the action of complement.

**EXAMPLE 2. Examination of Campylobacter jejuni putative adhesins as and Identification**
**of a new protein, designated FlpA, required for chicken colonization**

*Campylobacter jejuni* colonization of chickens is presumably dependent upon multiple surface
exposed proteins termed adhesins. Putative *C. jejuni* adhesins include OadF, CapA, JlpA,
MOM?, PEB1, Cj!2?9e, and Cj! 34e. The genetic relatedness of 97 *C. jejuni* isolates
recovered from human, poultry, bovine, porcine, ovine, and canine- sources was examined by
multiple sequence typing (MLST) and their profile of putative adhesin-encoding genes was
determined by dot bio* hybridization. To assess the individual contribution of each protein
in bacteria-host cell adherence, the C. jejuni genes encoding the putative adhesins were
interrupted 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, Cj! 1279c, and Cj! 1349c proteins were found to
play a significant role in the bacterium's in vitro adherence to chicken epithelial cells, while
CadF, PESI, and Q \(279c\) were determined to play a significant role in the bacterium's in vivo colonization of broiler chicks. Collectively, the data indicate that Cj 1279c is a novel adhesin. Because Cj 1279c harbors fibronectin type \(1\) domains, the protein was designated FlpA for Fibronectin-ti,ke protein \(A\).

**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 (data not shown). All human isolates were obtained from individuals with eibical symptoms of campylobacteriosis. *C. jejuni* F3801 I was isolated from an individual with bloody diarrhea, hi total, we used 43 human strains (F3801 I, 81-176, 81116, M129, H13H2, H4-7, H9-24, H26-32, and 1134-43), 41 poultry strains (RM 1221, Turkey, SI, S2B, USDA02-833L, A2a, A5a, A18a, D34a, GUa 3Iowa 2, Iowa 4-9, Tovva 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 ovU 2), and one canine strain (can 1979858). (*Z. jejuni* isolates were cultured at 37°C under microaerobic conditions (5% O\(_2\), 10% CO\(_2\), 85% N\(_2\)) on Mueller-Hinkm agar plates supplemented with 5% ciliated bovine blood (MH-blood agar plates). *C. jejuni* strains were subcultured to a fresh plate every 48 h. The *C. jejuni* F3801 I can (Kanamycin resistant, Kart\(R\)), c0pA (tetracycline resistant, Tet\(R\)), jlpA (Kan\(S\)), pehLA (Kaa\(R\), Cj\(j\) 278c (Tei\(R\)), Q 279c (K\(\alpha\) \(R\)), and Cj\(j\) 349c (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 let., 2.0 µg/ml (Sigma).

Motility assay. Motility was determined using MH medium supplemented with 0.4% Select agar (Invirogen, Carlsbad, CA). Briefly, 10 µl of each bacterial suspension in MM 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.

**M. hiiocicus** sequence typing. Genomic UNA was isolated from the *C. jejuni* isolates using phenol chloroform extractions. Briefly, bacteria were cultured on MH-blood agar plates and harvested in 5 nil of phosphate-buffered saline (PBS). After incubation for 1 h at 37°C with 500 µS SDS sodium dodecyl sulfate- (SDS) and 5 µl proteinase K (20 ing/ml), three phenol and isoamyi alcohol chloroform extractions (24 parts chloroform and 1 part isoamyi alcohol) were performed with the aqueous layer retained each time. An equal volume of eoid isopropano! and

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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 OD260/OD280 ratio > 1.8, was determined using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE).

*C. jejuni* housekeeping genes aspA ("aspa"), glutamine synthetase ("gfaA"), citrate synthase ("cit4"), serine hydroxymethyltransferase ("glyA"), phosphoglycerate mutase ("p.gm"), transketolase ("tkt"), and the ATP synthase alpha subunit ("uncA") were amplified and sequenced, using the primers described elsewhere (26). 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× Master Amp PCR buffer (Epicentre, Madison, WI), 1× Master Amp PCR enhancer (Epicentre), 2.5 mM MgCl₂, 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). Amplification products were visualized by agarose gel electrophoresis and purified on a BioRobot 800Q workstation (Qiagen, Valencia, CA). Cycle sequencing reactions were performed on a Terratrace nucleotidyl (Bio-Rad, HCRules, CA), using the ABI® BigDye terminator cycle sequencing kit (ver. 3.1; Applied Biosystems, Foster City, CA) and standard primers. Cycle sequencing extension products were purified using Dye-Ex 96 plates (Qiagen). EJNA 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 Sequenc 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 FubMLST 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*, *pehLA*. The sequence of each gene from C jejuni NCTC 11168 was obtained from on-line resources (web site located at sattger.ac.uk/Projects/Cjejuni/). Gene-specific probes were generated as outlined below. An internal fragment of each gene was amplified via PCR using the primers listed in Table 2. The amplifications were performed using high fidelity Taq DNA polymerase (Taq DNA polymerase) 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 FCR fragments were ligated into the vector pCR2.1 according to the manufacturer’s directions (Original TA Cloning Kit, Invitrogen) and electroporated into *Escherichia coli* InvAF. The purified plasmids were nkk-traaslated using a Nick Translation Kit according to the manufacturer’s directions (Roche Applied Science, Indianapolis, IN). Oligonucleotidng of *C. jejuni* genomic DNA, isolated via phenol chloroform extractions as described above, were vacuum transferred to a gene-screen membrane (PerkinElmer, Waltha MA) using a Schleicher and Schuell Minifold 11 Slot-Blotter (Jencom, United Kingdom). Depurinating solution (0.25 M HCl) was added to each slot for 4 min, followed by denaturing solution (1.5 M NaOH and 0.5 NaCl) for 3 min, neutralizing 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, J5 m6 formamide, 2 m6 5x P buffer (1.5% BSA, 1.0% polyvinylpyrrolidone, 3.0% 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 (Robins Scientific, Hudson, NM) overnight. Membranes were washed twice with 2x SSC at 25°C for K min, and twice with a 2x SSC and 0.1% 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* *ndaF, pA, peblA, Cj1279c, and CjB49c* stn vectors. The PCR amplimcons 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 pBlueScr (Invitrogen), except that the original kaiA cassette was replaced with one that functions in both *C. jejuni* and *E. coli* (Labignc-Roussei, A. et al. (1987), *Campylobacter jejuni*. J. Bacteriol. 169:5320-5323). The resulting pBSK-Kan2 vectors (pMEK252-pME IC256) were confirmed by DNA sequencing, and were electroporated into *E. coli lmoF* electrocompetent cells.

Generation of *C. jejuni* *eapA* and Q1278c stn vectors. DNA regions upstream and downstream of the *C. jejuni* *eapA* and Cj1278c genes were amplified by PCR using *Taq* DNA Polymerase (Invitrogen) and the primers listed in Table 2. *C. jejuni* NCTC 1168 chromosomal
DNA was used for the amplification of DNA regions flanking capA, while C. jejuni F38031 chromosomal DNA was used for the regions flanking Q l278c. The reaction conditions were: 94°C for 2 min (1 cycle); 94°C for 45 sec, 63°C M 9°C per cycle) for 30 sec, 70°C for 4 min (8 cycles); 94°C for 45 sec, 5CPC 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 ρBSK-Ka2. 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 ODs450 of 1.0. Two-hundred ml of culture was centrifuged at 6,000 x 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 die 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 Mil-blood plates containing the appropriate antibiotic (50 µg/ml Kan or 2 µg/rai let). 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 Q l278c mutants by sequencing the DNA flanking regions. The motility of each C jejuni rautani was assessed prior to use.

Tissue culture. Chicken LMH Hepatocellular carcinoma cells (ATCC CR1.-21 17) were obtained from the American Type Culture Collection (Msnassas, VA). Stock cultures of LMH cells were grown in 2% flasks coated with 0.1% gelatin in Waymouih's MB 752/1 medium supplemented with 10% fetal bovine serum (FBS; HyClon Laboratories, Logan, UT). Cells were maintained at 37°C in a humidified, 5% CO2 incubator.

C jejuni-hMA binding mmy. LMH cells were seeded to a cell density of 3.0 x 105 cells/ml and incubated for 24 h at 37°C in a humidified, 5% CO2 incubator. The cells were rinsed once with Minimal Essential Medium (MEM; Invitrogen) supplemented with 1% FBS and inoculated with approximately 3.0 x 107 CFU bacteria. Each plate was then subjected to centrifugation at 600 x g for 5 min to promote bacteria-host cell contact and incubated at 37°C for 30 min. To qitantitate cell adherence, the C. jejum-inaaxl&i&d cells were rinsed three times
with PBS, and lysed with a solution of 0.1% (v/v) Triton X-100 (Caibiociem, La Jolla, CA) in PBS. Ten-fold serial-dilutions of the samples were made and plated on MH-hia+d agar plates to determine the number of adherent bacteria. The reported values represent the mean counts ± standard deviation from triplicate wells.

Table 2. Genes targeted for mutagenesis

<table>
<thead>
<tr>
<th>Locus Tag (Gene Designation)</th>
<th>Gene Product (protein)</th>
<th># of nucleotides/residues</th>
<th>Amplified Fragment(s) in nucleotides</th>
<th>Primers</th>
</tr>
</thead>
</table>
| Cj1478c (cadF)               | CadF                   | 960/320                   | 620                                  | cadF-F<sup>+</sup>
|                              |                        |                           |                                      | cadF-R<sup>+</sup> |
| Cj0628 / Cj0629 (capA)       | CapA                   | 3436/1145                 | 1321, 1635                           | capA-F<sup>+</sup>
|                              |                        |                           |                                      | capA-R<sup>+</sup> |
|                              |                        |                           |                                      | capA-SalI-F<sup>+</sup>
|                              |                        |                           |                                      | capA-SalI-R<sup>+</sup> |
|                              |                        |                           |                                      | pATGAGCTCGAGAGGGTAACGAGTAAATAGAAG (SEQ ID NO: 89) |
|                              |                        |                           |                                      | CCCATTCTGATCCTACATAACCT (SEQ ID NO: 88) |
|                              |                        |                           |                                      | capA-SalI-F<sup>+</sup>
|                              |                        |                           |                                      | capA-SalI-R<sup>+</sup> |
|                              |                        |                           |                                      | AATACGCAGGCAGACCATATACATAACGAGTAAATAGAAG (SEQ ID NO: 90) |
|                              |                        |                           |                                      | TCCCTTTC (SEQ ID NO: 90) |
|                              |                        |                           |                                      | capA-SalI-F<sup>+</sup>
|                              |                        |                           |                                      | capA-SalI-R<sup>+</sup> |
|                              |                        |                           |                                      | ATACCACGGATCCAGTTAATATACATAACGAGTAAATAGAAG (SEQ ID NO: 91) |
|                              |                        |                           |                                      | capA-SalI-F<sup>+</sup>
|                              |                        |                           |                                      | capA-SalI-R<sup>+</sup> |
|                              |                        |                           |                                      | ATATCGACAGCATATACGAGCTTACATATACATAACGAGTAAATAGAAG (SEQ ID NO: 92) |
| Cj0983 (jlpA)                | JlpA                   | 1119/373                  | 868                                  | jlpA-F<sup>+</sup>
|                              |                        |                           |                                      | jlpA-R<sup>+</sup> |
|                              |                        |                           |                                      | TTCAGGAGGACTCTGGAATAAAAGATGG (SEQ ID NO: 93) |
|                              |                        |                           |                                      | GCTGCTTAGTAGTCACAACGAGGATG (SEQ ID NO: 94) |
| Cj0921c (peb1A)              | PEB1                   | 780/260                   | 560                                  | peb1A-F<sup>+</sup>
|                              |                        |                           |                                      | peb1A-R<sup>+</sup> |
|                              |                        |                           |                                      | TCTAGGAGGACTCTGGAATAAAAGATGG (SEQ ID NO: 95) |
|                              |                        |                           |                                      | GCTGCTTAGTAGTCACAACGAGGATG (SEQ ID NO: 96) |
| Cj1279c (FliA)               | Cj1279c (FliA)         | 1233/411                  | 832                                  | Cj1279c-F<sup>+</sup>
|                              |                        |                           |                                      | Cj1279c-R<sup>+</sup> |
|                              |                        |                           |                                      | TCAGAAGATGGCAAGTTATAGAAG (SEQ ID NO: 97) |
|                              |                        |                           |                                      | GTTATATGCTATATGATACACGGAG (SEQ ID NO: 98) |
| Cj1349c                      | Cj1349c                | 1308/436                  | 1115                                 | Cj1349c-F<sup>+</sup>
|                              |                        |                           |                                      | Cj1349c-R<sup>+</sup> |
|                              |                        |                           |                                      | TATATGCTATTGATACACGGAG (SEQ ID NO: 99) |
|                              |                        |                           |                                      | Cj1349c-F<sup>+</sup>
|                              |                        |                           |                                      | TATTATGCTATTGATACACGGAG (SEQ ID NO: 99) |
| Cj1278c                      | Cj1278c                | 1179/393                  | 1033, 991                            | Cj1278c-BamHI-F<sup>+</sup>
|                              |                        |                           |                                      | Cj1278c-BamHI-R<sup>+</sup> |
|                              |                        |                           |                                      | ATATAAGATACGAGGACTCTGAATGATG (SEQ ID NO: 100) |
The gene number is from the genome sequence from C. jejuni NCTC 1168. 
Indicates the primers used to amplify a DNA fragment for the generation of a suicide vector (gene knock-out) and the probe for dot-blai hybridization. 
Indicates the primers used to amplify the DNA fragments for construction of the vectors to generate the m-tjants (Le., capA and QJ2Sc) via a double-crossover event; the two fragments were cloned into :>BSK-Kan2, and disrupted by the insertion of a tetracycline resistance cassette.

**Chicken colonization experiments.** Ali 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 (HorsfaSI Bauer isolators) on wire mesh. Water and a commercial ehkk starter feed were provided *ad libitum.* Each isolator was equipped with two removable metal trays. Fecal matter was collected and autolavved before disposal. The chicks were inoculated with C. jejuni by oral gavage with (.15 ral of a bacterial suspension (10^-7 bacteria); the C. jejuni F380I 1 strain was cultured in Bolton’s broth at 42°C for 16 h under microaerobic conditions prior to inoculation of the birds. One group of 10 chicks was kept as the imirtoueled control group. The remaining groups of chicks were inoculated with the C. jejuni F380I 1 strains: 1) wild-type strain; 2) cadF mutant; 3) capA mutant; 4) jjLpA mutant; 5) pebt A mutant; 6) Cj! 279c mutant; and 7) Cj!$49c mutant. After the chicks were inoculated, the remaining bacterial suspensions were serially diluted and plated on Carapy-Cefex agar (30) to confirm the CFTr of each inoculum. The chicks were euthanized and neeropsied at 10 days post-inoculation (DPI). The cecum was dissected from each chick, weighed dilute 1:10 (wt/v) in Bolton’s broth media, and thoroughly stomached. For enumeration, serki 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 37°C and the CFUs were counted after 72 h of incubation. PCR was performed with C. jejuni cadF and capA specific primers (Table 2) to confirm that the counted colonies were C. jejuni.
RESULTS

The *C. jejuni* strains used to signify are genetically diverse.

Multilocus sequence typing (MLST) is commonly used for molecular typing of *C. jejuni* isolates (Dingle, K. E. *et al.* (2001). 1. Clin. Microbiol. 39: 14-23; Levesque, S. *et al.* (2008). 1. Clin. Microbiol 46:3404-3411). A total of 97 isolates from humans, poultry, bovine, porcine, ovine, and canine sources were collected and *thmr* genetic relatedness was assessed by MLST. The *C. jejuni* isolates comprised 45 sequence types (data not shown). 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 1, 1, 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, H19, Iowa 11, Iowa 13, Iowa 15, Iowa 35, and 29. The second most common ST was ST-50, represented by isolates H6, H34, H36, H37, H40, Sl, 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, F3801, 1H1, 11H1, and 1H30, and one poultry isolate USDA02-833L. In total, 103 alleles were identified amongst the seven loci, and a new *pgm* allele gm431 was reported. Based on the MIST analysis, we concluded that the *C. jejuni* strains used in this study were genetically diverse.

The adhesin-encoding genes, except *ctxA*, 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 3. Six of the seven putative adhesin-encoding genes, *i.e.*, *cadF*, *jlpA*, *pebiA*, *pon-t*, *Cf1229cF*, and *Cf1349c*, 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, *C. jejuni capA* was absent in 17 of the 43 (40%) human isolates and in 21 of the 54 (09%) 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*.

Table 3. *C. jejuni* genes encoding putative adhesins

<table>
<thead>
<tr>
<th>ORF</th>
<th>BLAST</th>
<th>Signal Peptide Cleavage Sites</th>
<th>Genes Within the Putative Operon&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>porA</em>/Cj1259</td>
<td>Major outer membrane</td>
<td>Residues 22-23,</td>
<td>None Identified</td>
</tr>
</tbody>
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CadF, CapA, Cj1279c, and Cj1349c coexpressed 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 6). A mutation in the pmrA gene was not attempted, as a mutation of this gene is hypothesized to be lethal due to its critical structural and pore activity (Amako, K. et al. (1996), Microbiol. Immunol. 40:749-754). All of the C. jejuni mutants (i.e., cadF, capA, jlpA, and pehlA) 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 pehlA 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, cupA, Cj!279c, and Q1349c mutants to LMH cells when compared with the C. jejuni wild-type strain. In addition, C. jejuni isolates were genetically matched (Hi 1 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 Towa 80 isolates did not. 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, PEbl, and Cj!279c contribute to C. jejuni colonization of broiler chickens. To determine the relative importance of each putative adhesin in chicken colonisation, one-week-old chicks were inoculated with the defined C. jejuni mutants. Eighty chicks were divided into groups, with each group consisting of ten chicks (Figaro 7j). 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 Q1349c 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, pehIA, and Cj!279c mutants demonstrated a marked impairment in their ability to colonize chicks, as only two of seven chickens inoculated with the C. jejuni cadF and Cj!279c mutants were colonized. None of the ten chicks inoculated with the C. jejuni pehIA mutant were colonized.

Cj!279c is required for efficient cell adherence and chicken coloabtation. In silico analysis of Cj!279c revealed that this gene is located within a putative operon consisting of 13 genes (website located at wwwv.iTiicrobeson1ine.org). The Cj!279c gene is situated downstream of Cj!HBOc that encodes a putative tifoosomai pseudouridme 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 Cj!278c gene downstream of Cj!279c was mutated. Adherence assays performed with chicken LMH cells demonstrated that the observed phenotype of the Cj!279c mutant was not due to a polar effect, as a difference in binding was not observed with the Cj!278c mutant relative to the wild-type strain (Figure 8). Although variations were observed from one experiment to another in the number of C. jejuni that bound to the chicken LMMT cells (Figures 6 and 8), these results appeared to be due to fluctuations in the MOI. Regardless, the C. jejuni cadF and Cj!279c mutants consistently showed reductions in cell-binding when compared to the wild-type strain in all experiments performed. Because Cj!279c has not been previously characterized, we propose that it is a novel adhesin. As indicated above, the Cj!279c mutant demonstrates a reduction in both adherence to chicken LMK cells and in the colonization of chickens. Based on these findings and the fact that
CjI 279c contains Fn type ITI domains, the CjI 279c gene is referred to as "y(pA" for hroneefin-like protein A from this point forward.

EXAMPLE 3. Campylobacter jejuni FlpA binds hroneefrhli and is required for maximal host eel! adherence.

The goal of this study was to characterize the binding properties of FlpA, and to determine if Uis protein is a member of the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) family. Experimental evidence showed that C. jejuni FipA is surface exposed, promotes the bacterium's attachment to host epithelial cells, and has Fn binding activity. Assays were also performed to determine if CadF and FlpA act cooperatively to promote binding of C. jejuni to host cells and Fn. The identification of FlpA as a second MSCRAMM in C. jejuni highlights the importance of Fn binding in host colonization and disease.

MATERIALS AND METHODS

Bacterial strains. All Campylobacter jejuni strains were cultured on Mueller Hbiton agar supplemented with 5% bovine blood (MH—blood agar) under microaerobic conditions (5% O2, 10% Xh, 85% KV) at 37°C. Strains were passed to fresh plates every 24 to 48 h. The C. jejuni F3801 strain was recovered from an individual with eanipylbactcriosis. The C. jejuni F3801 C/ lp (tetacycline-resistant, TetR) and cudF Jlp (KanR, TetS) mutants were generated as outlined below. The C. jejuni F3801 C/ lp (karsamycin-resistant, KanR) mutant was generated as outlined elsewhere (5). When appropriate, growth media were supplemented with chloramphenicol (ChL 8 µg/ml), kanamycin (Kan, 50 µg/ml), tetracycline (Tet, 2 µg/ml), or eefoperazone (Cef, 30 µg/ml). Escherichia coli XLI-Blue MRF (TetR) (Stratagene, Garden Grove, CA), E. coli BL21 (Novagen, Madison, WI), and E. coli LMG 194 (streptomycine, SmR and TetR, Invitrogen, Carlsbad, CA) were grown aerohjcaUy at 37°C on Luria-Bertani (LB) agar plates or in LB broth. When necessary, growth media were supplemented with ampicilU m (Amp, 100 µg/ml), kanamycin (50 µg/ml), tetracycline (12.3 µg/ml), or chloramphenicol (20 µg/ml).

Analysis of the FlpA operon. The C. jejuni F3801 strain was grown to mid-exponential phase in M U broth, and total cellular RNA was extracted using the KiboPurc-bacterial kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Genomic DNA was degraded by treatment with 31 units of RQI ENase-free DNase at M X ' for 30 min, cDNA was synthesized from 500 ng of RNA using random hexaer priraers and the ThermoScripti reverse-transcriptase CRT) PCR system (Invitrog S) according to the manufacturer’s directions,
As a negative control, RT-PCR reactions were performed without RT enzyme. Two separate RNA extractions and cDNA synthesis reactions were performed on different days.

Table 4 lists all primers used in this study. PCR was performed to determine which genes are co-transcribed with fliP using 1 µl of a 1:10 dilution of cDNA as template in a total volume of 25 µl. As a positive control, the reactions were performed using C. jejuni 3FS01 1 genomic DNA as a template, DNA fragments were amplified using Taq DNA polymerase (Invitrogen, Carlsbad, CA) with the following parameters: 94°C for 4 min, 1 cycle; 94°C for 45 sec, 60°C for 30 sec (-1°C per cycle), and 2 min at 70°C, 10 cycles; 94°C for 45 sec, 60°C for 30 sec, and 2 min at 70°C, 25 cycles. PCR products spanning the junctions between genes Q 128uc/clpA, Q J27sc, Q J27sc, Q J27sc, ami Q i27sc were amplified using the following primer pairs; MEK2386 and MHK2387, MBK2388 and MEK2389, MEK2412 and MEK241 1, MEK2420 and MEK242 1, and MEK2422 and MEK2423. The resulting PCR amplicons were analyzed by electrophoresis in a 1% agarose gel.

Table 4. Primers used in this study

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<th>Primer</th>
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Generation of the C. jejuni FM&lt;{eq}flpA{eq} and cadF βpA mutant. A mutation in the βpA gene of C. jejuni F3801 was generated by homologous recombination using a suicide vector harboring a disrupted copy of the flpA gene. The 5’ flanking region of the flpA gene was PCR amplified using HiFi Taq (Invitrogen) with the primers MEK1 672 and MEKI 671 containing BamHI and SstII restriction sites, and ligated into pCR2.1 (Invitrogen). The 3’ flanking region of the flpA gene was PCR amplified using primers MEK3 673 and MEK4 674 containing SstII and BamHJ restriction sites, and Ugaied into pCRII. The 3’ fragment was restricted with the SstII and BamHJ re&iright;striction enzymes, gel-purified, and ligated to the 5’ fragment in the pCR2.1 vector. The resultant vector was digested with SstII, and the tefO gene conferring Tet resistance was inserted. This vector was that digested with BamHII to liberate the fragment containing the 5’ and 3’βpA flanking fragments with the tefO gene, which was subsequently ligated into the suicide vector pBSK (Stratagene, La Jolla, CA). The pBSK vector had previously been modified to include an apkA-3 gene cassette encoding Kan resistance. This vector was electroporated into the C. jejuni F3801 wiid-type strain and C. jejuni F3801I cadF mutant, and colonies, were picked that were Tet resistant. The C. jejuni flp A mutants were confirmed by FCR ushtg flpA gene specific primers.

Complementation of the C. jejuni βpA mutant. The flp A ORF with 0 bp of upstream sequence and 15 bp of downstream sequence was PCR amplified from C. jejuni F3801 genomic DNA using WiFi Taq and the primers MEK1 681 and MEK1 883 harboring the NdeI and Kpnl restriction enzymes. The mcp promoter sequence was amplified for a C. jejuni NCTC! 168 using primers MEK1 657 and MEK1 688 harboring BamHI and NdeI restriction enzymes. The metK promoter βpA gene product was cloned into the MCS of the pRYH 1 shuttle vector using BamHI and Kpnl sites, The meIK ptoniizer-βpA in pRYH 1 was confirmed by DNA sequencing, and the resultant vector was electroporated into E. coli S17-1 λ-pir for conjugation into the C. jejuni F3801I flpA mutant. The conjugations were performed with overnight cultures of the C. jejuni F3801I flpA mutant grown in MH broth supplemented with
Kan and *E. coli* S 17-1 λ-pir harboring the pRY1 i 1 metK promoter- flp-1 construct grown in LB broth supplemented with ChL. The bacteria (the equivalent of 1 OD{\text{m}}, units) were pelleted via centrifugation at 6,000 x g for 2 min, and the supernatant was discarded. The *E. coli* S 17-1 λ-pN pejiet was re-suspended in 50 μl of Mi broth and combined with the *C. jejuni* F3801 XflpA mutant pellet. The cells were pelleted again, and the supernatant discarded. The combined pellet was spotted onto a MH-bløod agar plate and incubated at 37°C in a microaerophilic environment for 14 h. The conjugation spot was then streaked onto MH-blood agar plates supplemented with Chi and Cef, and incubated for 48 h. Isolated transformants were selected and the presence of the recombinant vector in the *C. jejuni* βpA mutant was confirmed by PCR. The complemented *HpA* mutant was designated the *C. jejuni* JlpA (flpA-1) complemented strain.

**Construction of flpA-pET24b, flpA-pBADA, and βpA-pGBT recombinant vectors.**

Recombinant histidine tagged FlpA protein was generated using the pET Expression System from Movagen. A fragment of the *flpA* gene was PCR amplified using the gene specific primers MEK.1691 and MEK.1692 harboring BaroRland XhoI restriction enzymes, and cloned into the pET24b (KaiI) vector using standard molecular biology techniques. The recombinant plasmid, φ /pET24b, was introduced into *E. coli* BL21(DE3). The His-tagged FlpA protein was purified using TALON Metal Affinity Resin (Clontech, Mountain View, CA) according to the manufacturer’s directions.

To determine if FlpA facilitates the binding of *E. coli* to epithelial cells, we expressed the *flpA* gene in *E. coli* using the pBAD-Express vector. The pBAO/Myc-His A vector (Amp^R_), referred to as the pBADA from this point forward, was obtained from Trevigen. A fragment of the *flpA* gene was PCR amplified using the gene specific primers MEK.1691 and MEK.1692 harboring Ncol and Kpal restriction enzymes and cloned into the pBADA vector using standard molecular biology techniques. The recombinant plasmid, 1279c pBADA, was introduced into *E. coli* LMG 194. Expression of the *flpA* gene in *E. coli* LMG 194 was induced by the addition of L-arabinose as outlined by the supplier.

The ability of FlpA to bind Fn was determined by BLISA using purified GST-tagged FlpA protein. The *flpA* gene was PCR amplified using gene specific primers MEK.1691 and MEK.1692 harboring the BamIII and XhoI restriction enzymes, and ligated into the pGEX-5x-1 vector using standard cloning procedures. The FlpA-GST T protein was purified using Glutathione Sepharose 4G affinity resin (GE Healthcare/Amersham) according to the manufacturer's instructions. The caIF gene fragment was cloned into the pGEX-Sx-1 vector...
using primers MEK2522 and MEK2523. The GST-tagged CadF protein was purified as described for FlpA.

**Generation of FlpA-specific serum.** Female New Zealand White rabbits were subcutaneously and intramuscularly injected with 500 μg of purified Mis-tagged FlpA protein in TierMax® Gold (CyRx Corporation, Noreross, GA). Two booster injections, each containing 50 μg of protein in Freund’s incomplete adjuvant (Sigma), were given at 4 and 6 weeks after the primary injection. Blood was collected prior to all immunizations, and 7 days after the second booster injection. The serum was prepared using standard laboratory procedures, and stored at -80°C. FlpA-specific antibody was generated in a New Zealand White rabbit using a protocol approved by the Institutional Animal Care and Use Committee (IACUC protocol #2433) at Washington State University.

**Outer membrane protein extracts.** C jejuni outer membrane proteins (OMPα) were extracted using N-iauroyl-sarcosine as described by de Melo and Peehere (2) with modifications. C jejuni were grown in MH broth under microaerobic conditions overnight, pelleted by centrifugation, and suspended in H2O, Tris, NaCl and Tween-20. The bacterial cell suspensions were sonicated with a Branson Sonifier Cell Disruptor (model 250; Branson Sonic Power Co., Danbury, CT) rive times for 30 s each with a 30 s cooling period in ice between each pulse. Cell debris was removed by two successive centriguations, each at 6,000 x g for 10 min. The crude membrane extracts were obtained by centrifugation at 500,000 x g at 4°C for 2 h. The resulting pellets were suspended in 10 mM Tris (pH 7.5), and the protein concentration of each sample was determined using the bicinchoninic acid (BCA) assay as outlined in the manufacturer’s instructions (Pierce, Rockford, IL). N-iauroyl-sarcosine (Sigma) was added to the crude extracts at a protein to detergent ratio of 1:4 (w/w). The samples were incubated at room temperature with gentle rocking for 30 min, and centrifuged at 100,000 x g at 4°C for 2 h. The pellets were washed with 50 mM Tris (pH 7.5), suspended in the same buffer, and stored at -20°C. The protein concentration of the OMP extracts was determined by BCA assay.

**SDS-PAGE and Western analysis.** Whole cell lysates (the equivalent of 0.1 OD540 units) of the C jejuni F3801 wild-type strain and mutants were solubilized in single-strength electrophoresis sample buffer and incubated at 95°C for 5 min. The proteins were separated in 12.5% polyacrylamide gels using the discontinuous system described by Laemmli (7).
Following electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (CBB R-250, Bio-Rad Laboratories, Hercules, CA). For immunoblot analysis, proteins were electrophoretically transferred to a poly(vinylidene difluoride) (PVDF) membrane from Bio-Rad Laboratories, Bedford, MA. Immoblotts were performed by incubating the membrane overnight at 4°C with a 1:500 dilution of the α-FlpA serum in phosphate buffered saline (PBS (20 mM sodium phosphate and 150 mM sodium chloride, pH 7.5) containing 0.01% Tween 20 (v/v)) with 9% non-fat dry milk. After 3 washes with PBS-Tween, a HRP-conjugated goat α-rabbit IgG (whole molecule) diluted 1:5000 in PBS-Tween was added as a secondary antibody and incubated at room temperature for 1 h. Following two washes with PBS-Tween and a final wash with PBS, indirect immunofluorescence assays were performed using Kodak BioMax MR film and Western Lightning Chemiluminescence (PerkinElmer, Boston, MA) according to manufacturer's directions.

Indirect immunofluorescence assays. The C. jejuni F3B01 wild-type strain and α-flpA mutant were harvested from MH-blood agar plates in PBS and 20 μl of the bacterial suspension was air-dried on a glass microscope slide. The air-dried samples were quickly passed over a flame and PBS added onto the surface of the slides. The bacteria were incubated for 45 min at 37°C in a humidified chamber with either a 1:20 dilution of a rabbit α-C. jejuni whole-cell polyclonal serum (PVDF) (6), rabbit α-FlpA serum, or rabbit pre-bleed serum in PBS containing 0.75% bovine serum albumin (BSA). The slides were washed 3 times with PBS and then incubated for 45 min at 37°C in a humidified chamber with a 1:100 dilution of a Cy2-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA). Following incubation, the samples were rinsed 10 times with PBS, placed on a glass slide with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA), and visualized using a Nikon Eclipse TE2000 inverted epifluorescence microscope. DAPI, a fluorescent stain that binds to DNA, was used to visualize all bacteria. Images were captured using the imaging software MetaMorph version 5 and processed using Adobe Photoshop 3.0.4.

Tissue culture. INT 407 human intestinal epithelial cells (ATCC CCL22) were maintained in minimal essential media (MEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (10% BS; HyClone Laboratories, Logan, UT) and 5% L-glutamine (8 mM). The cells were cultured at 37°C in a humidified, 5% CO2; incubator and passed every 48 to 72 h.

Bacteria-Most cell adherence assay. Each well of a 24-well tissue culture tray was seeded with INT 407 cells (1.5 x 10^5 cells/well) and incubated for 18 h at 37°C in a humidified, 5%
CQjj incubator. The cells were rinsed with the appropriate medium and inoculated with approximately \(5 \times 10^7\) CFU of the various \(C. jejuni\) strains. Bacteria-host cell contact was promoted by centrifugation at 600 x g for 5 min. To determine the viable number of bacteria that adhered to the INT 407 cells, the trays were incubated for 30 min at 37°C in a humidified, 5% CO\textsubscript{2} incubator. Following this incubation period, the epithelial cells were rinsed three times with PBS to remove non-adherent bacteria. The epithelial cells were then lysed with a solution of 0.1% (v/v) Triton X-100 in PBS. The suspensions were serially diluted and the number of viable, adherent bacteria determined by counting the resultant colonies on MH-blood agar plates. The results represent the mean counts ± standard deviations derived from triplicate wells.

To determine if antibodies against FlpA reduce the binding of \(C. jejuni\) to INT 407 cells, different dilutions of the FlpA-specific and pre-bled sera were added to bacterial suspensions containing approximately \(1 \times 10^7\) CFU. The bacterial suspensions were then incubated for 30 minutes at 37°C under microaerobic conditions (5% O\textsubscript{2}, 10% CO\textsubscript{2}, 85% N\textsubscript{2}). The binding assay was performed as outlined above.

Binding (adherence) assays were also performed with \(E. coli\) LMG 194 \(htwbtmngflpA-pBAD\) and \(E. coli\) LMG 194 harboring pBAOA without a DNA insert. For these assays, the bacteria were cultured overnight at 37°C in LB broth supplemented with Amp. The following morning, 5 µl of LB broth containing Amp was inoculated with 250 µl of the overnight culture and incubated with shaking for 90 min at 37°C. Expression of \(flpA\) was induced by the addition of L-arabinose; 0.0002% L-arabinose was added to all the cultures for 2 h. The amount of L-arabinose added to the bacterial cultures was determined based on preliminary experiments that examined relative FlpA protein levels versus time and bacterial viability. The adherence assays were performed as described above for the \(C. jejuni\) strains, with the exception that the INT 407 cells were inoculated with approximately \(2 \times 10^7\) CFU of each \(E. coli\) isolate.

ELISA. The wells of a 96-well plate were coated overnight at 4°C with either 1 µg of plasma fibronecin (Fn) (Sigma, St. Louis, MO) or BSA-coated wells which served as a negative control. The following day the wells were rinsed with wash buffer [PBS containing 0.005% Tween 20 (v/v)] and blocked with 1% BSA for 1 h at 25°C and rinsed once with wash buffer. To determine the Fn binding activity of each protein, 2-fold serial dilutions of FlpA-GST and CadF-GST proteins were made in PBS, added to the wells, and incubated for 90 min at 25°C. The CadF-GST protein was used as a positive control for Fn binding affinity. After the wells were washed 3 times with wash buffer, a 1:1000 dilution of rabbit anti-GST antibody (Sigma) in incubation buffer was added to the wells and the plate incubated at 25X for 90 min. The
wells were washed 3 times with wash buffer, a 1:5000 dilution of horseradish peroxidase labeled goat anti-GST antibody (Sigma) diluted in PBS was added to the wells and the plate incubated at 25°C for 90 rain. The wells were washed, and bound antibodies were detected by addition of TMB Substrate Solution (Thermo Scientific, Rockford, IL). Binding was quantitated by colorimetric detection at 492 nm.

*C. jejuni-Fn binding* assay. The wells of %-weli flat-bottom plates (Costar, Corning, NY) were coated with a 1 mg/ml solution of Fn in 0.05 M Tris Buffered Saline, pH 7.5 (Sigma) overnight at 4°C. For a control, wells were also coated with 1% BSA in PBS. The *C. jejuni* tm F3801 wild-type, *fipA*, *cadF*, and *cadFip* mutants were harvested from overnight plate cultures and resuspended in PBS at an OD540 of 0.150 (approximately 10⁸ CFU). Wells were rinsed with PBS, and 100 µl of the bacterial suspensions were added to each well and incubated at 37°C, 5% CO₂ for 1 h. The wells were washed three times with PBS and adherent bacteria were removed by the addition of 0.25% trypsin (Gibco. Invitrogen). To enumerate the number of adherent bacteria, serial dilutions of the trypsin suspension were plated on MH-blood agar.

**RESULTS**

**Salient features of thr βpA ORF and the protein it encodes**

Analysis of the *fip* A gene and predicted operon structure from four *C. jejuni* sequence strains (*Le.,* NCTC 11168, RMI 221, 81-176, and 811116) revealed conserved features (Figure 9). The order of the genes flanking *fipA* in the four *C. jejuni* sequenced strains are identical (*Le., C. jejuni* QJ 1280c, CjJ1279c QEpAl CjJ278c, CjJ277c, and CjJ 276c). Apart from in *silico* analysis of *fipA*, little is known regarding the operon structure in which this gene resides. To determine the number of genes in the operon in which *fipA* resides, PCR was initially performed using gene specific primers to determine the gene order in the *C. jejuni* FSS01 strain (clinical strain). The sizes of the PCR fragments were in agreement with that predicted from NCTC 11168 genome analysis, suggesting the *C. jejuni* F3801 strain likely owns the same gene order (*i.e., C. jejuni* Q1280c, Q1279c (fipA), QjJ278c, QJ 1277c, and CjJ1276c) as the four *C. jejuni* strains indicated above. RT-FCII analysis was then performed with gene specific primers to experimentally determine the number of genes in the *fip* operon. In the *C. jejuni* F3801 strain, *fipA* is the second gene in an operon consisting of *C. jejuni* Q1280c, Q1279c (fipA), QJ277c, and CjJ277c.

The βpA gene in *C. jejuni* NCTC 11168 is 1236 nucleotides. The ORF from *C. jejuni* NCTC J160S (CjJ71c) begins with two AUG codons in tandem followed by an AAA eodon [L$_{277}$ (K) residue] and is terminated by an XJAO termination codon. One discrepancy in the
annotation of h& β p A ORF from C. jejuni NCTC i 1i>8 versus other sequenced strains is that
the ORF begins with a single AIGG codon followed by an AAA codon in the ORF from C
jejuni strains RM1221, SI-1 76, and SH 16. The proposed methionine start codon in all die C
jejum sequenced strains is preceded by a typical Shine-Dalgamo sequence (AGA). The ORF
from C. jejuni NCTC i 1i>8 is 411 amino acids and is predicted to synthesize a protein with a
calculated mass of 46,124 Da (Table 2). hi aiUco analysis of the FlpA deduced amino acid
sequence further revealed that the protein shares greater than 99% identity at the amino acid
level among the four C j φ ni strains (i.e., NCTC 11168, KM 1221, 81-1 76, and 811116) (Table
2). Other than the ORF additional methionine at the amino terminus of FlpA from NCTC
11168, only eleven residues differed within the entire deduced amino acid sequence of the four
strains. The nucleotide sequence of the flpA gene in the C. jejuni F380 11 strain is identical to
that of the C. jejuni HCJC 11168 strain, except for a single silent nucleotide difference at base
ES2 (i.e., C in strain F3801 versus a T in strain 11168). Examination of the C. jejuni NCTC
11168 FlpA predicted amino acid sequence identified an L - S - A - C motif at residues 18-21,
which matches the prokaryotic lipoprotein signal consensus [LVmASTVIlGASIIC! (Figure
10). This consensus sequence, found at the C-terminal end of a lipoprotein signal peptide, is
referred to as the lipobox. The invariant OγS residue is lipid modified, and presumably
inserted into one leaflet of the lipid bilayer. The FlpA deduced amino acid sequence also harbors Fn
type III domains.

FlpA is s roembrnae-associated protein with surface exposed domains.

A C. jejuni βpA mutant was generated as outlined in Materials and Methods, and
demonstrated have similar growth rates as the C. jejuni wild-type strain (not shown). To
determine the cellular location of FlpA, whole cell lysates (WCL) and outer membrane protein
(OMP) extracts were prepared from a C. jejuni wild-type strain, flpA mutant, andΔflpA βpA°)
complemented strain and analyzed by SDS-PAGE coupled with immunoblot analysis using a
FlpA-specific serum. A band with a $M_\text{r}$ of 46 kDa was readily observed in WCL extracts of the
C. jejuni FISSt) 11 wild-type and flpA (flpA°) complemented strains but not in the isopnik jip A
knockout (Figure 10). Consistent with the notion that HpA is a membrane-associated protein,
as suggested by its anααααααaemiinal leader, a 46 kDa immunoreactive band was also observed in the
OMP extracts of the C. jejuni F38011 wild-type strain and ihejipA ijlipA° I complemented
strain.

To determine if domains of the FlpA protein are surface exposed, C. jejuni were
incubated with the HpA-spe-eic serum and indirect immunofluorescence microscopy was
performed. AH bacteria were incubated with a rabbit α-C. jejuni whale-cell serum for a positive control. After the bacteria were incubated with either the rabbit α-C jejuni whole-cell or rabbit FlpA-specific sera, they were incubated with a Cy2-conjugated goat anti-tabbit secondary antibody and examined. The rabbit α-C jejuni whole-cell serum stained both the wild-type and flpA xamimii bacteria (not shown). In contrast, the rabbit FlpA-specific serum only stained the wild-type bacteria (Figure 15). Together, these results indicate that FlpA is a membrane-associated protein with surface exposed domains.

FlpA promotes the binding of C. jejuni to ttumia epithelial cells

Previous work demonstrated that FlpA plays a role in C. jejuni colonization of broiler chickens, as only 2 of 10 chickens inoculated with the C. jejuni jlpA mutant were colonized (3). To build on this initial work, in vitro adherence assays were performed with human INT 407 cells and a C. jejuni wild-type strain, cidF mutant, jlpA mutant, arαjlpA jlpA + ) complemented strain (Figure 12). The C. jejuni αdf mutant was included in these assays as a negative control (5). At a multiplicity of infection of 30; 1, the C. jejuni JlpA mutant showed a 62% reduction in adherence to INT 407 cells when compared with the C. jejuni wild-type strain. In contrast, the rjlpA cadF mutant showed a 72% reduction in adherence to INT 407 cells when compared with the C. jejuni wild-type strain. The reduction in the binding of the C. jejuni UpA mutant was judged to be specific, as complementation of the mutant in trctm with a wild-type copy of the gene driven by the mci promoter restored the organism’s binding to the INT 407 cells. To alleviate the concern of a polar effect and to further demonstrate that the phenotype displayed by the C. jejuni jlpA mutant was due to the presence of the FlpA protein, we tested if the binding of C.jlpA mutant to INT 407 cells could be blocked with the FSpA-specific serum (Figure 13). The FlpA-specific serum reduced the binding of C. jejuni to INT 407 cells in a dose-dependent fashion, reaching a maximum value of 77% inhibition at a 1:1.25 dilution of the serum. In contrast, a statistically significant difference was not observed in the binding of C.jlpA mutant to TNT 407 cells treated with the rabbit pie-bleed serum. Together, these findings demonstrate that FlpA mediates adherence to epithelial cells.

FlpA promotes the binding of E. eott to imman epithelial cells

While a C.jlpA mutant exhibited a reduction in binding to INT 407 cells when compared with the wild-type strain, and the FlpA-specific serum blocked adherence, it remained possible that other proteins could act indirectly to potentiate the adhesive property of FlpA. To determine if FlpA is sufficient to promote the binding of bacteria to epithelial cells, adherence assays were performed with E. coli expressing FlpA. More specifically, we tested whether
binding properties of an E. coli LMG 194 strain harboring the pBADA plasmid containing fip A (E. coli pA-pBADA) and the E. coli LMG 194 strain harboring pBADA without a DNA insert. Prior to these assays, experiments were performed to determine the minimal concentration of α, αafainose and time sufficient to hζaztfipA expression. A 46 kDa band was readily visible in the whole cell lysates of the E. coli pA-pBADA strain that had been cultured in medium containing 0.0002% of L-arabkose for 2 h as judged by SDS-PAGE (not shown). A statistically significant difference was observed in binding of the E. coli pA-pBADA isolate to ENT 407 cells (1 ± 0.2 x 10^5) versus E. coli harboring an empty pBADA vector (4.13 ± 1.1 x 10^5), This finding anther demonstrates that FlpA is an adhesin.

FlpA binds to Fibronectia

Each Fn monomer has a molecular weight of 250 kDa and contains, type I, II and III repeat units. Sequence analysis of FlpA revealed the presence of at least three domains with similarity to the Fn type III (Fn III) domain (see Figure 9). The Fn DII domain mediates Fn-Fn interactions (9). Based on the presence of the Fn type I domain, HJLSAs were performed to determine whether FlpA has FII binding activity. The C. jejuni CadF protein was included in these assays as a positive control because its Fn binding activity is well documented (4, 5). As a negative control, wells were coated with BSA. In addition, we assessed the binding of GST alone. FII binding activity was evident with both the FlpA-GST and CadF-GST lagged proteins as judged by EJSA (Figure 14). The specificity of these interactions was demonstrated in that Ac binding was both dose-dependent and saturable at concentrations between 5 to 10 μg. However, under the conditions used, more CadF bound to Fn than FlpA, suggesting that the two proteins have different affinities for Fn, GST alone did not demonstrate significant Fn binding affinity; background absorbance values of 0.1 were obtained over a range of concentrations (not shown). In addition, all of the GST fusion proteins demonstrated only low-level nonspecific binding to BSA-coated wells. The reason for using the GST-recombinant proteins was to alleviate the concern of using different antibodies to detect the hound proteins. The FII binding activities of FlpA and CadF were also confirmed using FlpA and CadF-specific antibodies (not shown). Based on these results, we concluded that FlpA has Fn binding activity.

Both CMP and FlpA are required for C. jejuni in bind to lost elf s and Fn

Based on the data shown above, FlpA is a MSCRAMM family member. To determine if FlpA and CadF binding to host cell and Fn is independent of each other, C. jejuni-host coli adherence and Fn binding assays were performed with a C. jejuni wild-type strain, C. jejuni cadF mutant, C. jejuni βpA mutant, and C. jejuni auif βpA double mutant (Figure 1S). Each
of the *C. jejuni* mutants (*i.e.*, *emiF*, *flpA*, and *cadF β pA*) demonstrated a statistically significant reduction in binding to INT 40? cells and FB coated wells when compared with the wild-type strain (Figure 16). In addition, the *C. jejuni mdF* *flpA* double mutant exhibited a similar reduction in binding to INT 407 cells and Fn coated wells as compared with the individual *C. jejuni unia cadF* and *flpA* mutants. Collectively, these date indicate that *FlpA* and *CadF* are both needed to facilitate the maximal binding of *C. jejuni* to Fn and host cells.

**DISCUSSION**

*FlpA is a member of the MSCRAMM family*

Previous work indicates that *C. jejuni* adherence to gastrointestinal cells and extracellular matrix components is crucial for host colonisation and subsequent disease. Move specifically, a *C. jejuni cadF* mutant shows a significant reduction in adhesion to human INT 40? intestinal cells when compared to a wild-type strain (8). Similar to *cadF*, disruption of *Q. 1279c* *β pA*) results in a *C. jejuni* mutant impaired in its ability to bind to chicken LMH hepatocellular carcinoma epithelial cells and to efficiently colonize broiler chickens when compared with a wild-type strain (3). The product encoded by the *Q. 1279c* gene is termed *FlpA* for FibrinOnechtylike protein A, based on the fact that the protein's deduced amino acid sequence harbors Fn type II domains. Here we conclude that *FlpA* is associated with outer membrane components as judged by SDS-FAGE coupled with mummbiot analysis using *FlpA*-specific serum and is a surface exposed as judged by immunofluorescence microscopy. We also conclude that *FlpA* acts as an adhesin based on the following experimental findings: 1) The binding of the *C. jejuni* *flpA* mutant strain INT 40? epithelial cells was significantly reduced when compared with a wild-type strain; 2) Rabbit polyclonal serum generated against *FlpA*-blocked *C. jejuni* adherence to INT 40? cells in a dose-dependent manner; and 3) The expression of *β pA* in *E. coli* significantly increased the bacterium's binding to INT 407 cells when compared with *E. coli* containing an empty vector. Finally, we submit that *FlpA* is a member of the M.SCRAMM family because it binds to Fn in a dose-dependent and saturable fashion, as demonstrated by FITC-ISA. Based on the sum of *in vitro* and *in vivo* assays, we conclude *FlpA* is a novel *C. jejuni* adhesin.

*FlpA is a putative outer membrane apoprotein*

While the primary focus of this research was to demonstrate the adhesive properties of *FlpA*, multiple observations indicate that *FlpA* is associated with the *C. jejuni* outer membrane. We visualized a 46 kDa band in OMP extracts prepared from *C. jejuni* F3801 tssig a *FlpA*-specific serum. In addition, a 46 kDa band was apparent in the OMP extracts prepared from the *C. jejuni* *flpA* *β pA*) complemented strain. Noteworthy is that the BIpA protein (*i.e.*, CJSt 176-
J 295) was detected by IXT/MALDI/TOF-TOF in C. jejuni 81-176 OMP extracts previously (1, 10). We also found that FlpA is exposed on the surface of the bacterium as judged by immunofluorescence microscopy using the FlpA-specific antibodies. Consistent with the notion that the domains of FlpA are surface exposed, the FlpA-specific antibodies used for the immunofluorescence assays reduced the adherence of C. jejuni to INT 407 cells in a dose-dependent manner.

Inspection of the amino terminus of FlpA indicated the presence of lipoprotein signal consensus sequence. Although a few experimental methods are available to conclusively demonstrate that a protein is lipid modified, presumptive evidence for the identification of a lipoprotein is evident from inspection of its deduced amino acid sequence. The amino terminal signal sequence of a lipoprotein is characterised by a tripartite structure of positively charged residues at the amino terminus, a hydrophobic core region, and the lipobox with the invariant Cys residue at the carboxy terminus of the signal. The FlpA deduced amino acid sequence contains each of these key features. The presence of a prokaryotic lipoprotein signal consensus sequence strongly suggests that FlpA is a lipoprotein.

**MmM of CadF and FlpA Binding to Fibronectin**

Adherence assays were performed to determine the contribution of FlpA in the binding of C. jejuni to human INT 407 epithelial cells. A C. jejuni βpA ramuani showed a 62% reduction in adherence to INT 407 cells when compared with the C. jejuni wild-type strain. In comparison, the C. jejuni cadF mutant showed 72% reduction in adherence to INT 407 cells. Given that both proteins demonstrate binding activity, the ability of a C. jejuni cadFβpA mutant to exhibit a greater reduction in binding to INT 407 cells than either the C. jejuni JlpA mutant or C. jejuni cadF mutant was tested. It was found that the reduction in binding of the C. jejuni cadFβpA double mutant was indistinguishable from a C. jejuni cadF mutant alone. Subsequently, purified FlpA and CadF were tested for competitive binding to Fn-coated wells by ELISA. However, conditions under which the two proteins compete for Fn binding were not identified. Based on these data, it appears likely that CadF binds to one portion of Fn and FlpA binds to another portion, and that both interactions are required for intimate host cell and Vn attachment. Regardless of the specifics of these interactions, it is noteworthy that C. jejuni possess at least two Fn binding proteins (i.e., MSCRAMMs).

**Swmmwry**

In this example, experimental evidence is provided that demonstrates that FlpA promotes the attachment of C. jejuni to host epithelial cells and has Vn binding activity. The identification
and characterization of FlipA, along with CadF, highlights the potential importance of *C. jejuni* binding to Fn for host colonization and disease.

**REFERENCES** for Example 3


**EXAMPLE 4. Characterization of the FlipA Fibronectin-binding Domain**

In this study, enzyme-linked immunosorbent assays (EUSAs) were used to determine the sites of FSpA and Fn adherence, E-LISAs using recombinant proteins encoding each of the

59
three FlpA domains demonstrated FlpA–D2 contained the Fn–binding domain. Using an array of synthetic pepHdeβ spasming the FlpA–D2 amino acid sequence, seven amino acids A^PMPDPRV 164 (SEQ ID NO: 51) were identified within FlpA–D2 with maximal Fn-binding activity. Since FN3 repeat are involved in intramolecular interactions with the N-tailor of FB, the ability of FlpA top bind two thermolytic fragments generated form the N-terminus of FlpA–D2 the 30 kDa N-terminal domain (NTD) and the gelatin-binding domain (OBD) was determined. FlpA bound the N-gektio -binding domain (GBD) nor the NTTX. Furthermore, the amount of FlpA bound to the GBD and full-length Fn were similar, indicating the GBD is the primary site of FlpA adherence to Fn. Collectively, these data demonstrated residues 158^PHPDFRV K4 within FlpA–D2 mediate adherence to the GBD of Vn.

Materials and Methods.

Bacterial Strains/spkids ids. Escherichia coli XL-1 Blue (Stratagene, Garden Grove, CA) and BL21DE3 (Novagen, Madison, Wi) were maintained on Luria-Bertani (LB) agar plates or in LB broth aerobically at 37°C. Strains harboring pGEX-5X-1 (GE Healthcare) and pET-24b (Novagen) were grown on media supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin, respectively, Consta^ction and expression of the recombinant N-terminal glutathione S-transferase (GS^T )-tagged and C-termina6X-bistidine (His)-tagged proteins were performed using standard molecular biology techniques; described previously (fCortkd et al., 2010). The following primer sets were used to clone the DNA fragment encoding each recombinant peptide for expression; FlpA-His, MEKI 679 and MEK 1680; FlpA-GST (full-length), MEK 1691 and MEK 692; FlpA-D1-GST, MEK 691 and MEK 2494; FlpA-D2-GST, MEK2495 and MEK2496; FlpA-D3-GST, MEK2497 and MEK 692 (Table S1).

Table S. Primers used for expression of the recombinant proteins.

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SUBSTITUTE SHEET (RULE 26)
Protein purification. E. coli harboring the pGEX-5x-1 and pET24b expression vectors were grown aerobically in 1 L of broth cultures supplemented with appropriate antibiotics at 37°C to an OD$_{600}$ of 0.6 and induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) overnight at 22°C. Cells were harvested by centrifugation at 6,000 x g for 15 min, resuspended in ice-cold 20 mM NaCl, 150 mM NaCl, pH 7.4 buffer (PBS) and lysed by sonication in ice. Fractions were clarified by centrifugation and applied to the appropriate affinity resin for purification.

GST fusions were purified on Sepharose 4B GST affinity resin (GE Healthcare/ Amersham) according to the manufacturer’s instructions. His-tag fusions were purified on TALON® Metal-affinity Resin (Clontech, Mountain View, CA) using the native protein purification protocol in the TALON resin manual. Fractions containing the desired recombinant proteins were pooled, dialyzed in 25 mM Tris pH 7.5 or FBS and concentrated using an Amicon/Millipore concentrator (Millipore, Bedford, MA).

Peptide synthesis. All FlpA and OadF peptides were synthesized using standard N-9-fluoretyl methoxycarbonyl chemistry on an Applied Biosystems 431A Peptide Synthesizer using instruction supplied by the manufacturer (Applied Biosystems, Foster City, CA) by the School of Molecular Biosciences Laboratory for Bioanalysis and Biotechnology at Washington State University (Pullman, WA).

EIISA with GSTfimm proteins. Human plasma iibréneitin (Tn), and the 30-kDa and 40-kDa proteolytic fragments from human Fn were purchased from Sigma (St. Louis, MO.) Costar 96 well polystyretio plates (Corning, NY) were coated with 40 nM of Fn or Vn fragments (Sigma) in 20 mM NaPi, 150 mM NaCl, pH 7.4 (PBS) overnight at 4°C. Plates were washed once with FBS, 0.01% pH 7.4 (PBS) and then blocked with PBS, 1% BSA (fraction V, Sigma). While the plates incubated with block solution, serial dilutions of the FlpA-GST, FlpA-D3-GST, FlpA-I2-GST, and FlpA-D3-GST were made in PBS to produce concentrations that ranged from 1000 nM to 7.815 nM. After washing the wells with PBST, the GST fusion protein samples were added in triplicate and incubated for 2 h with shaking. Wells were washed three times with PBST and GST antibody (1:1000 in PBS, Sigma) was added for 2 h. Wells were washed again a horseradish peroxidase antibody specific to rabbit IgG (α-R-HRP, Sigma) was added at a 1:5000 dilution in PBS for 1.5 h. The wells were rinsed and developed using the TMB substrate kit (Thermo Scientific, T/L) according to the manufacturer’s instructions. Binding was quantitated spectrophotometry by measuring the absorbance at 450 rats (A450 nm). All samples were assayed in triplicate and the experiments were conducted at room temperature unless otherwise indicated. Each ELISA experiment was performed in triplicate on separate days with fresh reagents to ensure reproducibility. Absorbance measurements recorded from
wells coated with F but not the GST fusion proteins were subtracted the sample absorbances to control for nonspecific binding by the primary and secondary antibodies. Statistical significance was determined using Student’s t-test.

**Fn-binding EUSA.** To investigate the binding of Fn to FlpA, 96 well plates were coated with 250 µM solutions of FlpA-GST, FlpA-D1-GST, FlpA-D2-GST, and FlpA-D3-GST in PBS overnight at 4°C. For coating plates with the FlpA or CadF synthetic peptides a concentration of 2.5 µM was used. Plates were washed with FBST, 0.1% BSA (FBST-BSA) and blocked with 20 µg/ml PBS 1% BSA for 1 h. Serial dilutions of Fn were made in PBS containing 0.02 %, BSA such that the concentra lions ranged from 20 µg/ml to 9.8 ng/ml. Plates were washed with PBS-BSA and the FM solutions were added and incubated: for 2 h with shaking. Plates were washed extensively with Fn antibody (Sigma) was added at a 1:1000 dilution in PBS 0.02% BSA for 1 h. After another wash step, u-R-IP was added and the ELISA was developed as previously.

**Results:**

**FlpA Domain 2 contains the Fn-binding domain.**

FlpA binds to human fibronectin (Fn) and mediates adherence of *C. jejuni* to Fn coated surfaces and epithelial cells (Konkel et al/ 20H1). Bioinformatics analysis of the FlpA amino acid sequences indicates that FlpA contains three domains that resemble Fn type 3 CFN3) repeat’s: FlpA-D1, FlpA-D2, and FlpA-D3 (Konke 20iO). To determine which of the three FlpA FKJ-like domain harbors the Fa-binding domain we cloned each FlpA domain into the pGKX expression vector and produced three GST fusion proteins: FlpA-D1-GST (aa35-132), FlpA-D2-GST (aa135-226), and FlpA-D3-GST (aa232-410) (see Figure 17). The GST tag .ervfd two purposes: 1) purification of the GST fusion proteins, and 2) detection of the four GST fusion proteins with a single antibody. Serial dilutions of FlpA-D1-GST, FlpA-D2-GST, and FlpA-D3-GST fnajon. proteins were incubated in wells coated with Fn (Figure 1A). The relative amounts of GST fnajon proteins bound were determined by measuring the absorbance at 450 nm described in the materials and methods. Full-length FlpA-GST (aa35-410) protein was used as a positive control. Of the three FlpA D1 domain fusion proteins, only FlpA-D2-GST (aa135-226.) bound to the Fn-coated wells in similar amounts to the full-length FlpA-GST protein (aa35-410). Binding of the FlpA-D2-GST and FlpA-GST proteins was dose-dependent and saturable. The amount of FlpA-D1-GST and FlpA-D3-GST bound to Fn-coated wells was significantly less than the FlpA-GST and FlpA-D2-GST proteins. These data indicated FlpA-D2 contains the FlpA Fn-binding domain.

To confirm that FlpA-D1 contained the Fn-hfriding domain, a second ELISA was performed in which wells were coated with FlpA-GST, FlpA-D1-GST, FlpA-D2-GST, and
FlpA-D3-GST  Serial dilutions OfFn were added, and the amount of Fn bound was recorded (Figure ISB). Fn bound to wells coated with FlpA-OST and FlpA-D2-GST significantly greater than bound to wells coated with FlpA-D1-GST and FlpA-D3-GST. Again, the interaction between FB and FlpA-D2-GST was dose-dependent and saturable demonstrating specificity. Binding of Fn to wells coated with FlpA-D1-GST and FlpA-D3-GST was minimal. Collectively, these data demonstrate that the FlpA Fn-binding domain resides within FlpA-D2 (aa135-226).

Fip A amino acids N1 SQ-Ff79 have maximal Fn-hitidittg activity.

In addition to FlpA, C. jejuni has another Frs-biitdr-protein termed CadF. In a previous study, the CadF Fn-broeding domain was localized to four amino acids FRi-S (CadF aal34-137) using a series of synthetic peptides (Kaurkel et al., 2005). Identification of the amino acids required for FlpA binding to Fn was conducted with a similar approach. Five 30aaer peptides, with 15 amino acid overlaps spanning the FlpA-D2 sequence were synthesized: P1 R135-VI 64, P2 N J50-Fi 79, P3 D165-1194, P4 K180-I209, and P5 D195-V224 (Figure 17). Similar with the previous ELISA, microliter plates were coated with each of the five FlpA-D2 peptides and dilutions of Fn were added to the wells. The amount of Fn bound by each peptide was determined spectrophotometrically (Figure 19). Two defined CadF peptides, one with the FRLS domain (FRLT, aal25-14O) and one without the FRLS domain (FRLS', aal 18-1X3), were used as positive and negative controls respectively. Fn bound to the P1 and P2 peptides in significantly greater amounts than the other three FlpA-D2 peptides and the CadF FRLS' peptide. As with the futi-length FlpA and FlpA-D2 proteins, Fn adherence to wells coated with P1 (RI35-VI 64) and P2 (NI 50-Fi 79) was dose-dependent and saturable. These data revealed that the amino terminus of FlpA-D2 (RI 35-FI 79) harbored the FlpA Fn-binding domain.

Replicate ELISA experiments with the FlpA peptides revealed that Fl1 (RI 35-VI 64) and F2 (N 150-F 179) consistently had comparable affinity for Fn. This observation suggested two possibilities for the spatial distribution of binding site (amino acids) within FlpA-02 involved in Fn-binding: 1) the FlpA-D2 Fn-binding site is located in the overlapping region of P1 and P2, corresponding to N150-VI 64, or 2) P1 and P2 each have unique residues responsible for Fn binding, indicating that the amino acid sequence of the Fu-binding domain is extended beyond the overlapping region and possibly non-continuous. To evaluate the first of these possibilities additional FlpA-D2 peptides were synthesized and ELISAs were performed.

Amino acids P 1594-1 64 are required for maximal Fn-binding.

Fn FN3 repeats are composed of seven β-strands arranged in two anti-paralite! β-sheets connected by flexible loops (Dickinson et al., 1994). The secondary structure FlpA-D2 is predicted to contain regions of β-sirands that alternate with non-fkstrand regions (Figure 17).
The overlapping region of P1 and P2 (N150-V164) corresponds to a portion of FlpA-D2 predicted to contain a non-β-strand region at the N-terminal beginning at N147, which is adjacent in the C-terminal direction to a β-strand, and followed by a second non-β-strand region ending at S166 (Figure 17). Based on the secondary structure prediction, we synthesized a sixth peptide (P6) composed of N147-S166. Fn binding by wells coated with P6 was compared to wells coated with the P1 and P2 peptides, and wells coated with P5 as a negative control (Figure 20). FlpA bound to P5 in comparable amounts as P1 and P2, which demonstrated that N147-S166 harbored residues critical for maximal Fn-binding.

Data from the initial EUSA using P1 to P5 (Figure 19) demonstrated that Fn does not bind P3 (DJ 654194), which indicates amino acids required for FlpA Fn-binding do not extend much beyond the O-terminus of P5 (V164). To determine the N-terminal boundary of the FlpA binding domain a seventh peptide was synthesized. Again, secondary structure predictions were used to select the sequence of the seventh peptide. Sehware- U’nek et al. (Schwarz-Unek et al., 2003) demonstrated that tandem β-strands within Sββ of Streptococcus pyogenes interact with the triple stranded β-Sheets of the N-terminal FNL repeats of Fn using synthetic peptides. Therefore, the seventh peptide, P7 (Fl 4J-Ri 57), was designed to span two predicted β-strands and one non-β-strand region (Figure 17). However, Fn binding to wells coated with P7 minimal, similar to the amount of Fn bound negative control peptides FSpA F5 and Cadp FRLS- (data not shown), and significantly less than Fn binding to P1 and P2. These data indicate the N-terminal boundary of free FlpA Fn-binding domain does not extend significantly beyond P158, and that FlpA amino acids 155-S-V164 comprise the core of the FlpA Fn binding domain. High-resolution structural studies are currently underway to further characterize the precise details of the interaction between FlpA and Fn.

FlpA binds like gelatin'coHagen interaction domain of Fn.

Amino acid sequences alignments of FlpA-D2 with FN3 using ClustalW revealed that FlpA-D2 mostly closely aligned with FN3 with 22.9% sequence identity (Figure 2). FN3 binds the to FNL and FNL repeats located toward the M-iermnus Fn 2Mao and Schwarzbauer, 2005. Digestion of Fn with thermolysin produces defined fragments of Fn that retain their physiological activity (Figure 16) (Pankov and Yamada, 2002). One of the Fn thermolysin fragments produced, termed the N-terminal domain (NTL), is ~30 kDa in size and composed of FN1-5. Another fragment of 40 kDa contains the gelatin/collagen interaction domain (G8D) composed of FN7-9 and FN2-1 (Figure 16). To determine if FlpA bound the Fn NTD ELISA plates were coated with full-length Fn, the 30 kDa NTD fragment and the 40 kDa 13BD Fn fragment. Interestingly, both full-length FlpA and FlpA-D1 bound the 40 kDa GBD fragment in
amounts similar to full-length Fn, whereas binding wells coated with 30 kDa NTD and ovalbumin was minimal (Figure 22). As with previous assays, the FlpA-DI-GST and the RpA-D3-GST did not bind Fn or the FΩ GBD. These data demonstrate that FlpA-D2 binds a site on Fn within the GBD.

**Identification of the FlpA Fn-binding site.**

This study was conducted to further characterize the interaction between FlpA and Fn, FlpA is composed of three domains (DI, D2, and D3) that resemble FN3 repeats from Fn (Figures 1> at id 20) (Konkei et al., 2010). To determine which domain(s) bound to Fn we expressed each FlpA domain separately as a recombinant protein fused to a GST tag. A series of ELISAs were conducted to determine which of the three FlpA domains bound to Fn. FlpA-D2 was the only FlpA domain to demonstrate significant Fn binding, whereas binding of FlpA-D1 and FlpA-D3 was minimal. Furthermore, the amounts of Fn bound by FlpA-D2 and FlpA full-length were similar - suggesting the major Fn-binding site resides within FlpA-D2.

Previous studies with CadF employed a panel of synthetic peptides in ELISA experiments to localize the CadF Fn-binding domain to 114-FRLS 137 (Konkei et al., 2005). We used a similar method to determine the residues within FlpA-D2 bound Fn. Five 30mer peptides, with 15 amino acid overlaps, spanning the FlpA-D2 amino acid sequence were synthesized and assessed for Fn binding activity (Figure 17). FlpA peptides P1 (R135-164) and P2 (N150-Ft 79) bound Fn in amounts comparable to the positive control peptide (CadF FRLS). The amounts of Fn bound FSpA P1 and P2 were indistinguishable, thus we tested if the Fn-binding domain consisted of amino acids shared between P1 and P2, corresponding to N ISO-Y 164. FlpA secondary structure is predicted to contain β-strands that alternate with non-β-strand regions.

These characteristics of the FlpA secondary structure are consistent with the structure of the FN3 repeats in Fn, which are comprised of several β-strands arranged into two anti-parallel β-sheets (Dickinson et al., 1994, Mao and Schwarzbauer, 2005). The sequence shared by P1 and P2 (NI 50-VS 64) is predicted to contain a single β-strand region sandwiched between two less ordered (non-β-strand) regions. Previous work with other Fn-binding MSCRAMMs found disordered regions mediate adherence to Fn (Sehwarz-Lniek et al., 2004). Therefore, P6 was designed to span FlpA N147-Sl 66, which covered the β-strand sandwiched and two less ordered sequences, Fn bound to FSpA P6 in amounts comparable to P1 and P2. In addition, we found that Fn binding was minimal to peptides composed of residues in directions N-terminal to P158 and C-terminal to V164. This observation indicated that the core of the FlpA Fn-binding domain was composed of 158-PHPDFRV k+.
FlpA binds the GBD of Fn.

Fsi is a mosaic protein composed of FN1, FN2, and PNS repeats. The N-terminus region of Fn is composed of FN1 and FN2 repeats, whereas the C-terminus is composed predominantly of FN3 repeats and a few FKI repeats (Figure 16) (Pankov and Yaraada, 2002). Plasma FN is soluble and maintains a globular structure that is stabilized by interactions between N-terminal FN1 repeats and C-terminal FN3 repeats. For example, FN3 binds an N-terminal domain of Fn composed of FN1. The interactions between FN1 and FN3 repeats are also thought to prevent recognition of epitopes on FN3 domain by cell surface receptors (Mao and Schwabauer, 2005, Pankov and Yamada, 2002). For instance, in plasma Fn access to the RGD sequence is limited. This prevents sPvV integrin dependent signaling involved in Fn assembly into the ECM and cytoskeletal rearrangements (Mao and Schwabauer, 2005, Piersehbacher and Rnoslahti, 1984).

Since FlpA contains putative FN3 domains, we tested to see if FlpA bound the N-terminus of Fn. Digestion of Fn with thermolysin produces well-characterized Fn fragments that maintain their biological activity. Two fragments that comprise the N-terminus of Fn are produced: a ~30 kDa fragment termed the N-ferroininal domain (NTD) that is composed of FN1; and a ~40 kDa fragment termed gielisn bindhig domain (GBD) that is composed of FN1 and FN2 (figure 16) (Pankov and Yamada, 2002). In ELJSAs FlpA bound to the GBD at levels comparable to full-length Fn, whereas FlpA binding to the NTD was minimal. ITC experiments were conducted to determine the affinity of the FlpA-Fn interaction.

The impact of FlpA on C. jejuni pathogenesis.

Previous work in our lab established that FSpA is required for C. jejuni adherence to host tissues (Flanagan et al., 2009, Konkel et al., 2010). Host cell adherence is a prerequisite for C. jejuni invasion, and invasion is associated with the development of acute disease (Babakhani et al., 1993, Konke! et al., 2001). CadF-mediated adherence of C. jejuni to Fn in required for maximal invasion efficiency. The reduction in invasiveness by C. jejuni cadF mutant is beyond what can be explained by reduced adherence alone (Montevi! et al., 2003). This result may be explained by the observation that C. jejuni inva&ion of epithelial cells coincides with the phosphorylation of paxillin and the activation of the Ral and Cdc42 (Krause-Gruszczynska et al., 2007, Montevi! et al., 2003). Paxillin, Ral and Cdc42 are proteins associated wish host cell focal complexes (FCs). FCs are composed of integrin receptors, adaptor proteins, and signaling proteins. In response to Fn-integrin engagement, FCs assemble on the cytoplasmic tails of the integrin receptors (Gil, 2007, Small and Kaverina, 2003). Paxillin is one of the first proteins recruited during FC assembly, where it is
phosphorylated by F0-associated kinases FAK and Src. FC-mediated signaling can activate the Rho GTPases. Racl and Cdc42, through several downstream effectors. Activated Rael and Cdc42 control the formation of actin-based membrane protrusion termed lamellipodia and filopodia, respectively (Broussard et al., 2005, Ridley, 2006, Small and Kaverina, 2003). The transient phosphorylation of paxillin and the activation of the Racl and Cdc42 observed during *C. jejuni* infection is dependent CadF adherence to Fn. In addition, *C. jejuni* localized to actin protrusions prior to internalization (Krause-Gruszczyńska et al., 2007, Monte-Vule et al., 2003). These observations support a model of *C. jejuni* invasion in which bacterial adherence to Fn stimulates host eel! signaling for cytoskeletal rearrangements required for bacterial internalization.

FlpA binding the Fn-CiBD has several potential impacts on cell FC-dependent model of *C. jejuni* internalization. FlpA adherence to the Fn-GBD may disrupt intramolecular interactions between the N-terminus and the C-terminus ofFn to expose Fn domains involved in Fn fibril assembly, activation of cell surface receptors, or CadF adherence. Assembly of plasma Fn into the ECM has been studied extensively using FN null (−/−) mouse embryonic fibroblasts. Assembly of plasma Fn into the ECM was found to be a cell-dependent process that is initiated at specialized cell-surface sites characterised by active integrin receptors. The display of cell-surface assembly sites is stimulated by cell adherence to O-terminal FN3 repeats (i.e., FN310 ROD). Activated integrin receptors associated with the cell-surface assembly sites recognize and bind the N-terminal Fn1 and Fn2 repeats ofFn, which results in conformational changes in the structure of plasma Fn and incorporation into the ECM (Mao and Schwarzbauer, 2005, Xu et al., 2009). FCs fib半 at the sites of fibril assembly composed of αβ integrin receptor, FAK, vinculin, and paxHlio, and attach the newly formed fibrils to the actin cytoskeleton (Mao and Schwarzbauer, 2005). Therefore, if FlpA adherence to Fn promotes Fn fibril assembly, host eel! FC components would be brought proximal to sites of *C. jejuni* adherence and participate in bacterial internalization. Alternatively, FlpA may induce conformational changes in Fn to promote CadF adherence to Fn and promote stimulation of FCs-dependent invasion processes. Experiments are currently being conducted to determine if FlpA changes the affinity of CadF for Fn in solution.

**FlpA is unique among Fn-binding AiSCK AζMs.**

The rod well-characterised Fn-binding MSCRAMMs belong to a group of proteins produced by *Streptococcus pyogenes* (SfbD, *Staphylococcus aureus* (FnBPA), and *Borrelia Burgdorferi* fBBK.32) collectively referred to as FnBPs (for Fn-binding proteins) (Schwarz-Liptack et al., 2004). Analyses of the FnBPs have identified conserved Fn-binding domains.
FKBPS harbor C-termin tandem repeats that bind the KTD of Fn by a tandem β-sdpper mechanism. In this mechanism structurally disordered Fn-binding repeats of FnBPs form short anti-parallel β-strands, which interact with the triple stranded β-sheets of sequential FNi modules, resulting in high affinity binding to the NTD of Fn (Schwann Linek et al., 2003). Taïay et al. (2000) assessed the role of the tandem repeats and a spacer in SM (Talay et al., 2000). The C-terminal tandem repeats of SfbJ were sufficient to confer bacteria adherence, whereas invasion was dependent on adherence of the spacer domain that bound the GBD of Fn. Interestingly, adherence of the SiW repeats to the Fn NTD was required for binding of the SiM spacer domain (Ozeri et al., 1996, Taïay et al., 2000).

FipA is a putative lipoprotein composed of three FN3-Hke domains. The primary sites of interaction between FipA and Fn reside within FipA-D2 and the GBD of FB. The PH?DFRV sequence resides within a region of FipA-D2 that is predicted to be less ordered and adjacent to β-smmds. This putatively disordered structure of the FipA FN-binding domain is consistent with the disordered structure of the Fn-binding repeats from SfbJ, which may suggest that like SfbJ the FipA-C domain of FipA undergoes a disordered to ordered conversion upon binding to Fn (Schwarz-Linek et al., 2004). However, FipA-D2 does not appear to harbor the C-terroinai repeat domains characteristic of FnBPs. The tandem repeats of FnBPs that bind the Fn NTD, and upstream spacer domains of FnBPs, that bind the Fn GBD, are organized over a span of ~60 amino acids (Schwarz-Linek et al., 2004). The FipA-Fn binding domain identified in this study consists of a relatively short peptide (^PHPOFNV^) that does not share sequence identity with any of the FipB Fnt-binding domains. It is difficult to define the eXac1 residues, involved in FipA adherence without detailed structural information, but the results of this study indicate that we have identified critical residues that comprise the core of FipA Fn-binding site.

In summary, FipA is a novel MSCRAMM composed of three FN3 repeats that binds a site within the GBD of Fn. Studies are currently underway to: 1) characterize the structure of FipA, 2) determine the affinities of interactions between FipA, CadF and Fn, and 3) assess the impact of FipA-Fn interactions on CadF adherence to Fn.

REFERENCES for Example 4


EXAMPLE 5. Effect of Lactobacillus on colonization of j in commercial broiler chickens

In this example, the effect of four Lactobacillus strains (L. acidophilus NCFM, Lactobacillus crtcatus JCM 5810, Lactobacillus gallinarum ATCC 33199 and Lactobacillus henricus CNRZ32) on colonization of C. jejuni in commercial broiler chickens was evaluated. Potential mechanisms responsible for competitive exclusion, including production of antagonistic metabolites, modulation of antibody responses and manipulation of the cecal microbiota were also evaluated.

MATERIALS AND METHODS

Bacterial .stratus attic growth conditions. The bacterial strains used in this study are listed in Table 6. C jejuni strains were cultured under microaerobic (85% nitrogen, 10% CO2, 5% oxygen) conditions in Mueller-Binto (MH) (Difco, Detroit, MI) broth at 37°C on MH agar plates supplemented with 5% bovine blood (MHB agar plates) at 37°C. Cultures were subcultured to a plate every 24 to 48 h. Motility of C. jejuni culture was determined prior to inoculation in ehkkens. Lactobacillus strains were propagated statically at 37°C in deMan, Rogosa and Sharpe (MRS) broth (Difco) or on MRS agar plates under microaerobic conditions.

Table 6. Bacterial Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Releyat CharacterisUcs</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C jejuni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F380S 1</td>
<td>Human clinical isolate</td>
<td></td>
</tr>
<tr>
<td>81-176</td>
<td>Human clinical isolate</td>
<td></td>
</tr>
<tr>
<td>S1116</td>
<td>Human clinical isolate</td>
<td></td>
</tr>
<tr>
<td>RM1221</td>
<td>Poultry isolate</td>
<td></td>
</tr>
<tr>
<td>S2B</td>
<td>Poultry isolate</td>
<td></td>
</tr>
</tbody>
</table>
Turkey

<table>
<thead>
<tr>
<th>Escherichia coli TOPIOF</th>
<th>Laboratory strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus NC-FIVt</td>
<td>Human isolate</td>
</tr>
<tr>
<td>l. crispatus JCM 5810</td>
<td>Chicken isolate</td>
</tr>
<tr>
<td>L. gallinarum ATCC 33199</td>
<td>Chicken isolate, Neotype Strain</td>
</tr>
<tr>
<td>L. helveticus CNRZ32-Str</td>
<td>Dairy starter strain</td>
</tr>
<tr>
<td>L. acidophilus NCFM -Str</td>
<td>Stř 200 µg/ml</td>
</tr>
<tr>
<td>L. asipatus JCM SSKKStr</td>
<td>Stř 200 µg/ml</td>
</tr>
<tr>
<td>L. gallinarum ATCC 33199-Stt</td>
<td>Stř 200 µg/ml</td>
</tr>
</tbody>
</table>

1 Japan Collection of Microorganisms
2 American Type Culture Collection
3 Centre National de Recherche Zootechnique

**Growth curve analysis.** *Lactobacillus* strains were inoculated into from overnight cultures into MRS broth at %%. Growth was monitored by during a BioscreenC analyzer (Growth Curves USA, Inc., Piscataway, NJ). Maximum growth rate (μₘ) was determined by fitting the growth curves to a modified Gompertz model (Zwietering, M, et al. (1990) Appl Envkofi Microbiol 56; 1875-1881) using Prism 5.0 (Graphpad Software, Inc., La Jolla, CA).

**C. jejuni inhibition assays.** Inhibition of *C. jejuni* cultures by kctobarilli was evaluated using spotted cultures and supernatants. For spotted cultures, overnight cultures of lactobacillus were spotted onto Brain Heart Muskm Agar (Difco) supplemented at 0.1% with Tween 80 (Fisher Scientific, Hampton, NH) (BRI-T) and incubated overnight under microaerobic conditions. Subsequently, plates were overlaid with molten Mil soft agar (0.75% agar) inoculated at % with overnight *C. jejuni* cultures standardized to O.D.540 in MH broth. Plates were incubated for 24 h at 37°C under aermaerobic conditions. Inhibition was evaluated by measuring the zone of inhibition around the *Lactobacillus* cultures and expressed as the ratio of the zone of inhibition to the zone of growth in mm. In order to determine if baciocins were contributed to inhibition, plates were treated with proteinase K (20 µg/µl) (mvironogen, Carisbad, CA) or trypsin (Sigma-Aldrich, St. Louis, MO) (10 µg/µl) prior to being overlaid with *C. jejuni*. In order to determine if peroxides contributed to inhibition plates were treated with catalase (10 µg/µl) (Sigma) prior to being overlaid.

Supernatant from *Lactobacillus* cultures were boiled for 6 min, neutralized to pH 7 with 6N NaOH (Fisher), treated with catalase, or left untreated. Supernatants were
subsequently filter sterilized (0.22 µM) and spotted onto solidified MH soft agar inoculated at 1% with overnight C. jejuni culture and incubated overnight.

Selection of antibiotic resistant Lactobacillus strains. To generate antibiotic-resistant Lactobacillus strains for use in further assays, serial transfers of 0.01% were performed in MKS broth containing increasing concentrations of streptomycin (Sigma). Cultures resistant to 200 µg/µl streptomycin were selected for further assays.

Broiler chickens. A total of 200 newly hatched chicks were subdivided into ten groups of 20 chicks; the chicks were then placed into isolation chambers (Horsfall-Batier isolators) on wire mesh. Water and a commercial chick starter feed were provided ad libitum. Fecal matter was collected and autoclaved before disposal. All animal studies were performed using protocols approved by the Institutional Animal Care and Use Committee (IA(XJC: protocol no. 3248) at Washington State University.

Bacterial cultures and eiskkea Inoculation. Lactobacillus cultures were grown statically in MRS at 37°C for 18 h. C. jejuni MTI 8301 were cultured in MH broth at 37°C for 18 h prior to inoculation. One group of 20 chickens was kept as the unmodified control group. The remaining 9 groups of chickens were inoculated as follows: group 2 and 6, L. acidophilus NCFM-Str; group 3 and 5, L. acidophilus JCM580-Str group 4 and 8, L. galiiwarum ATCC 33199-Str; groups 5 & 9, L. heiferkm CNRZ32-Str; and group 10, C. crescenticus. Group 10 was administered C. euscenum, a non-probiotic bacterium, as a treatment control to demonstrate any observed reduction in C. jejuni colonization was due to the probiotic lactobacilli specifically (i.e., positive control for C. jejuni colonization, referred to from this point forward as the C. jejuni control). Lactobacilli or caulobacter were administered on Days 1 and 4 post-hatching by oral gavage with 0.3 ml bacterial suspension 10^8 CFU/ml. At 14 days post hatching, Groups 5-10 were administered C. jejuni F3801 1 by oral gavage with 0.5 ml bacterial suspension 10^8 CFU. After each inoculation, remaining bacterial suspensions was serially diluted onto appropriate media to confirm the number of CFU in each dose.

Bacterial enumeration. Half of the chickens in each group were euthanized and necropsied at day 21 and the remaining chickens on day 28 of the study. A cecum and intestine were dissected from each chicken. The samples were weighed, diluted in an equal volume of MFI broth for enumeration of Lactobacillus and C. jejuni, respectively. The MRS dilutions were plated onto Rogosa SL (Difco) agar plates supplemented at 200 µg/ml with streptomycin for enumeration of lactobaciH while MH dilutions were plated on Campy Cefex (Difco) agar plates for
enumeration. Plates were incubated microaerobically at 37°C and CFU counted after 96 h of incubation. To confirm the identity of recovered Lactobacillus, PCR was performed on cultures of colonies isolated from plates used for enumeration of lactobacilli using surface layer protein specific primers (Table 1).

Construction of 16S rRNA done libraries. Total DNA was isolated from cecal contents using the UltraClean Fecal DNA kit (MoBio Laboratories, Inc., Carlsbad, CA). 16S rRNA genes were amplified with PCR Super Mix High Fidelity (TaqPrep) previously described using three sets of primers: 8F and 1492R (Set A), 8F and 1522R (Set B), and 8F and 926R (Set C) (Table 5) (Lu, J. et al. (2003), Appl Environ Microbiol 69:681-6-24). PCR products were pooled and purified using the QiaQuick PCR cleanup kit (Qiagen, Valencia, CA). Purified products were ligated to pCR2.1 (Invitrogen) and transformed into chemically competent Escherichia coli UTOPIOP. Clones were screened for u-complementation of p-galactosidase by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) with IPTG (isopropyl-β-D-thiogalactoside) (Ausubel, F. M. et al. (2007), Current Protocols in Molecular Biology: hum Wiley and Sons, Inc., New York, NY).

Table 7. Primers Used in Tiis Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>AGAGTTTGATCTGGCTCAG</td>
<td>16S rRNA Gene</td>
</tr>
<tr>
<td>926R</td>
<td>ACCCTTTGTCGCGCrCCC</td>
<td>16S rRNA Gene</td>
</tr>
<tr>
<td>149211</td>
<td>TACGGYTACCTFGTTACGAaT</td>
<td>16S rRNA Gene</td>
</tr>
<tr>
<td>1522R</td>
<td>A AGGGTATCCACCCAGC A</td>
<td>16S rRNA Gene</td>
</tr>
<tr>
<td>UVX</td>
<td>GCAAGATTTTTTGAAGTATTGTGC</td>
<td>L. acidophilus sipA</td>
</tr>
<tr>
<td>MEK</td>
<td>TGAACACTCACGATAAGG</td>
<td>L. acidophilus sipA</td>
</tr>
<tr>
<td>MEK</td>
<td>CAACATAATTCTCTTCTCTCTCTCTTG</td>
<td>L. casei tcm ts chsA</td>
</tr>
<tr>
<td>MEK</td>
<td>TTAAATACTGCTAGAGT AAC'AGC</td>
<td>L. casei tcm ts chsA</td>
</tr>
<tr>
<td>MBK</td>
<td>CCGTTACCCTGTGTIFSCTAATGG</td>
<td>lgsB galilariitin</td>
</tr>
<tr>
<td>MEK</td>
<td>GTATTGGATCTKJTAATTACTATCC</td>
<td>L. helveticus sipA</td>
</tr>
<tr>
<td>MEK</td>
<td>TGTGCTGCAA^VGTACTTAGAGG</td>
<td>L. helveticus sipA</td>
</tr>
</tbody>
</table>

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SUBSTITUTE SHEET (RULE 26)
DNA sequencing and analysis. Sequencing of constructed libraries was performed at Functional BioSciences, Inc. (Madison, WI) using M13P(-20) and M13R(-27) primers. The resulting sequences were processed and aligned using the RiboSoma'i Database Project (RDP) pipeline tool (website located at rdp.cme.msu.edu) (Cole, J. R. et al. (2009), Nucleic Acids Res 37:D541-5). Aligned sequences were taxonomically classified using the RDP Classifier (Wang, Q. et al., 2007, Appl Environ Microbiol 73:5261-7). Sequences were assigned to operational taxonomic units (OTUs) at 1% sequence dissimilarity using DOTUR (Schloss, P. D., and J. Haudelsman, 2005, Appl Environ Microbiol 71:1501-6) on the RapidOTU server (website located at genepjot3y.imn.fr/rapidotu). DOTUR was also used to generate the Shannon-Weaver (H) and Simpson (D) diversity indices for the eight libraries. Evenness (E) was calculated as described previously CKrebs, C. J. (1989), Ecological Methodology. Harper & Row, Publishers, Lac, New York, NY). Libraries were compared using KDP Library Compare (Cole, J. R. et al., 2009, Nucleic Acids Res 37:0141-5).

Detection of anti-C jejuni antibodies in cfeick sera, ELISA plates were coaled plates with 10 µl of 2 µg/ml C. jejuni F3505 1 whole cell lysate diluted in PBS. After incubating plates overnight at 4°C, the well were washed twice with PBST wash buffer (PBS with 0.05% Tween 20) and blocked with 150 µl of PBS, 0.05% Tween 20, and 0.25% gelatin (PBST-G) at 25°C for 2 h. The plates were washed three times. The chick sera were diluted 1:200 in PBST-G and 100 µl of each serum sample was added in triplicate. After incubation for 2 hours at 25°C, the wells were washed three times and 100 µl of anti-chicken IgG antibody horseradish peroxidase conjugate diluted 15000 in PBST-G was added for 2 h at 25°C. WeSIs were washed three times with PBS and 50 µl of tetramethylbenzidine (TMB) substrate (Pierce-Endogen) was added to the wells. The reaction was stopped with 0.18 N H2SO4 after 10 min of development. Absorbances at 490 nm (A490) within wells were determined at 492 nm with an EL_i_x808 Ultra Microplate Reader (BioTek instruments. Inc., Winooski VT).

RESULTS
Lactobacilli InMtIt C jejuni growth in vitro. Growth curves were performed in order to characterise the ability of Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus gallimim, and Lactobacillus Helvetians to grow on MKS (Figure 22). The µw were determined as follows: L acidophilus, 0.281 ± 0.002 per h; L crispatus, 0.30S ± 0.003 per hi L.
gallinarum, 0.275 ± 0.002 per h; and L helveicus, 0.265 ± 0.002 per h; and were not.

The ability of lactobacilli to inhibit growth of C. jejuni in vitro was evaluated. Spotted cultures of all Lactobacillus strains tested appeared to inhibit C. jejuni F3S01 while their ability to inhibit growth of other C. jejuni strains was variable. Overnight cultures of Lactobacillus were spotted onto BHI-T and allowed to grow O/N at 37°C. Overnight cultures of C jejuni were standardized to CXD. sT)), 100) and inoculated at 1% into 10 ml MH soft agar, overlaid, and incubated 24 h at 37°C. Supernatant was; from overnight cultures of Lactobacillus were left untreated, neutralized with 6.25 N NaOH, or boiled for 6 min. Supernatants from overnight cultures of Lactobacillus were left untreated or caiaise treated for 1 h. Ali supernatant were filter sterilized (0.22 μm) and spotted onto 20 ml MH soft agar inoculated at 1% with C jejuni. Lactobacillus strains used are indicated as follows: (A) L. acidophilus, (B) L. crispatus, (C) L. gallinarum and (D) L. helveicus. Zone of Inhibition (G) and Zone of Growth (G) as used in Table 8 are indicated. L acidophilus and L. crispatus were able to effectively inhibit growth of all C. jejuni strains tested while L. gallinarum and L. helveicus were only able to effectively inhibit C. jejuni F3S01. Additionally, C. jejuni F3S01 appeared to be the most susceptible strain to inhibition by lactobacilli in vitro.

Table 8. Inhibition of C. jejuni by lactobacilli

<table>
<thead>
<tr>
<th>LNB Strains</th>
<th>C. jejuni Strains</th>
<th>P3801 I</th>
<th>S1-176</th>
<th>81116</th>
<th>RMI 221</th>
<th>S2B</th>
<th>Turkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>L acidophilus</td>
<td>2.6 ± 0.33</td>
<td>1.6 ±0.1</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>L crispatus</td>
<td>4.1 ± 0.8</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L gallinarum</td>
<td>2.4 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L helveicus</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as Ratio of Zone of Inhibition (mm) / Zone of Growth (mm)

In order to determine whether bacteriocins produced by Lactobacilli were involved in inhibit itio of C. jejuni, plates spotted with Lactobacillus cultures were treated with trypsin and proteinase K prior to being overlaid with C. jejuni. Neither protease treatment reduced inhibition of C. jejuni (not shown), suggesting that inhibition was not due to the production of a proteinaceous component and, thus, not due to the production of bacteriocins. Additionally, beat-treatment of supernatants did not effect inhibition, confirming that bacteriocins produced by lactobacilli were not responsible for inhibition of C. jejuni. To determine if production of
organic acid or hydrogen peroxide contribute to the inhibitory ability of these lactobacilli, supernatants of Lactobacillus cultures were neutralized to pH 7 with NaOM or treated with catalase, respectively. Neutralization with NaOH abolished inhibition. Treatment of supernatants with catalase also reduced inhibition of C jejuni, suggesting that peroxides produced by lactobacilli contribute to inhibition. These data suggest the ability of Lactobacillus cultures to inhibit growth of C jejuni m in vivo is due, at least in part, to the production of organic acids and hydrogen peroxide.

**Lactobacilli reduce C jejuni coliform infection of chickens.** Lactobacilli were administered to commercial broiler chicks as potential competitive exclusion organisms on day-of-hatch and 4 days post-hatching. In order to evaluate the effectiveness of these lactobacilli the chickens were challenged with C jejuni F3801 I 14 days post-hatching. Half the chickens were euthanized and necropsied at 7 days post-inoculation with C jejuni. The remaining chickens were euthanized and necropsied at day 14 days post-inoculation. C jejuni present in the cecum of each chicken were enumerated (Figure 23). The experiment illustrated in Figure 23 was performed as follows. Broiler chicks were administered Lactobacillus by oral gavage (10^8 CFU/ml) at days 1 and 4 post-hatching. Chicks receiving C jejuni challenge were administered C jejuni F3801 I by oral gavage (10^6 CFU/ml) at day 14 post-hatching. Half of the chickens were euthanized and necropsied at day (A) 2! post-hatching and the remaining chickens were day (B) 2$ post-hatching. A cecum was dissected from each chicken, weighed, diluted in an equal volume of MH broth, and thoroughly homogenized. Sample were serially diluted and plated onto Campy Cefex agar for enumeration. C jejuni was not detected in the ceca of on-challenged birds indicating containment procedures were effective. In challenged birds receiving L acidophilus, C jejuni was detected in 7 of 9 birds at both 7 and 14 days post challenge. Colonization of chickens administered L gallinarum and L helveticum by C jejuni appeared to decline from 7 to 14 days post-challenge. Colonization of birds receiving L gallinarum and L helveticum decreased from 9 of 9 to 5 of 9 and 7 of 9 to 3 of 9, respectively. Chickens receiving L crispatus had a low rate of colonization by C jejuni at 4 of 10 birds at both 7 and 14 days post-challenge.

Colonization of chickens by administered Lactobacillus strains was also determined (Table 3). Chicken receiving I helveticus displayed the highest rate of colonization by lactobacilli (94.4%), followed by birds receiving L crispatus (90%), L acidophilus (72.5%), and L gaUttiarum (51.7%). In chickens that were administered L acidophilus, L crispatus and L.
kelveticus, lactobacilli were recovered in more birds when challenged with C jejuni than when not.

PCR using strains specific primers (Table 5) was used to ascertain whether recovered presumptive lactobacilli were of the same strain administered to the chickens (data not shown). 10 isolates from each group were selected for this analysis. Of all the groups tested, only those groups administered L cr? atus JCM5810 were positive for the administered species. Additionally, several presumptive Lactobacillus isolates from other groups were also positively identified as L. erispans, as shown in Table 9.

Table 9. Cecal Colonization by lactobacilli

<table>
<thead>
<tr>
<th>JLAB Treatment</th>
<th>L. mimi w?hha</th>
<th>L. ctispa/us</th>
<th>L. g?lUnanun</th>
<th>j. Lli?vetjcm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>72.5%</td>
<td>90.0%</td>
<td>51.7%</td>
<td>94.4%</td>
</tr>
<tr>
<td>c. jejuni Treatment</td>
<td>78.3%</td>
<td>95.0%</td>
<td>36.7%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

* Shown as percentage of cecal specimens in which lactobacilli were detected

16S rDNA microbiome analysis. Specimens receiving various treatments were selected for cecal microbiome analysis by 16S rDNA sequencing (Table 10). The 16S rDNA clones were classified using the RDF Clas?ifer. Of the 74? 16S rDNA clones. 644 (86%) were classified as Firmicutes, 64 (13%) were classified as Bacteroidetes, 5 (1%) were unclassified, and a single clone was classified as a Proteobacteria. The Firmicutes were the dominant phylum with the Clostridia being the major class across all the specimens accounting for 64% of the total clones in the libraries. While, clones classified as Lactobacillus were identified in specimens from groups receiving L. cr? paws, L. gallinamn, and L. hehaicus, Lactobacillus was only appreciably identified from the specimens receiving L cr? atus. The single Proteobacteria clone was identified as belonging to the genus Salmonella and found in the specimen receiving L g?lnarum and C jejuni challenge. No clones were classified as belonging to the genus Campylobacter. Grain-positive flora were dominant across all specimens and regardless of treatment with Lactobacillus. While there were some shifts of flora from Bacteroidetes to Firmicutes, the normal flora, aside from the increased number of Lactobacillus clones identified, predominate regardless of treatment and the dominant flora was not disrupted by the microbial treatments.
### Table 10. Features of Specimens Selected for Cecal Microbiome Analysis

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Treatment</th>
<th><strong>Bacterial Counts</strong></th>
<th><strong>Lactobacillus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Campylobacter</td>
<td>Cecum</td>
</tr>
<tr>
<td>6</td>
<td>Uninoculated ed</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td><em>L. crispatus</em></td>
<td>ND</td>
<td>NB</td>
</tr>
<tr>
<td>31</td>
<td><em>L. gallinarum</em></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>41</td>
<td><em>L. helveticus</em></td>
<td>ND</td>
<td>KD</td>
</tr>
<tr>
<td>68</td>
<td><em>L. crispatus</em> + C jejuni</td>
<td>2.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>78</td>
<td><em>L. helveticus</em> + C jejuni</td>
<td>2.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>89</td>
<td>+ C jejuni</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C. jejuni</td>
<td>2.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>Counts shown as CFU/gm of cecal or ileal contents
<sup>b</sup>Positive control for *C. jejuni* colostrum receiving only *C. jejuni*
<sup>c</sup>-ND - not detected, limit of detection is 1 × 10<sup>3</sup> CFU/gm

Determination of serum antibodies. Levels of anti-*C. jejuni* antibodies in sera at 21 and 28 days post-hatch were determined by ELISA (Figure 24). Anti-*C. jejuni* antibodies were detected in the sera of challenged birds at 14 days post-challenge but not at 7 days post-challenge, while anti-*C. jejuni* antibodies were not detected in the sera of naïve chickens. In challenged birds receiving *L. crispatus*, anti-*C. jejuni* antibodies were detected in the fewest birds. This is consistent with their level of colonization. Additionally, the strain of *Lactobacillus* administered did not appear to affect levels of anti-*C. jejuni* antibodies. Natural antibodies to *Clostridium perfringens* present in the chicken sera were also determined (Figure 25). Antibodies were not detected in significant quantities and did not appear to be affected but the *Lactobacillus* strain administered.

**EXAMPLE 6:** Use of a probiotic *Lactobacillus* strain as an oral vaccine to reduce Campylobacter jejuni in chickens

Mainpi Inacio of the gene (slpA) encoding the S-layer protein of *Lactobacillus* bacterium.

The data below relates to a cloning experiment used to test whether epitopes can be inserted into surface exposed sites of the S-layer protein. The *slpA* gene from *L. helveticus* CNR232 was amplified by PCR using gene specific primers and cloned into the pCR 2.1 vector using the Original TA Cloning Kit (hwilrogen). The promoter and start codon were not
included to prevent expression of the slpA gene in E. coi, which is toxic (Avall-Jaaskelainen, S. et al (2002) Appl. Environ. Microbiol. 68:5943-5951). Using inverse PCR, the entire pCR 2.1 vector piasrmd containing the slpA gene fragment was amplified using primers whose 5’ end each contained one half of the 36 base pair sequence for the Fn-BD from CadF. Blunt-ended ligation of the PCR product resulted in the desired slpA modified construct, with the CadF epitope inserted within the shuttle vector pSA3. This vector has a temperature-sensitive origin of replication, which aDows replication in LAB at 37°C but not 44°C (Chrlsteisen, J. E., and J. I. Steele (2003); J. BacterioL 185:297-3306: Dao, M. L., and J. s. Ferreni (1985); Appl. Environ. Microbiol. 49:131-19), Following eleiroporation. L. h扡etricus isolates containing the recombinant pSA3 vector were recovered at 37T by the pour-plate method using MRS supplemented with 500 ng/ml erythromycin (ERY) and 0.75% agar. The transformants were suspended in MRS broth, plated on MRSfEKYSO plates, and incubated at 44°C. Because pSA3 is unable to replicate at 44°C, the only colonies that were recovered contained the pSA3 plasmid with the modified slpA gene incorporated in the chromosome. To promote excision of the plasmid, the single-crossover isolates were subcultured daily in MRS broth, for 20 days, at 37°C. The broth cultures were then plated on MRS agar plates containing 10 ng/ml HRY. This concentration of antibiotic is inhibitory but not lethal, causing isolates that were cured of pSA3 to appear as pinpoint colonies, whereas the isolates harboring the pSA3 vector were 2-3 cm in diameter. Isolates containing the modified slpA with the CadF Fn-BD were confirmed by PCR amplification and sequencing of the modified slpA gene.

CoSectio<ge>rat<em> of serum containing C jejuni protective antibodies. The immune response of two groups of birds is analysed. The first group represents serum samples collected from breeder chickens located at two farms, and the second group represents chickens that have been immunized with Campylobacter extracts or whole bacteria. This first experiment has three groups of chickens; 1) non-immunized, non C.jejuni challenged; 2) non-immunized, C. jejuni challenged; and 3) C. jejuni whole cell lysate (we!) immunized by subcutaneous (SubQ) injection, C. jejuni challenged. The we! was mixed with an adjuvant obtained from a commercial poultry producer (the adjuvant was obtained with the understanding that we would not share the company’s name or formulation/composition of the adjuvant with others). The chickens were immunized (primary immunization) at 5 days post-hatch, and again at 12 days post-hatch (boost). At 19 days of age, one half of the chickens (n = 10) within group L group 2, and group 3 are euthanized and blood collected to assess the antibody responses, whereas the
remaining chickens (n = 10) jα groups 2 and 3 are challenged via oral gavage with *Uf* *ctiu* of *C. jejuni*. At 26 days of age, the chickens were euthanized and the number of *C. jejuni* in the intestinal and cecal contents is determined and compared with chickens that did not receive prior immunization. Immunisation of the chickens results in overall reductions in *C. jejuni* colonization of chickens.

A second experiment is performed to compare and contrast two different immunization strategies. Groups 1 and 2 are immunized by SubQ injection with a *C. jejuni* wcl as above. However, as represented by groups 3 and 4, a total of 40 chickens are immunized by oral administration of formalin-fixed as outlined by Rice et al. (1997) Vaccine. 15: 1922-1932 and Black et al. (1987) Infect. Immun. 55:1 116-1 1201. Groups 5 and 6 serve as non-immunized controls, where only group 6 is being challenged with *C. jejuni* in it. The load of *C. jejuni* in groups 2 and 4 is less than that of the chickens in group 6 (non-immunized, *C. jejuni* were challenged). Serum is obtained that contains antibodies protective against *C. jejuni*.

**Determination of** *C. jejuni* **proteins against which antibodies are generated**. Sera obtained from chicks immunized with *C. jejuni* were (SubQ) and formalin-fixed *C. jejuni* (oral administration), as well as non-vaccinated breeder chickens, are analyzed using the following protocol. First, *C. jejuni* were and outer membrane protein (omp) preparations are separate by SDS-PAGE and then transfected to polyvinylidene fluoride membranes. The membranes are rinsed and incubated with chicken sera diluted in PBS (pH 7.4) containing 0.01% Tween 20 and 9% dried milk. Bound immunoglobulin are detected with either α- and γ-chicken IgG horseradish peroxidase conjugates. 4-μmol-1-naphthol is used as the cinnamoylgluc substrate. An example of the expected result is shown in Figure 26. The identity of the *C. jejuni* outer membrane proteins against which the chickens generated protective antibodies is identified by coupling SDS-PAGE, immunoblot, and nano-LC/MS/MS tandem mass analysis.

**Selection of epitopes for incorporation into the Lactobacillus S-layer protein.** Two methods are used to select the region of a protein (α- or γ- residues) that will be incorporated into the S-layer of *Lactobacillus*. One method relies on determining the specificity for reactivity of the antibodies generated in the chickens and the second method utilizes a molecular biology approach to determine the conserved regions of a protein. Ideally, the residues incorporated into the S-layer are highly immunogenic and conserved in nature.

**Immunoblot and enzyme-linked immunosorbent** assays (ELISAs). The region within a protein that the antibodies bind is identified via immunoblot analysis of protein fragments. The
protein fragments are generated by expression of various gene segments in an expression system (Le., pET24b, His-tagged) or via enzymatic digestion. The identity of the digested fragment can also be determined using LC/MS/MS/ion trap analysis. Fine-mapping of a protein's immunoreactive regions is performed by ELISA. As an example, the procedure with the FlaA filament protein is outlined here. FlaA derived synthetic peptides, oligomers (30-mers) are ordered that span the desired region of the protein. Each successive oligomer will overlap the previous oligomer by 30-residue$. The oligomers are used to coat the wells of the microliter plates. As a positive control, wells are coated with the His-tagged recombinant protein. After blocking with FBS/0.5% BSA (wt/vol), chicken sera is added in two-fold serial dilutions (1:50 to 1:6400) and incubated for 90 min at 25°C. The wells are rinsed three times with IX PSS-Tween and incubated for 90 min with an α-ehiekes IgG/hgM-HRP antibody. The plates are developed using laboratory-established protocols. This method will define the region(s) within a protein to which the antibodies ate generated.

Molecular Approach. Pour C. jejuni genomic sequences are currently available. Moreover, the NCBI databases contain numerous entries for any given C. jejuni protein. For the proteins of interest, sequences are aligned to identify regions with greater than 95% identity at the amino acid level An example of the type of data generated is shown in Figure 27. In preferred embodiments, epitopes (residues) that are highly immunogenic and conserved in nature are incorporated into the S-layer.

Creation of a strain of Lactobacillus that synthesizes a recombinant S-hem protein containing epitopes from C. jejuni antigens, An exemplary cloning strategy using L. keiicm as a model is described below. A difference between the three Lactobaciillius species is that the S-layer protein is synthesized by genes unique to the individual species (Avalijaaskeiainen, S., and A. Palva (2005) FEMS Microbiol, Rev. 29:55 1-529; Boot, H. I et al (1996), Microbiology, 142 (Tt 9):2375-2384). As L. acidophilus has two genes that encode the S-layer, it is necessary to modify both or generate a knockout in one of the two genes. However, the approach is customized depending on the species and strain of Lactobacillus chosen. Figure 28 shows a hydropathy profile of the L. helvetiats SlpA protein. The lower (more negative) hydropathy values correlate with hydrophobic regions of the proteins that are likely to be solvent-exposed, while higher (more positive) hydropathy values indicate more hydrophobic regions that are less likely to be solvent exposed. The four putative solvent-exposed insertion sites are located at residues 104, 259, 333, and 370. Insertion of the CadF 12-mer at site I, residue 104, would increase the hydropathy value, but the value would remain less

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Thus, based on the results of the membrane protein hydrophobicity prediction tool Membrane Protein Explorer (http://blaHC0.bicatmol.uic.edu/mpred/), we have selected four putative epitope insertion sites in the _L. heheticus_ SlpA S-layer protein. Each of these sites is located immediately downstream of a hydrophobicity peak, as these regions are the most likely to be solvent-exposed. The insertion sites were not placed directly at the peak of hydropathility, as this could result in the region being more hydrophobic. The four insertion sites are tested both independently and in combination to determine the most effective sites for surface display of epitopes.

To demonstrate the feasibility of this approach, a 36 base pair DNA fragment encoding the Fn-binding domain (Fn-BD) from residues 130-541 of CadF = AGVKFLRSLSLA (SEQ ID NO: 166), encoded by GCTGGTGTATG σiTrCG Π TATCAGACACITGCT, SEQ ID NO: 167) was inserted into target site 1 located in the slpA gene of _L. heh-eticus_ CNRZ32 (Figure 28, and Figure 29). A similar protocol is used to clone other C. _jejuni_ epitopes into the S-layer protein. Where an epitope spans larger regions (i.e., 30 residues, 90 nucleotides), restriction sites are inserted at the desired location in the S-layer gene. The epitopes are PCR amplified with gene specific primers containing restriction sites incorporated into the 5’ end. digested with the appropriate enzyme, and then Hgaied into the S-layer gene. Ali epitopes inserted into the slpA gene are in-frame with the coding sequence and eodon-bias optimized for efficient expression in _Lactobacillus_.

**Analysis of the surface-layer in the modified Lactobacillus strain.** The CadF epitope is individually inserted into each of the four insertion sites, and the recombinant S-layer is examined for surface display of the epitope and proper conformation. The other _C. jejuni_ epitopes are inserted into the S-layer gene after it is confirmed that an insertion at the four sites will not result in an altered S-layer conformation.

**Examination of the S-layer protein for C. jejuni epitope synthesis.** ELISAs are used to confirm incorporation of a _C. jejuni_ epitope (i.e., the CadF Fn-BD) in the S-layer. Both whole-cell IAB and purified S-layer protein (wild-type and recombinant) are tested. The S-layer protein is extracted from a culture of _Lactobacillus_ bacteria using 6M LiCl as described previously (Johnson-Henry, K. C, et al. (2007) Cell. Microbiol. 9:356-367). The ELISA is performed in microliter wells coated with suspensions of the wild-type and recombinant _Lactobacillus_ bacteria and purified S-layer protein. In the case of the S-layer-CadF Fn-BD, the plates are incubated with α-CadF antibody at 25°C for 90 min. Bound α-CadF antibody is detected as described previously.
Determining the level of recombinant S-layer synthesis. To determine the level of S-layer synthesis in the modified *Lactobacillus* strain, S-layer extracts from both wild-type and recombinant strains are purified and subjected to SDS-PAGE- Brief!-) cultures of the *Lactobacillus* wild-type and recombinant strains are grown m equivalent densities, and the S-Sayer extracted (Johnson-Henry, K. 0. et al. (200?) CeH. Microbiol 9:356-367). Equal volumes of the extracted proteins are subjected to protein quantitation assays (i.e., bicinehoram acid assay) and SDS-PAGE. The protein bands are visualized by staining with Oornassie Brilliant Blue R-250. The amount of S-layer protein in each sample is determined using densitometry scans over a limited range of proteins concentration. Both the modified *Lactobacillus* bacteria and purified, recombinant S-layer protein will also be examined by microscopy to determine if introduction of the epitopes has resulted in morphological or conformational changes to the bacteria or protein, respectively. Scanning electron microscopy of purified S-layer extracts are performed as outlined by Johnson-Heary et al. to determine if the S-layer pracsin will auto-aggregate into characteristic sheets and helices. Transwissien electron microscopy analysts of the *Lactobacillus* bacteria is performed as described in Avail-Jaaskieainen et al. ((2002) Appl. Environ. Microbiol 68:5943-595 1). The bacteria are placed on a Formvar coated grid, stained with 1% phosphovungsric acid (PTA, pH 7.0), and examined for morphological irregularities in the S-layer protein.

Assessment of *C. jejuni* epitope surface display. Surface display of the *C. jejuni* epitopes in the S-layer protein is determined by imrøimo-Oouresenee (W) microscopy. If we do not possess antibodies reactive against a specific *C. jejuni* protein, they are generated by immunisation of rabbits with a protein purified from *C. jejuni* or from *E. coli* harboring a recombinant plasmid. IF microscopy is performed with viable bacteria incubated first with a *C. jejuni*-specific antibody and then with α-rabbit IgG conjugated to fluorescein isothiocyanate. The bacteria are visualized using a UV microscope.

Determination of the efficacy of *Lactobacillus* vaccine strain in preventing and reducing *C. jejuni* colonization and whether an immune response is generated against *C. jejuni* epitopes. The goal is to bridge the protective response provided from the maternal antibodies with thai of the newly produced *C. jejuni* specific-IgA antibodies generated in response to the modified *Lactobacillus* strain. Chickens are inoculated with a strain of *Lactobacillus* thai displays three *C. jejuni* epitopes, including portions of the CadF, FlaA, and FlpA proteins. As illustrated in Figure 30, chickens are inoculated with the modified *Lactobacillus* strain at day 5, which will allow the level of maternal antibodies to begin to decline. Blood is collected, and
the sera screened for \textit{a-Campyphhacter} IgG and IgM antibodies. IgA antibodies will also be extracted. Briefly, an extraction solution (PBS containing 0.05% Tyviers 20, 0.1 mg/ml soybean trypsin inhibitor, 0.05 mg/ml BDTA, and 0.35 mg/ml phenylmethylsuiftmyl fluoride) is mixed with an intestinal lavage and shaken for 2 hours at $4^\circ$C. Following centrifugation (20,000 x g) at $4^\circ$C for 30 min, the supernatant is harvested and stored at $-20^\circ$C. Detection of \textit{a-Campyhhaaer} antibodies is performed by EOSA and immunobiot analysis.

ELISAs are performed with the \textit{C jejuni} proteins that contain the antigenic epitopes (purified via a Mis-tag), as well as a \textit{C. jejuni} we! The chicken sera is serially diluted added to microtiter wells coated with we! and purified proteins, and incubated at 25$^\circ$C for 90 min to allow the antibodies to bind to the \textit{C. jejuni} proteins. After rinsing the wells twice with PBS, $\phi$-chick E IgG, IgM, and IgA antibodies conjugated to horseradish peroxidase are added to the wells and incubated at 25$^\circ$C for 90 min. Bound antibodies are detected as described previously. Immunoblot analysis is used to confirm the results of the ELISAs. The same we! used in the ELISA, as well as the purified \textit{C jejuni} proteins, is probed with chicken sera. The proteins are separated by SDS-12.5% PAGE, and transferred to PVDF membranes. The membranes are washed 3 times in PBS, and incubated with each serum diluted 1:100 in PBS pH 7.4 containing 0.01% Tween 20 with 20% fetal bovine serum. Bound antibodies are detected using \textit{a-chicken antibodies OlgG, IgM, and IgA} conjugated to HRP.

\textbf{Comparison of the number of Campylobacter colonizing chickens inoculated with a Lactobacillus vaccine strain versus non-vaccinated chickens.} As part of the experiment outlined in Figure 30, the number of \textit{Campylobacter} colonizing chickens inoculated with a \textit{Lactobacillus} vaccine strain versus non-vaccinated chickens is compared. One week after inoculation with the modified \textit{Lactobacillus} strain, the chickens are challenged with low $10^3$ cfu) and high (MF cfti) doses of \textit{C jejuni}. One week after the (I jejuni challenge, the chickens are euthanized. The \textit{C jejuni} in the digestive tracts of the birds is enumerated, and compared with one another. The recombinant (modified) strain of \textit{Lactobacillus} colonizes the digestive tract of chickens at densities comparable to that of a \textit{Lactobacillus} wild-type strain. The modified \textit{Lactobacillus} strain is recovered at $10^7$ CFU/g ia ileal contents and at $\sim 10^5$ CFU/g in cecal contents (Wise, M. G., and G. R. Siragus (2007) J. Appi. Microbiol. 102: 1138-1149). If the modified \textit{Lactobacillus} sixain is unable to persist in the chickens, it will most likely be because antibodies have been generated against the \textit{C jejuni} epitopes. For this to occur, antibodies reactive against the organism will have been produced, and these are effective in reducing the amount of \textit{Campylobacter} colonizing the ceca of chickens. Low and high C

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jejuni challenge doses are used because the dose that chickens are exposed to in the natural environment is not known; also, many vaccines will fail if the challenge dose is increased beyond the capacity of the immune system to respond effectively. Although not outlined herein, additional experiments are performed to determine if the number of bacteria (inoculum) and day of delivery will affect the efficiency of LAB colonization. Ultimately, the efficacy of the modified Lactobacillus strain in reducing the microbial load of Campylobacter organisms in chickens is tested with a minimum often genetically distinct C. jejuni strains. All experiments are performed in a stepwise fashion where each experiment is designed and carried out based on the resJ-iH of a previous experiment. Should C. jejuni neutralizing antibodies not be generated during a one-week time frame (Figure 30), the experimental design is modified. First, the modified LAB is administered earlier (i.e., day 2, 3, and 4 post-hatch). Although this strategy may result in the clearance of a majority of the modified LAB from the ileum, some bacteria are likely to survive and the number of bacteria in the digestive tract will increase as the level of maternal antibodies decreases. Second, the chickens are challenged with C. jejuni at a later date (i.e., day 14, 16, 18, and 21 post-hatch). Either modification will effectively extend the time between when the chickens receive the modified Lactobacillus strain and the C. jejuni challenge. The goal is to generate an antibody response against C. jejuni prior to 3 weeks of age, by which time most chickens become colonized.

EXAMPLE 7: Competitive exclusion of Campylobacter jejuni eionkation of chickens with recombinant Caulobacter crescentis expressing C. jejuni antigens

The purpose of this experiment was to modify the surface layer of Caulobacter crescentis by adding i) a CadF fibronectin binding domain, ii) a PorA conserved epitope, and iii) a conserved HageiHn domain, Caulobacter (whole bacterial cells) were administered on day of hatch and 4 days post-hatching by oral gavage with 0.5 ml bacteria! Suspension (-10^9 CFU). At 14 days post hatching, the chicks were administered C. jejuni P38011 by oral gavage with 0.5 ml bacterial suspension (-10^9 CFU), Half of the chickens in each group were euthanized and neeropsied at Day 21 f1 week post-inoculation) and the remaining chickens on Day 28 (2 weeks post-inoculation) of the study.

MATERIALS AND METHODS

Bacteria! strains, growth conditions, plasmids, and reagents.

Escherichia coli strain DHS alpha (Invitrogen, Carlsbad, CA) was grown at 37°C in Luria Broth (% tryptone, 0.5% NaCl, 0.5% yeast extract) The C. crescentHn strain JS 4022 (Nomeilini 1. F. et, at 200?S-layer mediated display of the αG-binding domain of Streptococcal Protein G on the surface of Caulobacter crescentis- Development of an immuno-
active reagent. Appi. And Emir. Microb. 73:3245-3253) was propagated in liquid peptone-
yeast extract (0.2% peptone, 0.1% yeast extract, 0.01% CaCl₂, 0.02% MgSO₄), at 30°C. For
growth on solid medium, agar was 3% (wt/Vol). Where necessary, media contained
chloramphenicol (CM) at 20 g/ml (E. coli) or 2 g/ml (C. crescentus). Bectmprotion of f.
cremcentus was performed as previously described (Gilchrist, A., and J. SmH. 1991.
Transformation of freshwater and marine eaulobacters by eктопротопariations, J. Bacteriol.
17^:921—925.). Fragments were recovered from agarose gels using a QIAEX ii gel extraction
kit (QIAGen). The piastnid DNA was isolated using a QIAprep τπininprei piasmid isolation kit
(QiAGen), and DNA segments to construct the 30 peptide epitopes were constructed by
GEMEART AG (Regerburg, Germany).

The epitopes were:

\[ \text{CadF} \ \\text{(HYG AGVKI}LSDSLALRLETR QIN}^{\text{RNI-TAN}) (SEQ \text{ ID NO: } 156); \]

\[ \text{FiaA2 (INAVKDTGVEASIDANGOLVLTS ADGRCI}1) (SEQ \text{ ID NO: } 4); \text{ and} \]

\[ \text{PorA (YGAAAVGSYDiACGQFPNPQWtAyW DQVAF) (SEQ \text{ ID NO: } 153).} \]

The top strand DNA segments are

\[ \text{CadF: } 5^\prime \ AGATCTACTAGTCACrACGCGCGXGGCCTCAAGGTCCGCTG } \tau \text{ CGGACTC}
\]

\[ \tau \text{uctkiccn } XGXjCCTGGAGACCCCGCGAG AGATCAAC } \pi \text{ CAACr ACGCC AACGCTA}
\]

\[ \text{GCGCTGCAO } T (SEQ \text{ ID NO: } 154); \]

\[ \text{Fia}^\lambda 2:5^\prime \ AGATCTACTAGTA TCAACGCCTGTAAGAC ACCACCGCGTGCAGGCGTC
\]

\[ \text{GATCGAOKTC AACGKX } \chi \text{AGCTGCTGTXrGACGTCGGCCGACGCGCCGGGTA} TXGCT
\]

\[ \text{ACICGCCTGCAO } T (SEQ \text{ ID NO: } 155); \]

\[ \text{PorA: } 5^\prime \ AGATCTACTAGTTATGGCGCCCGCXGCCGCTGGCT } \alpha \text{ TA}XrACCTGGCCGG
\]

\[ \text{CCKiCCAGTTX ACCCCGCAGC } ^\tau \text{Gi } XjXCTGGCCCTACTG(\alpha GACCAGGirGCX'T
\]

\[ \text{TCGCTAGCGCTGCAG } r (SEQ \text{ ID NO: } 156). \]

The segments were engineered with BglI and Spe! sites on the 5' end and NheI and PstI
sites on the 3' end. The restriction sites arrangement allowed the segments to be directionally
cloned into p4ARsaA(223)/GRCC with BglI/PstI at Jindividuai epitopes or could e multireadered as desired (NomelHni J. F. et al 2007S-kyer mediated display of the IgG-binding
domain of Streptococal Protein G on the surface of Caulobacter ren?)Sremus-
Pevelopment of an immuno-active reagent. AppL And Envir. Microb, 73:3245-3253. AEM
reft. Moltimerizaion led to the p4ARsaA(723)/CadF/FkA2/PorA clone.
ELISA

EIISA plates were coated plates with 1 µg of the CadF peptide, amino acids 128-142 diluted in PBS (i.e. 100 µl of 10 µg/ml solution). After incubating plates overnight at 4°C, the well were washed twice with PBST wash buffer (PBS, 0.05% Tween 20) and blocked with 150 µl of PBS, 0.05% tween 20, and 0.25% gelatin (FBST-G) at 25°C for 2 hours. The plates were washed three times. The chick sera were diluted 1:50 and 1:200 in PBST-G and 100 µl of each serum sample was added in triplicate. After incubation for 2 hours at 25°C, the wells were washed three times and 100 µl of anti-chicken IgG antibody horseradish peroxidase conjugate (Sigma) diluted 1:5000 in PBST-G was added for 2 hours at 25°C. Wells were washed three times with PBS and 50 µl of tetramethybenzidine (TMB) substrate (Pierce-Endogel) was added to the wells. The reaction was stopped with 0.18 N H2SO4 after 10 min of development. The absorbances (A1/2) within wells were determined at 492 nm with an ELx808 Ultra Miroplia Reader (BioTek Instruments, Inc., Whiooski, VT). Figures 33S and 37 summarize results from this experiment. Only 4 of the 10 birds that received the C. crescentus CadF/FlaA/PorA strain were colonized, whereas 9 of the 10 birds that received the C. crescentus wild-type strain were colonized. Due to the fact that only 4 of the 10 birds that received the vaccine strain were colonized, the median colony forming units for the C. crescentus CadF/FlaA/PorA strain summarized in Figure 31 is below a detectable level. ELISA assays performed show that the Caulobacter crescentus CadF/FlaA/PorA vaccine strain stimulates a specific antibody response against a CadF peptide (Figure 32). The data of Figure 32 is presented in tabular form in Table 11.

Table 11. Competitive exclusion of C. jejuni colonization of chickens with recombinant Caulobacter crescentus

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<tr>
<td>Week 1</td>
<td>RsA</td>
<td>0.047</td>
<td>0.013</td>
<td>0.035</td>
<td>0.163</td>
<td>0.0022</td>
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In summary, this experiment shows that the S-layer of Caulobacter crescentus can be genetically modified to mediate multiple C. jejuni epitopes. In doing so, a Cmtiohacier
crecentus vaccine strain has been generated that reduces the load of _C. jejuni_ in the eeea of broilers.

EXAMPLE 8. Comparative analysis of FlaA sequences from several _C. jejuni_ strains

The sequences of the FlaA protein from several _C. jejuni_ strains were compared and a consensus sequence was developed (see Figures 33A-C). As can be seen, certain domains of regions within the protein are more highly conserved. A highly conserved region was selected as a vaccine antigen in order to provide protection against the greatest number of _C. jejuni_ strains. These residues are predicted to be surface exposed and accessible to protective antibodies. Based on these results, a highly conserved 30 amino acid peptide (residues 27S-307 of FlaA: INAVKDTTGEASIDANGQLVLTSGRGI (SEQ ID NO: 4) was identified. This 30mer represents a candidate antigenic sequence for use in the present invention.

* * *

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.
What is claimed is:

1. A method for preventing or treating *Campylobacter Jejuni* colonization in an animal comprising the step of
   providing to said animal
   one or more *C. jejuni* polypeptides: or
   a host genetically engineered to contain and express nucleic acid sequences encoding one or more *C. jejuni* polypeptides.

2. The method of claim 1, wherein said one or more *C. jejuni* polypeptides is selected from the group consisting of CacIF, FlpA, FlaA, CmeA, CmeC, OjaA, CjaC, COS176_0126, α J8U76_0128, CJJ81_176_0164, α JSI 176_0586, CJJSI i76_1185, CJJSI 176J295, CJJ8U76J525, FlaB, FlgO, PBB2, PEB3, PorA, MapA, SdhB, and antigenic fragments thereof.

3. The method of claim 2, wherein said one or more *C. jejuni* polypeptides is selected from the group consisting of CadF, FlpA, FlaA, and antigenic fragments thereof.

4. The method of claim 3, wherein said one or more antigenic fragments are selected from the group consisting of polypeptide sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, RHQ IO NO: 4.

5. The method of claim 1, wherein said animal is a bird.

6. The method of claim 1, wherein said bird is a chiekerh

7. The method of claim 1, wherein said host is a bacterial host cell or a virus.

8. The method of claim 7, wherein said host is attenuated.

9. The method of claim 7, wherein said bacterial host cell is a *Lactobacillus*.

10. The method of claim 9, wherein said nucleic acid sequences encoding one or more *C. jejuni* polypeptides are inserted into an S-layer protein of said *Lactobacillus*. 

*
11. The method of claim 1, wherein said step of providing includes providing said one or more 
*C. jejuni* polypeptides; or said host genetically engineered to contain and express nucleic acid 
sequences encoding one or more *C. jejuni* polypeptides in drinking water of said animal.

12. The method of claim 1, wherein said step of providing includes providing said one or more 
*C. jejuni* polypeptides; or said host cell genetically engineered to contain and express nucleic 
ad sequences encoding one or more *C. jejuni* polypeptides as an aerosol.

13. A host genetically engineered to contain and express nucleic acid sequences encoding one 
or more *C. jejuni* polypeptides.

14. The host of claim 11, wherein said one or more *C. jejuni* polypeptides is selected from the 
group consisting of CadF, FIpA, FkA, CmeA, CmeC, CJaA, QaC, CJS1 176J1 26, 
CJJ81176H28, CJJ81 176J_O364, CJJ81 176J86, OJJSi 176J 3S5, CJS1 176J295, 
CJJ81 176J525, FlaB, FlgE2, PEB2, PEB3, PorA, MapA, StlhB, and antigenic fragments 
thereof.

15. The host of claim 14, wherein said one or more *C. jejuni* polypeptides is selected from the 

group consisting of CadF, FIpA, FlaA, and antigenic fragments thereof.

16. The host of claim 15, wherein said one- or more antigenic fragments are selected from the 

group consisting of polypeptide sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SF:Q NO: 
X and SEQ ID NO: 4.

17. The host of claim 13, wherein said host cell is a bacterial host cell or a virus.

18. The host of claim 13, wherein said host is attenuated.

19. The host of claim 17, wherein said bacterial host cell is a *Lactobacillus*.

20. The host of claim 19, wherein said nucleic acid sequences encoding one or more *C. 
jejuni* polypeptides are inserted into an S-layer protein of said *Lactobacillus*. 

11. 'The method of claim L wherein said step of providing includes providing said one or more
*C. jejuni* polypeptides; or said host genetically engineered to contain and express nucleic acid
sequences encoding one or more *C. jejuni* polypeptides in drinking water of said animal.

12. The method of claim 1, wherein said step of providing includes providing said one or more
*C. jejuni* polypeptides; or said host cell genetically engineered to contain and express nucleic
acid sequences encoding one or more *C. jejuni* polypeptides as an aerosol.

13. A host genetically engineered to contain and express nucleic acid sequences encoding one
or more *C. jejuni* polypeptides.

14. The host of claim 11, wherein said one or more *C. jejuni* polypeptides is selected from the
group consisting of CadF, FIpA, FkA, CmeA, CmeC, CJaA, QaC, CJS1 176J1 26, 
CJJ81176H28, CJJ81 176J_O364, CJJ81 176J86, OJJSi 176J 3S5, CJS1 176J295, 
CJJ81 176J525, FlaB, FlgE2, PEB2, PEB3, PorA, MapA, StlhB, and antigenic fragments
thereof.

15. The host of claim 14, wherein said one or more *C. jejuni* polypeptides is selected from the
group consisting of CadF, FIpA, FlaA, and antigenic fragments thereof.

16. The host of claim 15, wherein said one- or more antigenic fragments are selected from the
group consisting of polypeptide sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SF:Q
NO: X and SEQ ID NO: 4.

17. The host of claim 13, wherein said host is a bacterial host cell or a virus.

18. The host of claim 13, wherein said host is attenuated.

19. The host of claim 17, wherein said bacterial host cell is a *Lactobacillus*.

20. The host of claim 19, wherein said nucleic acid sequences encoding one or more *C. jejuni*
polypeptides are inserted into an S-layer protein of said *Lactobacillus*. 

90
21. A modified bacterial S-layer protein having an internal insertion of at least one heterologous polypeptide from a *C. jejuni* bacterium.

22. The modified bacterial S-layer protein of claim 21, wherein said at least one heterologous polypeptide is selected from the group consisting of CadF, CmeA, CmeC, CjaA, CjaC, CJJ8U76JH26, CJJSI 176...0128, CJJ81 Î‘76_0164, CJJSi 176_0586, CJJ8i π 6J Î·85, CJJ81 176J295, CJJ81 176J52S, FlAa, FlAB, FlpA, FlgE2, PEB2, PEB3, PerA, MapA, SdhB, and one or more antigenic fragments thereof.

23. The modified bacterial S-layer protein of claim 21, wherein said modified bacterial S-layer protein is from a bacterial genus selected from *Lactobacillus* and *Carnhacfer*.

24. An antigenic composition for generating an immune response to *C. jejuni* in an animal comprising,

- at least a first polypeptide selected from the group consisting of *C. jejuni* CadF, FlAa, FlpA, and antigenic fragments thereof; and
- at least a second *C. jejuni* polypeptide that is different from said first polypeptide.

25. The antigenic composition of claim 24, wherein said second *C. jejuni* polypeptide is selected from the group consisting of CadF, FlAa, FlpA, CmeA, CmeC, CjaA, CjaC, CJJSI 176...0126, CJJSI i76...0118, CJIÎ‘ 1176...0164, CJJSI 176...0586, CJJS 1176...I!85, CJJ8U76J295, CJBI 176...1525, FlAB, FlgE2, FEB2, PEB3, PorA, MapA, SdhB, and antigenic fragments thereof.

26. The antigenic composition of claim 24, wherein said antigenic composition comprises one or more polypeptide sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 4.

27. The antigenic composition of claim 24, further comprising an adjuvant.

28. The antigenic composition of claim 24, wherein said antigenic composition is formulated for administration to a bird.
29. The antigenic composition of claim 24, wherein said antigenic composition is formulated for oral administration.

30. The antigenic composition of claim 24, wherein said antigenic composition is formulated for oral administration to a chicken.

31. The antigenic composition of claim 24, wherein said first polypeptide and said second C. jejuni polypeptide are recombinant or synthetic and inhibit C jejuni colonization of said animal.

32. A method for delivering a substance of interest to a subject, comprising the steps of:
   providing the subject with a fusion product containing a fibronectin binding protein of C. jejuni and a substance of interest, wherein said fibronectin binding protein and said substance of interest are associated with one another; and
   permitting said fusion product to bind fibronectin in cells or tissues of said subject.

33. The method of claim 32, wherein the fibronectin binding protein is FipA.

34. The method of claim 32, wherein the substance of interest is a polypeptide antigen for one or more human infectants.
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\(^{a}\) Proteins reactive with one or more of the sera were identified and given numerical values: 1-16.

\(^{b}\) Detection of a strong reactive band = X

\(^{c}\) Detection of a faint reactive band = O

**Figure 2**
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* a The score derived from Protein predict searches of LC/MAIDI/TOF-TOF data compared to *C. jejuni* 81-176 proteins.

* b The percentage of the protein sequence covered by the peptides from LC/MAIDI/TOF-TOF.

* c The predicted subcellular location of the protein (as defined by PSORT2.0), SP - signal peptide present.

**Figure 4**
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<sup>a</sup> The score derived from Protein predict searches of LC/MAIDI/TOF-TOF data compared to C. jejuni 81-176 proteins.

<sup>b</sup> The percentage of the protein sequence covered by the peptides from LC/MAIDI/TOF-TOF.

<sup>c</sup> The predicted subcellular location of the protein (as defined by PSORT2.0), SP - signal peptide present.
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<th>Protein identification</th>
<th>Total scorea</th>
<th>% coverageb</th>
<th>Subcellular location (PSORTb)c</th>
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a The score derived from Protein predict searches of LC/MAIDI/TOF-TOF data compared to C. jejuni 81-176 proteins.
b The percentage of the protein sequence covered by the peptides from LC/MAIDI/TOF-TOF.
c The predicted subcellular location of the protein (as defined by PSORT2.0), SP - signal peptide present.

Figure 4 (Continued)
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<sup>a</sup> The score derived from MASCOT searches of nano-LC/MS/MS data against the C. jejuni 81-176 strain.

<sup>b</sup> Percentage of the sequence covered by the sequenced peptides from nano-LC/MS/MS.

<sup>c</sup> Refers to the number of sequenced peptides from nano-LC/MS/MS.

<sup>d</sup> Not determined, identification was performed through immunobLOTS using anti-F1aB antibodies raised in rabbits (Fig. 3).

<sup>e</sup> Not determined, identification was performed through immunobLOTS using anti-C1dF antibodies raised in goats (Fig 2A).

<sup>f</sup> Not determined, lipooligosaccharide (LOS) is a common antigen found below 19 kDa and is characterized by a large diffuse band (33).
<table>
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<tr>
<th>Band</th>
<th>Mass (kDa)</th>
<th>Protein ID</th>
<th>Protein Name</th>
<th>MASCOT&lt;sup&gt;a&lt;/sup&gt;</th>
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The score derived from MASCOT searches of nano-LC/MS/MS data against the *C. jejuni* 81-176 strain.

Percentage of the sequence covered by the sequenced peptides from nano-LC/MS/MS.

Refers to the number of sequenced peptides from nano-LC/MS/MS.

Not determined, identification was performed through immunoblots using anti-FlaAB antibodies raised in rabbits (Fig. 3).

Not determined, identification was performed through immunoblots using anti-CadF antibodies raised in goats (Fig 2A).

Not determined, lipooligosaccharide (LOS) is a common antigen found below 19 kDa and is characterized by a large diffuse band (33).

*Figure 5 (Continued)*
Figure 6

Figure 7
Figure 8

Number of adherent bacteria/well

- 6.0 x 10^6
- 5.0 x 10^6
- 4.0 x 10^6
- 3.0 x 10^6
- 2.0 x 10^6
- 1.0 x 10^6

F.3801
cadF
Gj1279c
Gj1279c mutants
wild-type
Figure 12

Figure 13
**Figure 15A**

Number of Adherent Bacteria

- Wild-type
- cadF mutant
- flpA mutant
- cadF flpA mutant

**Figure 15B**

Percent bacteria bound relative to the wild type F38011 strain

- Wild-type
- cadF mutant
- flpA mutant
- cadF flpA mutant
Figure 16A

Type I repeat (FN I₁₋₁₂)  Type II repeat (FN I₁₋₂)  Type III repeat (FN I₁₋₁₅)

Major thermolysin digestion sites

Extra domain A & B and the variable region

Figure 16B

Type I repeat (FN I₁₋₁₂)

~30 kDa NTD

Figure 16C

~40 kDa GBD

Type I repeat (FN I₁₋₁₂)

Type II repeat (FN I₁₋₂)
**Figure 18A**

Concentration of GST fusion protein (nM) added to Fn coated wells

**Figure 18B**

Micrograms of Fn added to wells coated with FlpA, D1, D2, or D3
Figure 21

FlpA 135-224
FN3-1

RLEAVP-FVQAVTNLPNRIKLIWR-PHPDFRVDYSIIEERTKGDDEFKKIAEVKNRLNAE 58
SSGDPVEVFITEETPSQPNSHPIQWNAPQPSH-ISKYILRWPKNSVGWRKEATIPGHN-S 58
.* *: ... ** : *. *:*: ..:*:*: . . .:*: *:* : .:*: 

FlpA 135-224
FN3-1

YIDSDLKPNENSSYRIIAVSFGIKGSSQVVSS 92 (SEQ ID NO: 70)
YTIKGLKPGVVYEGQLISIQYGHQ--------- 83 (SEQ ID NO: 71)
* .:* **: . :*: :* : .:* :
**Figure 22A**

**Figure 22B**
**Figure 22C**

- Fibronectin
- 45 kDa GBD
- 30 kDa NTD
- 1% Ovalbumin

**Figure 22D**

- Fibronectin
- 45 kDa GBD
- 30 kDa NTD
- 1% Ovalbumin
Figure 23A

- N = # of birds colonized
- N = median CFU/gm
- CFU/gm

Uninoculated
- L. crispatus
- L. helveticus
- L. acidophilus
- L. gallinarum
- C. jejuni

F38011 Challenged
- 0/10
- 0/9
- 4/10
- 7/9
- 9/10
- 10/10

Not Challenged
Figure 23B
Figure 24B

CFU / gram

Control
L. helveticus
L. acidophilus
L. crispatus
C. figulin
C. figulin + C. figulin
Uninoculated
Figure 28

Sequence Description: L. helveticus slpA

Hydropathy

Predicted Segments

Smoothed Hydropathy

Known TM Segments

Insertion sites = 1, 2, 3, and 4 = ○

Figure 29

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Figure 30

1/2/3

Inoculate with the modified Lactobacillus strain

4/5/6

No treatment

Necropsy 10 birds and collect blood

3

Challenge with 10³ (low dose) cfu of C. jejuni

2

Inoculate with PBS only

1

Inoculate with 10⁳ (high dose) cfu of C. jejuni

SAMPLES = 1) Intestine 2) Cecum

DAY 5

Inoculation

DAY 12

Necropsy and C. jejuni challenge

DAY 19

Necropsy

AM 3:1

AM 3:2
Figure 31

- Chick inoculated with C. crescentus wild-type
- Chick inoculated with C. crescentus CadF/FlaA/PorA

Colony forming units per gram of cecal contents

N = 9
N = 4
Figure 32
Figure 33A
Figure 33B (Continued)
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Figure 33C (Continued)