The fibronectin-binding motif within FlpA facilitates *Campylobacter jejuni* adherence to host cell and activation of host cell signaling

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*Campylobacter jejuni* is a gram-negative, curved and rod-shaped bacterium that causes human gastroenteritis. Acute disease is associated with *C. jejuni* invasion of the intestinal epithelium. Epithelial cells infected with *C. jejuni* strains containing mutations in the FlpA and CadF fibronectin (Fn)-binding proteins exhibit reduced invasion of host cells and a *C. jejuni* CadF FlpA double mutant is impaired in the activation of epidermal growth factor receptor (EGFR) and Rho GTPase Rac1. Although these observations establish a role for Fn-binding proteins during *C. jejuni* invasion, their mechanistic contributions to invasion-associated signaling are unclear. We examined FlpA, a *C. jejuni* Fn-binding protein composed of three FNIII-like repeats D1, D2 and D3, to identify the interactions required for cellular adherence on pathogen-induced host cell signaling. We report that FlpA binds the Fn gelatin-binding domain via a motif within the D2 repeat. Epithelial cells infected with a *flpA* mutant exhibited decreased Rac1 activation and reduced membrane ruffling that coincided with impaired delivery of the secreted Cia proteins and reduced cell association. Phosphorylation of the Erk1/2 kinase, a downstream effector of EGFR signaling, was specifically associated with FlpA-mediated activation of $\beta_1$-integrin and EGFR signaling. *In vivo* experiments revealed that FlpA is necessary for *C. jejuni* disease based on bacterial dissemination to the spleen of IL-10$^{-/-}$ germ-free mice. Thus, a novel Fn-binding motif within FlpA potentiates activation of Erk1/2 signaling via $\beta_1$-integrin during *C. jejuni* infection.

**INTRODUCTION**

Pathogen adherence to the surface of epithelial cells is often critical for host colonization and infection. There are currently $\sim$100 known bacterial outer surface adherence proteins (adhesins) that bind fibronectin (Fn), suggesting that adherence to Fn confers specific benefits to pathogens. Fn is a dimeric multidomain glycoprotein composed of 12 Fn type I repeats (FNI), two Fn type II repeats (FNII) and 15–17 Fn type III repeats (FNIII). Pathogen binding to Fn is thought to induce integrin clustering and recruitment of numerous regulatory molecules to cytoplasmic integrin domains. The resulting multiprotein complexes formed, termed focal complexes (FCs), act as signaling platforms that physically link Fn in the extracellular matrix to intracellular signaling machinery and the actin cytoskeleton. By initiating signaling through FCs, Fn on the cell surface can stimulate cytoskeletal and membrane rearrangements involved in cell motility, division, and phagocytosis. In addition, Fn provides a surface exposed ligand for bacterial attachment enabling pathogens to engage cellular regulatory pathways, in particular signaling events that promote bacterial internalization.

*Campylobacter jejuni* is a gram-negative bacterium and the causative agent of campylobacteriosis, a debilitating gastrointestinal illness associated with abdominal cramps, fever and diarrhea. *C. jejuni* encodes two known Fn-binding proteins, an outer membrane protein termed CadF, and a recently identified lipoprotein termed FlpA that harbors three FNIII-like domains. Needed are studies to dissect the contribution ofFn binding in *C. jejuni*–host cell interactions.

Our understanding of the mechanism utilized by *C. jejuni* to invade cells is incomplete. Specifically, complex interactions at the bacterial host cell interface resulting in *C. jejuni* internalization are ill defined. We conducted this study to better define the nature of *C. jejuni* adherence to Fn and the contribution of this interaction to activating host signaling responses to promote invasion. Here, we identify the FlpA Fn-binding residues, the sites on Fn bound by FlpA, and the specific invasion-associated host cell signaling responses to the FlpA–Fn interaction, which contribute to acute disease.

**MATERIALS AND METHODS**

Bacterial strains/plasmids/primers

*C. jejuni* were cultured on Mueller-Hinton agar plates supplemented with 5% bovine blood (Mueller-Hinton blood agar) under microaerobic conditions. *Escherichia coli* XL-1 Blue (Stratagene, Garden Grove, CA, USA) and BL21(DE3) (Novagen, Madison, WI, USA) were maintained on Luria-Bertani agar plates or in Luria-Bertani broth aerobically at 37°C. Strains harboring pGEX-5X-1 (GE Healthcare, Pittsburgh, PA, USA) were grown on medium supplemented with 100 μg/mL ampicillin. Construction and expression of the recombinant N-terminal...
glutathione S-transferase (GST)-tagged protein was performed using standard molecular biology techniques described previously. The primers used in this study are provided in Supplementary Table S1.

Protein purification
Protein purification was performed as previously described. Briefly, E. coli pGEX-5X-1 strains were grown in Luria-Bertani broth supplemented with antibiotics at 37 °C to an OD_{540}=0.6 and induced with 1 mM Isopropyl-β-d-thio-galactoside (Sigma, St Louis, MO, USA) overnight at 22 °C. Cells were harvested by centrifugation (6000 g for 15 min at 4 °C), resuspended in ice-cold 20 mM NaP, 150 mM NaCl, pH 7.4 buffer (phosphate-buffered saline (PBS)), lysed by sonication and centrifuged at 15 000 g for 30 min at 4 °C to remove insoluble debris. GST-tagged proteins were purified from the clarified lysates using Sepharose 4B GST affinity resin (GE Healthcare) according to the manufacturer’s instructions. Fractions containing recombinant GST fusion proteins were pooled, dialyzed in 25 mM Tris pH 7.5 or PBS and concentrated using Amicon Ultra centrifugal filter units (Millipore, Bedford, MA, USA).

Peptide synthesis
FlpA peptides were synthesized using conventional fluorenylmethoxycarbonyl chloride solid-phase peptide chemistry on an Applied Biosystems 431A Peptide Synthesizer using protocols supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA) by the School of Molecular Biosciences Laboratory for Bioanalysis and Biotechnology at Washington State University (Pullman, WA, USA). Synthesis of CadF FRLS<sup>+</sup> and FRLS<sup>−</sup> peptides was described previously.

Enzyme-linked immunosorbent assays (ELISAs) with GST-FlpA proteins
Human plasma Fn, the 30-kDa N-terminal domain, and the 40-kDa gelatin binding domain (GBD) were purchased from Sigma. To determine if FlpA or the FlpA domains bound to Fn, 96-well polystyrene plates (Costar, Corning, NY, USA) were coated with 40 nM of Fn or Fn fragments (Sigma) in 20 mM NaP, 150 mM NaCl, pH 7.4 (PBS) overnight at 4 °C. Plates were washed once with PBS, 0.01% Tween 20 pH 7.4 (PBST) and then blocked with PBS containing 1% bovine serum albumin (BSA) (fraction V; Sigma). While the plates incubated with block solution, serial dilutions of the GST-FlpA, GST-FlpA-D1, GST-FlpA-D2 and GST-FlpA-D3 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 PBS and the bacteria were harvested by treatment with 1% trypsin for 15 min. The bacteria were resuspended, serial diluted, and plated on Mueller-Hinton agar for enumeration.

Peptide competitive inhibition assay
Fn and BSA fraction V (Sigma) were diluted in 0.1 M sodium carbonate buffer pH 9.5 to a concentration of 250 µg/mL. A flat-bottomed maxisorp 96-well plate (NUNC, Rochester, NY, USA) was coated with 100 µl per well of the diluted protein and incubated overnight at 4 °C. Following incubation, the plate was rinsed three times with PBS. The synthetic peptides were diluted in PBS, added to the wells and incubated for 1 h at 20 °C. The plate was rinsed three times with PBS and 1×10<sup>5</sup> of the C. jejuni wild-type strain and the flpA mutant were added to each well and incubated for 1 h at 37 °C. The plate was then rinsed three times with PBS and the bacteria were harvested by treatment with 1% trypsin for 15 min. The bacteria were resuspended, serial diluted, and plated on Mueller-Hinton agar for enumeration.

C. jejuni Fn-binding and cell-binding assays
Measurements of C. jejuni adherence to Fn and epithelial cells were performed as previously described.

Generation of FlpA constructs with altered Fn-binding linear motif (FBLM) residues
The FlpA mutant and FlpA complemented strain of C. jejuni were previously generated using standard techniques. The pRY111 FlpA complementation plasmid was used as a template for PCR reactions. Primers MEK3316 and MEK3317 were used to generate full FlpA Fn binding domain mutants. MEK3318 and MEK3319 were used to generate proline and aspartic acid mutations. These primers contain a 5′ Sphl restriction site. PCR products were digested with Sphl and re-ligated to generate the pRY111 flpA Δ158–164 and pRY111 flpA P160A D161C mutants. Plasmid constructs were confirmed by DNA sequencing.

FlpA gels and immunoblots
Bacterial whole-cell lysates and outer membrane protein extracts were separated by SDS–PAGE and transferred to PVDF membranes (Millipore, Temecula, CA, USA) as described previously. The following antibodies were used: β1 integrin (4706), pErk (4370) (Cell Signaling Technology, Danvers, MA, USA), actin (sc-1616) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-FlpA polyclonal serum (Konkel lab) and rabbit anti-CysM polyclonal serum (Konkel lab).

Cia protein delivery assay
Cia protein delivery assays were performed 30 min post-infection as previously described.

Scanning electron microscopy
Scanning electron microscopy was performed as previously described. Quantification of membrane ruffling was done by two independent observers as described previously.
**Rho GTPase Rac1 activation**
Rac1 activation was quantified as previously described.16

**Erk1/2 phosphorylation and β1 integrin knockdown**
INT 407 cells were seeded in 24-well plates at a density of 1.5×10^5 cells per well. The following day, the cells were rinsed twice with PBS and serum starved for 3 h in minimal essential medium without fetal bovine serum. For protein depletion assays, INT 407 cells were transfected with 1 pmol siRNA of non-targeting (46–2000; Invitrogen, Grand Island, NY, USA) and β1 integrin-targeting siRNAs (ITGB1HS55559; Invitrogen) using Lipofectamine RNAi MAX (Invitrogen) according to manufacturer’s instructions and incubated for 24 h prior to an assay. β1-integrin depletion was confirmed by immunoblot. The C. jejuni infection assay on INT 407 cells was performed in the following manner. INT 407 cells were infected with C. jejuni suspended in minimal essential medium containing 1024 μg/mL chloramphenicol for 30 min prior to the assay. This same concentration of chloramphenicol was maintained throughout the course of the assay. Treatment of C. jejuni with 1024 μg/mL of chloramphenicol for 30 min completely blocks C. jejuni protein synthesis, as judged by the absence of [^35S]-methionine incorporation (not shown). The site(s) on Fn bound by FlpA was also examined. Amino acid sequence alignments of FlpA-D2 with the 1–5 FNIII repeats using ClustalW19 revealed that FlpA-D2 most closely aligned with 3FNIII (22.9% sequence identity) (Supplementary Figure S3).1FNIII is involved to Fn-binding, these data indicate that FlpA-D2 mediates the interaction with Fn and the amount of Fn protein bound was determined by ELISA. Full-length GST-FlpA (aa 35–410) protein was used as a positive control and wells coated with BSA were used to determine background adherence. Of the three FlpA domain fusion proteins, only GST-FlpA-D2 (aa 135–226) bound to the Fn-coated wells in amounts similar to the full-length GST-FlpA protein (aa 35–410) (Figure 2A). As with full-length GST-FlpA, the amount of GST-FlpA-D2 bound to the Fn-coated wells was dose-dependent and saturable, demonstrating specificity. Compared to GST-FlpA, significantly less GST-FlpA-D1 and GST-FlpA-D3 bound Fn-coated wells and did not exhibit attributes of specific binding. A second ELISA was performed in the opposite orientation, where wells were coated with GST-FlpA, GST-FlpA-D1, GST-FlpA-D2 and GST-FlpA-D3 and serial dilutions of Fn were added (Supplementary Figure S2). As expected, the amount of Fn bound to wells coated with GST-FlpA-D2 was similar to that of GST-FlpA and minimal binding to Fn was measured for D1 and D3.

### Domain 2 of FlpA binds the GBD of Fn
The site(s) on Fn bound by FlpA was also examined. Amino acid sequence alignments of FlpA-D2 with the 1–5 FNIII repeats using ClustalW19 revealed that FlpA-D2 most closely aligned with 3FNIII (22.9% sequence identity) (Supplementary Figure S3).1FNIII is involved in intramolecular interactions with the FN1 and FNII repeats within the N-terminal of Fn, suggesting FlpA may also bind the N-terminus.7 Digestion of Fn with thermolysin produces fragments that retain their biological activity.9 One of these Fn fragments, termed the N-terminal domain (NTD), is approximately 30 kDa in size and composed of 1–5FN1. Another fragment of approximately 40 kDa contains the GBD and is composed of 6–8FN1 and 1–2FNII.4 To evaluate the binding of FlpA to the N-terminal region of Fn (i.e., either the NTD or GBD fragment), 96-well plates were coated with full-length Fn, the 30 kDa NTD fragment or the 40 kDa GBD Fn fragment. Serial dilutions of the GST-FlpA, GST-FlpA-D1, GST-FlpA-D2 and GST-FlpA-D3 proteins were incubated in NTD- and GBD-coated wells and the amount of GST-FlpA fusion proteins bound to the Fn fragments was determined by ELISA (Figures 2B and 2C). FlpA and FlpA-D2 exhibited dose-dependent and saturable binding to wells coated with the 40 kDa GBD, indicative of a specific interaction, and low levels of nonspecific binding to wells coated with the 30 kDa NTD. The equilibrium binding constants (K_D) calculated from the saturation curves for FlpA and FlpA-D2 were 28.7 nM and 2.3 nM to immobilized full-length Fn and 11.5 nM and 5.5 nM to the immobilized GBD of Fn, respectively. These results indicate that FlpA and FlpA-D2 bind to Fn with high affinity, similar to that of other Fn-binding proteins such as BBK32 from B. burgdorferi.20 FlpA-D1 and FlpA-D3 did not exhibit specific or high affinity binding to the GBD, and the amount of Fn bound by FlpA-D1 and FlpA-D3 was significantly reduced compared to FlpA and FlpA-D2 (P<0.05). While we cannot exclude the possibility that FlpA-D1 and FlpA-D3 contribute to Fn-binding, these data indicate that FlpA-D2 mediates the interaction of FlpA with the GBD of Fn.
FlpA amino acids N150–F164 have maximal Fn-binding activity

Experiments were then performed to localize the Fn-binding residues within FlpA-D2. Five overlapping peptides were designed spanning the FlpA-D2 sequence: P1 (R135–V164), P2 (N150–F179), P3 (D165–I194), P4 (K180–I209) and P5 (D195–V224) (Figure 1C). As above, microtiter plates were coated with each of the five 30mer peptides, Fn was added to the wells, and the amount of Fn bound to peptide-coated wells determined (Supplementary Figure S4). Previously characterized CadF peptides, FRLS1 (aa 128–143) positive for Fn-binding, and FRLS2 (aa 118–143) negative for Fn-binding, were included as controls.15 The amount of Fn bound to wells coated with peptides P1 and P2 was comparable to FRLS1 control peptide, whereas the amount of Fn bound to peptides P3, P4 and P5 was equivalent or less than that bound by the FRLS2 peptide. No difference was observed in the amount of Fn bound by P1 (R135–V164) and P2 (N150–F179).

Together, these data suggest that: (i) the Fn-binding residues are shared by P1 and P2, which corresponds to residues N150–V164; or (ii) P1 and P2 each have unique residues responsible for Fn-binding.

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Figure 1  FlpA domain organization, GST fusion proteins and synthetic peptides used in this study. (A) Schematic of FlpA structural features. FlpA harbors a signal peptide ‘S’ at the amino terminus that is lipidated at A20 and three domains designated FlpA-D1, FlpA-D2 and FlpA-D3, homologous to Fn type three (FNIII) repeats. (B) N-terminally tagged GST fusion proteins used in this study. Each of the three FlpA FNIII-like repeats (FlpA-D1, FlpA-D2 and FlpA-D3) were expressed with GST tags. (C) Sequences of the synthetic peptides used in this study. Peptides are aligned relative to the FlpA-D2 sequence. The 30mer peptides P1–P5 overlap by 15 residues and span the entire FlpA-D2 sequence. Peptides P6 and P7 span predicted β-strands and flanking disordered regions of interest. White double arrowheads indicate the predicted β-strands and vertical gray boxes highlight their location (DOMpro 1.0). Peptides NP1–NP9 are 12mers that overlap by 9 residues. The sequence of the FlpA FBLM156WRPHPDFRV164 is highlighted in bold.
was bound to P7 (not shown). These data suggested that the Fn-binding activity is localized to a predicted β-strand or the flanking disordered regions (L148–I152 and P158–V164) (Figure 1C). Given that neither P7 nor P3 bound significant amounts of Fn, we concluded that the residues unique to P6 158PHPDFRV164 were likely involved in Fn-binding activity.

Identification of a FlpA peptide encoding residues required for *C. jejuni* adherence to Fn
To confirm the residues localized within N150–V164 of FlpA-D2 are required for Fn-binding, binding assays were conducted with a set of new peptides (NP1–9) of 12 residues. Each of the peptides overlapped by nine amino acids to localize the Fn-binding activity to three amino acids. The P6 30mer used above was included as a positive control, and the amount of Fn bound to NP1–9 was measured by ELISA (Figure 4A). Of the new peptides, NP5 bound the largest amount of Fn (P<0.05). NP3, NP4 and NP6 also bound Fn, but significantly less than NP5. The amount of Fn bound was statistically indistinguishable for the NP3, NP4 and NP6 peptides, but all three peptides bound more Fn (P<0.05) than for the NP1, NP2, NP7, NP8 and NP9 peptides. Collectively, these data indicate that the FlpA residues 156WRPHPDFRV164 bind to Fn. We termed these nine residues the FlpA FBLM.

The experiments to this point had been performed to localize the Fn-binding activity with the FlpA protein. We then performed experiments to determine the importance of the FlpA FBLM in *C. jejuni* adherence to Fn and host cells. We first examined whether NP5 could inhibit binding of *C. jejuni* to immobilized Fn. NP5 harbors all nine residues of the FlpA FBLM and had bound more Fn than the other 12mer peptides. Plates coated with Fn were incubated with *C. jejuni* and serial dilutions of NP5, NP2 (non-binding control) or no peptide. The number of *C. jejuni* bound to the peptide treated wells was determined (Figure 4B). NP5 blocked the adherence of *C. jejuni* to Fn-coated wells in a dose-dependent manner, whereas the highest concentration of the NP2 peptide tested (50 μM) did not inhibit *C. jejuni* adherence. Furthermore, the amount of *C. jejuni* bound to wells treated with 50 μM NP5 was statistically indistinguishable (P>0.05) from the amount bound by the *C. jejuni* flpA mutant. These findings indicate that the FlpA FBLM is required for *C. jejuni* binding to Fn.

Deletion of conserved residues abrogates FlpA-mediated adherence of *C. jejuni* to Fn and INT 407 epithelial cells
We previously showed complementation of the *C. jejuni* flpA mutant with *flpA* in trans restores bacterial adherence to INT 407 epithelial cells. Thus, we generated two constructs for the synthesis of FlpA proteins containing mutations in the FBLM residues and examined whether they could rescue the binding defect of the flpA mutant. In the first construct, FlpA Δ158–164, residues 158PHPDFRV164 were deleted and replaced with alanine and cysteine (Figure 5A). In the second construct, FlpA P160A D161C, a smaller mutation was made in central FBLM residues by substituting P160 and D161 with alanine and cysteine. This smaller alteration was made to examine the sensitivity of the residues unique to P6 158PHPDFRV164 bind to Fn. We termed these nine residues the FlpA FBLM.

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Emerging Microbes and Infections

Figure 3  Fn-binding is localized to residues 158-Pro-His-Pro-Asp-Phe-Arg-Val164 encoded within the P6 peptide. Binding of Fn to 96-well plates coated with peptides P1, P2, P5 and P6. Peptide-coated plates were incubated with serial dilutions of Fn and the amount of Fn bound was determined by ELISA. P1, P2 and P5 are 30mer peptides used above and P6 was designed to span predicted β strands and flanking disordered regions within the region shared by P1 and P2 (N150–V164). P6 was included as a negative control for Fn-binding. Data are displayed as mean values and standard deviations from triplicate samples. Statistical significance was determined at the 80 nM concentration by one-way ANOVA using Tukey’s post-test (*P<0.05).

Figure S5A). Introduction of cysteine residues within the altered FlpA proteins created the possibility of introducing disulfide bonds, but both wild-type and the altered FlpA proteins exhibited similar mobility under reducing and non-reducing conditions (Supplementary Figure S5B). Assays were then conducted to measure binding by flpA mutants synthesizing the FlpA, FlpA Δ158–164 and FlpA P160A D161C to immobilized Fn and epithelial cells (Figures 5C and 5D). The C. jejuni flpA mutant synthesizing FlpA wild-type protein bound to Fn-coated wells and epithelial cells at or above wild-type levels, indicating functional complementation (Figures 5C and 5D). Synthesis of the FlpA Δ158–164 and FlpA P160A D161C proteins did not restore the binding of the flpA mutant to Fn-coated wells or INT 407 cells. Failure of FlpA Δ158–164 and FlpA P160A D161C to complement the C. jejuni flpA mutant is consistent with the peptide inhibition results (Figure 4B), and support the conclusion that the FlpA FBLM is required for C. jejuni to bind to Fn.

FlpA-mediated adherence facilitates C. jejuni–host cell interactions

After identifying the Fn-binding residues in FlpA proteins created the possibility of introducing disulfide bonds, but both wild-type and the altered FlpA proteins exhibited similar mobility under reducing and non-reducing conditions (Supplementary Figure S5B). Assays were then conducted to measure binding by flpA mutants synthesizing the FlpA, FlpA Δ158–164 and FlpA P160A D161C to immobilized Fn and epithelial cells (Figures 5C and 5D). The C. jejuni flpA mutant synthesizing FlpA wild-type protein bound to Fn-coated wells and epithelial cells at or above wild-type levels, indicating functional complementation (Figures 5C and 5D). Synthesis of the FlpA Δ158–164 and FlpA P160A D161C proteins did not restore the binding of the flpA mutant to Fn-coated wells or INT 407 cells. Failure of FlpA Δ158–164 and FlpA P160A D161C to complement the C. jejuni flpA mutant is consistent with the peptide inhibition results (Figure 4B), and support the conclusion that the FlpA FBLM is required for C. jejuni to bind to Fn.

Adherence facilitates Cia protein delivery

To determine if FlpA is required for the delivery of Cia proteins to host cells, we performed protein delivery assays. To measure protein delivery, the C. jejuni isolates were transformed with a plasmid encoding CiaC fused to the adenylate cyclase domain (ACD) of the Bordetella pertussis protein CyaA.17 Translocation of CiaC-ACD into the host cell cytosol catalyzes the production of cAMP only when bound by calmodulin in the eukaryotic cell cytosol. For these experiments, translocation of CiaC into the host cell cytosol by the C. jejuni flpA mutant was compared to the C. jejuni wild-type strain. As a control, we also included a C. jejuni capA mutant.13,25 The C. jejuni capA mutant displays a similar reduction in epithelial cell adherence as the flpA mutant (Supplementary Figure S6A). The cells inoculated with the C. jejuni flpA and capA mutants synthesizing the CiaC-ACD resulted in significantly less cAMP than cells inoculated with the C. jejuni wild-type strain (P<0.05) (Supplementary Figure S6B). Moreover, equivalent reductions in cAMP concentrations were observed in response to the C. jejuni flpA and capA mutants. These data indicate that bacterial
proteins, host cell infections were performed in the presence of chloramphenicol, as the synthesis of the Cia proteins is completely inhibited by chloramphenicol.27 Cells infected with the C. jejuni wild-type strain and the capA mutant exhibited increased Erk1/2 phosphorylation compared to uninfected cells, while cells infected with the flpA mutant did not exhibit increased Erk1/2 phosphorylation (Figure 7A). These findings suggest that FlpA-mediated adherence to Fn contributes to the activation of Erk1/2.

While other integrin receptors also bind Fn, outside-in signal transduction is predominantly associated with the αβ1-integrin receptor.28 Thus, engagement of Fn during C. jejuni infection could activate Erk1/2 by a β1-integrin-dependent pathway. To examine the requirement of β1-integrin for Erk1/2 phosphorylation during C. jejuni infection, we knocked down cellular β1-integrin with specific siRNA and examined Erk1/2 activation upon C. jejuni infection. Cells treated with non-targeting siRNA, either infected with C. jejuni or uninfected, were used as positive or negative controls, respectively. Efficient siRNA knockdown of β1-integrin protein levels were confirmed by immunoblot (Figure 7B). Increased Erk1/2 phosphorylation was observed in cells treated with non-targeting siRNA and infected with the C. jejuni wild-type strain (Figure 7B). However, in C. jejuni infected cells depleted of β1-integrin, we did not observe an increase in Erk1/2 phosphorylation. Collectively, these results indicate that Erk1/2 is activated by FlpA-mediated adherence, and suggest that FlpA binding to Fn stimulates outside-in signaling.

FlpA is required for maximal disease progression in IL-10−/− mice
Since the C. jejuni flpA mutant is reduced in its ability to adhere to fibronectin, adhere to epithelial cells and to stimulate host cell signaling, we wanted to determine if FlpA contributes to disease in vivo. IL-10−/− germ-free mice were inoculated with a C. jejuni wild-type strain, flpA mutant and flpA complemented strain and housed for 14 days prior to necropsy. Uninfected mice (PBS sham-inoculated) were included as a negative control. We determined the number of bacteria in the colon in order to determine if there is a relationship between intestinal colonization and invasion of the spleen. The colonies of the mice inoculated with the C. jejuni wild-type strain and C. jejuni flpA complemented strain showed signs of edema and apparent stool softening compared to mice inoculated with the C. jejuni flpA mutant and un inoculated mice (Supplementary Figure S7). Noteworthy is that all of the C. jejuni inoculated mice were colonized at statistically indistinguishable levels (Figure 8). In contrast to the levels of colonization, fewer bacteria were recovered from the spleen of mice inoculated with the C. jejuni flpA mutant versus the C. jejuni wild-type and flpA complemented strains (Figure 8). Together, these data indicate that FlpA is necessary for the development of severe disease (bacterial dissemination) in IL-10−/− germ-free mice.

**DISCUSSION**

This study increases our understanding of the mechanism used by C. jejuni to bind to and invade epithelial cells. We demonstrated that FlpA binds the GBD of Fn via the FBLM within the second FNIII-like repeat (FlpA-D2) and that the phosphorylation of Erk1/2 requires FlpA-mediated adherence and β1-integrin engagement. These findings are consistent with the hypothesis that the FlpA–Fn interaction triggers outside-in signaling during C. jejuni infection of epithelial cells. FlpA is comprised of three FNIII-like repeats and is 99% conserved among C. jejuni sequenced strains (Supplementary Figure S3), implying that FlpA plays a critical role in pathogenesis. Other bacterial adhesins have been identified with multiple Fn-like domains. Brown
C. jejuni adherence requires the FlpA Fn-binding motif

CL Larson et al.

Figure 5  Mutation of the FlpA FBLM abrogates FlpA-mediated adherence. (A) Sequence modifications for generation of FlpA Δ158–164 and FlpA P160A D161C. Non-native residues (gray) result from enzyme restriction sites introduced for removal of the FBLM residues. (B) FlpA protein synthesis and outer membrane protein distribution localized to the FBLM likely accounts for the loss of FlpA mediated adherence. (C) Binding of FlpA to Fn is possible that FlpA-D1 and/or D3 provide structural support for binding to FlpA-D1 or FlpA-D3, the possibility remains that these domains contribute to FlpA Fn-binding. Given the scaffolding role of FNIII repeats for the display of binding motifs in other proteins, it is possible that FlpA D1 and/or D3 provide structural support for interactions between FlpA-D2 and the GBD of Fn.

We found that peptides harboring residues 156WRPHPDFRV164 (termed the FlpA FBLM) exhibited the greatest Fn-binding affinity and inhibited C. jejuni adherence to Fn and cells. FBLM residues 157RPHPD161 are flanked on either end by hydrophobic residues and comprise the core of the Fn-binding motif in which charged residues alternate with proline residues. Similar to the FBLM of FlpA, prolinerich motifs have been identified within eukaryotic proteins recognized by SH3 domains during the assembly of signal transduction complexes. The conformational constraints of the proline cyclic side chain confers secondary structure beneficial for proper alignment of residues within binding motifs. The substitution of proline and aspartate within the FBLM (P160A D161C) drastically impaired FlpA Fn-binding affinity. Disruption of secondary structure and charge distribution localized to the FBLM likely accounts for the loss of FlpA P160A D161C binding activity, as assessments of outer-membrane localization, surface exposure, and electrophoretic gel mobility indicated the altered FlpA protein behaves like wild-type protein. Sensitivity of the FBLM to minor changes in amino acid composition is a characteristic shared with the linear binding motifs in other systems. Together, these results are consistent with the conclusion that FBLM residues are required for FlpA-mediated adherence.

Bacterial adherence to Fn, in addition to having a critical role for host cell attachment, can initiate integrin-dependent activation of regulatory proteins that coordinate cytoskeletal rearrangements and bacterial uptake by non-phagocytic cells. Here, reduced epithelial cell membrane ruffling and Rac1 activation were observed in response to infection with the flpA mutant. Based on these results, we concluded that the loss of FlpA-mediated adherence is sufficient to disrupt the activation of host signaling pathways.

Epidermal growth factor receptor (EGFR) activation can be initiated by extracellular ligands or via an integrin-dependent mechanism. Interestingly, treatment of epithelial cells with inhibitors of...

Emerging Microbes and Infections
Figure 6 Decreased epithelial cell membrane ruffling and Rac1 GTPase activation in response to C. jejuni flpA mutant strains synthesizing FlpA with altered FBLM residues. (A) Representative scanning electron micrographs of INT 407 cell membrane ruffles stimulated in response to infection with C. jejuni strains: (a) cells only; (b) C. jejuni F38011 wild-type strain; (c) C. jejuni flpA mutant; (d) C. jejuni flpA complement; (e) C. jejuni flpA Δ158–164; (f) C. jejuni flpAP160A D161C; (g) C. jejuni ciaC mutant. The number of cells exhibiting membrane ruffles is indicated as a percentage of total cells (×7000 magnification, scale bar=10 μm). Boxes within images ‘a’ through ‘g’ indicate the magnified area shown adjacent to the right ‘a-1’ through ‘g-1’ (×50,000 magnification, scale bar=2 μm). Arrowheads indicate C. jejuni. (B) Rac1-GTP production in INT 407 cells infected with C. jejuni. INT 407 cells were infected with C. jejuni F38011 wild-type strain, C. jejuni flpA mutant, C. jejuni flpA complement, C. jejuni flpA Δ158–164 and C. jejuni flpA P160A D161C. Activated Rac1 (GTP bound) in lysates from infected cells was measured by G-LISA and expressed as relative optical density. Means and standard deviation of total Rac1-GTP are plotted. Statistical significance was determined by one-way ANOVA using Tukey’s post-test (*P<0.05).
EGFR phosphorylation (PD168393 and erlotinib) blocks *C. jejuni* invasion and the addition of exogenous EGF to cells rescues the invasiveness of a *C. jejuni* ciaC mutant. We examined Erk1/2, which is a signaling molecule downstream of the EGFR, in cells infected with *C. jejuni*. We found that Erk1/2 phosphorylation was reduced in cells inoculated with the *C. jejuni* flpA mutant, but not with the *C. jejuni* capA mutant. These findings indicate that Erk1/2 is phosphorylated in response to FlpA binding to Fn. As stated above, *C. jejuni* stimulates host signaling during infection by multiple mechanisms, but these data suggested that FlpA binding to Fn specifically contributes

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**Figure 7** FlpA and β₁-integrin are required for phosphorylation of Erk1/2 during *C. jejuni* infection. (A) Levels of Erk1/2 phosphorylation in INT 407 epithelial cells infected with *C. jejuni* treated with chloramphenicol. Cell lysates were immunoblotted with antibodies against pErk and actin (loading control). Uninfected cells treated with chloramphenicol served as a negative control. Band intensities were quantitated by densitometry (*n*=4, error bars indicate standard error of the mean). (B) Erk1/2 phosphorylation in epithelial cells depleted of β₁ integrin in response to *C. jejuni* infection. Uninfected cells treated with NT show basal levels of Erk1/2 phosphorylation, and *C. jejuni*-infected cells treated with NT were used as a positive control. Erk1/2 phosphorylation was measured by densitometric analysis (*n*=4, error bars indicate standard error of the mean) and statistical significance was determined by ANOVA using Tukey’s post-test (*P*<0.05). NT, non-targeting siRNA; pErk, phosphorylated Erk1/2.

**Figure 8** FlpA is required for *C. jejuni* dissemination to the spleen. IL-10⁻/⁻/ germ-free mice were inoculated with the *C. jejuni* wild-type strain, flpA mutant and the flpA complemented strain. No difference was observed in bacterial colonization of the colon. However, significant differences were observed in bacterial dissemination to the spleen between the *C. jejuni* wild-type strain and the flpA mutant. Similarly, the flpA complemented strain of *C. jejuni* had levels of dissemination to the spleen indistinguishable from the levels obtained for the *C. jejuni* wild-type strain. Results are expressed as mean±s.e.m. for at least seven animals per group (*n*≥7). Significance (*P*<0.05) between groups was determined by non-parametric Kruskal–Wallis one-way ANOVA, followed by Dunn’s multiple comparison test of the means. A second experiment, which was performed to ensure reproducibility, yielded identical results.
to Erk1/2 phosphorylation during *C. jejuni* infection. Consistent with this observation, knockdown of β1 integrin with specific siRNA also inhibited Erk1/2 phosphorylation in cells infected with the *C. jejuni* wild-type strain. While others have shown maximal *C. jejuni* invasion requires β1 integrin and Erk1/2 activation, we demonstrate that *C. jejuni* stimulates integrin-dependent signaling by a mechanism requiring FlpA adherence for Erk1/2 phosphorylation. Our findings suggest that outside-in signaling is initiated by FlpA–Fn binding, requiring FlpA adherence for Erk1/2 phosphorylation. Our findings demonstrate that FlpA is necessary for the maximal binding of host intestinal epithelial cells, and is required for the dissemination from the intestine and to dissect the complexity pathways; and (iv) invasion of epithelial cells. Other factors that may contribute to the loss in virulence of the *C. jejuni* flpA mutant include its inability to effectively: (i) translocate across the intestinal epithelium; and (ii) combat the host innate and acquired immune responses. While additional work is needed to define the route of systemic disease initiated by oral inoculation, we concluded that FlpA contributes to disease in the IL-10−/− germ-free mouse model. We identified the FBLM in FlpA and discovered that FlpA binds to the gelatin-binding domain of Fn. We discovered that FlpA is required for the maximal binding of host intestinal epithelial cells, and is required for the CIA-independent activation of Erk1/2. We demonstrate that FlpA is necessary for *C. jejuni* invasion of the spleen using IL-10−/− germ-free mice.

ACKNOWLEDGMENTS

We thank Dr Jason M Neal-McKinney (School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, USA) for proofreading this manuscript. We would also like to thank Dr Magnus Huk (Professor of Biochemistry and Biophysics, of the IIB-Houston, Veterinary Anatomy and Public Health, Director, Center for Extracellular Matrix Biology) and Dr Laurent Vuillard (BioXtal, PX Unit, c/o AFMB, UMR 6098, Case 932, 163 Avenue de Luminy, 13288 Marseille CEDEX 09, France) for helpful discussions. Finally, we thank Dr Maureen Bower at the Gnotobiotic Core Facility of the Center for Gastrointestinal and Dr Sue Tonkonogy at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC, USA).

This project was supported by Agriculture and Food Research Initiative Competitive (Grant No. 2011–67015–30772) from the USDA National Institute of Food and Agriculture awarded to Michael E Konkel. Derrick R Samuelson and Tyson P Eucker were supported by Award Numbers T32GM083864 and T32GM008336 from the National Institute of General Medical Sciences, respectively. Jason L O’Loughlin was supported by a National Institutes of Health T32 Training program in Infectious Diseases and Microbial Immunology (Award Number 5 T32 AI 7025–33). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIGMS or the National Institutes of Health.

Supplementary Information for this article can be found on Emerging Microbes and Infections’ website (http://www.nature.com/emi).

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