Characterization and antigenicity of recombinant *Campylobacter jejuni* flagellar capping protein FliD

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*Received 8 March 2013*  
*Accepted 18 January 2014*

**INTRODUCTION**

It is estimated that more than 400 million cases of human campylobacteriosis occur worldwide (2 million cases in the USA) (Scallan et al., 2011; WHO, 2009). The economic loss caused by this disease has been estimated at US$1.7 billion dollars annually in the USA (Hoffmann et al., 2012). The major bacterial aetiological agent is Gram-negative *Campylobacter jejuni*, which is a commensal in the chicken intestinal microbiota (Hermans et al., 2012b; European Food Safety Authority, 2010). Risk assessment studies have demonstrated that a reduction in the number of *Campylobacter* on poultry carcases or shedding in poultry farms results in a reduction of human campylobacteriosis cases (Rosenquist et al., 2003; Messens et al., 2007). Therefore, many strategies for reduction of this micro-organism in broiler chickens have been investigated (e.g. Bahndorff et al., 2013; Lin, 2009; de Zoete et al., 2007; Hermans et al., 2011, 2012a; Line et al., 2008; Robyn et al., 2013; Wagenaar et al., 2006). One of these strategies is through vaccination, and, prior to exposure, chicken anti-*Campylobacter* antibodies may reduce bacterial colonization and counts from the chicken intestinal tract (Cawthraw & Newell, 2010; Layton et al., 2011; Shoaf-Sweeney et al., 2008; Sahin et al., 2003).

Because *C. jejuni* flagella have been studied extensively, we first selected flagellar proteins as targets for potential vaccine development. Bacterial flagella are organelles required for their movement towards or away from various environments (Berg, 2003; Macnab, 2003). Like *Escherichia coli* and *Salmonella* flagella, the flagellum of *C. jejuni* consists of three distinct structural regions (composed of more than 35 proteins): (i) the basal body anchored in the cytoplasm and inner membrane; (ii) the curved hook region extended from periplasmic space to the outer surface; and (iii) extracellular filaments (reviewed by Gilbreath et al., 2011; Lertsethtakarn et al., 2011). The major components of the extracellular filaments are synthesized as flagellin monomers, which require another protein, called hook-associated protein 2 or flagellar capping protein (encoded by the *fliD* gene), to cap the monomers into the filament at the distal end (Blair & Dutcher, 1992; Homma et al., 1984; Ikeda et al., 1987, 1993; Tasteyre et al., 2001b). The flagellar capping protein has been implicated as being involved with adherence and colonization of *Clostridium difficile* and *Helicobacter pylori* in the intestinal mucosa (Kim et al., 1999; Tasteyre et al., 2001a). However, although the *fliD* gene is found in the *C. jejuni* genome (Parkhill et al., 2000), the role of this gene product in *C. jejuni* pathogenesis has not been investigated.

**Abbreviations:** HRP, horseradish peroxidase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry. The GenBank/EMBL/DDBJ accession number for the *Campylobacter jejuni* fliD gene sequence determined in this study is KC618388.
In this report, we describe the expression and characterization of the *fliD* gene product of the *C. jejuni* D1-39 isolate. The deduced amino acid sequence of the FliD protein (with a calculated molecular mass of 69.7 kDa) from the nucleotide sequence was highly homologous to the flagellar capping protein of *C. jejuni*. In addition, the recombinant FliD protein was tested to see whether it was antigenic using broiler chicken sera.

**METHODS**

**Bacterial strains, culture conditions and genomic DNA isolation.** The *C. jejuni* D1-39 isolate from chicken faeces in Georgia, USA, was propagated in Mueller–Hinton agar plates at 42 °C for 48 h in a microaerobic environment (5 % O₂, 10 % CO₂ and 85 % N₂) as described previously (Hiett et al., 2008). Competent *E. coli* for propagation and expression of recombinant DNA were cultured according to the manufacturer’s instructions (Lucigen). *C. jejuni* genomic DNA was isolated using a DNeasy Blood & Tissue kit in a QIAcube automation system (Qiagen) according to the manufacturer’s instructions. The genomic DNA in 10 mM Tris/HCl (pH 8.0) was stored in a −80 °C freezer.

**Expression of *C. jejuni* FliD, flagellar capping protein, in the *E. coli* expression system.** The *fliD* gene was PCR amplified from the genomic DNA, ligated in a pPHam expression vector and transformed in competent *E. coli* cells (Lucigen) according to the manufacturer’s instructions, as described previously (Yeh et al., 2013). The nucleotide sequence between the insert and expression vector junctions of the recombinant gene was verified by DNA sequencing (see below) to have the start and stop codons at the 5′ and 3′ end junctions, respectively. In screening, several colonies were randomly selected for cultures in the absence and presence of 0.2 % L-rhamnose. The total cultures from both conditions were harvested and solubilized in 2 × Laemmli sample buffer (Bio-Rad Laboratories), and proteins were separated in 10–20 % SDS-PAGE gels (see below). The positive clones were stored in 20 % glycerol for further study. The primers used for PCR amplification were: *fliD*-F (5′-GAAGGAGATACTATATGGCATTTGATAGTGCTTATCGGATTT-3′) and *fliD*-R (5′-CTGATGTTGCTATGATCGATTGATAATGTTGTTGCGGATACATCATCATCAT-3′).

**Purification of recombinant FliD protein.** For large-scale production of FliD, 10 ml seed culture from an overnight culture of one colony was added to 500 ml Luria–Bertani broth containing kanamycin (30 μg ml⁻¹) and incubated at 37 °C. After the OD₆₀₀ reached 0.4, L-rhamnose was added to the culture at a final concentration of 0.2 %. After incubation at 37 °C for 16–18 h, the cells were harvested for protein purification by cobalt-chelated affinity chromatography, as described previously (Yeh & Klesius, 2011, 2012; Yeh et al., 2013). Briefly, the bacterial cells were collected by centrifugation. Cell pellets were resuspended in 10 mM Tris/HCl (pH 8.0), followed by homogenization in Lysing Matrix B beads (MP Biomedicals) with a FastPrep-24 instrument (MP Biomedicals) according to the manufacturer’s instructions. The crude lysates containing inclusion bodies were separated from the beads by low-speed centrifugation. The resulting lysates were centrifuged at 10 000 g for 30 min to harvest inclusion bodies. These inclusion bodies were dissolved in 10 mM Tris/HCl (pH 8.0) containing 6 M urea solution, followed by incubation with cobalt-chelated agarose beads (Thermo Scientific) on an end-over-end rotator at room temperature. After 1 h incubation, the beads were washed extensively with 10 mM Tris/HCl/urea solution containing 20 mM imidazole. The bound FliD protein on the beads was eluted with the same solution as for the washing step but containing 250 mM imidazole. The eluted protein was dialysed against a series of decreasing concentrations of urea in 10 mM Tris/HCl buffer (pH 8.0). The purified protein was stored at −80 °C.

**DNA sequencing and bioinformatics.** DNA sequencing in both directions was carried out at the US Department of Agriculture, Agricultural Research Service, Genomics and Bioinformatics Research Unit (Stoneville, MS, USA). Sequence chromatograms were edited, trimmed to remove vector sequence and analysed using the Phred (Ewing & Green, 1998; Ewing et al., 1998) and Lucy (Li & Chou, 2004) programs. Amino acid sequences were deduced from the nucleotide sequences using the ExPaSy Translate tool (Gasteiger et al., 2003). Sequence alignment was performed using MUSCLE (Edgar, 2004; http://www.ebi.ac.uk/Tools/mus/muscle/). Phylogenetic analysis was carried out using MEGA version 5.2.1 (Tamura et al., 2011) based on the MUSCLE alignment results.

**Collection of chicken sera.** Sera from broiler chickens older than 4 weeks of age were withdrawn from the wing vein. Blood was clotted by incubation at 37 °C for 1 h, followed at 4 °C overnight. Sera were collected by low-speed centrifugation, aliquoted and stored at −80 °C. All broilers for serum collection were clinically healthy, and their bacteriological status was not checked.

The experimental uses of broiler chickens were approved by the Institutional Animal Care and Use Committee, Richard B. Russell Agricultural Research Center, Agricultural Research Service, US Department of Agriculture, Athens, GA (IACUC no. PMSRU-08-2013-A).

**SDS-PAGE and immunoblotting.** Proteins were solubilized in 2 × Laemmlı sample buffer (Bio-Rad Laboratories) and separated in 10–20 % SDS/Tris/HCl polyacrylamide pre-cast gels (Bio-Rad Laboratories). After electrophoresis, the proteins were stained with Bio-Safe Coomassie G-250 stain (Bio-Rad Laboratories) according to the manufacturer’s instructions.

For immunoblotting, proteins in the SDS-PAGE gels were electro-transferred onto Immobilon membranes in Towbin buffer (Towbin et al., 1979). The membranes were blocked with a solution of 10 mM Tris/HCl (pH 8.0), 0.5 M NaCl, 0.5 % Tween 20 and 5 % skimmed milk at room temperature for 2 h, followed by incubation with broiler chicken sera (1:500 dilution). To detect serum IgG, the membranes were incubated with goat anti-chicken IgG antibody conjugated with horseradish peroxidase (HRP; KPL) and 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (KPL). The membranes were washed extensively after each antibody incubation. The 6 × His tag fused to the recombinant protein was detected with a Nickel HisDetector Western blot kit (KPL) according to the manufacturer’s instructions. Images were recorded and processed with an AlphaImager HP System (ProteinSimple) and its associated software.

**Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).** After electrophoresis, the protein bands corresponding to FliD were excised and sent to the Mass Spectrometry/Proteomics Shared Facility of the University of Alabama at Birmingham for identification by MALDI-TOF MS. The protein was digested in gel with trypsin as described previously (Wang et al., 2011).

**RESULTS AND DISCUSSION**

The flagellum of *C. jejuni* is an important factor for this micro-organism’s adherence and colonization to host cells (Grant et al., 1993; Hiett et al., 2008; Nachamkin et al., 1993). Previously, we cloned and expressed a battery of *Campylobacter jejuni* flagellar proteins in an *E. coli* expression system (Yeh et al., 2013). Among these, we
further studied the FliD (flagellar capping protein) protein, which is located at the distal end of the flagellin and is essential for assembly of the functional flagella (Blair & Dutcher, 1992; Kim et al., 1999; Tasteyre et al., 2001a). The fliD gene of the D1-39 isolate had 1929 nt, potentially encoding 642 aa with a calculated molecular mass of 69.6 kDa. When the deduced amino acid sequence of our isolate was compared with its counterpart from other campylobacters, we noted that the degree of conservation of the FliD proteins ranged from >95% (vs Campylobacter jejuni) to 77% (vs Campylobacter coli) to <40% (vs Campylobacter lari, Campylobacter showae, Campylobacter fetus subsp. fetus, Campylobacter curvus and Campylobacter concisus). Phylogenetic analysis showed that the FliD protein from our D1-39 isolate fell in the well-segregated clade of C. jejuni (Fig. 1). It is well established that Campylobacter flagella undergo post-translational glycosylation, which may contribute to antigen specificity (Guerry et al., 2006; Logan, 2006; Ewing et al., 2009; Howard et al., 2009). The deduced FliD amino acid sequence of C. jejuni D1-39

![Phylogenetic analysis of C. jejuni FliD proteins](image-url)

**Fig. 1.** Phylogenetic analysis of C. jejuni FliD proteins. Amino acid sequences were retrieved from GenBank and aligned with MUSCLE (Edgar, 2004). The maximum-likelihood method in MEGA version 5.2.1 (Tamura et al., 2011) was carried out to infer the phylogenetic tree. The GenBank or NCBI Protein sequences of the FliD proteins used for this analysis are as follows: FliD G52, KC618388; C. jejuni subsp. jejuni LMG 23210, WP_002935293; C. jejuni subsp. jejuni, CP001876; C. jejuni subsp. jejuni 81-176, ZP_00270961; C. jejuni subsp. jejuni NCTC 11168, YP_002343979; C. jejuni subsp. jejuni 81116, YP_001482085; C. jejuni subsp. jejuni RM1221, YP_178667; Campylobacter coli Z163, EIA42544; Campylobacter upsaliensis RM3195, ZP_00370083; Campylobacter lari RM2100, YP_002575342; Campylobacter fetus subsp. fetus 82-40, YP_891302; Campylobacter showae, EKU10495; Campylobacter concisus 13826, YP_001466708; Campylobacter curvus 525.92, YP_001408376; Clostridium difficile QCD-32g58, ZP_01232795; Escherichia coli B7A, ZP_00715153; Helicobacter pylori Shi470, NC_010698; Helicobacter pylori 98-10, ZP_03439182; Helicobacter pylori B128, ZP_03437654; Helicobacter pylori 52, CP001680; Pseudomonas aeruginosa PAO1, NC_002516; Salmonella enterica subsp. enterica serovar Saintpaul str., ZP_02346581; Vibrio alginolyticus 12G01, ZP_01258802; Vibrio cholerae MO10, ZP_00759609; Vibrio harveyi ATCC BAA-1116, NC_009783. Bar, amino acid substitutions per site.
was subjected to analysis by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) to predict whether it had potential N-glycosylation sites. This protein had nine potential N-glycosylation sites at Asn\textsuperscript{43}, Asn\textsuperscript{104}, Asn\textsuperscript{131}, Asn\textsuperscript{132}, Asn\textsuperscript{287}, Asn\textsuperscript{417}, Asn\textsuperscript{514}, Asn\textsuperscript{521} and Asn\textsuperscript{548}. However, it seems that the consensus sequon for bacterial N-glycosylation requires a negatively charged side chain at position \(-2\) upstream of the Asn site (Kowarik \textit{et al.}, 2006). The sequence, therefore, is extended at the N-terminus to (D/E)XNX(S/T), where X is any amino acid but not proline (Scott \textit{et al.}, 2011). Based on this information, we re-examined the Flid amino acid sequence, and found three potential N-glycosylation sites at Asn\textsuperscript{43}, Asn\textsuperscript{152} and Asn\textsuperscript{548}. In addition, O-glycosylation is also present in bacteria (Abu-Qarn \textit{et al.}, 2008; Lees-Miller \textit{et al.}, 2013). Analysis of the Flid amino acid sequence by the NetOGlyc 4.0 program (http://www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft \textit{et al.}, 2013) revealed seven potential O-glycosylation sites at Thr\textsuperscript{44}, Thr\textsuperscript{232}, Ser\textsuperscript{233}, Ser\textsuperscript{234}, Ser\textsuperscript{250}, Thr\textsuperscript{325} and Thr\textsuperscript{597}. The flagellar proteins including Flid were not identified in the N-linked glycosylation study reported by Scott \textit{et al.} (2011). This may be due to the fact that glycosylation in \textit{C. jejuni} flagella is O-linked (Thibault \textit{et al.}, 2001). Whether the Flid capping protein is N- or O-linked needs further investigation.

To characterize Flid, it was expressed in and purified from recombinant \textit{E. coli} cells. As depicted in Fig. 2(a), the recombinant Flid protein was overexpressed in the presence of 0.2% of L-rhamnose (Fig. 2a, lane U vs lane I), and had a relative mobility of relevant size and position in the SDS-PAGE. Because a \(6\times\) His tag sequence was added during the expression vector construction, the Flid protein was purified with cobalt-chelating resins to high homogeneity (Fig. 2a, lanes E1–E3). To further confirm the purified Flid protein fused with a \(6\times\) His tag, this protein was electrotransferred to Immobilon membranes. As illustrated in Fig. 2(b), the Flid protein was one major dense band detected by nickel-nitritolriacetic acid conjugated to HRP on the blot, suggesting that this purified recombinant Flid is a fusion protein. Furthermore, to ensure that the band was the Flid protein, the band was excised from the Coomassie-stained SDS-PAGE gels and subjected to MALDI-TOF/TOF analysis. The \textit{MASCOT} score was 415, and the MS spectra of the peptide fragments with about 16% coverage of the sequence were identified as the \textit{C. jejuni} Flid protein after searching in the deposited database (Fig. 3). Together with the results of SDS-PAGE analysis, His tag detection and MALDI-TOF analysis, we concluded that this overexpressed recombinant protein is the \textit{C. jejuni} Flid protein.

Because the Flid protein locates at the tip of the flagellum, we wanted to determine whether the Flid protein was antigenic for broiler chickens. Sera from 18 broilers older than 4 weeks were collected and evaluated by immunoblot assay. All broiler sera reacted strongly to the Flid protein, indicating that this anti-Flid antibody may be prevalent in the poultry population (Fig. 4). This may be due to broiler chickens naturally harbouring this micro-organism and the Flid protein being expressed during colonization in the chicken caeca. Because the chickens were older than 4 weeks, we ruled out the possibility of maternal antibodies, which diminish within 2 weeks of age (Sahin \textit{et al.}, 2001; Wyszynska \textit{et al.}, 2004), being involved in the reaction to this protein. As the \textit{Campylobacter} status of the aforementioned broilers was undetermined, attempts were made to test broilers with no prior \textit{Campylobacter} exposure (theoretically \textit{Campylobacter}-negative sera). Specific-pathogen-free chickens were raised according to common industrial standards, tested and determined to be culture negative; however, further testing of their faeces using real-time PCR (Meinersmann \textit{et al.}, 1997) resulted in an amplicon, suggesting that the chickens had been exposed previously to \textit{Campylobacter}. As depicted in Fig. 5, the serum from a culture-negative chicken reacted strongly to the Flid protein (Fig. 5, lane 1). Several negative controls such as no serum, no HRP-conjugated anti-chicken antibody or no serum and anti-chicken antibody in the reactions revealed no band on the blot (Fig. 5, lanes 2–4, respectively). These results indicated that \textit{Campylobacter} antibody was present in this
culture-negative (PCR-positive) broiler, possibly as a result of the numbers of this micro-organism being at levels below the limit of detection using culture methods, colonization with ‘viable non-culturable’ Campylobacter (de Boer et al., 2013; Bullman et al., 2012; Rollins & Colwell, 1986; Yang et al., 2004) or previous exposure to Campylobacter (Hiett et al., 2013).

Because our analysis of the FliD proteins from various micro-organisms showed that the C. jejuni FliD amino acid sequence shared 26 and 27% identity with those of Escherichia coli and Salmonella, respectively, the possibility that broiler antibody to this recombinant FliD protein may have been elicited by other micro-organisms cannot be ruled out. Further studies on broiler immune response to this protein are undergoing.

Although previous studies have demonstrated that a battery of whole-cell lysate proteins of C. jejuni were identified by chicken maternal antibodies (Sahin et al., 2001; Shoaf-Sweeney et al., 2008), this extracellular FliD protein was not detected by the maternal antibodies for several possible

**Fig. 3.** Analysis of the recombinant C. jejuni FliD protein by MS peptide mapping and sequencing analysis. The protein band stained with Coomassie blue was excised from the SDS-PAGE gel and digested with trypsin. The resulting fragments were concentrated and eluted onto an anchor chip target for analysis on a Bruker Autoflex III MALDI-TOF/TOF instrument. The MS spectral data were used to search against the protein databases using the MASCOT software. The MASCOT score was 415, and the mass spectra of these peptide fragments with 16% coverage of the sequence were identified as C. jejuni FliD protein. The matched peptides corresponding to the amino acid sequence are indicated in bold red italic (GenBank accession no. KC618388).

**Fig. 4.** Reactivities of broiler chicken sera to the purified recombinant C. jejuni FliD protein analysed by immunoblotting. After SDS-PAGE, the protein was transferred to PVDF membranes, followed by probing with broiler chicken sera. Lanes: 1–4, sera from four different chickens; M, molecular mass markers (see Fig. 2).

**Fig. 5.** Reactivities of broiler chicken sera to purified recombinant C. jejuni FliD protein analysed by immunoblotting. After SDS-PAGE, the protein was transferred to PVDF membranes, followed by probing with broiler chicken sera. Lanes: 1, serum from a culture-negative, PCR-positive chicken with HRP-conjugated anti-chicken antibody; 2, chicken serum but no anti-chicken antibody; 3, anti-chicken antibody but no chicken serum; 4, no chicken serum or anti-chicken antibody; 5, serum from a broiler with unknown bacteriological status; M, molecular mass markers (see Fig. 2).
reasons. First, the stoichiometry of FliD is about five, which is relatively small compared with 20,000 filaments per flagellum (Berg, 2003; Macnab, 2003). Therefore, the amount of FliD protein in the lysate preparation may be too low to be detected by the antibodies or may produce a faint reactive band that is difficult to detect. Second, it is possible that the C. jejuni S3B strain used in the study of Shoaef-Sweeney et al. (2008) might not contain the fliD gene, and therefore no FliD protein is produced to stimulate an immune response in chickens. Many studies have demonstrated that humoral immunity on the mucosal surface in the intestine prevents C. jejuni colonization in chicken caeca (Buckley et al., 2010; Caithraw & Newell, 2010; Clark et al., 2012; Layton et al., 2011; Stern et al., 1990; Wyszynska et al., 2004). The results in this study demonstrated that all chickens tested had anti-FliD antibodies in their sera. Whether this antigen elicits mucosal responses in chicken intestinal tracts that may potentially be protective is yet to be determined.

In summary, the fliD gene of C. jejuni was successfully amplified and expressed in an E. coli expression system and the recombinant FliD protein was purified to homogeneity. This recombinant protein was confirmed by SDS-PAGE analysis, His tag detection and MALDI-TOF MS analysis. Sera from broiler chickens were used to test the antigenicity of the recombinant FliD protein, and the results of antibody studies suggested that anti-FliD antibody may be prevalent in the poultry population. These results also provide a rationale for further evaluation of this protein as a vaccine target for chickens and as a tool for investigating host–C. jejuni interactions.

ACKNOWLEDGEMENTS

We are grateful to Susan Q. Brooks, Johnna K. Garrish and Latoya T. Wiggins of Poultry Microbiological Safety Research Unit, Agricultural Research Service, US Department of Agriculture, Athens, GA, USA, for the excellent technical support. We also thank Dr Brian E. Scheffler and his Bioinformatics Group at the USDA ARS Genomics and Bioinformatics Research Unit in Stoneville, MS, USA, for sequencing and bioinformatics, and Dr James A. Mobley and his team at the Bioanalytical and Mass Spectrometry Shared Facility of the University of Alabama at Birmingham, AL, USA, for MALDI-TOF analysis. We are grateful to Dr Holly Sellers of Poultry Diagnostic & Research Center of the University of Georgia, GA, USA, for chicken sera. This study was supported by the USDA Agricultural Research Service CRIS project no. 6612-32000-060-00, and the US Poultry & Egg Association project no. 679. Mention of trade names or employer.

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