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Short communication

Construction, expression, purification and antigenicity of recombinant *Campylobacter jejuni* flagellar proteins

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ABSTRACT

Campylobacter jejuni, a flagellated, spiral-rod Gram-negative bacterium, is the leading etiologic agent of human acute bacterial gastroenteritis worldwide. The source of this microorganism for human infection has been implicated as consumption and handling of poultry meat where this microorganism is a commensal in the gut. Because the genomes of many *C. jejuni* isolates have been sequenced, our ultimate goal is to develop protein arrays for exploring this microorganism and host interactions. In this communication, we report cloning, expression and purification of *C. jejuni* flagellar proteins in a bacterial expression system. Twelve recombinant proteins were purified, which were confirmed by SDS–PAGE analysis and a His tag detection kit. The FlgE1, FlgG, FlgK, FliE, FlgH/FliH and FlaA recombinant proteins were further confirmed by LC–ESI-MS/MS. The purified recombinant proteins were tested whether they were immunogenic using antibodies from several sources. BacTrace anti-*Campylobacter* species antibody reacted to the FlaA recombinant protein, but not others. Rabbit anti-MOMP1 peptide antibody reacted strongly to FliE and weakly to FlaA, but not others. Rabbit anti-MOMP2 peptide antibody reacted strongly to the FlaA, FliG, FliE, FlhF, FlgG, FlgE1 and FliD recombinant proteins, less to FlgK and FlgH/FliH, and did not react to the FliY, FliS and FliH recombinant proteins. These antibody studies suggest that these recombinant flagellar proteins have potential for novel targets for vaccine development. It is also anticipated that these recombinant proteins provide us a very useful tool for investigating host immune response to *C. jejuni*.

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1. Introduction

Campylobacter jejuni is the leading reported food-borne bacterium that causes acute bacterial gastroenteritis in humans worldwide (Altekruse et al. 1999; Janssen et al. 2008; Kubota et al. 2011). In the United States, it is estimated that more than two million cases of human campylobacteriosis and more than eight thousand hospitalizations occur annually (Scallan et al. 2011). The clinical symptoms of human campylobacteriosis range from mild watery diarrhea, nausea and abdominal pain to severe Guillain–Barré syndrome and reactive arthritis (Allos 1997; Hughes and Cornblath 2005; Peterson 1994; Hannu et al. 2002; Humphrey et al. 2007). The main source of this microorganism for human infection has been linked to consumption and handling of poultry where *Campylobacter* is a commensal in the gut (Hermans et al. 2012; European Food Safety Authority 2010). Strategies for reducing abundance and prevalence of *Campylobacter* in poultry flocks to prevent contamination of human food supplies are in great demand (Lin 2009; Hermans et al. 2011).

C. jejuni is a Gram-negative, microaerophilic, thermophilic, spiral-rod bacterium with one or both polar flagella (Ryan et al. 2004; Ursing et al. 1994). The genomes of many *C. jejuni* isolates have been sequenced and are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genome). This information provides us with many opportunities to explore this microorganism in every aspect (Cliften 2004; Young 2001). Recently, an ORF expression library of *C. jejuni* has been constructed (Parrish et al. 2004), which has been used for identifying proteins for host interactions and virulence (Nielsen et al. 2012). Our ultimate goal is to develop the *C. jejuni* protein array, which enables us to monitor chicken immune responses, discover novel protein antigens, study protein–protein interactions, etc. (e.g. Burbelo et al. 2010; Kunnath-Velayudhan et al. 2010; Vigil et al. 2010).

We first selected flagellar proteins for expression and development of the protein array, because *C. jejuni* flagella have been extensively studied (Gilbreath et al. 2011; Lertsethtakarn et al. 2011, for review). In addition, the flagellar proteins from *Pseudomonas aeruginosa* have been used as a vaccine in the clinical trials for cystic fibrosis patients. The results showed the flagellar vaccine

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lowers the risk for infection with *P. aeruginosa* and contributes to a longer survival of patients (Döring et al. 2007).

Flagella are organelles for bacterial cell movement toward or away from various environmental conditions for their survival (Berg 2003; Macnab 2003). Synthesis of bacterial functional flagella requires strict coordination in hierarchy at both transcriptional and translational expression of more than 35 flagellar genes and their gene products (Berg 2003; Macnab 2003; Gilbreath et al. 2011; Lertsethtakarn et al. 2011). The structure of the bacterial flagella is very complex made up of (1) the basal body embedded in the cytoplasm and inner membrane, (2) the hook extended from periplasmic space to the outer surface, and (3) extracellular filament (Berg 2003; Macnab 2003; Gilbreath et al. 2011; Lertsethtakarn et al. 2011). In addition, the *Campylobacter* flagella undergo post-translational glycosylation, a process important for mediating filament–filament interactions, colonizing the microorganisms in hosts, and contributing to antigen specificity (Guerry et al. 2006; Logan 2006; Ewing et al. 2009; Howard et al. 2009).

In this communication, we report amplification, expression, and purification of *C. jejuni* flagellar proteins from a bacterial expression system. These recombinant proteins were also tested whether they were immunogenic using sera from several sources.

2. Materials and methods

2.1. Bacteria and growth conditions

C. jejuni D1-39, isolated from chicken feces in Georgia, USA, was propagated in Mueller–Hinton agar plates at 42 °C for 48 h in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) as described previously (Hiett et al. 2008). Competent *Escherichia cloni* 10G cells (Lucigen Corp., Middleton, WI) for propagation and expression of plasmid DNA were cultured according to the manufacturer's instructions.

2.2. Genomic DNA isolation

C. jejuni genomic DNA was isolated using a DNeasy Blood & Tissue kit in a QIAcube automation system (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The quality and yield of genomic DNA were determined by agarose gel electrophoresis and spectrophotometry, respectively. The genomic DNA in 10 mM Tris–HCl (pH 8.0) was stored at −80 °C.

2.3. PCR amplification and construction of C. jejuni flagellar protein expression plasmids

Flagellar genes were PCR amplified as previously described (Yeh and Klesius 2011). Briefly, 50 μl/reaction of the PCR mixtures contained (in final concentrations): 1× Hi-Fi reaction buffer (BIOLINE USA, Inc., Taunton, MA), 250 μM/each of dNTP (New England BioLabs, Inc., Ipswich, MA), 400 nM/each of forward and reverse primers, 1 U of proofreading Velocity™ DNA polymerase (BIOLINE USA, Inc.) and 150 ng of genomic DNA template. The amplification was carried out on a PTC-200 Peltier thermal cycler (MJ Research, Inc., Waltham, MA) with the following parameters: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min, and final extension at 72 °C for 10 min. The amplified products were examined by electrophoresis on 2% agarose gels.

The gene-specific oligonucleotide primers for PCR amplification were designed with complementarities to both ends of each open reading frame, but without the start and stop codon sequences at 5′- and 3′-end, respectively. The oligonucleotide primers were synthesized by Sigma–Aldrich Co. (The Woodlands, TX), and are listed in Table 1.

To construct expression vectors, the *Expresso*® Rhamnose Cloning and Protein Expression kit (Lucigen Corp.) was used according to the manufacturer's instructions.

2.4. DNA sequencing and bioinformatic analysis

To confirm the flagellar genes were inserted in the right direction, at least five colonies from each gene were randomly picked and grown in WU medium (http://www.plantgenetics.iastate.edu/protocols/plasmid_isolation.pdf) for DNA sequencing. DNA sequencing reactions in both strands were carried out at the USDA ARS MidSouth Genomics and Bioinformatics Research Unit (Stoneville, MS) with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) or Retrogen, Inc. (San Diego, CA). The pRham™ Forward and pETite Reverse primers provided in the kit were used in the DNA sequencing reactions. Sequence chromatograms were edited for quality and trimmed to remove vector sequences using Phred (Ewing and Green 1998; Ewing et al. 1998) and Lucy (Li and Chou 2004). Phylogenetic analysis of the flagellar genes was carried out using the maximum-likelihood phylogenetic reconstructions with PhyML (Guindon et al. 2009) based on the results of amino acid sequences aligned with MUSCLE (Edgar 2004) where default parameters were used. Sequences for comparison were retrieved by sequence similarity searches using BLASTp against the Integrated Microbial Genomes (IMG) database (Mavromatis et al. 2009).

2.5. Expression of C. jejuni flagellar proteins in E. cloni 10G cells

In the preliminary screening, at least three colonies containing in-frame flagellar gene inserts were selected to evaluate in small scales whether they were able to be induced to express recombinant proteins in the presence of 0.2% L-rhamnose. After 4–5 h induction, the cultures were solubilized in 2× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), and proteins were separated in 10–20% SDS–PAGE gels (see below). After identification of the positive clones, the *E. cloni* 10G cells were stored in LB-kanamycin (30 μg/ml) broth supplemented with 20% glycerol at −80 °C.

For large scale production of recombinant proteins, 10 ml of seed culture from one colony overnight culture were added into 500 ml LB-kanamycin (30 μg/ml) broth, which was incubated at 36 °C until OD_{600nm} reached 0.4. L-Rhamnose was added to the culture in a final concentration of 0.2% (w/v), and the culture was incubated at 36 °C. After 16–18 h, the cells were harvested for recombinant protein purification by nickel-chelated affinity chromatography.

2.6. Purification of recombinant flagellar proteins

The *E. cloni* 10G cells after induction were harvested by centrifugation at 5000 × *g* for 20 min. The cells were resuspended in Tris–HCl buffer (pH 8.0) containing 0.5 mg/ml lysozyme (Sigma–Aldrich Company, St. Louis, MO) and 250 units/reaction of Benzonase® (Sigma–Aldrich), and were subjected to homogenization by sonication. The lysates were spun at 10,000 × *g* for 20 min. The pellets were solubilized in a Tris–HCl–6 M urea solution, and were subjected to homogenization by sonication. The mixtures were centrifuged as above. The supernatants were collected and incubated with nickel-chelated agarose beads (Thermo Scientific, Rockford, IL) at room temperature with mild rocking. After 1 h, the beads were extensively washed with a Tris–HCl–6 M urea–20 mM imidazole solution (wash solution) to remove non-specific proteins. The recombinant flagellar proteins that bound to the nickel beads were eluted with the wash solution, but in the presence of 250 mM imidazole. The eluates were further dialyzed against a series of decreasing concentrations of urea. The purity of the