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SHORT COMMUNICATION

Characterization and reactivity of broiler chicken sera to selected recombinant *Campylobacter jejuni* chemotactic proteins

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Abstract *Campylobacter jejuni*, a Gram-negative rod bacterium, is the leading causative agent of human acute bacterial gastroenteritis worldwide. Consumption and handling of raw or undercooked poultry are regarded as a major source for human infection. Because bacterial chemotaxis guides microorganisms to colonization and invasion in the host cells, proteins involved in chemotactic processes can be novel targets for vaccine development. In this communication, we report amplification, cloning and expression of the *C. jejuni* chemotactic proteins in an *Escherichia coli* expression system. A total of 15 chemotactic protein genes were successfully expressed. These recombinant proteins were confirmed by nucleotide sequencing, SDS-PAGE analysis and immunoblot analysis of six-His and hemagglutinin tags. Twelve recombinant chemotactic proteins were further tested whether they were antigenic using sera from broiler chickens older than 4 weeks. The immunoblot results show that each chicken serum reacted to a variety of the recombinant proteins, but all sera reacted to the Cjj0473 gene product (annotated as a methyl-accepting chemotaxis protein), suggesting that anti-*Campylobacter* antibodies may be prevalent in the poultry population. These antibody screening results provide a rationale for further evaluation of the Cjj0473 protein as a potential vaccine for broilers to improve human food safety.

Keywords *Campylobacter jejuni* · Poultry · Chemotaxis · ε-Proteobacteria · Foodborne pathogen · Zoonoses · Methyl-accepting chemotaxis proteins

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Introduction

Campylobacter jejuni, a Gram-negative flagellated, curve-rod bacterium, is an etiological agent of human campylobacteriosis worldwide (Janssen et al. 2008; Kubota et al. 2011; Zilbauer et al. 2008; Lawes et al. 2012). The clinical symptoms range from mild watery diarrhea, nausea and abdominal pain to severe Guillain–Barré syndrome and reactive arthritis (Hughes and Cornblath 2005; Hannu et al. 2002; Humphrey et al. 2007; Moore et al. 2005). Human infection is often associated with consumption and handling of raw or undercooked poultry where this microorganism is a commensal in the chicken gastrointestinal microbiota (Hermans et al. 2012; European Food Safety Authority 2010). Therefore, strategies for reducing abundance and prevalence of *Campylobacter* in poultry flocks to prevent contamination of human food supplies have been intensively investigated (Lin 2009; Hermans et al. 2011). Vaccination of broiler chickens with components of *C. jejuni* is an attractive approach. Because bacterial chemotaxis can guide bacteria to colonization sites and invasion in the host cells (Chang and Miller 2006; Hendrixson and DiRita 2004; Hartley-Tassell et al. 2010), proteins involved in chemotaxis can be targets for vaccine development.

Bacterial chemotaxis involves a cascade of signal transduction and needs a battery of proteins to carry out this process. The major components of this chemosensory

system include chemoreceptors [also called methyl-accepting chemotaxis proteins (MCPs)], the CheA kinase, the CheY response regulator and the CheW coupling protein (Wuichet and Zhulin 2010; Lertsethtakarn et al. 2011). *C. jejuni* has 10 chemoreceptors of which seven are as integral membrane proteins and three are as soluble forms (Lertsethtakarn et al. 2011; Marchant et al. 2002). Among MCPs, the CetA and CetB chemosensory proteins work together to detect pyruvate and fumarate (Elliott and DiRita 2008; Hendrixson et al. 2001). In addition, Hartley-Tassell et al. (2010) identified the Tlp1 chemoreceptor (Cj1506c) for aspartate. Tlp1 is universally conserved among isolates regardless of the isolation source (Day et al. 2012). The Cj0019c/DocB and Cj0262c chemosensory proteins play roles in *C. jejuni* colonization of chicken ceca (Hendrixson and DiRita 2004).

The canonical CheA kinase is a dimer, and each subunit has five separate functional domains (P1–P5) (Bilwes et al. 1999; Mourey et al. 2001; McEvoy et al. 1996; Wuichet et al. 2007). However, *C. jejuni* does not have the P2 domain and has an additional REC domain at the carboxyl terminus (Lertsethtakarn et al. 2011; Marchant et al. 2002). After the CheA kinase phosphorylates the CheY response regulator, the activated CheY interacts with the FliM and FliN proteins and subsequently regulates the flagellar motor rotation (Wadhams and Armitage 2004; Alex and Simon 1994; Hess et al. 1988). The coupling protein CheW is a cytoplasmic protein with two β-sheet domains (Griswold and Dahlquist 2002; Vu et al. 2012). This protein interacts with both the chemoreceptor and the CheA P5 domain that the kinase activity of the latter is modulated (Boukhvalova et al. 2002; Vu et al. 2012).

In this report, the chemotactic genes of *C. jejuni* D1–39 were amplified, cloned, and their gene products expressed in *E. coli*. These recombinant proteins were also assayed as to whether they were antigenic using broiler chicken sera.

Materials and methods

Bacteria, growth conditions and genomic DNA isolation

Campylobacter 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 condition (5 % O_2, 10 % CO_2 and 85 % N_2) as described previously (Hiett et al. 2008). Competent HI-Control™ 10G and BL21 (DE3) cells (Lucigen Corp., Middleton, WI) for propagation of plasmid DNA and expression of recombinant proteins, respectively, were cultured according to the manufacturer's instructions. *C. jejuni* genomic DNA was isolated using a DNeasy Blood and 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.

PCR amplification and construction of *C. jejuni* chemotactic protein expression plasmids

Chemotactic gene sequences were downloaded from GenBank as references for designing oligonucleotide primers, which were complementary to both ends of each open reading frame, but without the start and stop codon sequences at 5′- and 3′-end, respectively. In order to facilitate the downstream cloning, purification and quality control, a six-His-tag sequence (5′-CATCATCACCACCATCAC-3′) at 5′-end and a hemagglutinin (HA) tag sequence (5′-TGCATAATCTGGAACATCATATGGATA-3′) at 3′-end were included in the forward and reverse primers, respectively. The oligonucleotide primers were synthesized by Sigma-Aldrich Co. (The Woodlands, TX) and are listed in Table 1. These genes were PCR amplified from the genomic DNA of the *C. jejuni* D1–39 isolate as previously described (Yeh et al. 2013). The amplified products were examined by electrophoresis in 2 % agarose gels. To construct expression vectors, the *Expresso*® T7 Cloning and Expression System kit (Lucigen Corp.) was used according to the manufacturer's instructions.

DNA sequencing and bioinformatic analysis

To confirm that the chemotactic genes were inserted in the appropriate direction for expression, at least five colonies from each gene were randomly picked for DNA sequencing as previously described (Yeh et al. 2013). The pETite T7 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 chemotactic genes was carried out using the maximum likelihood phylogenetic reconstructions with MEGA version 5.10 (Tamura et al. 2011) based on the results of amino acid sequences aligned with MUSCLE (Edgar 2004) with default parameters. Sequences for comparison were retrieved by sequence similarity searches using BLASTp against the GenBank database (Schäffer et al. 2001).

Expression of *C. jejuni* chemotactic proteins in HI-control™ BL21 (DE3) cells

The plasmids harboring desired inserts were purified from HI-control™ 10G cells and transformed into competent

Table 1 Oligonucleotide primers used for PCR amplification of *Campylobacter jejuni* D1–39 chemotactic genes in this study

Primer	Sequence (5′ → 3′)
cheA-F	catcatcaccaccatcac GAAGATATGCAAGAAATACTTGAAGACTTT
cheA-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TCCTAGTTTCAAATTTTTTCTAACTAC
cheB-F	catcatcaccaccatcac AAGCTCATACTCATAGGATCTTCAACAGGT
cheB-R	gtggcggccgctctatta tgcataatctggaacatcatatggata ATCCTGATCAATAAAATTTACAATTTC
cheR-F	catcatcaccaccatcac GAAAAAAAAATAACTCCTAGCGAATTAGAA
cheR-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TACTTTTTCATAGTAAACACCTCTTGG
cheV-F	catcatcaccaccatcac TTTGATGAAAATATCGTGAAAACGGGTTCA
cheV-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CCCCTGTTCTTGAGATTGATGTTTTTT
cheW-F	catcatcaccaccatcac AGTAATGAAAAATTAGAGCAAATTTTGCAA
cheW-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAATTCGCGCTTAAGTAAAGCTTCTAC
cjj0046-F	catcatcaccaccatcac TTGATAAAATTTATTATTTTATCTACACTA
cjj0046-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CTGAAAGCTACTTAATTTTTCGGAGAG
cjj0180-F	catcatcaccaccatcac AAAAGCGTAAAATTGAAGGTTTCACTGATT
cjj0180-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAACCTTTTCTTCTTAACATCTTCTAA
cjj0289-F	catcatcaccaccatcac CAATCAATAAATTCAGGCAAATCCGTTGGA
cjj0289-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAACCTTTTCTTCTTAACATCTTCTAA
cjj0473-F	catcatcaccaccatcac TTTGGAAGTAAAATAAACCATTCTGATCTT
cjj0473-R	gtggcggccgctctatta tgcataatctggaacatcatatggata ATGATCTGACTCATCAAGCATTTCTTT
cjj0706-F	catcatcaccaccatcac AATAAAGCTTTTACTCTGCTTGAGCTTGTT
cjj0706-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TATCAGATCTTTGCATCTAGAACTTCC
cjj0842-F	catcatcaccaccatcac ATATTTTTCATAATAATTCTAGGGGCTTGT
cjj0842-R	gtggcggccgctctatta tgcataatctggaacatcatatggata GAATAATCCTAAGTTTTTATAAAATAA
cjj0975-F	catcatcaccaccatcac TTTAAAACTATAGGATTTAAGGTTTCTGCT
cjj0975-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AATTTGAAATTGGTTAAGTTCGCTTTC
cjj1128-F	catcatcaccaccatcac TTTGGTGCTAAGAAAAAATAATACTGAAATA
cjj1128-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TGACATCGCTTTAGCAACTTCAGCAGA
cjj1205-F	catcatcaccaccatcac GTAAAAAAATGGATTATTGTTAACTATTTTT
cjj1205-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TATTTTTAATTTTGCTAAGATTTCAGC
cjj1498-F	catcatcaccaccatcac TTTAAATCTTTAAATATAGGTTTAAAACTT
cjj1498-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAATCTTTTTTTACTCACATCTTCAAG
cjj1549-F	catcatcaccaccatcac AATGCTATTCATAAAATTTTTGAAGAATAC
cjj1549-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAACCTCTTCTTCTTAACATCTTCTAA
ctsD-F	catcatcaccaccatcac ATAAGATTAATATTAATTAACATTCTTTTT
ctsD-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CTCATAATGCTTAAATCCTAGATCTTT
ctsE-F	catcatcaccaccatcac GAAAGTAGAATGGATAAAATTTTTCAAGCT
ctsE-R	gtggcggccgctctatta tgcataatctggaacatcatatggata TCTTACAACCCCTTAAAAGCTCATCTAT
ctsF-F	catcatcaccaccatcac AAATTTTATGAAGTAGAATTTCTTAAAAAC
ctsF-R	gtggcggccgctctatta tgcataatctggaacatcatatggata AAAATTGACCCCCGAACTAAGCTCCCA
ctsG-F	catcatcaccaccatcac AGAAAAGCTTTTACTATATTAGAACTTGTT
ctsG-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CATTCCCCCTATTAAAAGATAAGTTTT
ctsP-F	catcatcaccaccatcac AGTAAAATTATTCCATTTAGAGAAGAAATT
ctsP-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CCTTAATAAGCCATTCTCTAAAGCACT
ctsX-F	catcatcaccaccatcac CAAGAAAGAATTAAAGAGCTTGAGCTTAGG
ctsX-R	gtggcggccgctctatta tgcataatctggaacatcatatggata CTTTCCATCTAATTCCATTAAACCATA

A six-His-tag nucleotide sequence was included in each forward (-F) primer, while an HA tag nucleotide sequence was added in each reverse (-R) primer

HI-control™ BL21 (DE3) cells (Lucigen Corp.). In the preliminary screening, at least four colonies were randomly selected to evaluate whether they were able to produce recombinant chemotactic proteins in small scales in the presence of 1 mM of isopropyl β-*d*-1-thiogalactopyranoside (IPTG). After 4–5 h induction, 100 µl of the cultures were solubilized in an equal volume of 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 corresponding *E. coli* BL21 (DE3) cells were stored in LB-kanamycin (30 μg/ml) broth supplemented with 20 % glycerol at −80 °C.

SDS-PAGE and immunoblot

Protein samples were solubilized in 2× Laemmli sample buffer (Bio-Rad Laboratories) and denatured for 5 min at 95 °C. Electrophoresis was carried out on 10–20 % SDS–Tris–HCl-polyacrylamide gels (Bio-Rad Laboratories) (Hames 1990). After separation, proteins in the gels were stained with Bio-Safe Coomassie G-250 Stain™ (Bio-Rad Laboratories) for 1 h at room temperature, followed by washing with double distilled water.

For immunoblot, proteins in the SDS-PAGE gels were electrotransferred into Immobilon membranes (Bio-Rad Laboratories) (Gershoni 1988). The membranes were incubated with appropriate primary antibodies, followed by appropriate peroxidase-conjugated secondary antibodies and TMB peroxidase substrate (KLP, Inc., Gaithersburg, MD). The six-His tag that was fused to the recombinant chemotactic proteins in the membranes was detected using a Nickel His Detection kit (KPL, Inc.) according to the manufacturer's instructions. Images were documented and processed by an AlphaImager HP System (ProteinSimple™, Santa Clara, CA) and its associated software.

Antibodies

Sera from broiler chickens 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. BacTrace anti-*Campylobacter* species antibody, which was purified pooled sera from goat immunized with heat-killed whole cells of various *Campylobacter* strains and recognizes 22 *Campylobacter* strains, was purchased from KPL. Goat anti-chicken IgG antibody conjugated to horseradish peroxidase was purchased from KPL, Inc.

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, U.S. Department of Agriculture, Athens, GA (IACUC no. PMSRU-08-2013-A).

Nucleotide sequences

Nucleotide sequences of the chemotactic genes have been submitted to the GenBank database and assigned the accession numbers KC692481–KC692494.

Results and discussion

After searching the genome sequence of *C. jejuni* subsp. *jejuni* 81-176 (GenBank accession no.: CP000538), 22 genes potentially involved in the chemotactic system were identified, including many MCPs. Except for the Cjj0180 gene, 21 chemotactic protein genes were successfully PCR amplified and ligated into the expression vector pETite. The sequences of the gene inserts and pETite vector junctions of the recombinant plasmids were verified by DNA sequencing in both directions. The results show that (1) all nucleotide sequences were in-frame and (2) all expression frames contained start and stop codons at the 5′- and 3′-end junctions, respectively. Further, based on the BLASTing against the GenBank database, both the nucleotide and amino acid sequences of the chemotactic protein genes from our D1–39 isolate were highly homologous to those of other *C. jejuni* deposited in GenBank.

The recombinant pETite plasmids containing the chemotactic protein genes were purified and transformed in *Escherichia coli* BL21 (DE3) competent cells, followed by induction with 1 mM of IPTG. A total of 15 *C. jejuni* chemotactic protein genes were successfully over-expressed in *E. coli* after IPTG induction, and each recombinant protein had its relative mobility of relevant sizes and positions in the SDS-PAGE. Here depicted in Fig. 1, we showed six (*cheA*, *cheB*, *cheR*, *cheV*, *cheW* and *Cjj1549*-MCP) chemotactic protein genes over-expressed after addition of IPTG (even numbers in Fig. 1) as examples. Other nine genes (*cjj0473*, *cjj0706*, *cjj1128*, *cjj1498*, *ctsD*, *ctsE*, *ctsG*, *ctsP* and *ctsX*) could also be induced by IPTG to generate recombinant proteins that had their respective relative mobility of relative sizes and positions in SDS-PAGE (data not shown).

Six genes (*cjj0046*, *cjj0289*, *cjj0842*, *cjj0975*, *cjj1205* and *ctsF*) were not expressed after IPTG induction. Two possible explanations for this discrepancy are: (1) these recombinant proteins may be toxic to the *E. coli* host cells, and/or (2) the codon use of these genes may differ significantly from that used by *E. coli* (codon usage bias). In the latter case, it is well known that codon usage affects heterologous protein production in other expression systems (e.g., Kane 1995; Fath et al. 2011; Burgess-Brown et al. 2008; and references therein). Our six non-expressed nucleotide sequences were subjected to further analysis by the Rare Codon Analysis Tool (www.genscript.com/cgi-bin/tools/rare_codon_analysis). We found that the codon usage in these sequences may play a role in their expression in *E. coli* from our preliminary analyses (Supplemental Table 1). In the former case, these phenomena have been observed in many proteins expressed in an *E. coli* expression system (e.g., Yeh and Klesius 2011; Bannantine et al. 2008b).

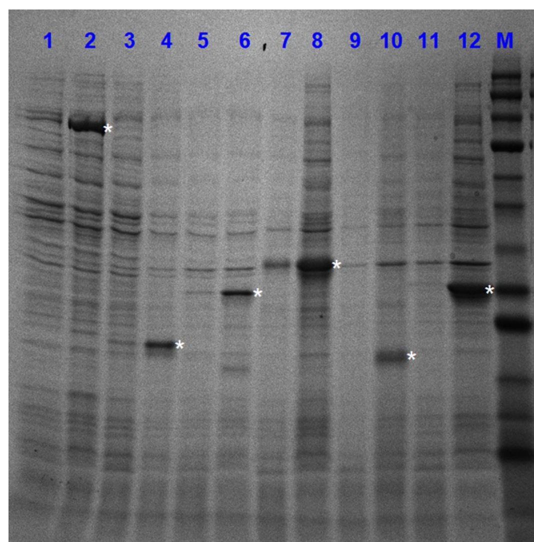

Fig. 1 SDS-PAGE analysis of six recombinant *Campylobacter jejuni* chemotactic proteins as examples expressed in the *Escherichia coli* expression system (Lucigen Corporation) in the absence (*odd number*) or presence (*even number*) of 1 mM of isopropyl β-*d*-1-thiogalactopyranoside (IPTG). Equal volumes of bacterial cultures and 2× Laemmli sample buffer (Bio-Rad Laboratories) were added to tubes, and the mixtures were heated at 95 °C for five min. Proteins were separated in 10–20 % SDS–Tris–HCl-polyacrylamide gels (Bio-Rad Laboratories) and stained with Bio-Safe™ Commassie G-250 Stain (Bio-Rad Laboratories). *Lanes 1* and *2* CheA; *3* and *4* CheB; *5* and *6* CheR; *7* and *8* CheV; *9* and *10* CheW; and *11* and *12* Cjj1549. M, prestained protein markers (New England BioLabs). Molecular masses (kDa) from *top* to *bottom* are: 230, 150, 100, 80, 60, 50, 40, 30, 25, 20, 15 and 10. *Asterisks* at the *right sides* of the *even-numbered lanes* indicate the recombinant chemotactic proteins

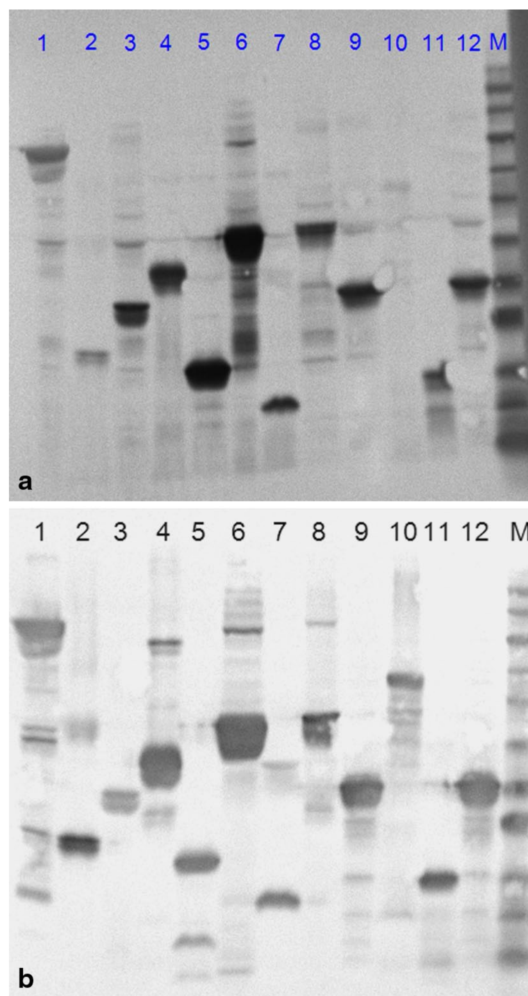

Fig. 2 Detection of recombinant *C. jejuni* chemotactic proteins. Western blot analysis by **a** HisDetector™ Western blot probed with nickel conjugated with horseradish peroxidase (KPL, Inc.) and **b** mouse anti-HA monoclonal antibody (Thermo Scientific) followed by goat anti-mouse conjugated with horseradish peroxidase (KPL). The six-His tag and HA tag sequences were added to the chemotactic protein genes at the 5′- and 3′-end, respectively, during recombinant plasmid construction. Proteins from induced bacterial cultures were separated in 10-20 % SDS–Tris–HCl-polyacrylamide gels and transferred to the PVDF membranes. The TMB peroxidase substrate was used for blot development. *Lanes 1* CheA; *2* CheB; *3* CheR; *4* CheV; *5* CheW; *6* Cjj0473; *7* Cjj0706; *8* Cjj1128; *9* Cjj1549; *10* CtsE; *11* CtsG and *12* CtsP. M, Prestained protein markers, which are the same as indicated in Fig. 1

Because a six-His tag and an HA tag were included in recombinant genes during the expression vector construction, the recombinant proteins were further confirmed by immunoblot analysis. The IPTG-induced lysates were electrophoresized in SDS-PAGE and electrotransferred to PVDF membranes. The HA tag was probed by mouse anti-HA monoclonal antibody, followed by incubation with goat anti-mouse antibody conjugated with horseradish peroxidase and development with TMB substrate, while the six-His tag was detected with a Nickel HisDetection kit. As illustrated in Fig. 2a, except for the CtsE protein, each recombinant protein showed one major dense band that corresponds to its respective expected protein size on the blot that was detected by the nickel-horseradish peroxidase conjugate. These recombinant proteins were also detected by anti-HA antibody (Fig. 2b), implying that these proteins are fusion proteins with six-His and HA tags. Together with nucleotide sequencing, SDS-PAGE analysis and immunoblot analysis, we conclude these recombinant proteins are very likely the *C. jejuni* chemotactic proteins. The CtsE protein (Fig. 2, lane 10) was barely detected by the nickel substrate, but strongly reacted to the anti-HA antibody. The reason of this discrepancy is not known, but may be due to the inaccessibility of the His-tag region in the CtsE protein for nickel binding after the protein transfer in the Towbin buffer. The purpose of double tagging the recombinant proteins is for future construction of an array of proteins that are able to be used as a tool for antibody detection. In our case, the six-His tag at the amino terminus binds the nickel coated glass surface, while the HA tag at the carboxyl terminus faces outside and can be used for quality control of the protein array on glass surface.

Table 2 The reactivity of chicken sera to the recombinant *C. jejuni* chemotactic proteins

Chicken No.[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CheA	+	−	−	+	−	+	−	+	−	−	+	+	+	+	+	+	+	+	+
CheB	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	+	+	−	+
CheR	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
CheV	−	−	−	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−
CheW	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−
Cjj0473[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cjj0706[b]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Cjj1128[b]	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Cjj1549[b]	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	+	+	+	+
CtsE	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CtsG	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CtsP	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−

[a] Chicken No. indicates sera were collected from 19 individual broiler chickens

[b] Cjj0473: methyl-accepting chemotaxis protein, Cjj0706: hypothetical protein, Cjj1128: methyl-accepting chemotaxis protein, and Cjj1549: methyl-accepting chemotaxis protein

Previously, we demonstrated that sera from broiler chickens contained anti-FliD (flagellar capping protein) antibody (Yeh et al. 2014). We wanted to determine whether broiler chicken sera contained antibodies that reacted to these recombinant proteins. Twelve recombinant proteins were separated by SDS-PAGE and electrotransferred to PVDF membranes. Immunoblot analysis was carried out with sera from 19 broilers older than 4 weeks of age. The results of reactivity of chicken sera to the recombinant chemotactic proteins are summarized in Table 2. Each serum reacted to various numbers of proteins. All broiler chicken sera did not react to the recombinant CtsE and CtsG proteins, but all sera reacted to the Cjj0473 (annotated as MCP). This result implies that the Cjj0473 may be one of the important common immunoreactive *C. jejuni* proteins among broiler chickens. Because the *Campylobacter* status of those broilers was not determined, we are not sure whether this anti-Cjj0473 antibody was protective or not at this point. The reasons for each serum reaction to various numbers of recombinant proteins may be due to different immunogenicity of the *C. jejuni* proteins, differences in immune responses among broiler chickens or both. These phenomena have also been reported in other systems (e.g., Bannantine et al. 2008a; Nielsen et al. 2012).

This Cjj0473 MCP from the D1–39 isolate had 365 amino acids with the calculated molecular mass of 40.5 kDa. It is well known that the *Campylobacter* proteins undergo posttranslational glycosylation (Logan 2006; Guerry et al. 2006; Ewing et al. 2009; Howard et al. 2009). The deduced MCP amino acid sequence of *C. jejuni* D1–39 was subjected to analysis by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) to predict whether it had the potential *N*-glycosylation site. This protein had six potential *N*-glycosylation sites at Asn^{6}, Asn^{19}, Asn^{65}, Asn^{110}, Asn^{116}, and Asn^{346}. Because *C. jejuni* also undergoes *O*-lined glycosylation (Thibault et al. 2001), the amino acid sequence of Cjj0473 was analyzed by the NetOGlyc 4.0 program (www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft et al. 2013). The result reveals that the Cjj0473 protein had three potential *O*-glycosylation sites Thr^{125}, Ser^{291}, and Thr^{322}. Either glycosylation may contribute to antigen specificity. Whether this protein has *N*- or *O*-lined glycosylation needs further investigation. The phylogenetic analysis shows that this MCP from our D1–39 isolate fell in the well-segregated clade of *C. jejuni* (Fig. 3).

The broiler sera reacted to these recombinant chemotactic proteins indicate that the anti-*Campylobacter* antibodies may be prevalent in commercial broiler flocks. Because the broilers used in this study were older than 4 weeks, the involvement of maternal antibodies, which diminish within 2 weeks of age (Sahin et al. 2003; Wyszyńska et al. 2004), was ruled out in the reaction to these proteins. This also suggests that broiler chickens acquire this microorganism from the environment during their maturation, and these chemotactic proteins were expressed and recognized by host immune cells during the microorganism's colonization and adhesion in the chicken ceca.

However, because our comparison of the *C. jejuni* Cjj0473 amino acid sequence with the counterpart from *E. coli* shows that they share 33 % identity each other, the possibility that broiler antibodies to this Cjj0473 and other recombinant proteins may have been elicited by other microorganisms cannot be ruled out.

Sahin et al. (2003) and Shoaf-Sweeney et al. (2008) used whole cell lysate of *C. jejuni* to identify a battery of proteins that reacted to chicken maternal antibodies. However, none of the chemotactic proteins were detected in their studies. The reason is not known, but it is possible that the amounts of these proteins are very low, which were not able to be detected, or these proteins are not expressed in standard in vitro cultures (Handfield et al. 2000; Hang et al. 2003; Harris et al. 2006; Rollins et al. 2008; Lowry et al. 2010; Alam et al. 2013).

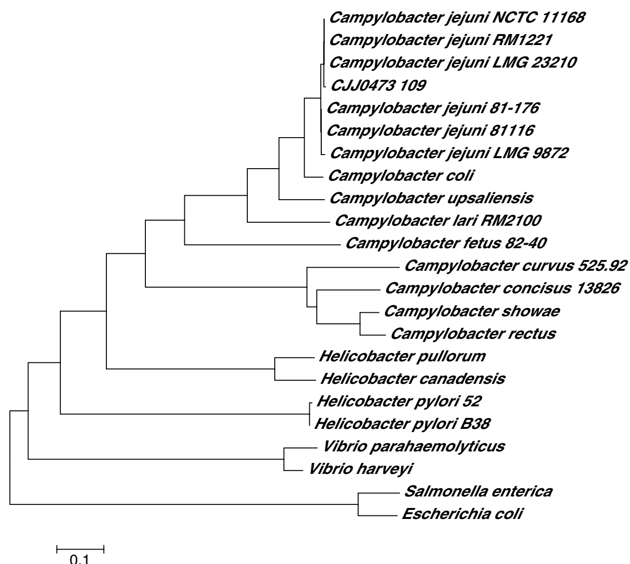

Fig. 3 Phylogenetic analysis of *Campylobacter jejuni* Cjj0473 (methyl-accepting chemotaxis protein). Amino acid sequences were retrieved from GenBank and aligned with MUSCLE (Edgar 2004) via www.ebi.ac.uk/Tools/msa/muscle/. The maximum likelihood method in MEGA version 5.2.1 (Tamura et al. 2011) was carried out to infer the phylogenetic tree. The amino acid sequences of the proteins for this analysis are as follows: Cjj0473 109, KC692485; *Campylobacter jejuni* subsp. *jejuni* LMG 23210, EIB94398; *Campylobacter jejuni* subsp. *jejuni*, LMG 9872, EIB87140; *Campylobacter jejuni* subsp. *jejuni* 81–176, YP_001000155; *Campylobacter jejuni* subsp. *jejuni* NCTC 11168, YP_002343882; *Campylobacter jejuni* subsp. *jejuni* 81116, YP_001481997; *Campylobacter jejuni* subsp. *jejuni* RM1221, AAW35085; *Campylobacter coli*, WP_002778453; *Campylobacter upsaliensis*, WP_004277195; *Campylobacter lari* RM2100, WP_012661737; *Campylobacter fetus* subsp. *fetus* 82-40, YP_892143; *Campylobacter showae*, WP_009493548; *Campylobacter concisus* 13826, YP_001466399; *Campylobacter curvus* 525.92, YP_001407852; *Campylobacter rectus*, WP_004319229; *Escherichia coli*, WP_001068997; *Helicobacter canadensis*, WP_006656891; *Helicobacter pullorum*, WP_005022854; *Helicobacter pylori* B38, YP_003057512; *Helicobacter pylori* 52, YP_005762254; *Salmonella enterica*, WP_001736440; *Vibrio parahaemolyticus* RIMD 2210633, NP_801203; *Vibrio harveyi*, WP_005429058

In conclusion, 21 chemotactic protein genes were amplified, ligated in the expression vector pETite, and transformed in *E. coli* BL21 (DE3) cells. A total of 15 recombinant genes were successfully induced by IPTG to express. The recombinant proteins were confirmed by SDS-PAGE analysis, six-His tag and HA tag detection on Western blot analysis. Sera from broiler chickens older than 4 weeks were used to test the antigenicity of these recombinant proteins. The results show that each broiler serum reacted to various numbers of recombinant chemotactic proteins, but all sera reacted to the Cjj0473 protein, suggesting this protein has potential as a novel target for vaccine development.

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