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Use of *In Vivo*-Induced Antigen Technology to Identify *In Vivo*-Expressed Genes of *Campylobacter jejuni* During Human Infection

Yuanqing Hu[†], Jinlin Huang[*], Qiuchun Li, Yuwei Shang, Fangzhe Ren, Yang Jiao, Zhicheng Liu, Zhiming Pan, and Xin-an Jiao[*]

Jiangsu Key Laboratory of Zoonosis, Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, Jiangsu 225009, P. R. China

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*Corresponding authors
X-a.J.
Phone: +86-514-8797-1803;
Fax: +86-514-8731-1374;
E-mail: jiao@yzu.edu.cn
J.H.
Phone: +86-514-8797-1803;
Fax: +86-514-8731-1374;
E-mail: sjxyhjl@yahoo.com.cn

[†]Present address: School of Biological Science and Biotechnology, Minnan Normal University, Zhangzhou, Fujian 363000, P. R. China

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Campylobacter jejuni is a prevalent foodborne pathogen worldwide. Human infection by *C. jejuni* primarily arises from contaminated poultry meats. Genes expressed *in vivo* may play an important role in the pathogenicity of *C. jejuni*. We applied an immunoscreening method, *in vivo*-induced antigen technology (IVIAT), to identify *in vivo*-induced genes during human infection by *C. jejuni*. An inducible expression library of genomic proteins was constructed from sequenced *C. jejuni* NCTC 11168 and was then screened using adsorbed, pooled human sera obtained from clinical patients. We successfully identified 24 unique genes expressed *in vivo*. These genes were implicated in metabolism, molecular biosynthesis, genetic information processing, transport, and other processes. We selected six genes with different functions to compare their expression levels *in vivo* and *in vitro* using real-time RT-PCR. The results showed that the selected six genes were significantly upregulated *in vivo* but not *in vitro*. In short, these identified *in vivo*-induced genes may contribute to human infection of *C. jejuni*, some of which may be meaningful vaccine candidate antigens or diagnosis serologic markers for campylobacteriosis. IVIAT may present a significant and efficient method for understanding the pathogenicity mechanism of *Campylobacter* and for finding targets for its prevention and control.

Keywords: *Campylobacter jejuni*, *in vivo*-induced antigen technology (IVIAT), *in vivo*-induced gene, foodborne pathogen

Introduction

Campylobacter jejuni has emerged as the leading cause of human bacterial foodborne diarrheal disease worldwide [17]. It can colonize the intestines of many hosts, including chickens, cattle, sheep, dogs, and wild birds. The pathogenic bacteria reside in the lower intestinal tract in humans and give rise to self-limiting diarrhea [25]. Owing to the wide distribution of *C. jejuni* in poultry and the broad consumption of poultry meat products [7], there is increasing pressure to control *C. jejuni* infections, although most cases of infection are sporadic [3]. An effective vaccination for *C. jejuni*, which may help to control infections, is lacking [11]. To identify targets for a vaccine to prevent *C. jejuni* infection, a better understanding of the biological characteristics of this pathogen, particularly its virulence mechanisms, is needed.

The completion of the sequencing of the *C. jejuni* NCTC 11168 genome in 2000 opened the door for studying its pathogenicity mechanisms. The most prominent feature of the sequence is its heteromorphism, which contributes to its strain-to-strain variability [17]. Moreover, the interactions between the host and pathogenic bacteria likely play a significant role in *Campylobacter* infection [27]. To elucidate the role of *C. jejuni* virulence-associated genes in infection, animal models, including chickens, rodents, and monkeys, have been developed and applied to investigate colonization or transmission. Unfortunately, these models do not entirely show the pathogenicity of *C. jejuni* in humans [15].

IVIAT (*in vivo*-induced antigen technology) is a screening

technique that identifies virulence-associated genes during host infection by different types of pathogens [20]. Using IVIAT, we can identify pathogenicity-related genes that are activated and expressed *in vivo* when a strong interaction occurs between the host and microbe. The advantage of IVIAT is that it contributes to the identification of virulence-associated genes expressed specifically during infection instead of during growth in laboratory conditions. Virulence-associated genes identified using IVIAT could play an important role in virulence or pathogenesis in the particular host-pathogen system, and proteins encoded may be novel diagnostic targets and subunit vaccine candidates. To date, IVIAT has been used for a number of pathogens, including *Escherichia coli* [22], *Vibrio* [28], *Streptococcus* [5], *Salmonella* [9], and *Brucella* [13]. Virulence genes from these microbes are expressed *in vivo*. Some of the identified proteins are candidate antigens for vaccines, whereas others may be diagnostic markers [5, 20].

This identification of *in vivo*-expressed genes will help to elucidate the pathogenic mechanisms of human infection by *C. jejuni* and to develop novel prevention strategies. In this study, a genomic expression library was constructed using random DNA fragments from the sequenced human *C. jejuni* strain NCTC 11168. *In vivo*-induced antigens were identified from human convalescent sera after being thoroughly absorbed with *C. jejuni* NCTC 11168 grown *in vitro* (scheme showed in Fig. 1).

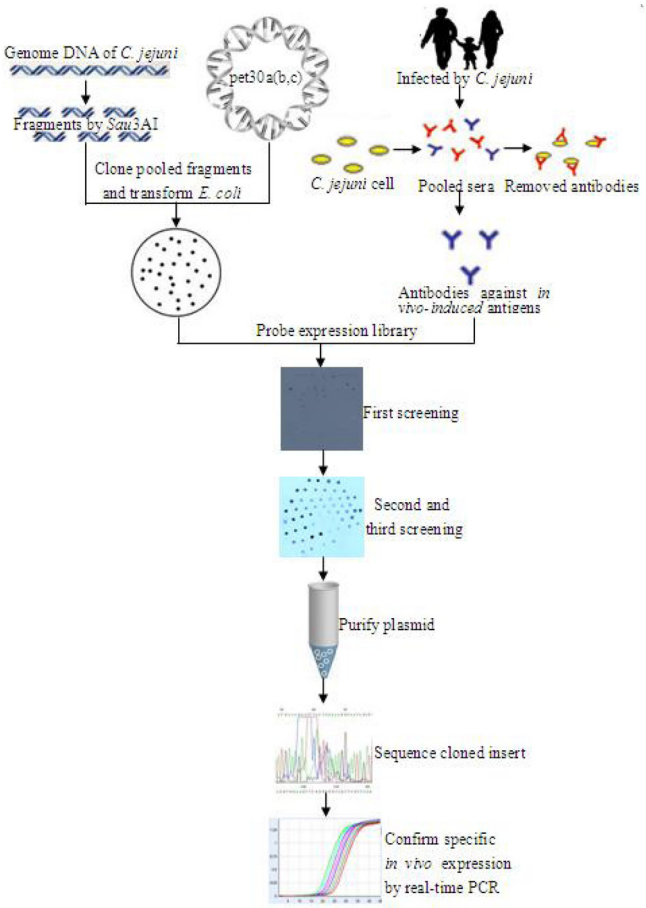

Fig. 1. The scheme of IVIAT in this study.

Materials and Methods

Bacteria, Recombinant Proteins, and Culture Conditions

C. jejuni NCTC 11168 was kindly provided by Dr. Wangbang Sun from Zunyi Medical College in China. The proteins FlaA370 (N-terminal 370 aa of FlaA) and CjaA have been previously expressed in our laboratory. The strain used for genomic DNA preparation and serum adsorption *in vitro* was cultured on Campy blood-free selective medium (CCDA) (Oxoid Ltd., Basingtoke, Hampshire, UK) plates at 37°C under anaerobic conditions for 30 h. For determining the protein expression profiles of these *in vivo*-expressed genes, the *C. jejuni* NCTC 11168 strain was cultured on CCDA medium plus 0.1% sodium deoxycholate (DOC; Sigma, USA) [14] (referred to as DOC plates).

Preparation of Antisera

In this study, human sera were collected and used for screening the genomic expression library. Equal volumes of human sera were obtained and pooled from seven clinical patients (Subei Hospital of Yangzhou, China) who had been infected by *C. jejuni* diagnosed through the culture-confirmed method. The antibody titer was determined with an indirect ELISA kit (SERION ELISA classic *CAMPYLOBACTER JEJUNI* IgG; Virion\Serion, Wurzburg, Germany), and seven selected sera were positive for the *C. jejuni* antibody. These patients were more than 50 years old; they suffered from repeated episodes of diarrhea, but they had not received any medical treatment before the collection of the sera. Human sera experiments were conducted in accordance with national guidelines, approved by the Subei Hospital of Yangzhou, and consented to by each patient.

Pooled human sera were adsorbed using *C. jejuni* NCTC 11168 whole cells cultured on CCDA plates on a rocking platform (Incubator Shaker; Crystal, China) at 4°C for 1 h. The sera were adsorbed with *C. jejuni* whole cells six times and then with *E. coli* BL21 (DE3) whole cells six times. Adsorption was performed on a PVDF membrane coated with the ultrasonically disrupted lysates of *C. jejuni* or *E. coli* BL21 (DE3) cells, inactivated ultrasonically disrupted lysates of *C. jejuni* NCTC 11168 or *E. coli* BL21 (DE3) cells, secreted proteins from NCTC 11168 (prepared referencing to [21]), and lysis-induced BL21 (DE3) cells (Fig. 2). Indirect ELISAs were conducted to assess the adsorption of the sera by coating the plates with *C. jejuni* NCTC 11168 and *E. coli* BL21 (DE3) whole cells, lysates, CjaA, FlaA370 recombinant protein, and extracted flagellum protein. To evaluate the reactivity of the antibodies to

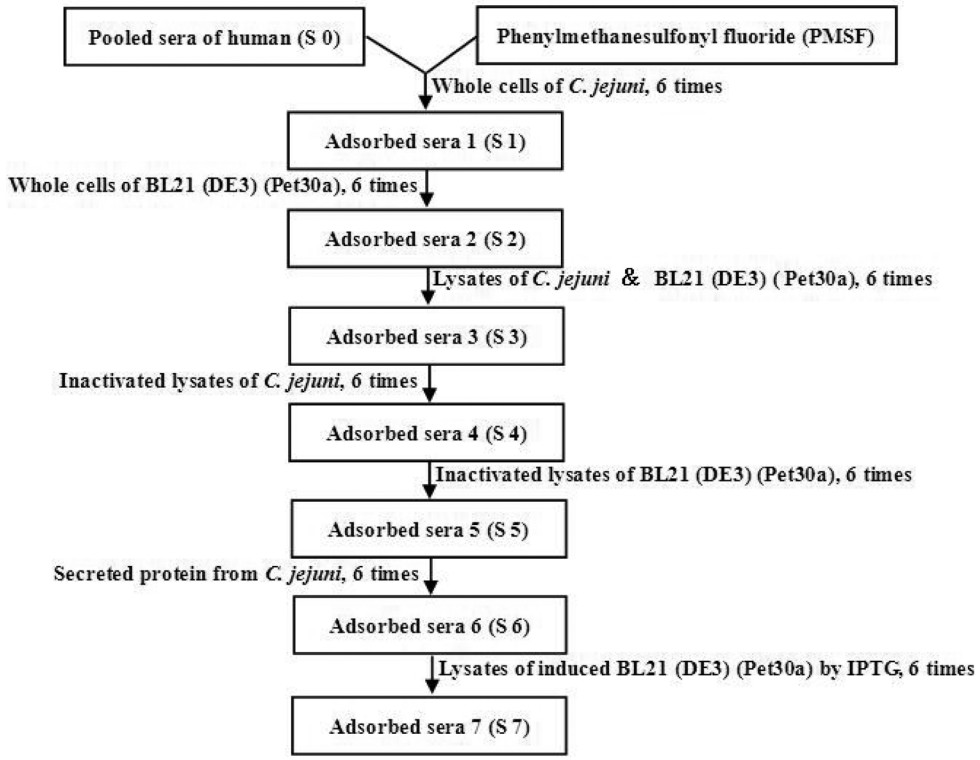

Fig. 2. The adsorption steps of human sera using several kinds of *in vitro* antigens.

the *C. jejuni* whole-cell lysates, successively diluted nickel affinity-purified lysate proteins were spotted onto a nitrocellulose (NC) membrane. A Dot-ELISA was conducted according to the standard protocol [16].

In Vivo-Induced Antigen Screening and Functional Prediction

We constructed an inducible expression library of genomic proteins from sequenced *C. jejuni* NCTC 11168 according to standard protocol [20, 22]. The IVIAT process was performed based on previously described methods [6, 20] with a few modifications. Plasmids from the library were transformed into *E. coli* BL21 (DE3) cells containing 50 μg/ml of kanamycin to obtain 300 to 400 colonies per plate after culturing the bacteria at 37°C overnight. These colonies were then replicated onto duplicate LB agar plates (containing 50 μg/ml of kanamycin and 1 mM of IPTG) using NC membranes and incubated for 5 h at 30°C to induce expression of the inserted genes. The NC membranes were removed, and the colonies were lysed in chloroform vapors for 15 min in a hermetic container. The membranes were then saturated with 1% BSA in PBS plus 0.1% Tween-20 (PBST) and allowed to incubate for 1 h with mild agitation at room temperature. Next, the membranes were incubated with the adsorbed sera (1:1,000 in PBST). Colony immunoblotting was performed using the pooled adsorbed sera as the primary antibodies. Reactive colonies were detected using goat anti-human–horseradish peroxidase (HRP) secondary antibodies (Sigma, USA) and were visualized using a chemiluminescent ECL substrate (Thermo, USA). Secondary and tertiary screens were performed by means as described for the primary screen. Clones that maintained reactivity through the three rounds of screening were considered positive identifications and were subjected to DNA sequencing using T7 primers that flanked the multiple cloning sites within the vectors. The sequences of the *in vivo*-induced (IVI) genes were analyzed by BLASTx alignment and a Conserved Domain Database (CDD) domain search (http://www.ncbi.nlm.nih.gov/BLAST/).

Real-Time PCR Analysis

The *in vivo* gene expression profiles were evaluated by comparing the level of RNA transcription on DOC and CCDA plates with the expression of virulence genes induced with sodium deoxycholate [10, 14]. The total RNA was extracted using TRIzol (TaKaRa, Dalian, China) according to the manufacturer's instructions after a 15 h culture of strain NCTC 11168 on CCDA or DOC plates. RNA was eluted with diethyl pyrocarbonate-treated water and treated with DNase I (TaKaRa). Next, the cDNA was synthesized from 500 ng of RNA using an RT-PCR kit (TaKaRa) according to the manufacturer's instructions. The real-time RT-PCR amplification of 2.0 μl of cDNA was performed using a reaction mixture containing SYBR Premix Ex Taq II (TaKaRa), 10 μM of the forward primer, 10 μM of the reverse primer, and diethyl pyrocarbonate-treated water. The real-time RT-PCR analysis was performed using a Gene Amp 7500 thermocycler

Table 1. Primers used for real-time RT-PCR.

Primer	Genes	Sequence (5′-3′)
Cj0100-F	Cj0100	CGCAAGCTAATGCGACTACAGG
Cj0100-R	Cj0100	CTTCCAAGAGCAGGCGGAGA
Cj0788-F	Cj0788	TTACCACCTATGGAGTTTGTATGCG
Cj0788-R	Cj0788	TCATTAAGCGTTTGAACCTCTGTCA
Cj0328c-F	*fabH*	GGTGCTGGAGTTGTAAGTTTGGAT
Cj0328c-R	*fabH*	GAAAGTGTTTGACATAGGCTTGATT
Cj1200-F	Cj1200	GAGTAATCCCTAATGTAATGGTTGG
Cj1200-R	Cj1200	TATTGGGTATAGAAATGCTTTGTCC
Cj0640-F	*aspS*	TCTTACCAAAGCTACTCCCGA
Cj0640-R	*aspS*	AACGATCAAAACCGCTACACA
Cj0064-F	*flhF*	CGCAGTGCGCTTTATGAGGTT
Cj0064-R	*flhF*	AGGTAGAGGATGGTTTTGGCGAG
Cj0402-F	*glyA*	CGATGGAACGGATAATCACC
Cj0402-R	*glyA*	AATACCTGCATTTCCAAGAGC

(Applied Biosystems, Carlsbad, CA, USA) with the following PCR parameters: 2 min at 50°C, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing at 60°C for 34 sec. Ten IVI genes (primers are shown in Table 1) were selected, and their transcription levels in the NCTC 11168 strain cultured on DOC plates were compared with those on CCDA plates. Gene Cj0402 was used to normalize these samples because this housekeeping gene is expressed both on DOC and CCDA plates [14]. Duplicate reactions were performed, and three biological replicates were used for each sample. The threshold cycle values were determined using 7500 software, ver. 2.0.1 (Applied Biosystems).

Results

Serum Selection and Adsorption

Seven human positive sera from patients were determined using a SERION ELISA IgG kit (ELISA data not shown). Pooled human sera were detected after each adsorption step using ELISA plates coated with *C. jejuni* NCTC 11168 and *E. coli* BL21 (DE3) whole cells, lysates, CjaA, FlaA370 recombinant protein, and extracted flagellum protein (Fig. 3A). These data showed that the immunoreactivity of human sera with *in vitro*-cultured *C. jejuni* antigen gradually decreased with the series of adsorptions. A Dot-ELISA showed that the levels of antibodies against *in vitro* antigens in human sera were drastically reduced (Fig. 3B).

Identification of *C. jejuni* Antigens by IVIAT

In the primary screening, approximately 25,000 clones from the *C. jejuni* NCTC 11168 genomic expression library were probed using extensively absorbed human sera. In

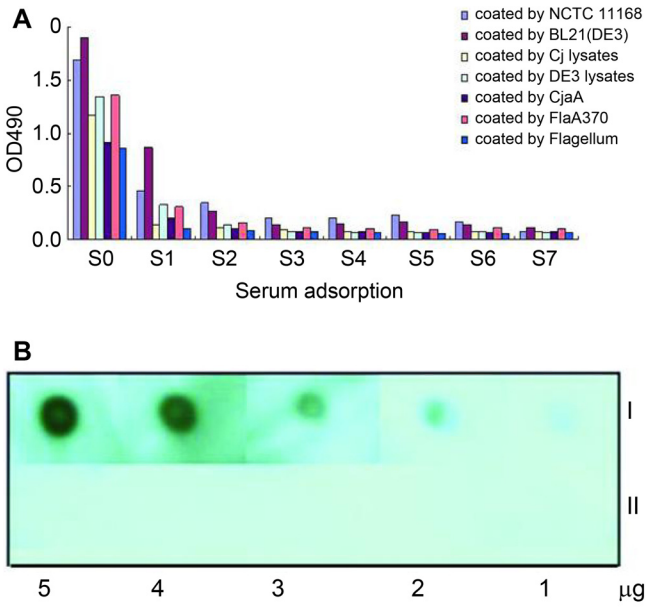

Fig. 3. Evaluation of adsorption for pooled sera using ELISA. (**A**) The results of ELISA for human sera with seven coated antigens after each step of adsorption. The whole cells of *C. jejuni* NCTC 11168, whole cells of *E. coli* BL(DE3), lysates from *C. jejuni*, lysates from BL21(DE3), recombinant CjaA, FlaA370 protein, and extracted flagellum protein were used as coating antigens for assaying the adsorption of sera. (**B**) Dot-ELISA results of reactivities of pooled unadsorbed (I) and adsorbed (II) human sera against *C. jejuni* whole-cell lysates. The proteins of *C. jejuni* whole-cell lysates were quantified using a UV spectrophotometer and diluted from 5 μg to 1 μg for each colony.

total, 157 immunoreactive clones were identified by PCR using pET30-specific primers, and the encoded proteins of the selected clones were analyzed in the SDS-PAGE assay after the primary screening. Of the initial clones, 31 were positive upon secondary screening. Finally, 24 unique protein-expressing ORFs were ascertained by nucleotide sequencing and homology analysis.

Nucleotide sequencing was performed in both directions, and homology and functional analyses were performed on the inserted sequence based on the published nucleotide sequence and the known functions of the proteins from the *C. jejuni* NCTC 11168 strain [17]. The majority of these 24 proteins have a defined or suspected role in the pathogenesis of *C. jejuni* infection *in vivo*. These proteins are implicated in metabolism, molecular biosynthesis, genetic information processing, transport, and other processes (Table 2).

Analysis of *In Vivo* Gene Expression Profiles

To evaluate the *in vivo* expression of the *C. jejuni* genes identified using the IVIAT method, we selected six genes

Table 2. Twenty-four genes of *C. jejuni* identified by IVIAT from human hosts.

Gene (ORF)	Function of gene product	Predicted cellular location
Metabolism		
fabH (Cj0328c)	3-Oxoacyl-ACP synthase	Cytoplasmic
hemL (Cj0853)	Glutamate-1-semialdehyde aminotransferase	Cytoplasmic
leuC (Cj1717c)	Isopropylmalate isomerase large subunit	Cytoplasmic
ptmB (Cj1331)	Acylneuraminate cytidylyltransferase (flagellin modification)	Cytoplasmic
eno (Cj1672c)	Phosphopyruvate hydratase	Cytoplasm
guaB (Cj1058c)	Inosine-5′-monophosphate dehydrogenase	Cytoplasmic
fcl (Cj1428c)	GDP-L-fucose synthetase	Cytoplasmic
gltD (Cj0009)	Glutamate synthase subunit beta	Cytoplasmic
sodB (Cj0169)	Superoxide dismutase (Fe)	Periplasmic
serC (Cj0326)	Phosphoserine aminotransferase	Cytoplasmic
Cj0874c	Putative cytochrome C	Periplasmic
Cj1365c	Putative secreted serine protease	Unknown
Molecular biosynthesis		
glf (Cj1439c)	UDP-galactopyranose mutase	Cytoplasmic
tufB (Cj0470)	Elongation factor TU	Cytoplasm
moeA (Cj0857c)	Molybdopterin biosynthesis protein signal peptidase I	Cytoplasm
flhF (Cj0064c)	Flagellar biosynthesis regulator FlhF	Multiple localization sites
Cj1200	NLPA family lipoprotein	Cytoplasmic membrane
Genetic information processing		
aspS (Cj0640c)	Aspartyl-tRNA synthetase	Cytoplasmic
recG (Cj0028)	Single-stranded-DNA-specific exonuclease	Cytoplasmic
Cj1710c	Putative metallo-beta-lactamase family protein	Cytoplasmic
Transport		
ctsE (Cj1471c)	Type II protein secretion system E protein	Cytoplasmic
Cj1587c	Multidrug transporter membrane component	Cytoplasmic membrane
Others		
Cj0100	ParA family protein	Cytoplasmic membrane
Cj0788	Hypothetical protein Cj0788	Cytoplasmic

according to their functional category to compare their expression levels *in vivo* and *in vitro* using real-time RT-PCR. An *in vitro* DOC plate culturing method was used for analyzing the RNA transcription, to mimic *in vivo* conditions. The real-time PCR results showed that these genes were upregulated under *in vivo*-like conditions (>1-fold) (Fig. 4).

Discussion

IVIAT is a rigorous method that can identify virulence-associated genes, which are expressed instantaneously *in vivo* when hosts are infected by pathogenic bacteria [20]. We used IVIAT to identify *in vivo*-induced genes of *C. jejuni* to better understand the pathogenicity mechanism of *Campylobacter* in humans. *C. jejuni* NCTC 11168 was used to construct a genomic expression library because this isolate was originally derived from infected humans. Moreover, the sequence of NCTC 11168 has been annotated. This information supports our IVIAT screens and analysis of *in vivo*-induced genes. We identified two genes, including Cj0100 and Cj0788, through IVIAT, that were found to have products that were upregulated in the *C. jejuni* F38011 strain in the presence of 0.1% sodium DOC. These culture conditions have been reported to mimic the *in vivo* environment [14].

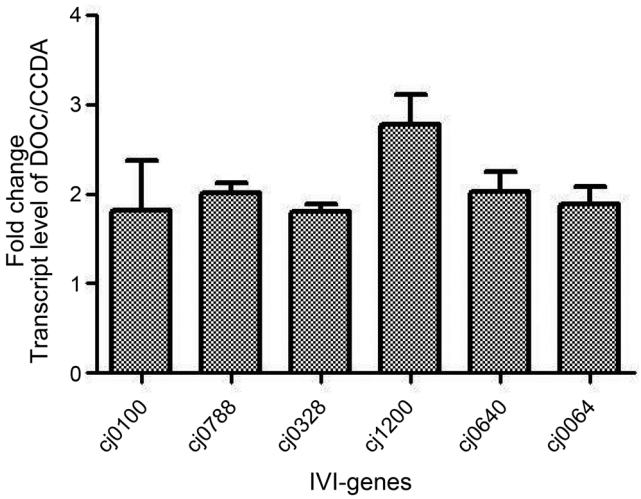

Fig. 4. *In vivo* gene expression relative to the level of expression *in vitro* by real-time PCR.
C. jejuni NCTC 11168 was cultured in plates of CCDA (control *in vitro*) and DOC (*in vivo*-like condition) for 15 h, and the transcription levels of mRNA were assessed by real-time PCR. The levels of transcription above 1-fold were considered upregulated in contrast with *in vitro*.

Twelve *in vivo*-induced genes that were classified with a metabolic function were identified in this study and may have important roles in bacterial growth or pathogenicity *in vivo*. Several of the genes, including *leuC*, *ptmB*, *eno*, and *fcl*, participate in special carbohydrate metabolism, such as C5-branched dibasic acid metabolism, amino sugar and nucleotide sugar metabolism, and glycolysis and fructose mannose metabolism. Previous studies [8] have shown that some strains of *C. jejuni* can utilize glutamine or glutathione to enhance their ability to colonize the intestines of their hosts. Because serine catabolism is required for colonization of the intestinal tract [8], these results indicate that the *serC* and Cj1365c genes identified by IVIAT may contribute to the colonization of *C. jejuni*. Cj1365c, a putative secreted serine protease, could be a significant marker for strains following environmental transmission because of its primary distribution in clinical and livestock isolates [26]. The protein FabH could catalyze the condensation of acetyl-CoA with malonyl-ACP to initiate cycles of fatty acid elongation [24]. The gene *fabH* (Cj0328c) was upregulated (3.09-fold by microarray) during chicken colonization, indicating that the protein encoded by *fabH* may be an important virulence-associated gene *in vivo* [24].

Five genes, including *glf*, *tufB*, *moeA*, *flhF*, and Cj1200, were classified as having a molecular biosynthesis function. Gene *tufB*, which encodes outer membrane translation elongation factor TU (Ef-Tu), was more abundant in the Cj0596 mutant (upregulated 2.4-fold). These results indicate that the fraction of outer membrane localization of Ef-Tu was increased. The altered outer membrane protein suggests that Ef-Tu is possibly involved in the pathogenesis of *C. jejuni* [12, 18]. The gene *flhF* encodes the flagellar biosynthesis regulator FlhF, which is a putative GTPase that is necessary for the development of the flagellar organelles in polarly flagellated bacteria [1]. FlhF is essential for σ^{54}-dependent flagellar gene expression and flagellar biosynthesis but participates in an independent pathway that converges with or works downstream of the flagellar export apparatus-FlgSR pathway [1, 2]. The characteristics of the other genes remain unknown.

Three *in vivo*-induced genes (*aspS*, *recG*, and Cj1710c) were assigned to a genetic information processing function, but their specific functions have not been determined. All bacteria have DNA repair mechanisms that act to reduce DNA damage and maintain genetic structures and stability [4]. For *C. jejuni*, genomic polymorphisms are important for its adaptation to diverse environments. However, DNA repair is essential for its survival in response to significant environmental stresses. We hypothesize that these nine DNA repair genes identified as *in vivo*-associated genes may be important when *C. jejuni* lives in human or chicken tissues.

We identified two virulence-associated genes, *ctsE* and Cj1587c, that were assigned to a transportation function. The gene *ctsE*, which encodes a putative type II secretion system E protein and is essential for DNA uptake and natural transformation, was classified as having a *Campylobacter* transformation function [19]. This protein is an important virulence-associated factor because its encoding gene is similar to several genes responsible for the transport of pilus subunits, toxins, and other exoenzymes [23].

In this study, real-time PCR was performed to assess the expression profiles of IVI-virulence genes by comparing the transcription levels of genes in DOC and CCDA cultures. All six genes selected were upregulated under the DOC culture conditions, which were considered to be *in vivo*-like conditions. Out of these six genes selected, Cj0100 and Cj0788 were previously found to be upregulated in the presence of 0.1% DOC by a microarray analysis [14], which is consistent with the results of this study. IVIAT identified 24 *C. jejuni* NCTC 11168 proteins expressed *in vivo* during the human infection with *C. jejuni*. This study is the first profile of virulence-associated genes of interaction between human hosts and *C. jejuni*. Several of the genes identified by IVIAT were closely correlated with the virulence and

pathogenesis of *C. jejuni*, and the coded proteins may be candidates for vaccines.

Acknowledgments

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