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Identification of Immunogenic and Virulence-Associated *Campylobacter jejuni* Proteins

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With the aim of identifying proteins important for host interaction and virulence, we have screened an expression library of NCTC 11168 *Campylobacter jejuni* genes for highly immunogenic proteins. A commercial *C. jejuni* open reading frame (ORF) library consisting of more than 1,600 genes was transformed into the *Escherichia coli* expression strain BL21(DE3), resulting in 2,304 clones. This library was subsequently screened for immunogenic proteins using antibodies raised in rabbit against a clinical isolate of *C. jejuni*; this resulted in 52 highly reactive clones representing 25 different genes after sequencing. Selected candidate genes were inactivated in *C. jejuni* NCTC 11168, and the virulence was examined using INT 407 epithelial cell line and motility, biofilm, autoagglutination, and serum resistance assays. These investigations revealed *C. jejuni* antigen 0034c (Cj0034c) to be a novel virulence factor and support the usefulness of the method. Further, several antigens were tested as vaccine candidates in two mouse models, in which Cj0034c, Cj0404, and Cj0525c resulted in a reduction of invasion in spleen and liver after challenge.

The food-borne pathogen *Campylobacter jejuni* is a Gram-negative, microaerophilic, spiral-shaped, and motile bacterium. It is the most common cause of food- and waterborne gastroenteritis worldwide, causing approximately 500 million human infections every year (10, 28). Infection is often associated with consumption and handling of undercooked poultry meat, but water and other food sources also play a great role in the transmission of *C. jejuni* (10). The symptoms of campylobacteriosis range from mild noninflammatory, watery, self-limiting diarrhea to severe abdominal cramps and bloody diarrhea with fever and vomiting. Also, postinfectious complications such as reactive arthritis and Guillain-Barré syndrome are found to be associated with *C. jejuni* (3).

To colonize hosts, microorganisms require adherence factors, which are often surface structures such as pili that are expressed by many bacteria. However, genome annotations of *C. jejuni* strains have not revealed obvious pilus or pilus-like open reading frames (ORFs) (23). Other bacterial surface structures can also interact with host tissue, and they are likely responsible for the ability of *C. jejuni* to colonize the gastrointestinal tract of humans, which is believed to be essential for infection. A study has shown that *C. jejuni* isolated from patients with fever and diarrhea revealed a high level of binding to epithelial cells compared to isolates from patients without fever and diarrhea (8). Several mechanisms involved in the survival and persistence of the bacteria in the gut are known. Colonization of the gut is promoted by flagellum-mediated motility and binding to host tissue such as fibronectin mediated by CadF and FlpA (9, 17). Furthermore, several other outer membrane proteins (OMPs) are implicated in colonization, including major OMP (MOMP) (22), PEB1 (19), Omp50 (5), lipoproteins Omp18 (6, 18) and JlpA (13), and Cia proteins (27). In addition, some of the surface-exposed proteins are found to be immunogenic (6, 25), which opens the possibility of vaccine development. Humoral immune response to a number of *C. jejuni* antigens is developed in most people upon an infection, and epidemiological studies indicate that the immunity is crucial for the development of protection against *Campylobacter* disease (30).

The purpose of this study was to identify novel *C. jejuni* antigens and potential new virulence factors by screening a *C. jejuni* ORF expression library (24) with serum from rabbits immunized with a clinical *C. jejuni* human isolate. Selected candidates of the identified genes were examined for their role in virulence and tested as potential vaccines by subcutaneous immunization followed by oral challenge with *C. jejuni* in mouse colonization and invasion models.

MATERIALS AND METHODS

Bacterial strains and plasmid. The bacterial strains used in this study included *Escherichia coli* SURE (Stratagene) and *E. coli* BL21(DE3) (Stratagene), and the plasmid was pTLJ03. Strains and plasmid originate from an NCTC 11168 *C. jejuni* ORF library (24) available from Geneservice. The expression clone set comprises >1,600 *C. jejuni* ORFs, and the expression vector pTLJ03 generates N-terminal glutathione *S*-transferase (GST)-His-tagged fusion proteins. Strains were grown in LB medium or the expression medium MagicMedia (Invitrogen) at 37°C. pTLJ03-containing strains were grown in media containing 50 μg/ml ampicillin unless otherwise specified. The *C. jejuni* strains used in this study included *C. jejuni* NCTC 11168, *C. jejuni* NCTC 11168H, *C. jejuni* 81116, and *C. jejuni* 72Dz/92 (32). *C. jejuni* NCTC 11168H is a stable hypermotile variant of the reference strain *C. jejuni* NCTC 11168 (16). *C. jejuni* strains were grown at 37°C microaerobically on blood plates (BaseII and 5%

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blood), in brucella broth, brain heart infusion (BHI) broth, or biphasic (blood agar overlaid with BHI or brucella broth) with antibiotic when needed (30 μg/ml kanamycin and/or 50 μg/ml streptomycin).

Expression library. The library was originally created in *E. coli* SURE for optimal storage. The strain does not contain the T7 polymerase, and for that reason the *E. coli* BL21(DE3) expression strain was used. The clones were grown separately in microtiter plates in 200 μl of LB medium containing ampicillin overnight, and subsequently the plasmids were purified as a pool. The chemically competent *E. coli* BL21(DE3) strain was then transformed with the pool of vectors and plated on selective plates. This revealed an expression library consisting of 2,304 clones (24 microtiter plates).

Immunoblot assay. Individual clones were grown for 16 to 20 h in microtiter plates in MagicMedia for optimal expression. Two microliters of the culture was spotted on nitrocellulose membranes. The membranes were blocked in blocking buffer for 30 min, washed in phosphate-buffered saline (PBS)-Tween, and then incubated in *C. jejuni* primary antibody raised in rabbits (1:1,000) at 4°C for 16 to 20 h. The membranes were then washed in PBS-Tween and incubated in secondary antibody (polyclonal goat anti-rabbit immunoglobulins/horseradish peroxidase [HRP]; Dako) for 1 h. The reaction was visualized by chemiluminescence (chemiluminescent substrate; Invitrogen). The *C. jejuni* primary antibody was raised in a rabbit immunized with a heat-killed (100°C for 1 h) *C. jejuni* Penner serotype 2 isolate from a human patient (29). This serotype was chosen since it corresponds to that used for creating the commercial library (NCTC 11168). The serum was preincubated with *E. coli* BL21(DE3) before use to minimize background reaction. To verify that the antigens also reacted against human serum, dot blotting with 10 selected clones expressing antigens and serum isolated from a patient infected with *C. jejuni* Penner serotype 2 (29) was carried out as described above.

Clone sequencing. Plasmid DNA was isolated from 100 ml *E. coli* BL21(DE3) cultures using MidiPrep (Qiagen). Sequencing was conducted by Macrogen Inc., and the primer 5′GCT ATC CCA CAA ATT GAT AA 3′ was used.

Recombinant DNA techniques. *C. jejuni* 11168H knockout mutants were kindly provided by the London School of Hygiene and Tropical Medicine, University of London. Mutants were constructed via insertion of the Km cassette into unique sites present in pUC18-based recombinant plasmids containing random 1- to 2-kb fragments from the *C. jejuni* NCTC 11168 genome library (23). The NCTC 11168H knockout mutants provided for this study were the *C. jejuni* 0034c (Cj0034c) mutant and the Cj0645, Cj0917, and Cj1371c mutants.

Subsequently, the gene knockouts were transferred from *C. jejuni* strain 11168H to *C. jejuni* strain 11168 to restore motility and spiral morphology. Natural transformation was performed as described previously (31) with some modifications. *C. jejuni* cultures grown overnight on BHI agar plates were collected and resuspended in 12 ml BHI broth to an optical density at 600 nm (OD_{600}) of 0.001. Bacterial suspensions in three dilutions were transferred to sterilized petri dishes and incubated at 37°C with no shaking under microaerobic conditions overnight. Cultures (200 μl) with OD_{600}s of 0.2 to 0.3 were transferred to sterilized tubes containing 1 ml BHI and incubated at 37°C with shaking under microaerobic conditions for 2 h. Then, 10 ng of genomic DNA (purified with a Qiagen blood and tissue kit) of the mutants was added to each tube. After additional incubation for 3 h, bacterial cultures were serially diluted and plated on BaseII agar plates with antibiotics (50 mg/liter kanamycin). The agar plates were incubated at 37°C under microaerobic conditions for 3 days. The mutants were checked for curved shape and motility before being tested in assays.

INT407 adhesion assay. INT407 cells (ATCC CCL6 [derived from human embryonic jejunum and ileum]) were grown in minimum essential medium (MEM) (plus GlutaMAX) (Invitrogen) containing 25 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum in 5% CO_2. Cells were seeded at 2.5×10^5 per well in 24-well plates, incubated overnight, and checked for a 100% confluent monolayer. The *E. coli* clones were grown overnight in MagicMedia broth (Invitrogen) at 37°C, and *C. jejuni* was grown on blood agar plates microaerobically at 37°C. Immediately before testing, the OD_{600} of the bacteria was adjusted to 1 in PBS, and 1 ml of bacterial culture was added to the INT407 cells, which were then incubated for 2 h at 37°C. The cells were then resuspended and diluted in PBS and spotted on agar plates with appropriate antibiotics.

Electron microscopy. To investigate whether the *C. jejuni* mutant strain differed morphologically from the wild-type strain, transmission electron microscopy was conducted. Initially, the bacterial cultures were fixated in 1% glutaraldehyde (ethyl methanesulfonate [EMS]; Hatfield) for 30 min. To improve the adhesion of the bacteria, Formvar-coated 400-mesh copper grids were treated for 5 min with alcian blue (Sigma-Aldrich). Grids were placed on top of cultures of *C. jejuni* NCTC 11168 and *C. jejuni* NCTC 11168ΔCj0034c, respectively; after 5 min of incubation, most of the suspensions were removed from the grids with filter paper and the grids were stained for 30 s with phosphotungstic acid (BDH Chemicals). The grids were allowed to air dry and then viewed in a Morgagni 268D transmission electron microscope, and pictures were taken using a Mega-view III digital camera.

Motility assay. A motility assay was carried out to ensure no altered motility for the NCTC 11168ΔCj0034c mutant. To 0.25% soft agar plates was added 1 μl bacterial culture (OD_{600} adjusted to 0.1) in the middle of the plate, and diameter was measured over a time period of 24 h.

Serum resistance assay. Serum sensitivity assays were performed by a modification of the method of Blaser et al. (4). *C. jejuni* NCTC 11168 and *C. jejuni* NCTC 11168ΔCj0034c were grown overnight in *Brucella* biphasic cultures at 37°C, washed in PBS, pH 7.4, and adjusted to a concentration of 10^3 CFU/ml. *C. jejuni* cells (10-μl aliquots) were incubated in 240-μl pools of whole human blood (venous blood), human serum (whole blood incubated at 25°C 30 min, centrifuged 1,000 × *g* for 10 min at 4°C, and supernatant isolated), and heat-inactivated human serum (56°C for 30 min) for 30, 60, 90, and 120 min. Following the incubation period, CFU were enumerated on BHI agar.

Biofilm and autoagglutination. Cell-to-cell autoagglutination was assayed in PBS as described by Misawa and Blaser (20). Biofilm assays were performed in 50-ml centrifuge tubes containing 25 ml of inoculated brucella broth with *C. jejuni* NCTC 11168 and the Cj0034c knockout mutant. A glass slide was added to each tube and incubated microaerobically for 48 h. Then, the slides were stained with crystal violet and biofilm formation was visualized.

Predictions of protein localization. Prediction of protein localization and amounts of transmembrane helices was made by the TMHMM 2.0 server (21). The SignalP 3.0 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences. The method incorporates a prediction of cleavage sites and a signal peptide/nonsignal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. The LipoP 1.0 server produces predictions of lipoproteins and discriminates between lipoprotein signal peptides, other signal peptides, and N-terminal membrane helices in Gram-negative bacteria (14). All three servers are available at http://www.cbs.dtu.dk/services/.

Protein purification. His tag purification was made with the already GST-His-tagged constructed vector from Geneservice. An overnight preculture of *E. coli* BL21(DE3) containing the vectors was 50-fold diluted to inoculate 1,000 ml of LB medium containing appropriate antibiotics. The cultures were incubated with shaking at 37°C to an OD_{600} of 0.5, induced with 10 mM IPTG (isopropyl β-D-thiogalactopyranoside), and incubated with shaking for 16 h at 30°C. After induction, cells were lysed on ice in 20 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol) by the addition of 1 mg/ml lysozyme followed by sonication. Lysates were cleared by centrifugation at 15,000 × *g* for 30 min. Proteins were purified by nickel affinity chromatography using Ni-nitrilotriacetic acid (NTA) resin (Qiagen) equilibrated with lysis buffer and eluted with 250 mM imidazole. Eluted proteins were concentrated and dialyzed against 25 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol.

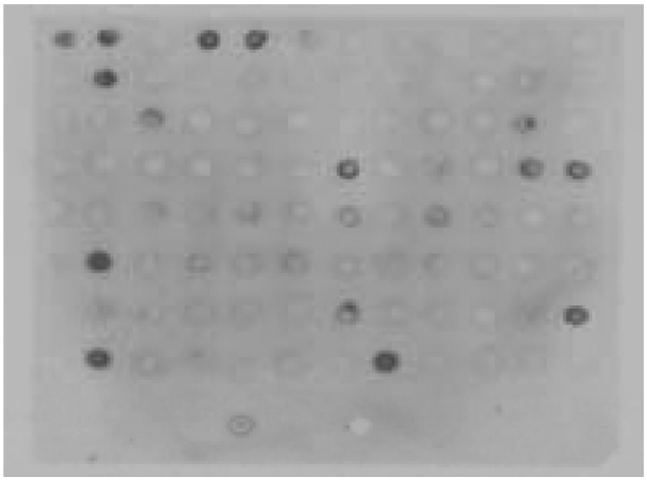

FIG 1 Immunoblot assay screen. The figure shows a representative immunoblot assay with 96 *E. coli* clones each expressing a *C. jejuni* gene reacting against rabbit serum. Dots with a strong reaction were isolated and the genes sequenced. A total of 52 clones were sequenced, representing 25 genes. The lower left dot is the BL21 expression strain with no insert and the right a medium control.

Mouse vaccination and challenge studies. Five antigens, Cj0525c, Cj0404, Cj1371, Cj0034c, and Cj1382c, were tested for their ability to protect against *C. jejuni* infection in 2 mouse models: (i) prevention of invasion of internal organs (liver and spleen) in CH3/HeN mice and (ii) prevention of intestinal colonization in BALB/c mice. Mice (10 in each group) were subcutaneously immunized with a 5-μg protein/dose (1.6 μg for Cj1371c) along with adjuvant GNE (a nonbacterial oil/water adjuvant; proprietary product; Intervet, Ltd., Boxmeer, The Netherlands). Four weeks later, the C3H/HeN mice were treated for 3 days with streptomycin (5 g/liter in drinking water) and challenged orally 1 day later with strain 72Dz/92 (32) (5×10^7 CFU). The strain was selected for its virulence in our model because invasion in organs was better compared to *C. jejuni* 81-176 and *C. jejuni* RM1221 (data not shown). Six days later, mice were euthanized and approximately 1/10 of liver and spleen was homogenized in 0.75 ml of physiological salt solution in the Percellys homogenizer and used to determine the number of viable *Campylobacter* cells. In the study of prevention against colonization strain *C. jejuni* 81116 (6×10^5 CFU) was orally inoculated in BALB/c mice. Subsequently, one fresh fecal dropping was collected regularly from each mouse and resuspended in medium, and dilutions were made in order to determine CFU/gram feces. Statistical significance ($P < 0.05$) was calculated using the Student *t* test (2-tailed with equal variance).

RESULTS

Identification of *C. jejuni* antigens. With the aim of identifying immunoreactive *C. jejuni* proteins, plasmid DNA was isolated from a pooled mixture of commercial library clones expressing *C. jejuni* NCTC 11168 ORFs in the plasmid pTLJ03 (21) and transformed *E. coli* BL21 to allow expression from the T7 promoter. The resulting transformants were individually spotted on a nitrocellulose membrane and reacted with serum isolated from a rabbit immunized with a *C. jejuni* human clinical isolate (serotype 2). The screening revealed several immunogenic *E. coli* clones that selectively reacted with the serum as shown in Fig. 1. Inserts in plasmids isolated from the transformants that repeatedly proved as highly immunogenic were selected for sequencing, and from a total of 2,304 clones, 52 inserts were sequenced representing 25 genes encoding potential antigens (Table 1). The identified *C. jejuni* genes were classified according to their predicted function (20) as shown in Table 1.

TABLE 1 Sequenced highly immunogenic clones

ORF (NCTC 11168)[a]	Annotation	Classification metabolism	Function location
Cj0014c	Putative integral membrane protein	Macromolecule	Membranes, lipoproteins, and porins
Cj0034c (4)	Putative periplasmic protein	Macromolecule	Miscellaneous periplasmic proteins
Cj0111 (3)	Putative periplasmic protein	Macromolecule	Miscellaneous periplasmic proteins
Cj0203	Putative transmembrane transport protein	Cell processes	Protein and peptide secretion
Cj0383c	*ribH*, 6,7-dimethyl-8-ribityllumazine synthase	Small molecule	Riboflavin
Cj0404 (3)	Putative transmembrane protein	Macromolecule	Membranes, lipoproteins, and porins
Cj0408	*frdC*, fumerate reductase cytochrome B subunit	Small molecule	Tricarboxylic acid cycle
Cj0477	*rplL*, 50S ribosomal protein	Macromolecule	Ribosomal protein synthesis
Cj0525c (3)	*pbpB*, putative penicillin binding protein	Macromolecule	Murein sacculus and peptidoglycan
Cj0645 (2)	Putative secreted tranglycosylase	Macromolecule	Murein sacculus and peptidoglycan
Cj0774c (5)	ABC transport system ATP binding protein	Cell processes	Protein and peptide secretion
Cj0811	*lpxK*, tetrasyldisaccharide 4′-kinase	Macromolecule	Surface polysaccharides, lipopolysaccharides, and antigens
Cj0917c	*cstA*, carbon starvation protein A homolog	Macromolecule	Membranes, lipoproteins, and porins
Cj0965c	Putative acyl coenzyme A thiosester hydrolase	Other	Miscellaneous
Cj1092c	*secF*, protein export membrane protein	Cell processes	Protein and peptide secretion
Cj1094c	*yajC*, preprotein translocase subunit	Cell processes	Protein and peptide secretion
Cj1163c (4)	Putative cation transport protein	Cell processes	Cations
Cj1174 (3)	Putative efflux protein	Other	Drug/analogue sensitivity
Cj1292	*dcd*, dCTP deaminase	Small molecule metabolism	2′-deoxyribonucleotide biosynthesis
Cj1364c	*fumC*, fumerate hydratase	Small molecule metabolism	Tricarboxylic acid cycle
Cj1371 (2)	Putative periplasmic protein (*vacJ* homolog)	Cell processes	Pathogenicity
Cj1382c (4)	*fldA*, flavodoxin	Small molecule metabolism	Respiration
Cj1529c (5)	*purM*, phosphoribosylaminoimidazole synthase	Small molecule metabolism	Purine ribonucleotide biosynthesis
Cj1628	*exbB2*, putative *exbB*/*tolQ* family transport protein	Cell processes	Other
Cj1632c	Putative periplasmic protein	Miscellaneous periplasmic proteins	Miscellaneous

[a] Numbers in parentheses are the times the indicated ORF products were identified as strong reactors against the rabbit antiserum and sequenced.

TABLE 2 Localization of the proteins and possible signal peptide molecule predicted by TMHMM, SignalP, and LipoP

Protein	Localization	Transmembrane helices	Statistical significance (probability)	Predicted signal peptide lipoprotein[a]
Cj0014c	Inside and outside	3	Yes (0.951)	TMH
Cj0034c	Inside and outside	1	Yes (0.999)	SpI
Cj0111	Inside and outside	1	No	CYT
Cj0203	Inside and outside	12	No	TMH
Cj0383c	Outside	0	No	CYT
Cj0404	Inside and outside	1	No	CYT
Cj0408	Inside and outside	5	No	TMH
Cj0477	Outside	0	No	CYT
Cj0525c	Inside and outside	1	No	TMH
Cj0645	Outside	0	Yes (0.992)	SpI
Cj0774c	Outside	0	No	CYT
Cj0811	Inside and outside	1	No	TMH
Cj0917c	Inside and outside	16	Yes (0.940)	TMH
Cj0965c	Inside	0	No	CYT
Cj1092c	Inside and outside	4	No	TMH
Cj1094c	Inside and outside	1	No	CYT
Cj1163c	Inside and outside	6	No	CYT
Cj1174	Inside and outside	4	No	TMH
Cj1292	Outside	0	No	CYT
Cj1364c	Outside	0	No	CYT
Cj1371	Outside	0	Yes (1.000)	SpI
Cj1382c	Outside	0	No	CYT
Cj1529c	Outside	0	No	CYT
Cj1628	Inside and outside	3	No	TMH
Cj1632c	Outside	0	Yes (0.999)	SpI

[a] TMH, transmembrane helices; SpI, signal peptidase I; SpII, lipoprotein signal pepdidase II; CYT, cytoplasmic or all the rest.

Prediction of localization of the proteins and other structural features was performed by the THHMM 2.0 server (21) and results are shown in Table 2. Fourteen out of 25 proteins were predicted to contain one or more membrane helices, 1 of these 14 with a signal peptide (predicted with The SignalP 3.0 server). Ten of the proteins were predicted to be located externally, where three of these harbor a signal peptide. One protein was predicted to be located inside the cell. None of the proteins were predicted to contain a lipoprotein signal peptidase.

Antigens are antigenic in humans. To confirm that the identified antigens are also recognized in human infection, we selected 10 clones for Western blot analysis with human antiserum obtained from a patient infected with *C. jejuni* Penner serotype 2. The clones were chosen to cover various cellular predicted localizations and functions and were found in all cases to react with the antiserum (Fig. 2).

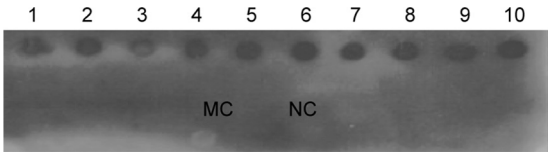

FIG 2 Immunoblot assay with human serum. The *E. coli* clones Cj0034c (lane 1), Cj0203 (lane 2), Cj0404 (lane 3), Cj0525c (lane 4), Cj0645 (lane 5), Cj0917c (lane 6), Cj1094c (lane 7), Cj1371 (lane 8), Cj1382c (lane 9), and Cj1632c (lane 10), from left to right, were tested for its reactivity against the antiserum isolated from a human patient infected with *C. jejuni* serotype 2. All the clones reacted more or less with the serum. Lower left is a medium control (MC) and lower right a negative BL21(DE3) control (NC).

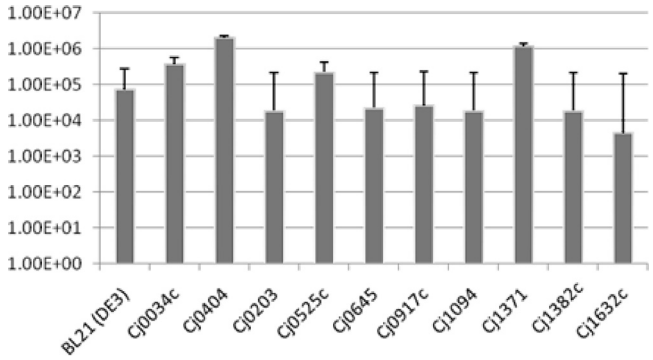

FIG 3 INT407 cell line adhesion assay. The *E. coli* clones Cj0034c, Cj0203, Cj0404, Cj0525c, Cj0645, Cj0917c, Cj1094c, Cj1371, Cj1382c, and Cj1632c were examined for their ability to adhere to INT407 epithelial cells. BL21 is used as negative control and standard bars indicate standard deviations.

Several antigens support host cell adhesion. Adhesion of *C. jejuni* to host cells forms the first important step in the infection process. With the aim of addressing if the identified antigens contribute to host cell adhesion, the clones reacting with the human antiserum were investigated for their ability to adhere to the intestinal epithelial cell line INT407 (Fig. 3). Interestingly, expression of three of the *C. jejuni* antigens, namely, Cj0034c, Cj0404, and Cj1371, enhanced the ability of *E. coli* BL21 to adhere to INT407 cells. Subsequently, the gene-specific *C. jejuni* mutants, available from Campylobacter Resource Facility, London School of Hygiene & Tropical Medicine, were examined in the same cell adhesion assay. While the absence of Cj0404 and Cj1371 did not affect the ability of *C. jejuni* to adhere to INT407 cells, inactivation of Cj0034c dramatically reduced adhesion, suggesting that Cj0034c may contribute to the establishment of *C. jejuni* in host organisms (Fig. 4). Thus, the Cj0034c gene may encode a novel *C. jejuni* adhesion factor.

Characterization of Cj0034c, a new *C. jejuni* virulence factor. Since Cj0034c reduces the ability of *C. jejuni* to adhere to host cells, we attempted to obtain more information about the gene product using the structural prediction and homology search tool HHpred (http://toolkit.tuebingen.mpg.de/hhpred). This search predicted

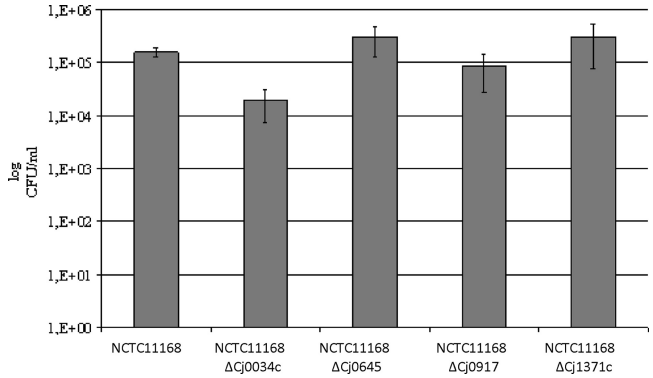

FIG 4 INT407 cell line assay. Knockout mutants of *C. jejuni* 11168, the ΔCj0034c, ΔCj0645, ΔCj0917, and ΔCj1371c mutants, were tested for their ability to adhere to INT407 epithelial cells compared to the NCTC 11168 wild type. Standard bars indicate standard deviations.

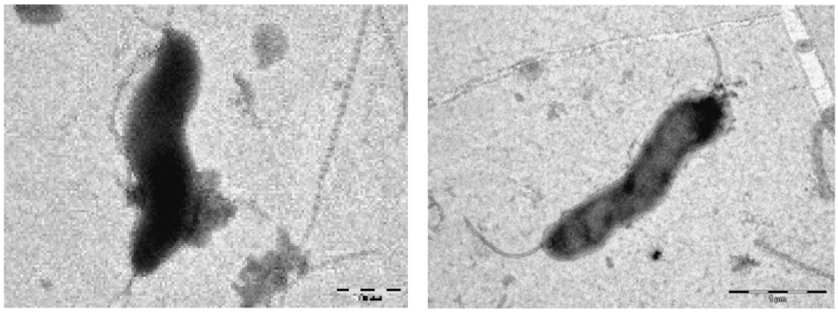

FIG 5 Electron micrographs of *C. jejuni* NCTC 11168 (left) and *C. jejuni* NCTC 11168ΔCj0034 (right).

homology to the *E. coli* YggE, which plays a role in the defense of oxidative stress imposed by the toxic salt potassium tellurite. With the anticipated location and the potential adhesive properties of Cj0034c, inactivation of the corresponding gene might influence the cell surface. This was examined by electron microscopy, and the result revealed that the mutation does not result in major structural changes to bacterial cell morphology (Fig. 5). Also, the inactivation of Cj0034c did not influence other properties associated with cell surface composition such as serum resistance, motility, autoagglutination, and biofilm formation compared to the wild-type strain (data not shown).

Antigens as vaccine candidates. With the aim of examining the identified antigens as vaccine candidates, we attempted to express and purify the gene products from the 10 characterized clones. However, after several attempts, it was possible to obtain sufficient quantities of only five of the antigens. These were tested in two *Campylobacter* oral challenge mouse models; one in C3H/HeN mice, in which invasion of strain 72Dz/92 in liver and spleen was measured, and the other in Balb7c mice, in which shedding of strain 81116 in feces was measured. The combination of mice and *Campylobacter* challenge strain was chosen based on trial experiments, and those were selected that gave the most reproducible results (data not shown). The challenge study (Fig. 6) showed the absence of invasion in spleen for at least two of the proteins, Cj0525c and Cj1382; however, this was not statistically significant. Invasion in the liver was significantly reduced only in the group immunized with Cj0404. When invasions in both liver and spleen were combined as one parameter, antigens Cj0525c, Cj0404, and Cj0034c showed significant protection against invasion. Challenge colonization results are shown in Fig. 7, but no significant protection against colonization by any of the proteins was observed.

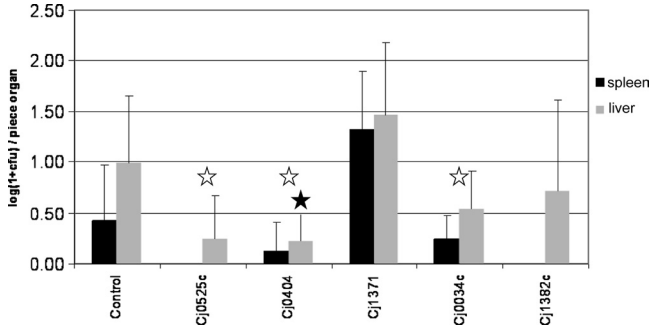

FIG 6 Challenge invasion study. Invasion of *C. jejuni* 72Dz/92 in spleen and liver of C3H/He mice ($n = 10$), 6 days after challenge. Values were determined individually, log transformed (log 1+CFU), and calculated as averages per group. Error bars indicate standard deviation. The mice were immunized with the indicated antigens before challenge. Statistical significance ($P < 0.05$) was found for invasion in liver for Cj0404 (filled star) and for combined invasion in liver and spleen for Cj0525, Cj0404, and Cj0034 (open stars).

DISCUSSION

In this study, we have successfully identified immunoreactive proteins of the important human pathogen *C. jejuni.* An *E. coli* library expressing single *C. jejuni* open reading frames was screened using specific *C. jejuni* antibodies, resulting in the identification of immunoreactive *C. jejuni* proteins. Of the 25 identified antigens, 14 were predicted to include one or more membrane helices, of which three carry a predicted signal peptide. Another three proteins were predicted to express a signal peptide, and only one candidate was proposed to be located in the cytoplasm. In a previous study, Cordwell et al. (7) examined surface-located *C. jejuni* proteins and described several proteins also identified in our study, e.g., Cj0034c, Cj0404, Cj0917c, Cj0774c, Cj1092, and Cj1094. Thus, our data suggest that *E. coli* is able to express and translocate *C. jejuni* cell surface proteins and that the *E. coli* library is suitable for the identification of *C. jejuni* antigens. Surprisingly, we did not detect the most immunodominant and well-characterized antigens described for *C. jejuni*, namely, FlaA, Peb1A, and PorA. This may be a consequence of the library construction, as a pool of library clones was used for transforming the T7-expressing BL21 cells and this pool may be biased, with some genes being present in more copies than others. Alternatively, some *C. jejuni* extracellular proteins may be less well expressed and secreted by *E. coli*, and if so they will not be detected in the Western blot analysis.

One of the interesting genes identified in our study, the Cj0034c gene, may encode a new adhesion factor in *C. jejuni*, as deletion of this gene resulted in reduced adhesion to epithelial cells, and, conversely, overexpression of Cj0034c in *E. coli* enhanced the ability of the bacteria to adhere to INT407 epithelial cells. The gene product is grouped within the 3.C.5 miscellaneous periplasmic proteins (Table 1) together with other important antigen and virulence genes, such as PEB2, 3, and 4 (23). A homology search using http://toolkit.tuebingen.mpg.de/hhpred predicted the secondary structure of Cj0034c to be homologous to YggE in *E. coli*. YggE defends *E. coli* against oxidative stress caused by the toxic salt tellulite, but the mechanism remains obscure (1). In general, other studies of *C. jejuni* have suggested a link between oxidative stress survival and virulence. Haddad et al. (12) discovered an increase of bound *C. jejuni* to epithelial cells when exposed

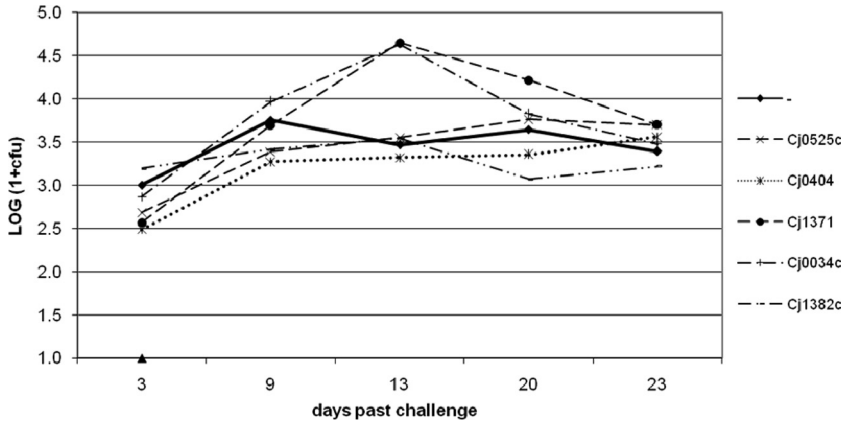

FIG 7 Challenge colonization study. Colonization of BALB/c with *C. jejuni* strain 81116 in days after challenge. Individual colonization was determined per fecal pellet, log transformed (log 1+CFU), and calculated as average per group. The mice were immunized with buffer, Cj0525, Cj0404, Cj1371c, Cj0034c, and Cj1382c before challenge. No statistically significant differences were found.

to oxidative stress, and Gundogdu et al. (11) found Cj1556 to be involved in resistance to oxidative stress and adherence to epithelial cells. We examined if Cj0034c similarly influences oxidative stress tolerance in *C. jejuni*, but preliminary results indicate that Cj0034c mutant and wild-type cells survive oxidative stress equally well (data not shown).

Cj0034c was also predicted to contain one helix and one signal peptide, suggesting that it is surface exposed and therefore an accessible target for the immune system. A surface location may also stimulate interactions with host cells by adhering to epithelial cells, for example. Despite the lack of identifiable pili and other adherence organelles, several proteins promote adherence of *C. jejuni* to eukaryotic cells. These include CadF, which binds to fibronectin and is required for maximal binding and invasion (17), and JlpA and CapA, needed for adherence to epithelial cells (2, 13).

A study by Prokhorova et al. (26) used proteomics for the identification of *Campylobacter* surface proteins and demonstrated that some of them were also protective in a *C. jejuni* challenge study. Flavodoxin (Cj1382c) was among the identified proteins but it was not examined in a mouse vaccination-challenge experiment. Flavodoxin and Cj1292 (dCTP deaminase) were identified as being among the proteins expressed in an efficient chicken colonizing strain when compared to a poor colonizer (15). Furthermore, this study determined that Cj0203 was overexpressed along with important surface adhesins such as CadF and FlaA (9, 15).

Vaccination challenge experiments showed that Cj0525c, Cj0404, and Cj0034c offered the best protection against invasion of spleen and liver. Cj0525c is a large antigen, not likely to be expressed by a vaccine strain (the *Salmonella enterica* serovar Typhimurium Δ*recA* Δ*htrA* strain (not published)). Cj0034c is a smaller antigen that might be suitable for expression in a vaccine strain as is Cj0404, and both were easily expressed by *E. coli* BL21(DE3) and purified. Interestingly, of the examined antigens, Cj0404 gave the best protection against invasion of *C. jejuni*. The protein Cj0404 is grouped with membrane proteins, lipoproteins, and porins and is predicted to contain one transmembrane helix and most likely be surface exposed. Therefore, we propose that Cj0404 is a vaccine candidate against *C. jejuni*.

Our screen for immunoreactive proteins resulted in identification of a new protein important for *C. jejuni* virulence, Cj0034c, and a possible new vaccine candidate, Cj00404.

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