

Characterization of Genetically Matched Isolates of *Campylobacter jejuni* Reveals that Mutations in Genes Involved in Flagellar Biosynthesis Alter the Organism's Virulence Potential[∇]

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Phenotypic and genotypic evidence suggests that not all *Campylobacter jejuni* isolates are pathogenic for humans. We hypothesized that differences in gene content or gene expression alter the degree of pathogenicity of *C. jejuni* isolates. A *C. jejuni* isolate (Turkey) recovered from a turkey and a second *C. jejuni* isolate (CS) recovered from a chicken differed in their degrees of *in vitro* and *in vivo* virulence. The *C. jejuni* Turkey isolate invaded INT 407 human epithelial cells and secreted the Cia (*Campylobacter* invasion antigen) proteins, while the *C. jejuni* CS isolate was noninvasive for human epithelial cells and did not secrete the Cia proteins. Newborn piglets inoculated with the *C. jejuni* Turkey isolate developed more severe clinical signs of campylobacteriosis than piglets inoculated with the *C. jejuni* CS isolate. Additional work revealed that flagellin was not expressed in the *C. jejuni* CS isolate. Microarray and real-time reverse transcription-PCR analyses revealed that all flagellar class II genes were significantly downregulated in the *C. jejuni* CS isolate compared to the *C. jejuni* Turkey isolate. Finally, nucleotide sequencing of the *flgR* gene revealed the presence of a single residue that was different in the FlgR proteins of the *C. jejuni* Turkey and CS isolates. Complementation of the *C. jejuni* CS isolate with a wild-type copy of the *flgR* gene restored the isolate's motility. Collectively, these findings support the hypothesis that critical differences in gene content or gene expression can alter the pathogenic potential of *C. jejuni* isolates.

Campylobacter jejuni strains are genetically diverse. The diversity of this organism has been observed by a number of techniques including pulsed-field gel electrophoresis (PFGE) (82), subtractive hybridization (2), microarray analysis (16, 42, 61, 64, 65), and multilocus sequence typing (MLST) (71). Some differences in genetic content between strains can be explained by the presence of polynucleotide tracts in hypervariable loci, including those encoding the lipo-oligosaccharide (LOS), flagellar biosynthesis, and the polysaccharide capsule (60). Such genetic diversity has been problematic in studies attempting to distinguish pathogenic from nonpathogenic isolates. Aeschbacher and Piffaretti (1) reported that there was no clustering of animal and human isolates based on multilocus enzyme electrophoresis using nine polymorphic loci. Based on this result, the authors concluded that every animal isolate is a potential human pathogen. Manning et al. (47) performed MLST on 266 *C. jejuni* isolates and found that the populations of *C. jejuni* veterinary and human isolates overlapped among the 19 clonal complexes identified.

The ability of *C. jejuni* to cause disease is a complex, multifactorial process. Potential virulence determinants include toxins (7, 11, 26, 34, 40, 46, 48, 63, 69, 79, 85), adherence factors

(adhesins) (13, 19, 20, 35, 38, 49, 53, 62), and entry-promoting molecules (invasins) (5, 18, 36, 55, 57, 70, 75). Also of interest are strain-variable genes, which encode factors involved in iron acquisition, DNA restriction/modification, sialylation, flagellar biosynthesis, LOS biosynthesis, and capsular biosynthesis (60, 81). Investigators have proposed that strain-variable genes encode factors that contribute to different disease presentations and enable the organism to survive in unique ecological niches. Regardless, an established set of *C. jejuni* virulence genes and a determination of their contribution to disease production have yet to emerge.

To better understand the metabolic capacity and virulence properties of *C. jejuni*, the genome sequence of strain NCTC 11168 was determined (60). Shortly thereafter a comparison of *C. jejuni* isolates by whole-genome microarray analysis revealed extensive genetic diversity (16). Based on the comparison of 11 *C. jejuni* isolates, 21% of the genes in the *C. jejuni* NCTC 11168 strain sequence were proposed to be dispensable as they were either absent or highly divergent among the other isolates included in the study. Dorrell et al. (16) also noted that many of the virulence genes identified to date are conserved among *C. jejuni* strains.

Published reports suggest that not all *C. jejuni* strains have the same virulence potential. Everest et al. (18) noted that 86% of *Campylobacter* isolates from individuals with colitis were able to translocate across Caco-2 polarized cells versus 48% of strains isolated from individuals with noninflammatory disease. Fauchère et al. (20) found that *C. jejuni* recovered from individuals with fever and diarrhea adhered to cultured cells with greater efficiency than those isolated from asymptomatic indi-

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viduals. The relative ability of *C. jejuni* to invade cultured cells also appears to be strain dependent (18, 38, 54). Newell et al. (54) found that environmental isolates were much less invasive for HeLa cells than clinical isolates as determined by immunofluorescence and electron microscopy examination of *C. jejuni*-infected cells. Moreover, a statistically significant difference in the level of invasion between *C. jejuni* isolates from individuals with colitis and those from individuals with noninflammatory diarrhea has been observed (18).

Poly et al. (65) compared two unrelated *C. jejuni* strains (ATCC 43431 and NCTC 11168) by a shotgun genomic DNA microarray technique. Not surprisingly, a large number of genes unique to ATCC 43431 encoded proteins involved in LOS and capsular synthesis. Interestingly, 36 of 130 DNA fragments unique to ATCC 43431 showed similarity to DNA from the *Helicobacter hepaticus* Imp locus, located on a putative pathogenicity island. In other organisms, the Imp locus has been proposed to modulate host responses (9). More recently, Poly et al. (64) compared the genomic content of *C. jejuni* strain 81-176 with that of *C. jejuni* strain NCTC 11168 and found divergence at LOS, capsular biosynthesis, and restriction/modification system loci.

In this study, we assessed the putative virulence properties of *C. jejuni* isolates recovered from poultry. More specifically, we analyzed the virulence attributes of two *C. jejuni* isolates, designated Turkey and CS, because they were indistinguishable by PFGE, MLST, and genotyping. The *C. jejuni* Turkey isolate showed greater pathogenic potential than the *C. jejuni* CS isolate based on both in vitro and in vivo assays. The lack in pathogenic potential of the *C. jejuni* CS isolate, relative to the *C. jejuni* Turkey isolate, was attributed to a loss in the synthesis of a functional flagellum. The flagellum and associated motility are a known *C. jejuni* virulence determinant (8, 51, 53, 77, 78, 84). In addition, host cell invasion and the secretion of the Cia (*Campylobacter* invasion antigen) proteins require a functional flagellar export apparatus (39). We identified a point mutation in the *flgR* gene of the *C. jejuni* CS isolate. The *flgR* gene in *C. jejuni* encodes the response regulator of the FlgS/FlgR two-component signal transduction system that participates in flagellar biosynthesis. Phosphorylation of the C-terminal output domain of the FlgR response regulator protein presumably results in its binding to DNA, whereby it interacts with σ^{54} to initiate the expression of flagellar class II genes. The class II flagellar genes encode proteins that are required for the assembly of a functional flagellum (32, 33).

While other investigators have examined differences in *C. jejuni* isogenic strains or passage variants, we are unaware of another study undertaken to examine the virulence attributes of naturally occurring clonally matched isolates. We identified a difference in the nucleotide sequences of the *flgR* genes in *C. jejuni* Turkey and CS isolates, which resulted in the nonmotile and avirulent phenotype of the *C. jejuni* CS isolate. This mutation appears to provide the isolate a fitness advantage in the environment. More specifically, the *C. jejuni* CS isolate exhibited greater resistance to the antimicrobial activity of the bile salt deoxycholate and is thus able to reach a greater cell density in deoxycholate-supplemented medium than a genotypically matched isolate. Further, the *C. jejuni* Turkey isolate harboring a mutation in *flgR* mirrored the phenotype of the *C. jejuni* CS isolate.

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MATERIALS AND METHODS

Culture of bacterial isolates. Two *C. jejuni* human clinical isolates (*C. jejuni* F38011 and 81-176) and six *C. jejuni* poultry isolates were used throughout this study. Five of the six *C. jejuni* poultry isolates were recovered from chicken rinses (CS, S1, S2B, S3, and SC), and one isolate was recovered from the liver of a turkey (Turkey) exhibiting clinical signs of poultry enteritis. All *C. jejuni* isolates were cultured at 37°C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) on Mueller-Hinton (MH) agar plates supplemented with 5% citrated bovine blood (MH-blood agar). Cultures were subcultured to a fresh plate every 24 to 48 h. In addition to the six *C. jejuni* isolates used throughout this study, we also screened an additional 99 *C. jejuni* poultry isolates for motility. These isolates used were recovered from either poultry processing plants located in Kansas (A1a, A2a, A3a, A4a, A5a, A7a, A8a, A9a, A11a, A12a, A13a, A14a, A15a, A16a, A18a, A20a, A22a, A23a, A24a, A25a, and A37a), Iowa (D28a, D30a, D31a, D32a, D33a, D34a, D37a, D38a, D41a, D42a, D44a, D45a, D48a, and D49a), and Washington state (B13a, B19a, C11a, C18a, C20a, C24a, C25a, G9a, G11a, G12a, G13a, G15a, G17a, G21a, G22a, G26a, H1a, H2a, H3a, H4a, H5a, H8a, H29a, I6a, I7a, I16a, and I22a) or from cecal droppings collected at different broiler houses (2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 19, 21, 22, 23, 24, 25, 26, 33, 34, 35, 36, 39, 41, 42, 43a, 43c, 44, 77, 78, 79, 80, 81, and 83). All the *C. jejuni* poultry isolates used in this study were passaged less than five times in the laboratory. The primary stock of each of *C. jejuni* isolate was frozen in citrated bovine blood and stored at -80°C. *Escherichia coli* INVαF' (Invitrogen, Carlsbad, CA), S17-1 λ *pir*, and XL1-Blue MRF' (Stratagene, La Jolla, CA) were cultured on Luria-Bertani (LB) agar plates at 37°C.

Culture of INT 407 cells. A culture of INT 407 human epithelial cells (ATCC CCL6) was obtained from the American Type Culture Collection (Manassas, VA). Stock cultures of INT 407 cells were grown in minimal essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Cultures were maintained at 37°C in a humidified, 5% CO₂ incubator.

***C. jejuni*-INT 407 cell binding and invasion assay.** For experimental assays, each well of a 24-well tissue culture tray was seeded with 1.4×10^5 INT 407 cells and incubated for 18 h at 37°C in a humidified, 5% CO₂ incubator. The cells were rinsed with MEM-1% FBS and inoculated with a suspension of approximately 5×10^7 CFU of bacteria. Tissue culture trays were centrifuged at $600 \times g$ for 5 min and incubated at 37°C in a humidified, 5% CO₂ incubator. For binding, the infected monolayers were incubated for 30 min and rinsed three times with phosphate-buffered saline (PBS) and the epithelial cells were lysed with 0.1% (vol/vol) Triton X-100 (Calbiochem, La Jolla, CA). The suspensions were 10-fold serially diluted, and the number of viable, adherent bacteria was determined by counting the resultant colonies on MH-blood agar plates. To measure bacterial internalization, the infected monolayers were incubated for 3 h, rinsed three times with MEM-1% FBS, and incubated for an additional 3 h in MEM-1% FBS containing a bactericidal concentration (250 μg/ml) of gentamicin. The number of internalized bacteria was determined as outlined above. Unless otherwise stated, the reported values represent the mean counts \pm standard deviations derived from triplicate wells. All assays were performed a minimum of three times to ensure reproducibility. Significance between samples was determined using Student's *t* test following log₁₀ transformation of the data. Two-tailed *P* values were determined for each sample, and a *P* value <0.01 was considered significant.

Secretion assay. *C. jejuni* isolates were harvested from MH-blood agar plates in PBS, pelleted by centrifugation at $6,000 \times g$, washed twice in MEM, and suspended in MEM lacking methionine (labeling medium; ICN Biomedicals, Inc., Aurora, OH) to an optical density at 540 nm (OD₅₄₀) of 0.3 (approximately 5×10^8 CFU). Metabolic labeling experiments were performed in 3 ml of labeling medium with the addition of [³⁵S]methionine (PerkinElmer Life Sciences, Inc., Boston, MA) as described elsewhere (37). Following a 3-h labeling period, bacterial cells were pelleted by centrifugation at $6,000 \times g$ and supernatant fluids collected. The supernatant fluids were concentrated fourfold by the addition of 5 volumes of ice-cold 1 mM HCl-acetone followed by centrifugation. The pellets were air dried and resuspended in water. The secreted proteins were resolved in sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels using the discontinuous buffer system described by Laemmli (41). Gels were treated with

Amplify (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Autoradiography was performed with Kodak BioMax MR film at -70°C .

Motility assay. Motility assays were performed using MH medium supplemented with 0.4% Select agar (motility agar plates; Life Technologies, United Kingdom). A 10- μl suspension of each bacterial isolate was spotted on the surface of the semisolid medium. Motility plates were incubated at 37°C under microaerobic conditions for 48 h.

Macrorestriction enzyme PFGE. *C. jejuni* isolates were harvested from MH-blood agar plates in 3 ml PETF IV buffer (1 M NaCl, 10 mM Tris, 10 mM EDTA, pH 8.0) and cell densities adjusted to 0.75 using a Microscan turbidity meter (Dade Behring, West Sacramento, CA) in 12- by 75-mm Falcon round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ). Four hundred microliters of 1.4% (wt/vol) molten (50°C) pulsed-field grade agarose (Bio-Rad, Hercules, CA) was added to an equivalent volume of each bacterial suspension, the suspension was mixed gently, and a 100- μl aliquot was pipetted into agarose plug molds. The agarose plugs were removed from the molds and incubated in 1 ml of ESP buffer (50 mM Tris-HCl [pH 8.0], 50 mM EDTA, 1% [wt/vol] *N*-lauroylsarcosine, 0.5 mg/ml proteinase K) at 50°C for 1 h. Following cell wall lysis, the agarose blocks were washed three times in sterile water and three times in TE (10 mM Tris, pH 8.0, 1 mM EDTA). Each wash was performed at ambient temperature for 30 min. Individual agarose plugs were incubated with 100 μl of $1\times$ restriction endonuclease buffer containing 20 U of SmaI. The reaction mixtures were incubated at 25°C for a minimum of 4 h. Following incubation, the agarose plugs were loaded into an agarose gel. Restricted genomic DNA was separated in 1% (wt/vol) pulsed-field grade agarose prepared with $0.5\times$ TBE (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]). Run parameters consisted of a reorientation angle of 120° with a constant voltage of 120 V and a constant temperature of 14°C . An electrophoresis run time of 19 h and a ramped pulse time of 6.8 to 35.4 s were used. For plugs digested with SalI, a run time of 18 h and a ramped pulse time of 5.2 to 42.3 s were used. Gels were stained for 20 min in 3 $\mu\text{g}/\text{ml}$ ethidium bromide and destained for 20 min in water. Images were captured using a Bio-Rad FluorS system and processed using Adobe Photoshop.

MLST. MLST was performed as described by Dingle et al. (15). Portions of the housekeeping genes encoding aspartase A (*aspA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxymethyl transferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*), and the ATP synthase alpha subunit (*uncA*) were amplified and sequenced. Genomic DNA was recovered from *C. jejuni* isolates harvested from MH-blood agar plates. Amplification of each gene fragment was performed using approximately 10 ng of genomic DNA and 1.25 U of *Taq* DNA polymerase (Invitrogen) in a 50- μl reaction volume containing 1 μM forward and reverse primer, $1\times$ PCR buffer (Invitrogen), 1.5 mM MgCl_2 , and 0.8 mM deoxynucleoside triphosphates (dNTPs). Amplification reactions consisted of the following cycling conditions: an initial denaturation step (94°C , 2 min) followed by annealing (50°C , 1 min) and extension (72°C , 1 min) steps, which were repeated for 35 cycles. PCR amplicons were assessed for quantity and quality by agarose gel electrophoresis. Primers and unincorporated dNTPs were removed by passing amplicons through a commercially available batch column purification system (Qiaquick PCR kit; QIAGEN, Valencia, CA). The nucleotide sequences of PCR amplicons were determined using a modification of Sanger dideoxynucleotide sequencing, incorporating fluorescence dye technology, at the DNA sequencing facility of Washington State University. Sequencing was performed on both strands for a segment of each allele from each isolate. Nucleotide sequences for each allele were aligned using the program Clustal X, and the nucleotide sequences were assigned alleles from the MLST website (<http://mlst.zoo.ox.ac.uk>). Sequence types, based on the combination of assigned alleles to the seven housekeeping genes, were determined using the MLST website (<http://mlst.zoo.ox.ac.uk>).

Inoculation of piglets. Newborn piglets were obtained from sows at the time of farrowing. Feces from each sow were plated on selective Abeyta Hunt Bark agar plates containing sodium cefoperazone (32 mg/liter), rifampin (10 mg/liter), amphotericin B (2 mg/liter), and 4 ml/liter of FBP (62.5 g/liter sodium pyruvate, 62.5 g/liter ferrous sulfate, and 62.5 g/liter sodium metabisulfite). Suspect colonies were tested for *C. jejuni* as described by Nogva et al. (56). Only piglets obtained from *C. jejuni*-free sows were used for in vivo studies.

Each piglet was fed five times daily with 50 ml of Similac formula (Ross Laboratories). Overnight-grown cultures of *C. jejuni* were resuspended in Similac at a concentration of approximately 5×10^9 CFU/ml. Piglets were orally inoculated with approximately 20 ml of either the *C. jejuni* Turkey or CS isolate. Actual numbers of viable bacteria inoculated were determined by serial dilution and plating of the bacterial suspension.

Piglets were observed for diarrhea, and rectal swabs were plated on Abeyta Hunt Bark medium prior to inoculation and daily thereafter to determine the presence of *C. jejuni* shedding. Piglets were euthanized when morbidity was

observed or at 6 days postinoculation with 2 ml of Beuthanasia-D (Schering Corp.). Gross examinations of the small intestine, colon, and cecum were recorded, and tissue samples were fixed in 10% buffered formalin and examined for histological lesions at the University of Arizona Veterinary Diagnostic Laboratory (Tucson, AZ).

Preparation of whole-cell lysates, outer membrane proteins (OMP), and flagellin. *C. jejuni* whole-cell lysates were generated by harvesting bacteria grown overnight on MH-blood agar plates in PBS and subjecting the bacterial suspension to sonication on ice (five 30-s pulses).

OMP were prepared as described by deMelo and Pechère (13) with minor modifications. Briefly, bacterial sonicates were centrifuged at $8,000 \times g$ to remove whole bacterial cells. Supernatant fluids were centrifuged at $100,000 \times g$ for 2 h. The resulting pellets were resuspended in a solution of 1% Sarkosyl (Sigma, St. Louis, MO) in 10 mM Tris-HCl (pH 7.5) and allowed to incubate at ambient temperature for 30 min on a platform rocker. The solutions were centrifuged at $100,000 \times g$ for 2 h, and the supernatants were removed. The pellets were washed in 10 mM Tris-HCl (pH 7.5) and centrifuged at $100,000 \times g$ for 2 h. Finally, the pellets were resuspended in 50 mM Tris-HCl (pH 7.5), and the protein concentration was determined by the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL).

Flagella were purified as described by Alm et al. (3). Bacteria were resuspended in PBS and homogenized twice for 3 min. The bacterial suspensions were centrifuged at $8,000 \times g$ to remove whole cells. The supernatant fluids were centrifuged at $100,000 \times g$ for 1 h. The resulting pellets were resuspended in 1% SDS and allowed to incubate at ambient temperature. The solutions were centrifuged at $100,000 \times g$ for 1 h, and the resulting pellets were washed twice in distilled water and centrifuged as above. The final pellets were resuspended in 10 mM Tris-HCl (pH 7.0), and the protein concentration was determined as described above. All protein extracts were stored at -20°C .

Gel electrophoresis. Two-dimensional gel electrophoresis was performed using the parameters described elsewhere (37). One-dimensional gel electrophoresis was performed by mixing an equal volume of a bacterial suspension (an equivalent of 0.1 OD₆₀₀ unit) with double-strength electrophoresis sample buffer (37). The samples were placed in boiling water for 5 min and allowed to cool to ambient temperature. Proteins were resolved by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) using the discontinuous buffer system described by Laemmli (41).

Immunoblot analysis. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were washed three times in PBS and incubated for 18 h at 4°C with a 1:500 dilution of a rabbit anti-*C. jejuni* flagellin antibody in PBS (pH 7.4)-0.01% Tween 20 containing 9% dried milk. Bound antibodies were detected using peroxidase-conjugated rabbit anti-goat immunoglobulin G (Sigma) at a 1:1,000 dilution and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

RNA isolation. Total cellular RNA was isolated from the *C. jejuni* Turkey and CS isolates grown to mid-log phase using the hot-phenol method (74). Briefly, 5 ml of RNA degradation stop solution was added (10% phenol solution, buffered saturated with 0.1 M citrate buffer, pH 4.3 + 0.2, in 100% ethanol) to 50 ml of each bacterial culture. The bacterial suspension was then pelleted and resuspended in 3 ml of 1% buffer-saturated phenol solution. The culture was pipetted into 1.5 ml of boiling lysis buffer in a water bath until cell lysis occurred. This was followed by two extractions with phenol at 60°C and one extraction with phenol-chloroform, and the aqueous phase was precipitated with ethanol and resuspended in diethyl pyrocarbonate-treated water. Contaminating genomic DNA was removed by two consecutive treatments with RQ1-DNase (Promega, Rockford, IL). The absence of genomic DNA was confirmed via PCR using *C. jejuni* *ciaB* gene sequence-specific primers (CiaB-F, CTATGCTAGCCACTACTTA GGC; CiaB-R, GCCCGCCTAGAACTTAC). The PCR conditions used were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension time of 5 min (35 cycles in total).

Construction of the *C. jejuni* DNA microarray. DNA fragments of individual open reading frames (ORFs) were amplified using primers specific for ORFs present in strain NCTC 11168 (Sigma Genosys, The Woodlands, TX). Each PCR (total reaction volume, 100 μl) consisted of $1\times$ MasterAmp *Taq* PCR buffer, $1\times$ MasterAmp *Taq* enhancer, 2.5 mM MgCl_2 , 200 μM each dNTP, forward and reverse primers at 0.2 μM each, 0.5 U of MasterAmp *Taq* DNA polymerase (Epicenter, Madison, WI), and approximately 50 ng of genomic DNA from strain NCTC 11168. Thermal cycling was performed using a Tetrad thermal cycler (MJ Research, Waltham, MA) with the following amplification parameters: 30 cycles of 25 s at 94°C , 25 s at 52°C , and 2 min at 72°C and a final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis in a 1% (wt/vol) agarose gel (containing 0.5 μg of ethidium bromide/ml) in $1\times$ Tris-acetate-

EDTA buffer. DNA bands were examined under UV illumination. A total of 1,530 PCR products were successfully amplified and then purified on a QIAGEN 8000 robot using a Qiaquick 96-well Biorobot kit (QIAGEN), dried, and resuspended to an average concentration of 0.1 to 0.2 $\mu\text{g}/\mu\text{l}$ in 20 μl of 50% dimethyl sulfoxide containing 0.3 \times saline sodium citrate (SSC; 1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). All of the PCR probes were then spotted in duplicate on GAPSII slides (Corning, Acton, MA) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI) producing a final array that contained a total of 3,060 features.

Fluorescent labeling of genomic DNA and cDNA. For each comparative genomic microarray hybridization reaction, genomic DNAs from the reference strain NCTC 11168 and a test strain were fluorescently labeled with Cy5 and Cy3, respectively. Approximately 2 μg of DNA was mixed with 5 μl 10 \times NEBlot labeling buffer containing random sequence octamer oligonucleotides (New England Biolabs, Beverly, MA) and water to a final volume of 41 μl . This mixture was heated to 95°C for 10 min and then stored for 5 min at 4°C. After this incubation, the remainder of the labeling reaction components were added: 5 μl of 10 \times dNTP labeling mixture (1.2 mM each dATP, dGTP, dCTP; 0.5 mM dTTP in 10 mM Tris, pH 8.0; 1 mM EDTA), 3 μl of Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Piscataway, NJ), and 1 μl of Klenow fragment. The labeling reaction mixtures were incubated overnight at 37°C. Fluorescently labeled DNA was purified using Qiaquick PCR purification columns (QIAGEN, Valencia, CA) according to manufacturer's directions.

For the expression profiling arrays, an indirect comparison of gene expression levels was performed (83), where the levels of expression from the various *C. jejuni* isolates were measured separately on different slides. In this microarray experimental design, each labeled cDNA is combined with a fixed reference source (83), allowing the comparison of different experiments in which a common reference has been used, as in previous studies (17, 45). Sixteen micrograms of total RNA from each *C. jejuni* isolate was labeled during reverse transcription to cDNA with Cy5-dUTP using Stratascript (Stratagene, Palo Alto, CA). Following 16 h of labeling, RNA was degraded by the addition of NaOH to 0.3 M and incubation at 70°C for 10 min, followed by neutralization with an equimolar amount of HCl. The labeled cDNA was purified using Qiaquick PCR purification columns (QIAGEN) according to the manufacturer's directions. In all expression microarray hybridizations, genomic DNA from *C. jejuni* strain NCTC 11168 was labeled with Cy3-dUTP as described above. The labeled genomic DNA also served as a quality control for all the spots in the array. In this particular indirect comparison for gene expression, an analysis of repeatable residual color bias from Cy dye swap experiments was removed since the between-slide differences were taken into account (see below) (83).

Microarray hybridization. For each genomic indexing hybridization, Cy5-labeled reference DNA from *C. jejuni* strain NCTC 11168 was mixed with Cy3-labeled test DNA or cDNA in 45 μl of Corning hybridization buffer (Corning, Acton, MA) and heated to 95°C for 5 min. Then 15 μl of the hybridization mixture was put onto the microarray slide and sealed with a coverslip in a GeneMachine hybridization chamber (Genomic Solutions, Ann Arbor, MA) and incubated at 42°C for 18 h. This method, known as differential labeling, allows the hybridization of fluorescently labeled control and test DNA or cDNA to be measured for each probe on the microarray. As the genetic composition of the sequenced strain *C. jejuni* NCTC 11168 is known, it served as a control fluorescence signal for each probe and was used for comparison with the test DNA signal during the statistical analysis (see below). Following hybridization, microarray slides were washed for 2 min in 2 \times SSC and 0.1% SDS at 42°C to remove the coverslip and then washed twice for 5 min in each of the following buffers: (i) 2 \times SSC and 0.1% SDS at 42°C, (ii) 0.2 \times SSC, and finally (iii) 0.01 \times SSC. Microarray slides were dried by centrifugation at 300 \times g for 15 min before scanning.

Microarray data analysis. DNA microarrays were scanned using a GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA), and the data for spot and background intensities were processed using GenePix 4.0 software. Poor features were excluded from analysis if they contained abnormalities or a reference signal lower than background plus 3 standard deviations. As described by Eriksson et al. (17), fluorescence ratios were calculated after local background was subtracted from spot signals. To compensate for any effect of the amount of template and uneven Cy-dye incorporation, data normalization was performed as previously described (4, 17, 45) by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero using the following equation: $\ln(T_i) = \ln(R_i/G_i) - c$, where T_i is the centered ratio, i is the gene index, R and G are the red and green intensities, respectively, and c is the 50th percentile of all red/green ratios. Normalized data that passed the quality controls were analyzed using GENESPRING 6.2 software (Silicon Genetics, Palo Alto, CA). For comparison of levels of *C. jejuni* Turkey and CS

gene expression, at least four hybridization measurements were generated per biological experiment (two technical replicate arrays and two replicate features per array) and the experiment was repeated two times (biological replicate). The significance of the centered data at P values >0.05 was determined using a parameter-based statistical t test adjusting the individual P value with the Benjamini and Hochberg false-discovery rate multiple test correction within the GeneSpring analysis package.

Sequencing genes involved in flagellar biosynthesis. The genes of interest (*flhA*, *flhB*, *flhF*, *flhA*, *flhF*, *flhG*, *flhH*, *flhI*, *flhM*, *flhN*, *flhP*, *flhQ*, *flhR*, *flhY*, *flhR*, *flgS*, *rhoN*, Cj0667, Cj0668, and Cj0669) were PCR amplified using KOD DNA polymerase (Novagen, San Diego, CA) and sequenced using gene-specific primers. Both strands of every gene above from both the *C. jejuni* Turkey and CS isolates were sequenced. All sequencing primers were designed based on *C. jejuni* NCTC 11168 genomic sequences (60). Sequencing was performed using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and the DNA sequencer ABI373 (Applied Biosystems, Foster City, CA). The sequences were analyzed using the MultAlin multiple sequence alignment program.

Real-time RT-PCR. The relative expression of 26 genes (*clpB*, *cmeA*, *flaA*, *flaB*, *flaC*, *flgB*, *flgC*, *flgD*, *flgE*, *flgG*, *flgH*, *flgK*, *flhF*, *flhG*, *flhH*, *flhI*, *flhM*, *flhN*, *flhA*, *flhB*, *meta*, *rhoN*, Cj1514, Cj0667, Cj0668, and Cj0669) was determined by real-time quantitative reverse transcription-PCR (RT-PCR). cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's directions. Real-time RT-PCR amplification of 0.5 μl of cDNA was performed in a reaction mixture containing Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), a 300 nM concentration of forward and reverse primers, and diethyl pyrocarbonate-treated water. Real-time RT-PCR analysis was performed using a Gene Amp 7000 thermocycler (Applied Biosystems, Foster City, CA). PCR conditions included 1 cycle of 2 min at 50°C, followed by 40 cycles of denaturation at 95°C for 15 s and an annealing at 55°C for 1 min. Cycle threshold (C_T) values were determined using Prism SDS software version 1.0 (Applied Biosystems). The comparative threshold cycle ($\Delta\Delta C_T$) method was used to calculate change (n -fold) where samples were normalized to *glnA*, since *glnA* is a housekeeping gene and was not found to be differentially expressed by microarray analysis. Reactions were performed in duplicate, and two biological replicates were performed for each sample.

Construction of reporter vectors. The promoter regions of the *flaB* and *metK* genes were PCR amplified using the following primer sets: primer set 1, (*PmetK*-F, ATTTGGATCCCTTGTGCTCCTGTTGTGC, and *PmetK*-R, ATATGGATCAAAAGTCTTTCATTTAAATGAACC) and, primer set 2 (*PflaB*-F, AAGGATCCACCTTAAAGGCGCTATGGCTGTGATG, and *PflaB*-R, AAGGATCCGATGTTGGTGTATCTCTAAACC). Primers were designed to include a BamHI site at the 5' end. The PCR products were cloned into pCR 2.1 using the TOPO TA cloning kit (Invitrogen). The ligated vector was electroporated into *E. coli* INV α F', and the transformants were selected on LB agar supplemented with kanamycin (KAN; 50 $\mu\text{g}/\text{ml}$). *PflaB*-pCR2.1 and *PmetK*-pCR2.1 were digested with BamHI to give 0.8-kb and 0.78-kb fragments, respectively. Each fragment was gel purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). The pMW10 shuttle vector (80) was digested with BamHI, gel purified, and ligated with the BamHI-*PflaB* and BamHI-*PmetK* fragments for 18 h. The ligation mixture was ethanol precipitated and electroporated into *E. coli* INV α F'. The transformants were selected on LB-KAN agar and tested for insertion by blue-white screening. The sequences of the constructs were verified using a primer that anneals to the *aphA3'* gene encoding KAN resistance (TACACTCAATGGTTCGCTG) as described previously (80).

Electroporation of promoter shuttle vectors in the *C. jejuni* F38011 and CS isolates. The vectors (pMW10, *PmetK*-pMW10, and *PflaB*-pMW10) were introduced into the *C. jejuni* F38011 and CS strains by electroporation. The resultant colonies were selected on MH-blood agar plates supplemented with KAN (200 $\mu\text{g}/\text{ml}$).

β -Galactosidase assay. β -Galactosidase activity was determined as a measure of conversion of *o*-nitrophenyl- β -galactopyranoside (Sigma) to nitrophenol. *C. jejuni* transformants were grown for 16 h in MH broth. Harvested bacteria were diluted in PBS until the OD₆₀₀ was between 0.4 and 0.5. The assay was carried out in triplicate as previously described (80).

Construction of the complementation vectors. To construct the *flgR* pRY107 (KAN resistance) complementation vector, the coding region of the *flgR* gene along with its native promoter (350 bp upstream of the start codon) was PCR amplified using primers containing 5' BamHI restriction sites (*flgR*compF, TATATAGGATCCAATGGGTATGTGAAATTTCTATAGTG; *flgR*compR, TATATAGGATCCCCAACCACCAGTAGC). The PCR product was cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). The ligated vector was electroporated into *E. coli* INV α F', and the transformants were selected on LB-KAN plates (50 $\mu\text{g}/\text{ml}$). The pCR2.1 construct containing the BamHI *flgR*

fragment was digested with BamHI, gel purified, and cloned into the BamHI site of pRY107. The ligated vector was electroporated into *E. coli* INV α F', and transformants were selected on LB-KAN plates (50 μ g/ml).

To construct the *rpoN* pRY111 (chloramphenicol [CHL] resistance) complementation vector, the promoter region of Cj0667 was fused with the *rpoN* ORF. Noteworthy is that the *rpoN* gene is the fourth gene within the operon (5'-Cj0667-Cj0668-Cj0669-*rpoN*). The promoter region of Cj0667 was PCR amplified using primers containing 5' SstI and NdeI restriction sites (Cj0667-SstI-F, TATATAGAGCTCTCAAGGCGTCTATACCGTG; Cj0667-NdeI-R2, ATATATACATATGTTTCAAGCAAAAATACCCCAAG; *rpoN*-SstI-R, TATAATCCGCGGAATATTTAAAAACAGTTATTATTGTATCAC). The PCR amplicons were cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). The ligated vector was electroporated into *E. coli* INV α F', and transformants were selected on LB-KAN plates (50 μ g/ml). The *rpoN*-pCR2.1 construct was digested with NdeI and SstI, and the gel-purified fragment was ligated into the NdeI- and SstI-restricted PCj0667-pCR2.1 construct. The ligated vector was electroporated into *E. coli* INV α F', and transformants were selected on LB-KAN plates (50 μ g/ml). The PCj0776-*rpoN*-pCR2.1 fusion vector was digested with SstI, and the SstI-PCj0667-*rpoN* fragment was gel purified and cloned into the SstI site of pRY111. The resulting vector was electroporated into *E. coli* INV α F', and transformants were selected on LB agar plates supplemented with CHL (LB-CHL).

Conjugation. The complementation plasmids were electroporated into *E. coli* S17-1 λ *pir*. *E. coli* S17-1 λ *pir* harboring the complementation plasmid *flgR*-pRY107 was then conjugated with the *C. jejuni* CS isolate. The transconjugants were selected on MH-blood agar plates supplemented with KAN (200 μ g/ml) and tetracycline (TET, 50 μ g/ml). The *C. jejuni* CS isolate colonies resistant to both KAN and TET were screened for motility on motility agar plates (Life Technologies, United Kingdom).

E. coli S17-1 λ *pir* harboring the PCj0667-*rpoN*-pRY111 complementation plasmid was conjugated with the *C. jejuni* S2B isolate. The transconjugants were selected on MH-blood agar plates supplemented with CHL (20 μ g/ml) and TET (50 μ g/ml). The *C. jejuni* S2B isolate colonies resistant to both KAN and TET were screened for motility on motility agar plates.

Generation of the *C. jejuni* Turkey *flgR* mutant. A disrupted copy of the *flgR* gene was amplified from the *C. jejuni* F38011 *flgR*-*cat* mutant using *flgR* gene-specific primers (66), and the resultant product was cloned into pCR2.1. The *flgR*-*cat* fragment was digested using EcoRI, gel purified, and cloned into the EcoRI site of the pBluescriptII SK(+) vector. The ligated vector was electroporated into *E. coli* INV α F' and selected on LB-CHL. The mutagenesis vector was introduced into the *C. jejuni* Turkey isolate by electroporation and selected on MH-blood agar plates supplemented with CHL. The putative mutants were confirmed by PCR amplification of *flgR*, followed by digestion of the PCR product by NheI to release the *cat* cassette.

Growth curves. Overnight cultures of *C. jejuni* Turkey and CS isolates were used to inoculate 250 ml of MH broth at an OD₅₄₀ of 0.01. The flasks were incubated at 37°C under microaerobic conditions and shaken at 150 rpm. At different time points, 1 ml of bacterial culture was used to determine the OD₅₄₀ until both cultures reached stationary phase.

Sensitivity to deoxycholate. Overnight cultures of *C. jejuni* Turkey, the *C. jejuni* Turkey *flgR* mutant, and CS isolates were used to inoculate tubes at an OD₅₄₀ of 0.05. Different concentrations of sodium deoxycholate were added to each tube in triplicate, and tubes were incubated for 48 h at 37°C under microaerobic conditions and shaken at 150 rpm. At the end of the incubation period, the OD₅₄₀ for each tube was measured.

Other analytical procedures. Protein concentrations were determined by the bicinchoninic acid method, with bovine serum albumin as the standard, as outlined by the supplier (Pierce, Rockford, IL). To determine the identity of the protein present in the flagellar extract prepared from the *C. jejuni* Turkey isolate, the extract was mixed with an equal volume of double-strength electrophoresis sample buffer and separated by SDS-PAGE. The proteins were transferred to PVDF membranes and stained with Coomassie brilliant blue G-250 (CBB-G250). The amino-terminal sequence of the 62-kDa protein was determined at the University of British Columbia Biotechnology Laboratory (Protein Sequencing and Peptide Mapping, NAPS Unit, Vancouver, B.C., Canada).

RESULTS

***C. jejuni* isolates display marked differences in virulence phenotypes.** While there are several reports documenting the

TABLE 1. Binding and internalization of *C. jejuni* poultry isolates

Isolate	Origin	A ^a	I ^b	I/A (%) ^c
81-176	Human	(3.0 ± 0.8) × 10 ⁵	(2.2 ± 0.3) × 10 ⁴	7.3
CS	Chicken	(5.8 ± 1.0) × 10 ⁵	(5.0 ± 1.8) × 10 ²	0.09
S1	Chicken	(3.1 ± 0.7) × 10 ⁴	(9.3 ± 1.5) × 10 ²	3.0
S2B	Chicken	(3.5 ± 0.4) × 10 ⁵	(1.3 ± 0.5) × 10 ²	0.04
S3	Chicken	(3.9 ± 0.4) × 10 ⁵	(9.0 ± 2.0) × 10 ¹	0.02
SC	Chicken	(1.6 ± 0.2) × 10 ⁵	(1.5 ± 0.4) × 10 ³	0.9
Turkey	Turkey	(1.2 ± 0.2) × 10 ⁵	(4.3 ± 0.5) × 10 ³	3.6
<i>E. coli</i> XL1-Blue MRF'	Stratagene	(3.3 ± 1.0) × 10 ⁵	(2.1 ± 0.4) × 10 ²	0.07

^a Number of cell-associated bacteria per well of *C. jejuni*-inoculated monolayers.

^b Number of internalized bacteria per well of *C. jejuni*-inoculated monolayers treated with gentamicin for 3 h.

^c Percentage of cell-associated bacteria that are internalized.

variation in the efficiency of *C. jejuni* isolates to invade cultured cells (18, 38, 54), the molecular mechanisms for these variations have seldom been elucidated. To identify *C. jejuni* isolates with apparent differences in virulence potential, we performed INT 407 cell invasion assays and secretion assays with six *C. jejuni* poultry isolates. *C. jejuni* invasion of INT 407 cells was assessed using the gentamicin protection assay. *C. jejuni*-INT 407 host cell contact was promoted by a low-speed centrifugation step immediately following inoculation. The levels of invasiveness of the six *C. jejuni* poultry isolates were markedly different (Table 1). Relative to the *C. jejuni* 81-176 clinical strain, the *C. jejuni* Turkey strain was the most invasive environmental isolate tested and the *C. jejuni* S2B strain was the least invasive. Because *C. jejuni* binding is a prerequisite for host cell invasion, the data were transformed and presented as the ratio of intracellular to cell-associated bacteria, expressed as a percentage, to more directly assess the invasive potential of each isolate. Three of the six isolates (CS, S2B, and S3) were readily discernible from the other isolates tested in that they displayed lower invasive potential.

C. jejuni synthesizes and secretes a set of proteins upon coculturing with epithelial cells, which aid in the invasion of the host cells. The secreted proteins are referred to, collectively, as Cia proteins. Since three of the six *C. jejuni* poultry isolates showed low invasive potential, secretion assays were performed to determine the ability of the isolates to secrete Cia proteins. The assays were performed in the presence of FBS, which serves as an artificial signal to stimulate the synthesis and secretion of the Cia proteins (68). The Cia proteins were readily identifiable in the supernatant fluids of *C. jejuni* 81-176, S1, SC, and Turkey isolates but not detectable in the supernatant fluids of *C. jejuni* CS, S2B, and S3 isolates (Fig. 1). The *M_s* of the Cia proteins exported from the *C. jejuni* poultry isolates were consistent with those of the *C. jejuni* 81-176 human clinical isolate (Fig. 1) and with previous work with other *C. jejuni* clinical isolates (39, 68). Noteworthy is that the *C. jejuni* CS, S2B, and S3 isolates displayed a lower invasive potential than the *C. jejuni* poultry isolates (S1, SC, and Turkey) that secreted the Cia proteins.

Previous work indicates that the *C. jejuni* Cia proteins are secreted from the flagellar apparatus (39). As a screen to determine whether each isolate possessed a functional flagellar export apparatus, motility assays were performed with each of the six *C. jejuni* poultry isolates (not shown). In contrast to *C.*

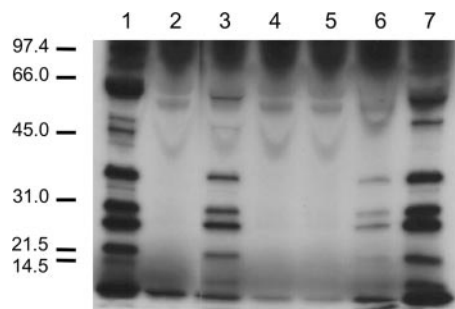


FIG. 1. Some *C. jejuni* isolates recovered from poultry do not secrete the Cia proteins. *C. jejuni* isolates 81-176 (lane 1), CS (lane 2), S1 (lane 3), S2B (lane 4), S3 (lane 5), SC (lane 6), and Turkey (lane 7) were incubated in MEM lacking methionine and containing 1% FBS and labeled with [³⁵S]methionine for 3 h. The supernatant fluids were analyzed by SDS-PAGE and autoradiography as outlined in Materials and Methods. Molecular mass standards, in kDa, are indicated on the left.

jejuni SC, S1, and Turkey, the *C. jejuni* CS, S2B, and S3 isolates were nonmotile. These findings raised the possibility that the *C. jejuni* CS, S2B, and S3 isolates were weakly invasive and did not secrete the Cia proteins because they did not possess a functional secretion apparatus/flagellum.

***C. jejuni* isolates Turkey and CS are genotypically indistinguishable.** To unravel the molecular basis of the differences in pathogenesis exhibited by the isolates, we wanted to determine their genetic relatedness. Since macrorestriction enzyme PFGE (MRP-PFGE) profiling is the currently accepted method to determine the relatedness of *Campylobacter* isolates (12, 14, 21, 22, 25, 28, 29, 67, 72, 73, 76), we used it to assess the genomic diversity of the *C. jejuni* isolates used in this study. Initially, *C. jejuni* isolates were analyzed by PFGE following digestion of chromosomal DNA with the restriction enzyme SmaI. A representative gel is presented in Fig. 2A. PFGE of SmaI-restricted *C. jejuni* chromosomal DNA yielded 4 to 10 fragments ranging in size from <97 kb to approximately 485 kb. The *C. jejuni* CS, SC, and Turkey isolates yielded indistinguishable macrorestriction profiles using the SmaI restriction enzyme. We chose to narrow the remainder of this study to the Turkey and CS isolates because the Turkey isolate was found

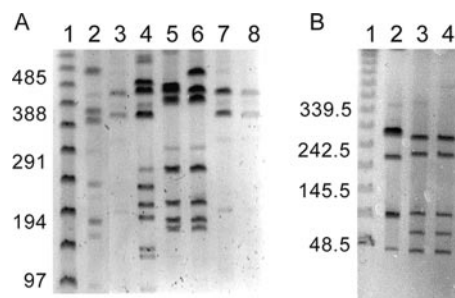


FIG. 2. MRP-PFGE profiles of *C. jejuni* isolates. Lanes 1 (A and B), PFGE lambda ladder (New England Biolabs, Beverly, MA). The sizes of the molecular mass markers are shown on the left of each panel in kilobase pairs. (A) MRP-PFGE of SmaI-digested DNA from 81-176 (lane 2), CS (lane 3), S1 (lane 4), S2B (lane 5), S3 (lane 6), SC (lane 7), and Turkey (lane 8). (B) MRP-PFGE of SallI-digested DNA from 81-176 (lane 2), Turkey (lane 3), and CS (lane 4).

TABLE 2. The *C. jejuni* Turkey isolate is more virulent than the CS isolate in the neonatal piglet model

<i>C. jejuni</i> strain	Fraction of piglets with diarrhea	Onset of diarrhea	Fraction of piglets that experienced:	
			Death (pre-necropsy)	Hemorrhage
Turkey	2/3	24 h	0/3	1/3
CS	0/3	Not detected	0/3	0/3

to be highly invasive for INT 407 cells and was Cia secretion positive whereas the CS isolate displayed a noninvasive phenotype for INT 407 cells and was Cia secretion negative. Noteworthy is that macrorestriction profiles of the *C. jejuni* Turkey and CS isolates were also indistinguishable using a second restriction enzyme, SallI (Fig. 2B).

MLST is a technique that characterizes isolates based upon combinations of alleles at seven "unlinked" housekeeping loci (15). Of interest was that the *C. jejuni* Turkey and CS isolates were found to belong to the same sequence type (ST-48).

We also examined the gene content of the *C. jejuni* Turkey and CS isolates by a comparative genomic hybridization analysis (genomotyping) using a *C. jejuni* NCTC 11168 DNA microarray. It should be noted that only genes that have a feature represented on the *C. jejuni* NCTC 11168 DNA microarray can be detected using this method. Moreover, some mutations, including frameshift and point mutations that might be present in one isolate and not the other, would not be identified by this method. With these caveats in mind, we found no observable differences between the signal intensities for the *C. jejuni* Turkey and CS isolates, suggesting that, among those genes conserved with *C. jejuni* strain NCTC 11168, all three isolates have an identical gene content (not shown). However, both the *C. jejuni* Turkey and CS isolates exhibited divergence from the NCTC 11168 strain for genes that are contained within the recognized hypervariable regions, including the capsular biosynthesis locus, LOS biosynthesis locus, and restriction modification locus. To confirm divergence in the LOS locus among the isolates, we used a recently described PCR method (59). Both *C. jejuni* isolates CS and Turkey possess the class B LOS biosynthetic locus (not shown), while strain NCTC 11168 possesses the class C LOS locus.

The *C. jejuni* Turkey isolate is more pathogenic for piglets. Because the *C. jejuni* Turkey and CS isolates were genetically indistinguishable as judged by MRP-PFGE, MLST, and comparative genomic hybridization analysis but differed significantly in their in vitro virulence capabilities, we focused our efforts on these two isolates to decipher the molecular basis for *C. jejuni* pathogenicity. However, we decided to perform in vivo experiments to assess the pathogenicity of the *C. jejuni* Turkey and CS isolates prior to comparing the protein and gene expression profiles. Noteworthy is that the infection of piglets with *C. jejuni* has been used previously as a model for *Campylobacter*-mediated enteritis, as infected piglets exhibit overt symptoms and histopathology that are characteristic of human infections (4). The *C. jejuni* Turkey isolate was more pathogenic than the CS isolate for piglets (Table 2). Diarrhea was noted in two of the three piglets inoculated with the *C. jejuni* Turkey isolate and none of the piglets inoculated with

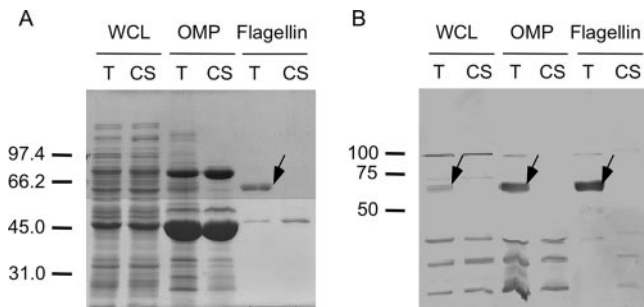


FIG. 3. Electrophoretic and immunoblot analysis of *C. jejuni* Turkey (T) and CS whole-cell lysates (WCL), OMP, and flagellin preparations. Proteins were separated in SDS-12.5% polyacrylamide gels and either stained with CBB-R250 (A) or transferred to PVDF membranes and reacted a 1:250 dilution of a *C. jejuni* anti-flagellin serum that contains antibodies reactive with both the FlaA and FlaB proteins (B). The arrows indicate the FlaA flagellar protein. Molecular mass standards, in kDa, are indicated on the left.

the *C. jejuni* CS isolate. A single Turkey-inoculated piglet developed hemorrhage in the colon, which was noted upon gross examination. Histological examination of intestinal samples was performed on piglets inoculated with the *C. jejuni* Turkey and CS isolates. Two of the three piglets inoculated with the *C. jejuni* Turkey isolate developed significant degenerative lesions in the small intestine. Also apparent in the small intestines of these two piglets were areas of congestion and cell necrosis. In the colon, the crypts were filled with eosinophilic debris and mucosal erosion was apparent, accompanied by a fibrinous exudate. One of the three *C. jejuni* CS-inoculated piglets exhibited minor pathological changes. This *C. jejuni* CS-inoculated piglet had congested vessels and slight villus degeneration.

The *C. jejuni* Turkey and CS isolates display differences in protein profiles. To determine if there were differences in the proteins synthesized by the *C. jejuni* Turkey and CS isolates in vitro, bacterial whole-cell lysates were analyzed by one- and two-dimensional gel electrophoresis and the proteins stained with CBB-R250 and silver stain (not shown). A minimum of 21 proteins varied in their relative amounts in the whole-cell lysate of the *C. jejuni* Turkey isolate versus the whole-cell lysate of the CS isolate as judged by two-dimensional electrophoresis coupled with silver staining (not shown). Further, CBB-R250 staining of the two-dimensional gels revealed a predominant band of 62 kDa in the whole-cell lysate of the *C. jejuni* Turkey isolate that was not observed in the CS isolate. As *C. jejuni* FlaA monomers are known to have a molecular mass of 62 kDa (30), this difference was further investigated. To confirm whether FlaA was indeed synthesized by the *C. jejuni* Turkey isolate and not by the *C. jejuni* CS isolate, bacterial extracts (e.g., whole-cell lysate, outer membrane protein, and flagellar protein) were prepared from both isolates using SDS-PAGE coupled with immunoblot analysis with the flagellin polyclonal antiserum. Again, a 62-kDa immunoreactive band was clearly visible in the various bacterial extracts prepared from the *C. jejuni* Turkey isolate and absent in the extracts prepared using the *C. jejuni* CS isolate (Fig. 3). The identity of the 62-kDa protein was confirmed to be FlaA filament protein by N-terminal sequence analysis. More specifically, the amino-terminal

amino acid sequence of the mature protein was determined after SDS-PAGE and electrophoretic transfer to PVDF membranes to be GFRINTNVAAL. This sequence corresponds to residues 2 through 12 of the deduced amino acid sequence of the FlaA protein. Collectively, these data indicate that the FlaA protein is synthesized in the *C. jejuni* Turkey isolate but not in the *C. jejuni* CS isolate.

Decreased expression of flagellum-related transcripts from the *C. jejuni* CS isolate. Based on the differences in the phenotypic properties and protein profiles of the *C. jejuni* Turkey and CS isolates, we anticipated that there would be differences in the gene expression profiles of the two isolates. From MH broth cultures, total RNA was isolated from the *C. jejuni* Turkey and CS isolates and fluorescently labeled as it was reverse transcribed to cDNA. The labeled cDNA was used to probe the *C. jejuni* NCTC 11168 DNA microarrays. The expression profiles for the isolates were distinct, showing that 47 genes had significantly reduced transcript levels of twofold or more in the *C. jejuni* CS isolate versus the Turkey isolate (Table 3). Most strikingly, 20 of the 47 genes are related to flagellar biosynthesis and motility, and this correlates with the decreased motility of the *C. jejuni* CS isolate. There were also 24 genes for which the transcript levels were increased twofold or more in the *C. jejuni* CS isolate versus the Turkey isolate (Table 4). Eight of these genes have annotated functions involving purine or amino acid biosynthesis. However, the role that the upregulated genes play in the phenotype displayed by the *C. jejuni* CS isolate is not known. To assess the accuracy of the microarray data, real-time RT-PCR was performed on four genes that were upregulated (*flaB*, *flgD*, *flgE*, and *flgK*) and three genes that were downregulated (*clpB*, *metA*, and Cj0414) in the array experiments. The expression profile of *C. jejuni* Turkey and CS isolates using real-time RT-PCR mirrored the microarray results.

Flagellar class II genes are downregulated in the *C. jejuni* CS isolate. Microarray analysis revealed that 20 of the 47 genes expressed at a higher level in the *C. jejuni* Turkey isolate than in the CS isolate were associated with flagellar biosynthesis. To elucidate the molecular mechanism associated with disruption of flagellar biosynthesis in the *C. jejuni* CS isolate, we performed real-time RT-PCR on eight class I genes (*flhA*, *flhB*, *fliF*, *fliG*, *fliH*, *fliI*, *fliM*, and *fliY*) and eight class II genes (*flaB*, *flaD*, *flgB*, *flgD*, *flgE*, *flgG*, *flgH*, and *flgK*) previously shown to be regulated by σ^{70} and σ^{54} , respectively (10). All class I genes were expressed at a higher level in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate. In contrast, the σ^{54} -regulated flagellar genes were expressed at a lower level in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate (Fig. 4A). Moreover, *flgG* and *flgH* were also found to be expressed at a lower level in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate, even though differential expression of these genes was not detected by microarray analysis.

The σ^{54} -regulated *flaB* gene is poorly expressed in the *C. jejuni* CS isolate. Real-time RT-PCR indicated that σ^{54} -regulated flagellar genes were downregulated in the *C. jejuni* CS isolate. To determine if σ^{54} is active in the *C. jejuni* CS isolate, we performed reporter assays with the pMW10 *lacZ* promoterless shuttle vector (80). The promoter regions of the σ^{54} -regulated *flaB* and σ^{70} -regulated *metK* genes were cloned upstream of the *lacZ* gene. *C. jejuni* F38011 was chosen as a

TABLE 3. Transcripts detected at lower levels in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate

Gene	Fold change	Description of encoded protein
Cj0040 ^{a,b}	4.30	Conserved hypothetical protein
Cj0041 ^{a,b}	3.01	Conserved hypothetical protein
<i>flgD</i> ^{a,b}	6.55	Flagellar hook assembly protein
<i>flgE</i> ^{a,b}	6.52	Flagellar hook protein
Cj0044 ^c	2.11	Conserved hypothetical protein
Cj0045 ^c	2.60	Conserved hypothetical protein, putative iron-binding protein
<i>aspA</i>	2.52	Aspartate ammonia lyase
<i>dcuA</i>	2.57	C4-dicarboxylate transporter, anaerobic
Cj0168c	3.50	Hypothetical protein
Cj0256	2.37	Conserved hypothetical integral membrane protein, putative sulfatase
<i>serB</i>	2.30	3-Phosphoserine phosphatase
<i>cheW</i> ^a	2.92	Purine-binding chemotaxis
<i>cheA</i> ^a	2.75	Chemotaxis protein
<i>cheV</i> ^a	2.84	Chemotaxis protein
<i>cmeC</i>	3.69	RND efflux system, outer membrane lipoprotein
<i>cmeB</i>	4.40	RND efflux system, inner membrane transporter
<i>cmeA</i>	4.01	RND efflux system, membrane fusion protein
Cj0391 ^{c,a,c}	2.77	Conserved hypothetical protein
Cj0420	3.52	Conserved hypothetical protein
<i>flgB</i> ^a	2.05	Flagellar basal body rod protein
<i>flaG</i> ^{a,b}	2.06	Possible flagellar protein
<i>fliD</i> ^{a,c}	2.48	Flagellar hook-associated protein 2
Cj0561c	5.97	Conserved hypothetical periplasmic protein
<i>peb-4</i>	2.14	Cell binding factor 2 precursor, major antigenic peptide
<i>fba</i>	2.78	Fructose-bisphosphate aldolase
<i>peb-2</i>	2.25	Peb2 accessory colonization factor AcfC (acfC)
Cj0805	2.08	Conserved hypothetical protein, putative peptidase/protease (M16 family)
<i>dapA</i>	2.98	Dihydrodipicolinate synthase
Cj0807	2.18	Conserved hypothetical protein, putative short chain dehydrogenase
<i>flaD</i> ^{a,b}	2.74	Flagellin family protein
Cj0950c	2.31	Conserved hypothetical lipoprotein
Cj0969	2.55	Unknown
Cj0998c	2.46	Conserved hypothetical protein
Cj1064	2.36	Unknown
Cj1159c	3.40	Unknown
Cj1242	3.30	Conserved hypothetical protein
Cj1279c	2.15	Conserved hypothetical protein, fibronectin domain-containing lipoprotein
<i>flaA</i> ^{a,c}	3.83	Flagellin
<i>flaB</i> ^{a,b}	4.15	Flagellin
Cj1407c	2.03	Phosphohexosemutase
<i>flgI</i> ^{a,b}	5.63	Flagellar P-ring protein
Cj1463 ^a	2.94	Conserved hypothetical protein
<i>flgM</i> ^{a,c}	2.00	Conserved hypothetical protein
Cj1465 ^a	2.07	Conserved hypothetical protein
<i>flgK</i> ^{a,b}	3.02	Flagellar hook-associated protein
Cj1656c	5.78	Unknown
<i>leuA</i>	2.36	2-Isopropylmalate synthase

^a Flagellar biosynthesis-associated gene.^b σ^{54} -regulated gene.^c σ^{28} -regulated gene.

positive control because we were unable to transform the *C. jejuni* Turkey isolate with a shuttle plasmid. Comparable levels of β -galactosidase activity were noted in the *C. jejuni* F38011 and CS isolates harboring the *PmetK*-pMW10 construct. In contrast, the *C. jejuni* CS isolates harboring the *PflaB*-pMW10

TABLE 4. Transcripts detected in greater levels in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate

Gene	Fold change	Description of encoded protein
Cj0091	2.05	Conserved hypothetical lipoprotein
Cj0203	2.04	Unknown
Cj0264c	2.04	Unknown
Cj0265c	2.19	Unknown
Cj0413	2.03	Conserved hypothetical protein, probable periplasmic protein
Cj0414	5.34	Conserved hypothetical protein
Cj0415	4.50	Conserved hypothetical protein, putative gluconate dehydrogenase
Cj0444	2.22	Unknown
<i>thiC</i>	2.01	Thiamine biosynthesis protein
Cj0501	2.61	Unknown
<i>clpB</i>	2.60	ATP-dependent Clp protease, ATP-binding subunit
<i>hypB</i>	2.10	Hydrogenase expression/formation protein
Cj0653c	2.37	Aminopeptidase (M24 family)
Cj0654c	3.02	Unknown
<i>glnA</i>	2.49	Glutamine synthetase
<i>putP</i>	2.04	Sodium/proline symporter
Cj1514c	2.01	Conserved hypothetical protein
<i>nuoC</i>	2.30	NADH-ubiquinone oxidoreductase, subunit C
<i>nuoB</i>	2.32	NADH-ubiquinone oxidoreductase, subunit B
<i>HisG</i>	2.53	ATP phosphoribosyltransferase
<i>hisD</i>	2.28	Histidinol dehydrogenase
<i>hisH</i>	2.35	Glutamine amidotransferase
<i>hisA</i>	2.19	<i>N</i> -(5'-Phospho-L-ribosyl-formimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide isomerase
<i>metA</i>	2.96	Homoserine <i>O</i> -succinyltransferase
<i>metB</i>	3.57	<i>O</i> -Acetylhomoserine sulfhydrylase

construct showed almost no β -galactosidase activity (Fig. 4B) compared to the *C. jejuni* F38011 isolate. This finding further suggests that σ^{54} (RpoN)-regulated flagellar genes are not expressed in the *C. jejuni* CS isolate.

To determine why the *flaB* flagellar gene is not expressed in the *C. jejuni* CS isolate, the entire *rpoN* operon (consisting of *rpoN*, Cj0667, Cj0668, and Cj0669) was sequenced in the *C. jejuni* Turkey and CS isolates to identify a frameshift or point mutation. No differences were found in the sequences of the genes contained within the *rpoN* operon of the *C. jejuni* Turkey and CS isolates. Furthermore, real-time RT-PCR indicated that all four genes in the *rpoN* operon of the *C. jejuni* Turkey and CS isolates were expressed at comparable levels.

Identification of point mutations in the *flgR* gene of the *C. jejuni* CS isolate. We sequenced the entire ORFs of genes whose products are involved in either flagellar gene regulation or biosynthesis in an attempt to identify the molecular mechanism associated with disruption of flagellar biosynthesis in the *C. jejuni* CS isolate. The class I genes sequenced in the *C. jejuni* Turkey and CS isolates were *flhA*, *flhB*, *flhF*, *fliF*, *fliG*, *fliH*, *fliI*, *fliM*, *fliN*, *fliP*, *fliQ*, and *fliR*. We also sequenced *fliA* (σ^{28}) and the genes known to interact or activate σ^{54} including *flgR* and *flgS*. No nucleotide alterations were noted in the *flhA*, *flhB*, *flhF*, *fliA*, *fliF*, *fliG*, *fliH*, *fliI*, *fliM*, *fliN*, *fliP*, *fliQ*, *fliR*, and *flgS* genes of the *C. jejuni* Turkey and CS isolates. However, a difference was noted in a single nucleotide in the *flgR* gene that

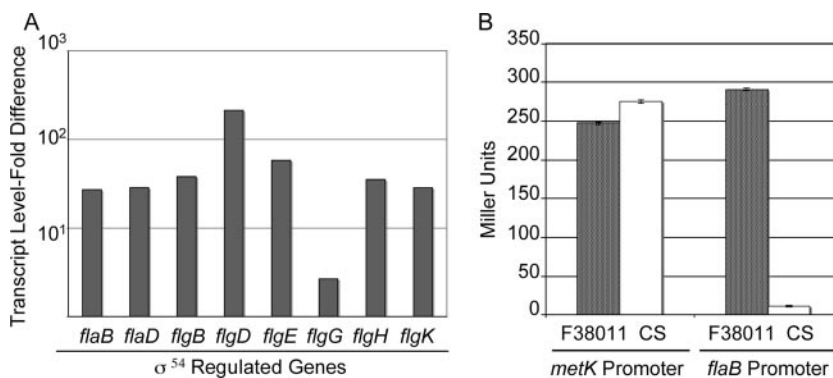


FIG. 4. (A) Transcript levels of σ^{54} -regulated genes in the *C. jejuni* Turkey isolate are increased relative to those of the *C. jejuni* CS isolate. The *flaB*, *flaD*, *flgB*, *flgD*, *flgE*, *flgG*, *flgH*, and *flgK* transcripts were measured by real-time RT-PCR using total RNA extracted from the *C. jejuni* CS and Turkey isolates grown to mid-log phase in MH broth. The change in transcript levels was measured using the comparative C_T method, where *glnA* was used as the internal control and ΔC_T of the *C. jejuni* CS isolate was used as the calibrator. (B) The *flaB* gene, which is σ^{54} regulated, is not expressed in the *C. jejuni* CS isolate. β -Galactosidase activity from the *C. jejuni* CS and F38011 isolates harboring the *PmetK*-pMW10 and *PflaB*-pMW10 constructs was measured. The bacteria were grown in MH broth for 16 h. The values are averages of three independent experiments, with the error bars representing the standard deviations.

resulted in an amino acid change in the *C. jejuni* CS isolate compared to the Turkey isolate (Fig. 5A). The *flgR* gene encodes the response regulator of the FlgS/FlgR two-component regulatory system associated with the flagellar regulon. The amino acid change resides within in the receiver domain of the FlgR protein.

Complementation of the *C. jejuni* CS isolate with a copy of the *flgR* gene from *C. jejuni* NCTC 11168 restores motility. To determine if FlgR is responsible for the phenotype of the *C. jejuni* CS isolate, the *flgR* gene from *C. jejuni* NCTC 11168 was cloned into the pRY107 shuttle vector and introduced into the *C. jejuni* CS isolate. The *C. jejuni* CS isolate transformed with the *flgR* pRY107 shuttle vector was motile (Fig. 5B). Collectively, these data indicate that a single nucleotide difference in the *C. jejuni* CS *flgR* gene, which resulted in an amino acid change, is responsible for the nonmotile phenotype displayed by the isolate. Subsequently, we generated a mutation in the *flgR* gene of the *C. jejuni* Turkey isolate. Analysis of this mutant revealed a phenotype that was indistinguishable from that of the *C. jejuni* CS isolate; the *C. jejuni* Turkey isolate is nonmotile and incapable of invading INT 407 epithelial cells (data not shown).

The *C. jejuni* CS isolate displays greater resistance to sodium deoxycholate than the *C. jejuni* Turkey isolate. *C. jejuni* flagellar mutants (i.e., *flgS*, *flgR*, and *rpoN* mutants) have been shown previously to multiply at a rate three times greater than an isogenic wild-type isolate (81). While we did not find any differences in growth rates of the *C. jejuni* Turkey and CS isolates, the *C. jejuni* Turkey isolate displayed a longer lag phase than the *C. jejuni* CS isolate. Also, the *C. jejuni* CS isolate achieved a greater maximum cell density in MH medium than the *C. jejuni* Turkey isolate (Fig. 6).

Microarray analysis revealed that some genes encoding membrane-associated proteins were expressed at different levels in the *C. jejuni* CS isolate compared to the *C. jejuni* Turkey isolate. Therefore, we studied the ability of the *C. jejuni* Turkey and CS isolates to resist the antimicrobial activity of the bile salt sodium deoxycholate. Both isolates were exposed to different concentrations of sodium deoxycholate, and the cell

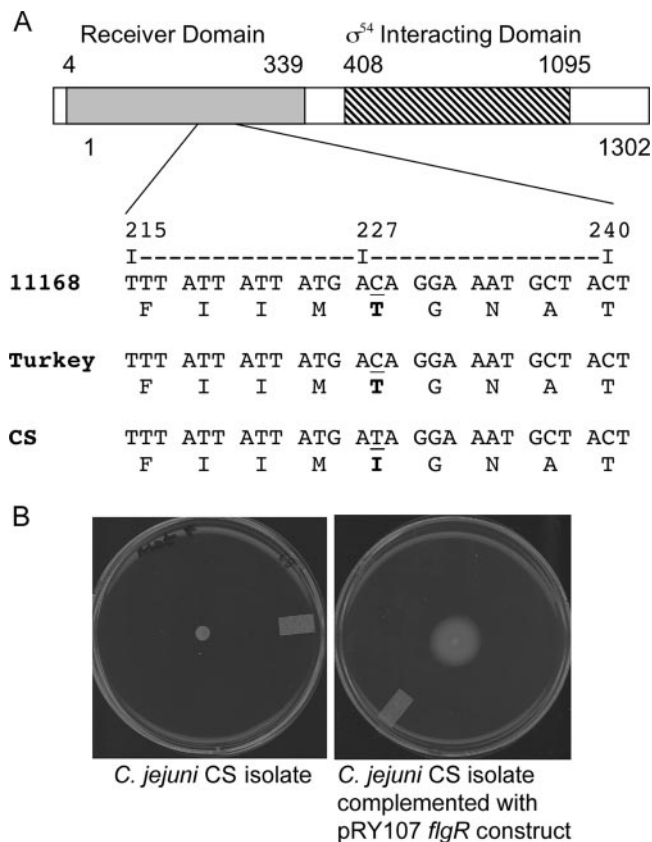


FIG. 5. (A) Multiple sequence alignment of the *flgR* gene from *C. jejuni* 11168, Turkey, and CS isolates. Shown is a segment of the nucleotide sequence of the *flgR* gene and its predicted translation from *C. jejuni* strains NCTC 11168 (11168), Turkey (Turkey), and CS (CS). The FlgR receiver and σ^{54} -interacting domains are indicated. Also highlighted (underlined) is the residue that differed in the *C. jejuni* CS isolate compared to the Turkey isolate. (B) Complementation of the *C. jejuni* CS isolate with a wild-type copy of *flgR* gene restores the isolate's motility. Both the *C. jejuni* CS wild-type and CS *flgR* pRY107-transformed isolates were spotted onto MH plates supplemented with 0.4% agar, and motility was assessed after 48 h of incubation.

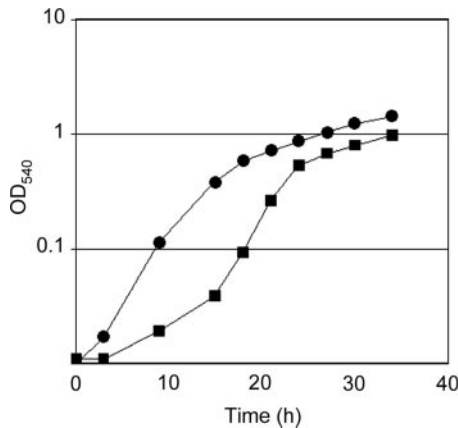


FIG. 6. Growth of the *C. jejuni* Turkey and CS isolates in MH broth. MH broth was inoculated with the *C. jejuni* Turkey and CS isolates to an OD_{540} of 0.01, and the cultures were incubated at 37°C and 150 rpm, under microaerobic conditions. The points represent the time points at which the optical density of each bacterial culture was determined at a wavelength of 540 nm. Circles, *C. jejuni* CS isolate; squares, *C. jejuni* Turkey isolate.

density achieved at 48 h was measured. The growth of the *C. jejuni* Turkey isolate was significantly inhibited by the presence of 0.05% and 0.1% sodium deoxycholate compared to the *C. jejuni* CS isolate (Fig. 7). This finding was reproduced with the *C. jejuni* Turkey isolate compared to a *C. jejuni* Turkey *flgR* mutant, where the *C. jejuni* Turkey *flgR* mutant was found to be more resistant to deoxycholate. Not known is why the *C. jejuni* Turkey and CS isolates differ in their sensitivities to deoxycholate.

Screening *C. jejuni* poultry isolates for nonmotile phenotype.

To assess how common are nonmotile isolates within a population of poultry isolates, we screened a total of 99 isolates to determine their motility phenotypes. We identified one addi-

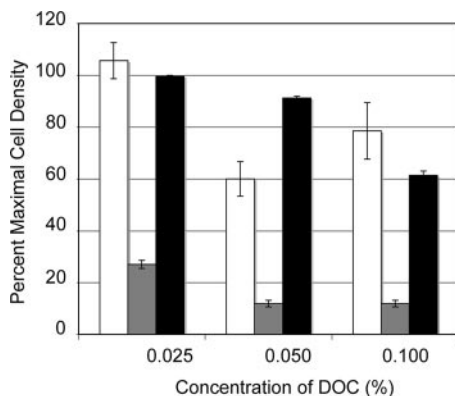


FIG. 7. The *C. jejuni* CS isolate and the *C. jejuni* Turkey *flgR* mutant display greater resistance to sodium deoxycholate (DOC) than the *C. jejuni* Turkey isolate. The *C. jejuni* CS isolate (open bars), *C. jejuni* Turkey isolate (gray bars), and *C. jejuni* Turkey *flgR* mutant (black bars) were incubated in MH broth with 0.1%, 0.05%, 0.025%, and 0% sodium deoxycholate under microaerobic conditions for 48 h at 37°C with shaking. The y axis represents the optical density of bacterial culture relative to the growth achieved without deoxycholate (0% sodium deoxycholate). The error bars represent the standard deviations.

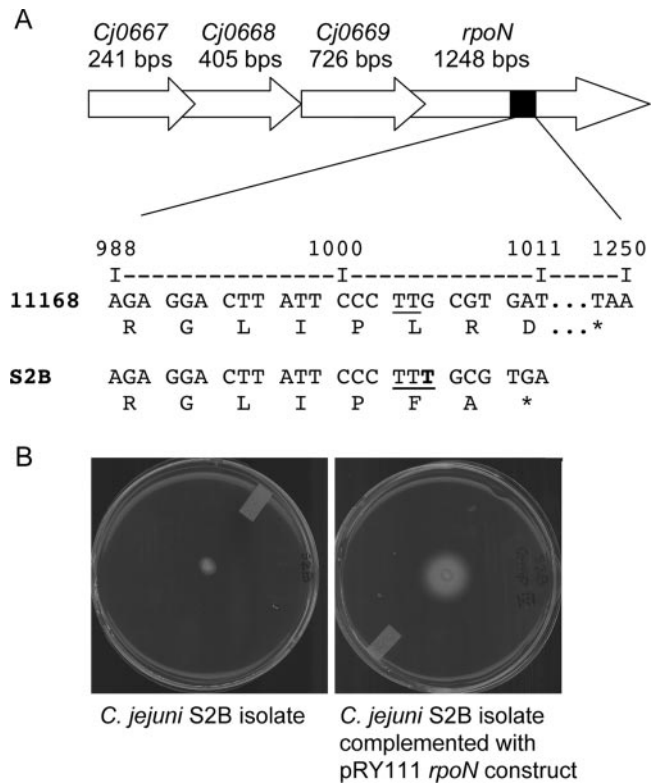


FIG. 8. (A) The *rpoN* operon of the *C. jejuni* S2B isolate was sequenced, and a single thymine base insertion was identified between bp 1004 and 1005. This base insertion results in a termination codon at bp 1011 as opposed to the normal termination codon at bp 1248. Shown is a segment of the nucleotide sequence of the *rpoN* gene and its predicted translation from the *C. jejuni* NCTC 11168 (11168) and S2B (S2B) isolates. Also shown (bottom) is the number of bp from the start codon of *rpoN*. *, termination codon. (B) Complementation of the *C. jejuni* S2B strain with a wild-type copy of *rpoN* restored the motility phenotype. The *C. jejuni* S2B wild-type and S2B *rpoN* pRY111-transformed isolates were spotted onto MH plates supplemented with 0.4% agar, and motility was assessed after 48 h of incubation.

tional nonmotile *C. jejuni* isolate, designated H8a. Counting the initial six *C. jejuni* isolates used in this study, a total of four nonmotile isolates were identified (CS, S2B, S3, and H8a).

To identify the molecular mechanism for the motility defects, we attempted to transform the *C. jejuni* S2B, S3, and H8a isolates with the pMW10 *flaB* promoter construct. Although we were unable to transform the *C. jejuni* H8a isolate with the pMW10 *flaB* construct, negligible β -galactosidase activity was detected in the S2B and S3 isolates. Upon sequencing the *rpoN* genes from each of these isolates, it was found that a thymine base was inserted between bp 1004 and 1005 in the *C. jejuni* S2B isolate (Fig. 8A). This insertion resulted in an early termination codon at bp 1011. To determine if this frameshift mutation was responsible for the loss in σ^{54} activity, a wild-type copy of the *rpoN* from *C. jejuni* NCTC 11168 was introduced into the *C. jejuni* S2B isolate using the pRY111 shuttle vector. The *C. jejuni* S2B isolate harboring the *rpoN*pRY111 shuttle vector was motile (Fig. 8B). The reason why the *C. jejuni* S3 and H8a isolates are nonmotile is not known currently.

DISCUSSION

The clinical presentation of campylobacteriosis varies extensively among individuals. Symptoms range from mild diarrhea to severe abdominal pain associated with diarrhea containing blood and leukocytes. In addition, infections in developing countries are more often reported in children, with symptoms limited to watery diarrhea, while infections in developed countries are associated with more dysentery-like symptoms. Explanations for the variation in the severity of campylobacteriosis include differences in the virulence of *C. jejuni* strains, repeated exposure to the organism, and host susceptibility.

Genetic variation among strains of *Campylobacter* spp. is well documented (2, 16, 42, 61, 64, 65, 71, 79). Dingle et al. (15) reported that *C. jejuni* cells form a weakly clonal population based on the distribution of strains among MLST sequence type complexes. In addition, various groups have shown that strains differ in their genomic contents using microarrays. Dorrell et al. (16) compared 11 *C. jejuni* strains with NCTC 11168 and found that at least 21% of the NCTC 11168 genome is divergent in one or more of the tested strains. Using a shotgun genomic microarray, Poly et al. (65) showed that there was significant variation between *C. jejuni* strains ATCC 43431 and NCTC 11168, especially at LOS, capsular biosynthesis, and restriction modification enzyme loci. Comparison of *C. jejuni* strains 81-176 and ATCC 43431 by the same method showed differences at similar loci (64). Finally, comparison of the whole genome sequences of NCTC 11168 and RM1221 identified the presence of phage insertion elements and polymorphisms at the LOS and capsular biosynthesis loci (23). Noteworthy is that both LOS and capsule biosynthesis have been implicated in the pathogenesis of *C. jejuni* (6, 27). Changes in the poly(G) tract of *cgtA* of *C. jejuni* strain 81-176 results in changes to the LOS core structure (27). A *cgtA* mutant in which the LOS mimics that of the GM₃ ganglioside rather than the GM₂ ganglioside demonstrates an increase in invasiveness in cultured epithelial cells. The *kpsM* gene is involved in the expression of the capsular high-molecular-weight glycan, and a *C. jejuni* 81-176 *kpsM* isogenic mutant is reduced in invasiveness and ability to cause diarrhea in the ferret model (6).

In addition to strain-to-strain genetic variation, intrastain variation has been observed in *Campylobacter* spp. For example, MRP-PFGE profile changes were found in five out of six *Campylobacter coli* isolates that had been passaged up to 50 times in the laboratory after initial isolation from separate pig herds (58). With respect to *C. jejuni*, Mixer et al. (50) observed that genomic changes occurred in *C. jejuni* strain F38011, as determined by SmaI MRP-PFGE, after intraperitoneal inoculation of mice. Carrillo et al. (10) reported that a *C. jejuni* NCTC 11168 strain that had undergone long-term passage in laboratory colonized chickens poorly and was less motile than the original stock strain (10). Although a specific mutation was not identified in a *C. jejuni* NCTC 11168 nonmotile variant, microarray analysis revealed an expression profile that was comparable to that for an *flhA* flagellar biosynthetic mutant. Gaynor et al. (24) also found gene expression differences between the genome-sequenced variant of *C. jejuni* NCTC 11168 and the original freezer stock; the two strains also showed differences in their abilities to colonize chickens.

Virulence-related phenotypic differences between *C. jejuni*

strains have been described. Multiple investigators have found differences in the invasion of *C. jejuni* strains into cultured epithelial cells (38, 54). Konkel and Joens (38) showed that the invasion of cultured epithelial cells by *C. jejuni* clinical isolates varied considerably. Overall, clinical isolates were more invasive than those obtained from culture collections (e.g., American Type Culture Collection, Manassas, VA). Everest et al. (18) found that *C. jejuni* isolates recovered from individuals with colitis were more invasive than those isolated from individuals with noninflammatory diarrhea. Newell et al. (54) observed that *C. jejuni* and *C. coli* isolates recovered from water sources were less invasive than clinical isolates. Variation in invasion efficiency among *C. jejuni* strains supports the notion that strain-to-strain genetic differences may play a role in the range of clinical symptoms of campylobacteriosis.

In this study, we examined the genotypic and phenotypic characteristics of *C. jejuni* poultry isolates. These isolates differed in both their abilities to invade INT 407 cells and levels of secretion of virulence proteins. All the isolates found to be nonmotile also showed lack of invasion and secretion potential. Conversely, all isolates that were able to secrete virulence proteins were also able to migrate through semisolid media, which is widely used as an indication of swarming motility. These data are consistent with previously published work demonstrating that the flagellar apparatus is required for the secretion of virulence proteins by *C. jejuni* (39). In addition, motility has been associated with *C. jejuni* pathogenesis both in terms of invasion of epithelial cells (77) and colonization of chickens (78).

We found that the *C. jejuni* Turkey and CS isolates used in this study were indistinguishable by MRP-PFGE and therefore constituted a clonally matched pair. MLST confirmed this finding, with both strains belonging to ST-48. Genomotyping using a microarray consisting of ORFs from NCTC 11168 revealed no differences in genomic content between the *C. jejuni* CS and Turkey isolates. Although clonally indistinguishable, the Turkey and CS isolates differed in their levels of putative pathogenic potential as judged by in vitro assays. The *C. jejuni* Turkey isolate secreted virulence proteins, invaded INT 407 epithelial cells at a high level, and was motile, while the *C. jejuni* CS isolate failed to secrete, invaded INT 407 epithelial cells at a low level, and was nonmotile. Also, the levels of pathogenic potential of *C. jejuni* isolates CS and Turkey were tested in the neonatal piglet model as described by Babakhani et al. (5). In agreement with our in vitro assays, piglets inoculated with the *C. jejuni* CS isolate did not develop diarrhea. Taken together, these results indicate a good correlation between the in vitro and in vivo models with respect to determining an isolate's pathogenic potential. Moreover, the *C. jejuni* Turkey isolate was assessed to be more pathogenic than the CS isolate in all assays.

No detectable differences in the genomic contents of the *C. jejuni* Turkey and CS isolates were noted by genomotyping or genomic subtractive hybridization (not shown), indicating that a difference in gene expression might result in the observed phenotypes. The protein profiles of the *C. jejuni* Turkey and CS isolates were examined. Based on the fact that the *C. jejuni* CS isolate was nonmotile, it is not surprising that the FlaA flagellin protein was not synthesized in the CS isolate, as judged by the absence of a 62-kDa protein in whole-cell extracts, outer membrane protein preparations, and a flagellar extract. The flagellin protein in the *C. jejuni* Turkey isolate, but not the *C. jejuni* CS

isolate, was confirmed to be FlaA by N-terminal sequence analysis. Total RNA was also extracted from the *C. jejuni* Turkey and CS isolates and used in an expression microarray to identify genes that differed in their expression. The *C. jejuni* Turkey isolate had significantly higher expression of genes involved in flagellar biosynthesis than the *C. jejuni* CS isolate.

Flagellar biosynthesis in *C. jejuni* is divided into three stages. The expression of class I genes is under the control of σ^{70} and is critical for the expression of class II genes. Class II genes are under the control of σ^{54} . Class III genes are under the control of σ^{28} . In addition to the participation of different sigma factors, the FlgS/R two-component signal transduction system participates in the regulation of the flagellar regulon. Real-time RT-PCR analysis of the isolates in this study indicated that all class I genes were upregulated in the *C. jejuni* CS isolate, whereas all the class II genes were downregulated. These findings suggested that a mutation might exist in either a class I flagellar biosynthesis gene (e.g., *flhA*) or a regulator of flagellar biosynthesis (*rpoN*, *flgR*, or *flgS*). To test this possibility, the ORFs of genes involved in flagellar gene regulation from both isolates were sequenced, including *flhA*, *flhB*, *flhF*, *fliA*, *fliF*, *fliG*, *fliH*, *fliI*, *fliM*, *fliN*, *fliP*, *fliQ*, *fliR*, *rpoN*, Cj0667, Cj0668, and Cj0669. Although we were unable to identify a point mutation in any gene contained within the *rpoN* operon, it was evident from the β -galactosidase reporter assay that the σ^{54} -regulated flagellar genes were not expressed in the *C. jejuni* CS isolate. Since FlgS and FlgR are required for σ^{54} activity, we sequenced the *flgS* and *flgR* ORFs. Comparison of the ORFs of the *flgR* genes of *C. jejuni* Turkey and CS revealed a difference in one nucleotide that altered the amino acid sequence. This change in the deduced amino acid sequence proved to be responsible for the nonmotile phenotype of the *C. jejuni* CS isolate, as indicated by the restoration of motility via complementation of the isolate with a wild-type copy of the *flgR* gene on the pRY107 shuttle vector. Hendrixson (31) reported a phase-variable mechanism responsible for modulating motility, which in turn helps the bacteria establish a commensal state. FlgR phase variation was achieved by the gain or loss of a nucleotide in the homopolymeric adenine or thymine tract. Noteworthy is that the region in which the mutation occurred in the *flgR* gene of the *C. jejuni* CS isolate was not identified previously as a phase-variable region (31).

To assess the frequency of nonmotile strains in a population of poultry isolates, we screened a total of 105 isolates and found 4 nonmotile strains (CS, S2B, S3, and H8a). Of the four nonmotile isolates recovered from poultry, we identified mutations in two distinct genes involved in flagellar biosynthesis. We were able to restore the motility of both the *C. jejuni* CS and S2B isolates and found a motile revertant of the S3 isolate (not shown). The latter finding indicates that the S3 isolate likely has a single point mutation that alters its motility phenotype. We have sequenced the *rpoN* and *flgR* genes from the *C. jejuni* S3 isolate and did not identify a nucleotide difference that altered the deduced amino acid sequence from that of the *C. jejuni* NCTC 11168 isolate. Thus, the S3 isolate possesses a mutation in a gene distinct from *rpoN* and *flgR*, indicating that a mutation of a third gene can also lead to a nonmotile phenotype. Based on these findings, it appears that a nonmotile phenotype likely offers a selective advantage to *C. jejuni* within the host or environment.

One of the differences noted in the phenotypes of *C. jejuni*

Turkey and CS isolates was in their resistance to the bile salt deoxycholate; the *C. jejuni* CS isolate is more resistant to deoxycholate. The difference in sensitivity appears to be specifically associated with the *flgR* mutation, as the same observation was noted for *C. jejuni* Turkey compared to a *C. jejuni* Turkey *flgR* mutant. It is presently not known why an *flgR* mutant is more resistant to deoxycholate than a wild-type isolate. In *C. jejuni*, the CmeABC multidrug efflux pump plays a major role in the organism's resistance to detergents, antimicrobials including different classes of antibiotics, and heavy-metal-containing salts (43, 44). A *C. jejuni* *cmeB* mutant is 64-fold more sensitive to sodium deoxycholate than a wild-type strain (44). In contrast to what one might predict, microarray analysis revealed that the *cmeABC* genes were downregulated in the *C. jejuni* CS isolates compared to the *C. jejuni* Turkey isolate. The reduction in expression of the *cmeABC* genes in the CS isolate was confirmed by real-time RT-PCR. Clearly additional work is needed to understand this phenomenon.

Microarray analysis of the *C. jejuni* CS isolate (*flgR* mutant) and *rpoN* mutant revealed that many more genes were not expressed in a *rpoN* mutant compared to its isogenic wild-type isolate, unlike the results of a similar comparison between the *C. jejuni* CS isolate and the Turkey isolate (data not shown). Of the nine flagellum-related genes whose expression was lower in the *C. jejuni* F38011 *rpoN* mutant than in the F38011 wild-type isolate, eight of the same nine genes had lower expression in the *C. jejuni* CS isolate than in the *C. jejuni* Turkey isolate. However, a total of 80 genes were downregulated in the defined *rpoN* mutant versus a total of 47 genes in the *C. jejuni* CS isolate. Based on this finding, we propose that FlgR is necessary for the expression of σ^{54} -regulated flagellar genes but that σ^{54} is responsible for the expression of other genes independent of the FlgR response regulator. Additional work is required to determine the genes of the σ^{54} regulon.

The role of motility in the pathogenesis of *C. jejuni* has been well documented. Newell et al. (52, 53) reported that, in a murine intestinal colonization model, a nonflagellated *C. jejuni* isolate colonized poorly and was cleared within 7 days postinfection. Nachamkin et al. (51) found that only *C. jejuni* isolates with intact and functional flagella colonized the 3-day-old chick model, while Wassenaar et al. (78) found that expression of the FlaA flagellin subunit maximized colonization of 1-day-old chicks by *C. jejuni*. Consistent with the hypothesis that motility is an important virulence attribute for *C. jejuni*, only motile variants were recovered from human volunteers challenged with a mixture of motile and nonmotile variants of *C. jejuni* (8). The results of this study also support the hypothesis that motility is an important *C. jejuni* virulence determinant as it affects an isolate's pathogenicity in the neonatal piglet model of campylobacteriosis. Our findings further underscore the need to assess whether a *C. jejuni* isolate is motile prior to assessing its in vitro and in vivo virulence potential.

In summary, we have compared the genetically matched *C. jejuni* Turkey and CS isolates and found that the differences in the pathogenicities of these two isolates are due to a point mutation in the *flgR* gene of the *C. jejuni* CS isolate. The *C. jejuni* CS isolate, compared to the *C. jejuni* Turkey isolate, exhibited a shorter lag phase in MH broth and achieved a greater maximum cell density in MH medium (Fig. 6). Inspection of the data published by Wösten et al. (81) also revealed

differences in lag phase and maximal cell density of a *flgR* mutant compared to a wild-type isolate, but the basis of these differences was not analyzed. It is unclear if the increased expression of certain nucleotide and amino acid biosynthesis genes by the *C. jejuni* CS isolate contributes to its higher maximal cell density. Nevertheless, it seems possible that a mutation in *flgR* may provide an organism with a selective advantage in a host (e.g., chicken intestinal tract) because it can reach a higher cell density. The resistance of a *C. jejuni* isolate to the bile salt deoxycholate might provide a selective advantage to the organism in the intestinal tracts of both humans and poultry. Finally, this study provides an approach to identify the molecular basis for *C. jejuni* pathogenicity. Moreover, we believe that it will be possible to identify novel *C. jejuni* virulence factors by comparing *C. jejuni* genetically matched isolates that display differences in virulence potential.

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REFERENCES

- Aeschbacher, M., and J.-C. Piffaretti. 1989. Population genetics of human and animal enteric *Campylobacter* strains. *Infect. Immun.* **57**:1432–1437.
- Ahmed, I. H., G. Manning, T. M. Wassenaar, S. Cawthraw, and D. G. Newell. 2002. Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. *Microbiology* **148**:1203–1212.
- Alm, R. A., P. Guerry, M. E. Power, and T. J. Trust. 1992. Variation in antigenicity and molecular weight of *Campylobacter coli* VC167 flagellin in different genetic backgrounds. *J. Bacteriol.* **174**:4230–4238.
- Anjum, M. F., S. Lucchini, A. Thompson, J. C. Hinton, and M. J. Woodward. 2003. Comparative genomic indexing reveals the phylogenomics of *Escherichia coli* pathogens. *Infect. Immun.* **71**:4674–4683.
- Babakhani, F. K., G. A. Bradley, and L. A. Joens. 1993. Newborn piglet model for campylobacteriosis. *Infect. Immun.* **61**:3466–3475.
- Bacon, D. J., C. M. Szymanski, D. H. Burr, R. P. Silver, R. A. Alm, and P. Guerry. 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol. Microbiol.* **40**:769–777.
- Belbouri, A., and F. Megraud. 1988. Enterotoxin-like activity produced by *Campylobacter jejuni* and *Campylobacter coli* isolated from patients and healthy controls in Algeria. *FEMS Microbiol. Lett.* **28**:25–28.
- Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. 1988. Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* **157**:472–479.
- Bladergroen, M. R., K. Badelt, and H. P. Spaik. 2003. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Mol. Plant-Microbe Interact.* **16**:53–64.
- Carrillo, C. D., E. Taboada, J. H. Nash, E. P. Lanthier, J. Kelly, P. C. Lau, R. Verhulp, O. Myktyczuk, J. Sy, W. A. Findlay, K. Amoako, S. Gomis, P. Willson, J. W. Austin, A. Potter, L. Babiuk, B. Allan, and C. M. Szymanski. 2004. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. *J. Biol. Chem.* **279**:20327–20338.
- Daikoku, T., M. Kawaguchi, K. Takama, and S. Suzuki. 1990. Partial purification and characterization of the endotoxin produced by *Campylobacter jejuni*. *Infect. Immun.* **58**:2414–2419.
- de Boer, P., B. Duim, A. Rigter, J. van Der Plas, W. F. Jacobs-Reitsma, and J. A. Wagenaar. 2000. Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. *J. Clin. Microbiol.* **38**:1940–1946.
- de Melo, M. A., and J.-C. Pechère. 1990. Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells in vitro. *Infect. Immun.* **58**:1749–1756.
- Dickins, M. A., S. Franklin, R. Stefanova, G. E. Schutze, K. D. Eisenach, I. Wesley, and M. D. Cave. 2002. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. *J. Food Prot.* **65**:957–962.
- Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden. 2001. Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**:14–23.
- Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G. Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W. Wren. 2001. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res.* **11**:1706–1715.
- Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton. 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.* **47**:103–118.
- Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knuttun, J. M. Ketley, and P. H. Williams. 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. *J. Med. Microbiol.* **37**:319–325.
- Fauchère, J.-L., M. Kervella, A. Rosenau, K. Mohanna, and M. Veron. 1989. Adhesion to HeLa cells of *Campylobacter jejuni* and *C. coli* outer membrane components. *Res. Microbiol.* **140**:379–392.
- Fauchère, J.-L., A. Rosenau, M. Veron, E. N. Moya, S. Richard, and A. Pfister. 1986. Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human feces. *Infect. Immun.* **54**:283–287.
- Fitzgerald, C., L. O. Helsel, M. A. Nicholson, S. J. Olsen, D. L. Swerdlow, R. Flahart, J. Sexton, and P. I. Fields. 2001. Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. *J. Clin. Microbiol.* **39**:2386–2390.
- Fitzgerald, C., K. Stanley, S. Andrew, and K. Jones. 2001. Use of pulsed-field gel electrophoresis and flagellin gene typing in identifying clonal groups of *Campylobacter jejuni* and *Campylobacter coli* in farm and clinical environments. *Appl. Environ. Microbiol.* **67**:1429–1436.
- Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. DeBoy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson. 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple campylobacter species. *PLoS Biol.* **3**:72–85.
- Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell. 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *J. Bacteriol.* **186**:503–517.
- Gibson, J. R., C. Fitzgerald, and R. J. Owen. 1995. Comparison of PFGE, ribotyping and phage-typing in the epidemiological analysis of *Campylobacter jejuni* serotype HS2 infection. *Epidemiol. Infect.* **115**:215–225.
- Goossens, H., J. P. Butzler, and Y. Takeda. 1985. Demonstration of cholera-like enterotoxin production by *Campylobacter jejuni*. *FEMS Microbiol. Lett.* **29**:73–76.
- Guerry, P., C. M. Szymanski, M. M. Prendergast, T. E. Hickey, C. P. Ewing, D. L. Pattarini, and A. P. Moran. 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infect. Immun.* **70**:787–793.
- Hänninen, M.-L., M. Hakkinen, and H. Rautelin. 1999. Stability of related human and chicken *Campylobacter jejuni* genotypes after passage through chick intestine studied by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* **65**:2272–2275.
- Hänninen, M.-L., P. Perko-Mäkelä, H. Rautelin, B. Duim, and J. A. Wagenaar. 2001. Genomic relatedness within five common Finnish *Campylobacter jejuni* pulsed-field gel electrophoresis genotypes studied by amplified fragment length polymorphism analysis, ribotyping, and serotyping. *Appl. Environ. Microbiol.* **67**:1581–1586.
- Harris, L. A., S. M. Logan, P. Guerry, and T. J. Trust. 1987. Antigenic variation of *Campylobacter* flagella. *J. Bacteriol.* **169**:5066–5071.
- Hendrixson, D. R. 2006. A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism. *Mol. Microbiol.* **61**:1646–1659.
- Hendrixson, D. R., and V. J. DiRita. 2003. Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Mol. Microbiol.* **50**:687–702.
- Jagannathan, A., C. Constantinidou, and C. W. Penn. 2001. Roles of *rpoN*, *flhA* and *flgR* in expression of flagella in *Campylobacter jejuni*. *J. Bacteriol.* **183**:2937–2942.
- Johnson, W. M., and H. Lior. 1986. Cytotoxic and cytotoxic factors produced by *Campylobacter jejuni*, *C. coli*, and *C. lariidis*. *J. Clin. Microbiol.* **24**:275–281.
- Kervella, M., J.-M. Pagès, Z. Pei, G. Grollier, M. J. Blaser, and J.-L. Fauchère. 1993. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. *Infect. Immun.* **61**:3440–3448.

36. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**:5–21.
37. **Konkel, M. E., and W. Cieplak, Jr.** 1992. Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect. Immun.* **60**:4945–4949.
38. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. *Infect. Immun.* **57**:2984–2990.
39. **Konkel, M. E., J. D. Klena, V. Rivera-Amill, M. R. Monteville, D. Biswas, B. Raphael, and J. Mickelson.** 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J. Bacteriol.* **186**:3296–3303.
40. **Konkel, M. E., Y. Lobet, and W. Cieplak, Jr.** 1992. Examination of multiple isolates of *Campylobacter jejuni* for evidence of cholera toxin-like activity, p. 193–198. In I. Nachamkin and M. J. Blaser (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, DC.
41. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
42. **Leonard, E. E., II, T. Takata, M. J. Blaser, S. Falkow, L. S. Tompkins, and E. C. Gaynor.** 2003. Use of an open-reading frame-specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. *J. Infect. Dis.* **187**:691–694.
43. **Lin, J., L. O. Michel, and Q. Zhang.** 2002. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* **46**:2124–2131.
44. **Lin, J., O. Sahin, L. O. Michel, and Q. Zhang.** 2003. Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infect. Immun.* **71**:4250–4259.
45. **Lucchini, S., H. Liu, Q. Jin, J. C. Hinton, and J. Yu.** 2005. Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen. *Infect. Immun.* **73**:88–102.
46. **Mahajan, S., and F. G. Rodgers.** 1990. Isolation, characterization, and host-cell-binding properties of a cytotoxin from *Campylobacter jejuni*. *J. Clin. Microbiol.* **28**:1314–1320.
47. **Manning, G., C. G. Dowson, M. C. Bagnall, I. H. Ahmed, M. West, and D. G. Newell.** 2003. Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* **69**:6370–6379.
48. **McCardell, B. A., J. M. Madden, and E. C. Lee.** 1986. *Campylobacter jejuni* and *Campylobacter coli* production of a cytotoxic toxin immunologically similar to cholera toxin. *J. Food Prot.* **47**:943–949.
49. **McSwegan, E., and R. I. Walker.** 1986. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. *Infect. Immun.* **53**:141–148.
50. **Mixer, P. F., J. D. Klena, G. A. Flom, A. M. Siegesmund, and M. E. Konkel.** 2003. In vivo tracking of *Campylobacter jejuni* using a novel recombinant expressing green fluorescent protein. *Appl. Environ. Microbiol.* **69**:2864–2874.
51. **Nachamkin, I., X. H. Yang, and N. J. Stern.** 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. *Appl. Environ. Microbiol.* **59**:1269–1273.
52. **Newell, D. G.** 1986. Monoclonal antibodies directed against the flagella of *Campylobacter jejuni*: production, characterization and lack of effect on the colonization of infant mice. *J. Hyg.* **96**:131–141.
53. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. *J. Hyg.* **95**:217–227.
54. **Newell, D. G., H. McBride, F. Saunders, Y. Dehele, and A. D. Pearson.** 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. *J. Hyg.* **94**:45–54.
55. **Newell, D. G., and A. Pearson.** 1984. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. *J. Diarrhoeal Dis. Res.* **2**:19–26.
56. **Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug.** 2000. Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol.* **66**:4266–4271.
57. **Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko.** 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc. Natl. Acad. Sci. USA* **90**:6884–6888.
58. **On, S. L. W.** 1998. *In vitro* genotypic variation of *Campylobacter coli* documented by pulsed-field gel electrophoretic DNA profiling: implications for epidemiological studies. *FEMS Microbiol. Lett.* **165**:341–346.
59. **Parker, C. T., S. T. Horn, M. Gilbert, W. G. Miller, D. L. Woodward, and R. E. Mandrell.** 2005. Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *J. Clin. Microbiol.* **43**:2771–2781.
60. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. M. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**:665–668.
61. **Pearson, B. M., C. Pin, J. Wright, K. l'Anson, T. Humphrey, and J. M. Wells.** 2003. Comparative genome analysis of *Campylobacter jejuni* using whole genome DNA microarrays. *FEBS Lett.* **554**:224–230.
62. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X.-Z. Huang, D. J. Kopecko, A. L. Bourgeois, J.-L. Fauchère, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infect. Immun.* **66**:938–943.
63. **Perez-Perez, G. L., D. L. Cohn, R. L. Guerrant, C. M. Patton, L. B. Reller, and M. J. Blaser.** 1989. Clinical and immunologic significance of cholera-like toxin and cytotoxin production by *Campylobacter* species in patients with acute inflammatory diarrhea in the USA. *J. Infect. Dis.* **160**:460–468.
64. **Poly, F., D. Threadgill, and A. Stintzi.** 2005. Genomic diversity in *Campylobacter jejuni*: identification of *C. jejuni* 81-176-specific genes. *J. Clin. Microbiol.* **43**:2330–2338.
65. **Poly, F., D. Threadgill, and A. Stintzi.** 2004. Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. *J. Bacteriol.* **186**:4781–4795.
66. **Raphael, B. H., S. Pereira, G. A. Flom, Q. Zhang, J. M. Ketley, and M. E. Konkel.** 2005. The *Campylobacter jejuni* response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization. *J. Bacteriol.* **187**:3662–3670.
67. **Ribot, E. M., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. J. Barrett.** 2001. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**:1889–1894.
68. **Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel.** 2001. Secretion of the virulence associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J. Infect. Dis.* **183**:1607–1616.
69. **Ruiz-Palacios, G. M., J. Torres, N. I. Torres, E. Escamilla, B. R. Ruiz-Palacios, and J. Tamayo.** 1983. Cholera-like enterotoxin produced by *Campylobacter jejuni*: characterization and clinical significance. *Lancet* **ii**:250–251.
70. **Russell, R. G., M. O'Donnoghue, D. C. Blake, Jr., J. Zulty, and L. J. DeTolla.** 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. *J. Infect. Dis.* **168**:210–215.
71. **Sails, A. D., B. Swaminathan, and P. I. Fields.** 2003. Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. *J. Clin. Microbiol.* **41**:4058–4067.
72. **Steinbrueckner, B., F. Ruberg, and M. Kist.** 2001. Bacterial genetic fingerprint: a reliable factor in the study of the epidemiology of human *Campylobacter* enteritis? *J. Clin. Microbiol.* **39**:4155–4159.
73. **Stephens, C. P., S. L. W. On, and J. A. Gibson.** 1998. An outbreak of infectious hepatitis in commercially reared ostriches associated with *Campylobacter coli* and *Campylobacter jejuni*. *Vet. Microbiol.* **61**:183–190.
74. **Tong, S., A. Porco, T. Isturiz, and T. Conway.** 1996. Cloning and molecular genetic characterization of the *Escherichia coli* *gntR*, *gntK*, and *gntU* genes of *GntI*, the main system for gluconate metabolism. *J. Bacteriol.* **178**:3260–3269.
75. **van Spreuwel, J. P., G. C. Duursma, C. J. L. M. Meijer, R. Bax, P. C. M. Rosekrans, and J. Lindeman.** 1985. *Campylobacter* colitis: histological, immunohistochemical and ultrastructural findings. *Gut* **26**:945–951.
76. **Vargas, A. C., M. M. Costa, M. H. Vainstein, L. C. Kreutz, and J. P. Neves.** 2003. Phenotypic and molecular characterization of bovine *Campylobacter fetus* strains isolated in Brazil. *Vet. Microbiol.* **93**:121–132.
77. **Wassenaar, T. M., N. M. Bleumink-Pluym, and B. A. van der Zeijst.** 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J.* **10**:2055–2061.
78. **Wassenaar, T. M., B. A. van der Zeijst, R. Ayling, and D. G. Newell.** 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* **139**:1171–1175.
79. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G₂-phase cell cycle block. *Infect. Immun.* **66**:1934–1940.
80. **Wösten, M. M. S. M., M. Boeve, M. G. A. Koot, A. C. van Nuene, and B. A. M. van der Zeijst.** 1998. Identification of *Campylobacter jejuni* promoter sequences. *J. Bacteriol.* **180**:594–599.
81. **Wösten, M. M. S. M., J. A. Wagenaar, and J. P. van Putten.** 2004. The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. *J. Biol. Chem.* **279**:16214–16222.
82. **Yan, W., N. Chang, and D. E. Taylor.** 1991. Pulsed-field gel electrophoresis of *Campylobacter jejuni* and *Campylobacter coli* genomic DNA and its epidemiological application. *J. Infect. Dis.* **163**:1068–1072.
83. **Yang, Y. H., and T. Speed.** 2002. Design issues for cDNA microarray experiments. *Nat. Rev. Genet.* **3**:579–588.
84. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and nonmotile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol. Microbiol.* **14**:883–893.
85. **Yeen, W. P., S. D. Puthuchery, and T. Pang.** 1983. Demonstration of a cytotoxin from *Campylobacter jejuni*. *J. Clin. Pathol.* **36**:1237–1240.