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Culture of *Campylobacter jejuni* with Sodium Deoxycholate Induces Virulence Gene Expression▽

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***Campylobacter jejuni*, a spiral-shaped gram-negative bacterium, is a leading bacterial cause of human food-borne illness. Acute disease is associated with *C. jejuni* invasion of the intestinal epithelium. Further, maximal host cell invasion requires the secretion of proteins termed *Campylobacter* invasion antigens (Cia). As bile acids are known to alter the pathogenic behavior of other gastrointestinal pathogens, we hypothesized that the virulence potential of *Campylobacter* may be triggered by the bile acid deoxycholate (DOC). In support of this hypothesis, culturing *C. jejuni* with a physiologically relevant concentration of DOC significantly altered the kinetics of cell invasion, as shown by gentamicin protection assays. In contrast to *C. jejuni* harvested from Mueller-Hinton (MH) agar plates, *C. jejuni* harvested from MH agar plates supplemented with DOC secreted the Cia proteins, as judged by metabolic labeling experiments. DOC was also found to induce the expression of the *ciaB* gene, as determined by β-galactosidase reporter, real-time reverse transcription-PCR, and microarray analyses. Microarray analysis further revealed that DOC induced the expression of virulence genes (*ciaB*, *cmeABC*, *dccR*, and *tlyA*). In summary, we demonstrated that it is possible to enhance the pathogenic behavior of *C. jejuni* by modifying the culture conditions. These results provide a foundation for identifying genes expressed by *C. jejuni* in response to in vivo-like culture conditions.**

Campylobacter jejuni is recognized as one of the leading bacterial causes of gastrointestinal disease in humans (1, 2). An estimated 2.4 million persons are infected by *C. jejuni* each year in the United States. Infection with *C. jejuni* results in symptoms that range from mild watery diarrhea to more severe diarrhea with blood and leukocytes. The most notable complication of campylobacteriosis is the development of Guillain-Barré syndrome, an acute demyelinating polyneuropathy. Approximately 1 in 1,000 diagnosed *C. jejuni* infections result in Guillain-Barré syndrome (6).

Bile is a digestive secretion that plays a major role in fat dispersion and absorption. Approximately 50% of organic bile consists of bile acids, which are synthesized in the liver from cholesterol by a multienzyme process. Bile acids, including cholates and deoxycholates (DOCs), are amphipathic molecules that act as detergents and possess potent antimicrobial activity. The average concentration of bile acids in the human intestine ranges from 0.2 to 2%, and DOC accounts for about 15% of the bile acids (7).

Bile has been shown to regulate virulence gene expression in several gastrointestinal pathogens (5, 12, 31–34, 38–40, 46). For example, *Shigella* spp. grown in the presence of bile show increased secretion of invasion plasmid antigens (Ipa) and enhanced invasion potential (36). Specifically, DOC stimulates the localization of IpaB to the tip of the type III secretion apparatus needle, where IpaB, in association with IpaD, is hypothesized to act as a sensor of host cell contact (31). In *Vibrio parahaemolyticus*, bile acids enhance the production of thermostable direct hemolysin (32, 33) and capsule and adherence to epithelial cells (34).

The ability of *Campylobacter* to cause illness is related to its ability to invade epithelial cells lining the intestinal tract (3, 9, 13, 48, 51). The proteins known to promote entry of the bacteria into eukaryotic cells are different from those that facilitate binding (15). In contrast to cellular adhesion, *C. jejuni* must be metabolically active to invade human epithelial cells. When it is cultured with epithelial cells, *C. jejuni* synthesizes and secretes a set of proteins that are required for maximal invasion of host epithelial cells (15, 16, 18, 44, 45). These proteins are termed *Campylobacter* invasion antigens (Cia). The secretion of the Cia proteins is dependent on a functional flagellum, indicating that this organelle has a dual function in motility and as a type III secretion system (17). To date, only one Cia, termed CiaB, has been identified. In contrast to the *C. jejuni* F38011 wild-type isolate, the host cell invasion of a *ciaB* null mutant is impaired and the mutant is secretion deficient (16). Further, the severity and time of onset of disease in piglets inoculated with a *C. jejuni ciaB* null mutant are retarded compared with the severity and time of onset of disease in piglets inoculated a *C. jejuni* wild-type isolate. Piglets inoculated with a *C. jejuni ciaB* null mutant developed diarrhea 3 days postinoculation, whereas piglets inoculated with a *C. jejuni* wild-type isolate developed diarrhea within 24 h (19).

Although a number of studies have highlighted the mechanism of resistance of *Campylobacter* to bile (20, 21, 24, 41), little is known about the effect of bile on *Campylobacter* virulence determinants. Bile acids, including DOC, cholate, and chenodeoxycholate, have previously been shown to induce synthesis of the Cia proteins (44). This study was undertaken to determine the role that bile plays in the temporal expression of *ciaB* and its effect on *Campylobacter* pathogenesis. More spe-

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cifically, we studied the effect of a physiologically relevant concentration of DOC on *Campylobacter* invasion potential, which is an important virulence determinant and contributes to the development of severe disease. We demonstrated that compared to bacteria grown on Mueller-Hinton (MH) agar, *C. jejuni* grown in the presence of DOC show (i) an increase in the kinetics of host cell invasion, (ii) an increase in *ciaB* gene expression, and (iii) an alteration in the expression of genes that play a role in *Campylobacter* pathogenesis. In summary, we demonstrated that it is possible to enhance the pathogenic behavior of *C. jejuni* in the laboratory by culturing this organism under conditions that resemble the in vivo environment.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* F38011 was recovered from an individual with clinical signs of campylobacteriosis. The *C. jejuni* F38011 strain was cultured on MH agar plates supplemented with 5% bovine citrated blood (MH-blood) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C and was subcultured to a fresh plate every 48 h. MH-blood agar plates were supplemented with 200 μg/ml of kanamycin (Kan) when appropriate. Where indicated, *C. jejuni* was cultured on plates containing MH agar and MH agar supplemented with 0.1% sodium DOC (MHD agar) (1 mg/ml; Sigma, St. Louis, MO). *Escherichia coli* INVαF′ (Invitrogen, Carlsbad, CA) was cultured in Luria-Bertani (LB) broth and on LB agar plates at 37°C. LB agar plates were supplemented with 50 μg/ml Kan when appropriate.

Tissue culture. Stock cultures of INT 407 epithelial cells (human embryonic intestinal; ATCC CCL 6) were grown in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone Laboratories Inc., Logan, UT) and were maintained at 37°C in a humidified, 5% CO_2 incubator.

Binding assay. For experimental assays, a 24-well tissue culture tray was seeded with 2×10^5 INT 407 cells per well and incubated for 18 h at 37°C in a humidified, 5% CO_2 incubator. *C. jejuni* F38011 was cultured on MH and MHD agar plates for 18 h. The bacteria were harvested in MEM supplemented with 1% FBS and washed twice. The optical density at 540 nm (OD_{540}) of the bacterial suspension was adjusted to 0.2. Tenfold serial dilutions of the initial suspension were used to inoculate INT 407 cells as described previously (29). Briefly, the INT 407 cells were washed with MEM, and 1 ml of a bacterial suspension was added to each well. The bacteria were centrifuged at $600 \times g$ to facilitate bacterium-host cell interaction. After 1 h of incubation, the nonadherent cells were removed by rinsing with phosphate-buffered saline (PBS). The INT 407 cells were lysed with a 0.1% Triton X-100 solution in PBS. 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. The initial inocula were also plated to determine the multiplicity of infection. The values reported below are the mean counts ± standard deviations derived from quadruplicate wells. The assay was repeated three times to ensure reproducibility.

Secretion assay. *C. jejuni* F38011 was cultured for 18 h on MH agar and MHD agar plates. The bacteria were harvested in MEM without methionine (labeling medium) (ICN Biomedicals, Inc., Aurora, OH), pelleted by centrifugation at $6{,}000 \times g$, and washed twice in MEM. For metabolic labeling, approximately 5×10^8 CFU was suspended in MEM without methionine. Six milliliters of the bacterial suspension was then used for labeling with [^{35}S]methionine (Perkin Elmer Life Sciences, Inc., Boston, MA) at a concentration of 50 μCi/ml (15). Both cultures were incubated for 30 min at 37°C under microaerobic conditions to allow incorporation of [^{35}S]methionine. To each bacterial suspension (harvested from MH and MHD agar plates), chloramphenicol (Cm) was added at a final concentration of 128 μg/ml (44). This concentration of Cm immediately halts protein synthesis, as judged by the absence of [^{35}S]methionine incorporation. The flasks were incubated for 30 min at 37°C under microaerobic conditions. The suspensions were divided into two flasks and incubated with and without 1% FBS (HyClone Laboratories Inc., Logan, UT) for 30 min at 37°C under microaerobic conditions to stimulate *C. jejuni* protein secretion. Prior to each secretion assay, the albumin was removed from the FBS using a SwellGel Blue albumin removal kit (Pierce, Rockford, IL). Following an additional 30 min of incubation, the supernatant fluids were harvested and samples were processed as described previously (15). The bacterial pellets were resuspended in water and mixed with an equal volume of double-strength sample solubilization buffer. The secreted proteins and whole-cell lysates (WCLs) (OD_{540}, 0.1) were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were exposed to phosphorimaging screens to detect secreted proteins and WCL proteins.

The *C. jejuni* secreted proteins and WCL were also probed with goat anti-CadF serum. Membranes were washed three times in PBS and incubated for 18 h at 4°C with a 1:500 dilution of the goat anti-*C. jejuni* CadF antibody in PBS (pH 7.4)-0.01% Tween 20 containing 5% dried milk. Bound antibodies were detected using peroxidase-conjugated rabbit anti-goat immunoglobulin G (Sigma, St. Louis, MO) at a 1:1,000 dilution and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

Internalization kinetics. The wells of 24-well tissue culture trays were seeded with INT 407 cells as described above for the binding assay. For the internalization assay, each well of a tray was inoculated with 1 ml of a bacterial suspension. The tissue culture tray was centrifuged at $600 \times g$ for 5 min and incubated at 37°C in a humidified, 5% CO_2 incubator for various periods of time. After 15, 30, 60, 90, and 180 min of incubation, the *C. jejuni*-infected INT 407 cells were washed with MEM and incubated with MEM supplemented with 1% FBS containing gentamicin at a concentration of 250 μg/ml. After 3 h of incubation, the cells were lysed, and the number of internalized bacteria was determined as described above.

Construction of P*ciaB*-pMW10 and P*porA*-pMW10 reporter vectors. The *ciaB* and *porA* promoter regions were PCR amplified from the *C. jejuni* NCTC 11168 strain using primers designed with BglII and BamHI sites, respectively, at the 5′ end and were cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The ligated vectors were electroporated into *E. coli* INVαF′, and the transformants were selected on LB agar supplemented with Kan (50 μg/ml). The P*ciaB*-pCR2.1 and P*porA*-pCR2.1 vectors were digested with BglII and BamHI to obtain 568- and 377-bp fragments, respectively. The fragments were gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). The promoter shuttle vector pMW10 (52) was digested with BamHI, gel purified, and ligated with the BglII P*ciaB* and P*porA* fragments. The ligation mixtures were electroporated into *E. coli* INVαF′. Transformants were selected on LB agar supplemented with Kan using blue-white screening. The sequences of positive clones were verified using gene-specific primers as described elsewhere (52). The P*ciaB*-pMW10 and P*porA*-pMW10 reporter vectors were electroporated into *C. jejuni* F38011, and the resultant colonies were screened on MH-blood agar plates with Kan (200 μg/ml). The presence of each vector was confirmed by PCR using primers designed to amplify a portion of the *aphA-3* kanamycin gene.

β-Galactosidase assay. To determine the levels of *ciaB* and *porA* promoter activities in the presence and absence of DOC, overnight cultures of the *C. jejuni* F38011 strain harboring the P*ciaB*-pMW10 and P*porA*-pMW10 constructs were subcultured in MH broth at an initial OD_{540} of 0.05. The cultures were then incubated and allowed to reach an OD_{540} of 0.2. Five-milliliter portions of the log-phase cultures were then used to inoculate MH and MHD agar plates. The plates were incubated for 9, 12, 15, and 20 h. At each time point the bacteria were harvested in cold PBS and washed twice. To determine the effect of DOC concentration on the promoter activities of *ciaB* and *porA*, 5-ml log-phase cultures were used to inoculate MH agar plates supplemented with 0.05, 0.1, 0.2, and 0.4% DOC. The bacteria were harvested after 15 h with cold PBS and washed twice. The β-galactosidase assays were performed as described previously (28).

RNA isolation. Total cellular RNA was isolated from *C. jejuni* F38011 cultured on MH and MHD agar plates after incubation for 3, 6, 9, 12, and 15 h using a RiboPure-bacterial kit (Ambion, Austin, TX) according to the manufacturer's instructions. The extracted RNA was treated twice with DNase at 37°C for 30 min to remove genomic DNA. The absence of genomic DNA was confirmed by PCR using *C. jejuni ciaB* gene sequence-specific primers CiaB-F (CTATGCTA GCCATACTTAGGC) and CiaB-R (GCCCGCCTTAGAACTTAC).

Real-time RT-PCR. To determine the temporal expression of *ciaB*, real-time quantitative reverse transcription-PCR (RT-PCR) was performed using RNA isolated from the *C. jejuni* F38011 strain cultured on MH agar and MHD agar plates for 3, 6, 9, 12, and 15 h. cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's directions and 500 ng of RNA. Real-time RT-PCR amplification of 2.5 μl of cDNA (1:100 dilution) was performed using a reaction mixture containing Power SYBR green PCR master mixture (Applied Biosystems, Foster City, CA), 300 nM forward primer, 300 nM reverse primer, and diethyl pyrocarbonate-treated water. The real-time RT-PCR analysis was performed using a Gene Amp 7000 thermocycler (Applied Biosystems) with the following PCR parameters: 2 min at 50°C and then 40 cycles of denaturation at 95°C for 15 s and annealing at 55°C

TABLE 1. Primers used for real-time RT-PCR analysis

Primer	Gene	Sequence (5′–3′)
Cj0181 RT-F	*tonB*	CTCAAGAAAAATCAAGTGGTGTTG
Cj0181 RT-R	*tonB*	CGATAGGAAACTCTGATACCATC
Cj0323 RT-F	Cj0323	TATACTCAAATAACTTCAAATCATAGTG
Cj0323 RT-R	Cj0323	CTCTTCTTGATTCTGTTCTAAAATTG
Cj0367c RT-F	*cmeA*	GTGTTGATTCGGCTTACGGAC
Cj0367cRT-R	*cmeA*	TCTAGCACTTGCTAGACTAGC
Cj0402 RT-F	*glyA*	CGATGGAACGGATAATCACC
Cj0402RT-R	*glyA*	AATACCTGCATTTCCAAGAGC
Cj0561 RT-F	Cj0561	TTGGCAAACAGTGATTATCTAAGC
Cj0561 RT-R	Cj0561	TAAAGTTCTGCACCGATAAAAGG
Cj0588 RT-F	*tlyA*	AATTTATGTTTCAAGAGCAGCTTT
Cj0588 RT-R	*tlyA*	CGTACTAGATCCTATATCAAGAC
Cj0706 RT-F	Cj0706	AATTAGACGCTGCAAATGATGAG
Cj0706 RT-R	Cj0706	CTTACTCTAATCTCGTTAATATTTTGC
Cj0786 RT-F	Cj0786	GGTGTTATTTTTGGAATTGATTATGTG
Cj0786 RT-R	Cj0786	CTATATATTCTTTTTTTTCTTTCTCTAAGC
Cj0793 RT-F	*flgS*	TGTTGCCTAGTGCGCTTTGG
Cj0793 RT-R	*flgS*	ATAAAACCTACCTTCAAATTCAAGC
Cj0862c RT-F	*pabB*	AAATGATACAAAAAATCTGAGTGAAAATG
Cj0862c RT-R	*pabB*	TTTGGTTTTTAAGCTTTTTTTTGTGATG
Cj0863c RT-F	*xerD*	AAGCAAAATGAAGAAGATGAAAAAGC
Cj0863c RT-R	*xerD*	TAATTTTACCCCTTTAGAACCTGC
Cj0914c RT-F	*ciaB*	AGACAAAGAAGATGTGGGTGA
Cj0914c RT-R	*ciaB*	AATCAATCAAACGCCTAAGTATGG
Cj0989 RT-F	Cj0989	TCTTTATCATCGTTACTCGCTATG
Cj0989 RT-R	Cj0989	TATCTTCTTTCATATTTTGTATGTTTTGG
Cj1212c RT-F	*rbn*	AGCAGCGCTTAGTTTTTATACTG
Cj1212c RT-R	*rbn*	GGAAATTTGCGTAAAAACAGAAAAAC
Cj1223 RT-F	*dccR*	GATATTTTGATCTTTGGATTTTAGATGT
Cj1223 RT-R	*dccR*	GGAGTTTGCTTTCCGCTTTTTC
Cj1224 RT-F	Cj1224	CAAAATGGGATAACAGCTATAGTG
Cj1224 RT-R	Cj1224	CTTCATTTTTACTTACAGATCTATCTG
Cj1314c RT-R	*hisF*	AATGCACGCAATGTTGATGAGC
Cj1314c RT-F	*hisF*	GCAGCCCTTGAGCCATCG
Cj1458c RT-F	*thiL*	GAACAAAGAAGATTTTATTATCAAAGC
Cj1458c RT-R	*thiL*	AAATCCTTACTAAAACACCAATCATC
Cj1530 RT-F	*coaA*	AACCGCTTCAATTGCTTGTGG
Cj1530 RT-R	*coaA*	CGATTTTGTCTGCGCTAATGC
Cj1531 RT-F	*dapF*	AGGTGCGGATGGCTTTATCG
Cj1531 RT-R	*dapF*	GCAGCCCTTGAGCCATCG

for 1 min. Threshold cycle values were determined using Prism SDS software, version 1.0 (Applied Biosystems). The comparative threshold cycle method was used to calculate changes, and samples were normalized to *glyA*, as this gene is a housekeeping gene that is not differentially expressed in the presence of DOC. We also performed real-time RT-PCR analysis to determine the change in *porA* in the presence of DOC as a control. The primers used for the analyses are shown in Table 1. Duplicate reactions were performed, and three biological replicates were used for each sample.

Construction of the *C. jejuni* DNA microarray. DNA fragments of individual open reading frames (ORFs) were amplified with the Sigma-Genosys (The Woodlands, TX) *C. jejuni* ORFmer primer set specific for strain NCTC 11168 coding sequences and with primers from Operon Technologies (Alameda, CA) specific for strain RM1221 unique sequences, as described previously (35). The PCR products were purified with a Qiagen 8000 robot by using a QIAquick 96-well Biorobot kit (Qiagen, Valencia, CA). A total of 1,530 ORFs from strain NCTC 11168 and 227 ORFs from strain RM1221 were PCR amplified, purified, and spotted in duplicate onto Ultra-GAPS glass slides (Corning Inc., Corning, NY) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI), as described previously (35). Immediately after printing, the microarrays were UV cross-linked at 300 mJ using a Stratalinker UV Cross-linker 1800 (Stratagene, La Jolla, CA) and stored in a desiccator. Before use, the microarrays were blocked with Pronto! prehybridization solution (Corning Inc.) used according to the manufacturer's specifications.

Microarray hybridization and analysis. For the expression profiling arrays, an indirect comparison of gene expression was performed, in which the expression profiles of *C. jejuni* F38011 cultured in the presence and absence of DOC were determined separately on different slides as described previously (26). Briefly, Cy5-labeled reference DNA from the *C. jejuni* F38011 strain was mixed with Cy3-labeled test cDNA (*C. jejuni* F38011 cultured in the presence and absence of DOC) and hybridized to the *Campylobacter* cDNA array (26) on separate slides. DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA), and the data for spot and background intensities were processed using the GenePix 4.0 software. To compensate for differences in the amount of template and uneven Cy3 or Cy5 dye incorporation, data normalization was performed as previously described (26).

Normalized data that passed the quality controls were analyzed using GENESPRING 7.3 software (Silicon Genetics, Palo Alto, CA). To compare genes differentially expressed in the presence and absence of DOC, at least four hybridization measurements were generated for each 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 a P value of <0.05 was determined using a parametric statistical t test, adjusting the individual P value with the Benjamini-Hochberg false discovery rate multiple test correction in the GeneSpring analysis package.

The microarray results were confirmed by real-time RT-PCR analysis. The genes upregulated in the presence of DOC were categorized into functional classes as described by the Sanger Center website (http://www.sanger.ac.uk/Projects/C_jejuni/). Genes in each functional class (a total of 19 genes) were selected, and the change in gene expression of the *C. jejuni* F38011 strain cultured in the presence and absence of DOC was confirmed using real-time RT-PCR analysis as described previously. The primers used for the analyses are shown in Table 1.

Microarray accession numbers. Details of the microarray have been deposited in the NCBI GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL6265. The microarray data set has been deposited in the NCBI Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE10110 (samples GSM255693, GSM255694, GSM255695, GSM255696, GSM255697, and GSM255698).

RESULTS

Culture with sodium DOC does not alter adherence or motility of *C. jejuni*. To determine if DOC affects the ability of *C. jejuni* to adhere to INT 407 cells, a binding assay was performed with bacteria harvested from an MH agar plate or an MH agar plate supplemented with DOC. DOC did not alter the ability of *C. jejuni* to bind to the INT 407 cells (Fig. 1A). In addition, DOC did not alter the motility of the *C. jejuni* F38011 strain, as judged by motility assays (Fig. 1B). Collectively, these results indicate that culturing *C. jejuni* in the presence of DOC does not alter the adherence potential or motility of this bacterium.

DOC stimulates the synthesis but not secretion of *Campylobacter* invasion antigens. Invasion of the host cell is required for maximal disease. To determine if DOC alters the phenotypic behavior of *C. jejuni* with respect to invasion, we initially examined the effect of DOC on the secretion of the Cia proteins (Fig. 2). The bacteria were harvested from MH and MHD agar plates and labeled with [^{35}S]methionine for 30 min. Following the metabolic labeling of proteins, Cm was added to inhibit protein synthesis. After an additional 30 min of incubation, FBS was added to the labeling medium. Previous studies have demonstrated that it is necessary to add FBS to the medium to induce secretion of the Cia proteins from *C. jejuni* (44). In contrast to the supernatants of the bacteria harvested from MH agar plates, the Cia proteins were clearly visible in the supernatants of bacteria harvested from MHD agar plates (Fig. 2A, lanes 1 and 2). To ensure that this finding was due to active protein secretion and reflected a bona fide difference in the phenotypic behaviors of *C. jejuni* harvested from MH and MHD agar plates, several controls were performed in parallel. First, samples in which FBS was not added to the labeling medium were included in the assay. Consistent with previous work, the Cia proteins were not present in the supernatants of bacteria harvested from both MH and MHD agar plates in the absence of FBS (Fig. 2A, lanes 3 and 4). Second, we harvested the bacteria after the labeling assay and assessed their meta-

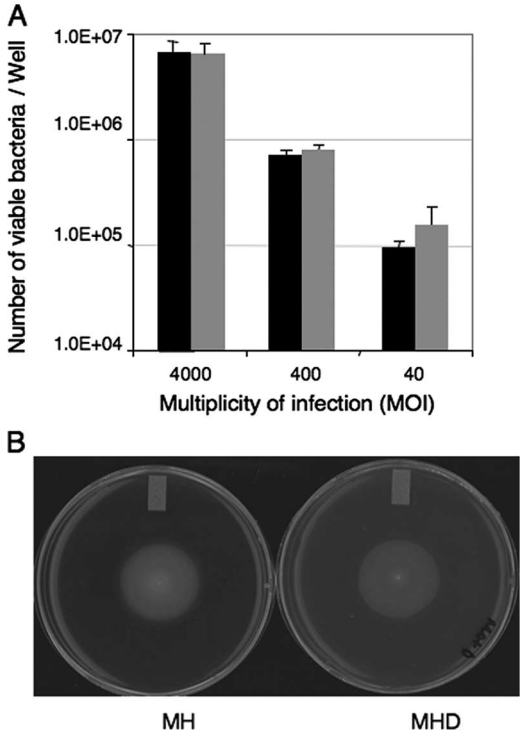

FIG. 1. Sodium DOC does not alter the adherence (A) or motility (B) of the *C. jejuni* F38011 strain. (A) Tenfold serial dilutions of the *C. jejuni* F38011 strain, cultured on MH and MHD agar plates for 18 h, were used to inoculate INT 407 cells. The data indicate the numbers of bacteria from MH agar plates (black bars) and MHD agar plates (gray bars) that bound to INT 407 cells 30 min postinoculation. The bars indicate the mean numbers of viable bacteria recovered per well of a 24-well tissue culture tray, and the error bars indicate the standard deviations. (B) *C. jejuni* cultures on MH and MHD agar plates with 0.4% agar displayed equivalent zones of migration.

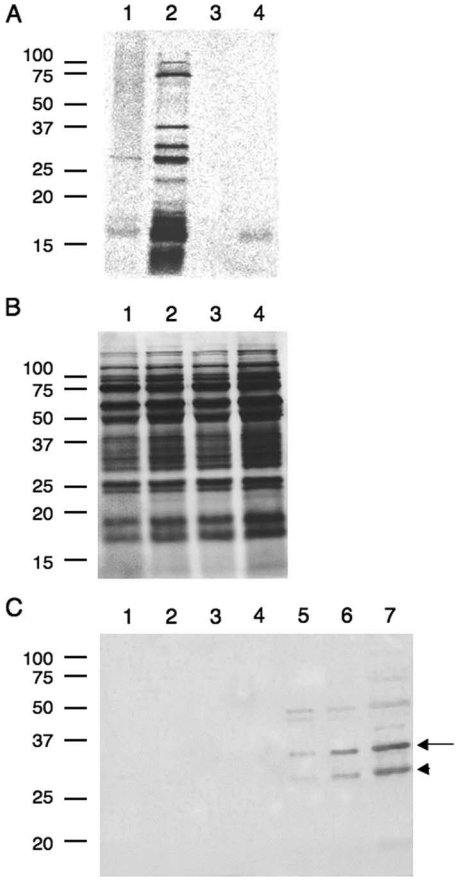

FIG. 2. Sodium DOC stimulates the synthesis of *Campylobacter* invasion antigens. (A) Proteins secreted by *C. jejuni* as determined by SDS-PAGE coupled with autoradiography of the supernatants. (B) Proteins synthesized by *C. jejuni* as judged by SDS-PAGE coupled with autoradiography of the WCLs (OD_{540}, 0.1). (C) Control, in which the proteins secreted by *C. jejuni* were probed with an antibody prepared against the *C. jejuni* CadF outer membrane protein. The metabolic labeling assays, preparation of supernatants and WCLs, and autoradiography were performed as described in Materials and Methods. Lane 1, *C. jejuni* F38011 harvested from an MH agar plate and radioactively labeled in medium supplemented with 1% FBS; lane 2, *C. jejuni* F38011 harvested from an MHD agar plate and radioactively labeled in medium supplemented with 1% FBS; lane 3, *C. jejuni* F38011 harvested from an MH agar plate and radioactively labeled in medium without 1% FBS; lane 4, *C. jejuni* F38011 harvested from an MHD agar plate and radioactively labeled in medium without 1% FBS; lane 5, 1:4 dilution of *C. jejuni* WCL; lane 6, 1:2 dilution of *C. jejuni* WCL; lane 7, 1:1 dilution of *C. jejuni* WCL. The migration of CadF as two protein species, indicated by the arrow (37-kDa species) and the arrowhead (32-kDa species), was due to the protein's heat-modifiable property.

bolic state by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis coupled with autoradiography of the WCLs (Fig. 2B). As expected, the intensities of the protein bands were similar for the bacterial samples, indicating that the bacteria were metabolically active (Fig. 2B, lanes 1 to 4). Third, we probed the secreted proteins with an antibody prepared against the *C. jejuni* CadF outer membrane protein to ensure that the proteins detected in the supernatants were not due to bacterial cell lysis (Fig. 2C). In contrast to the *C. jejuni* WCLs, the CadF protein was not detected in the supernatants of any of the samples. Based on these results, we concluded that DOC is capable of stimulating the synthesis of the Cia proteins.

DOC alters the invasion kinetics of the *C. jejuni* F38011 strain. We tested the effect of DOC on the ability of *C. jejuni* F38011 to invade INT 407 cells as judged by a gentamicin protection assay. Here, the bacteria were incubated with INT 407 cells for 3 h, after which medium containing gentamicin was added for a 3-h period to kill the extracellular bacteria. Compared to *C. jejuni* harvested from MH agar plates, *C. jejuni* harvested from MHD agar plates was more invasive with the INT 407 cells (not shown). To further assess the specificity of DOC for stimulating the synthesis of the *C. jejuni* Cia proteins, an additional gentamicin assay was performed, in which the bacteria harvested from MH and MHD agar plates were suspended in medium containing Cm prior to inoculation of the INT 407 cells. The concentration of Cm used in these assays immediately halted *C. jejuni* protein synthesis, thereby preventing additional protein synthesis by *C. jejuni* in the presence of epithelial cells (not shown). In the presence of Cm, an 11-fold increase in the number of intracellular *C. jejuni* cells harvested from MHD agar plates was observed compared with bacteria harvested from MH agar plates. Collectively, these data suggest that culture of *C. jejuni* with DOC

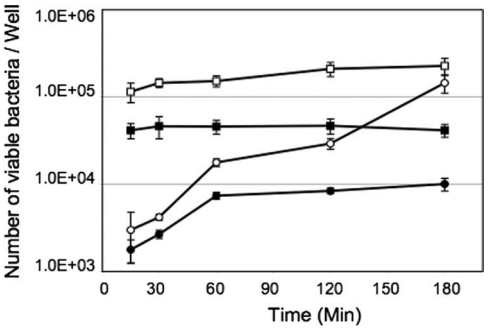

FIG. 3. Culturing the *C. jejuni* F38011 strain with sodium DOC alters the kinetics of invasion of INT 407 cells. Gentamicin protection assays were performed as described in Materials and Methods with *C. jejuni* F38011 cultured on MH and MHD agar plates for 18 h. The bacteria were incubated with INT 407 cells in the absence and presence of 128 μg/ml of Cm to inhibit protein synthesis. The numbers of internalized bacteria are indicated for *C. jejuni* F38011 cultured on an MH agar plate (○), *C. jejuni* F38011 cultured on an MHD agar plate (□), *C. jejuni* F38011 cultured on an MH agar plate and then incubated for 30 min with Cm (●), and *C. jejuni* F38011 cultured on an MHD agar plate and then incubated for 30 min with Cm (■). The symbols indicate the mean numbers of viable bacteria recovered per well of a 24-well tissue culture tray, and the error bars indicate standard deviations.

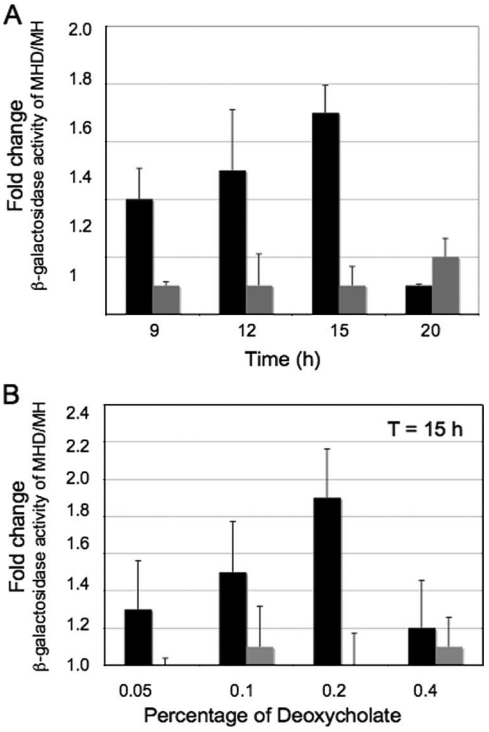

FIG. 4. Stimulation of *ciaB* promoter activity by sodium DOC is time (A) and dose (B) dependent. The data shown represent the ratios of the β-galactosidase activity of *ciaB* (black bars) and *porA* (gray bars) for the *C. jejuni* F38011 strain cultured on an MHD agar plate to the β-galactosidase activity of *ciaB* and *porA* for the *C. jejuni* F38011 strain cultured on an MH agar plate. The bars indicate the means of two separate experiments, and the error bars indicate the standard deviations; each experiment was comprised of triplicate samples.

induces the synthesis of proteins that facilitate the organism's invasion of epithelial cells.

Since culture with DOC stimulates Cia protein synthesis and enhances the ability of *C. jejuni* to invade INT 407 cells, an invasion assay was performed to determine the effect of DOC on the kinetics of invasion. Based on the observation that DOC stimulates the bacterium to synthesize the Cia proteins, we hypothesized that culturing *C. jejuni* on MHD agar plates would increase the invasive potential compared to that of bacteria harvested from MH agar plates. As predicted, *C. jejuni* F38011 harvested from MHD agar plates was able to maximally invade INT 407 cells within 15 min after infection (Fig. 3). An increase in INT 407 cell invasion was also observed at 15 min after infection with *C. jejuni* F38011 harvested from MHD agar plates and suspended in medium with Cm compared to the invasion efficiency of *C. jejuni* F38011 harvested from plates containing MH agar alone. For *C. jejuni* cultured on MH agar plates, the number of intracellular bacteria increased over time, and the level reached was similar to that observed for the bacteria harvested from MHD plates after a 3-h period. As expected, the invasion of INT 407 cells was significantly reduced when bacteria that were harvested from MH agar plates and resuspended in medium containing Cm were used. Based on these results, we concluded that culturing *C. jejuni* in the presence of DOC alters the invasion kinetics of *C. jejuni* such that maximal invasion is achieved as early as 15 min postinfection.

DOC induces the *ciaB* promoter. To determine if DOC induces *ciaB* gene expression, the *ciaB* promoter was cloned upstream of the β-galactosidase gene (*ciaB* promoter–β-galactosidase) in the pMW10 vector. A log-phase culture of *C. jejuni* F38011 harboring the P*ciaB*-pMW10 construct was then used to inoculate MH and MHD agar plates, and β-galactosidase assays were performed. The *ciaB* promoter activity gradually increased in the presence of 0.1% DOC and reached a maximum at 15 h postinoculation, after which it decreased (Fig. 4A). The ability of DOC to induce the promoter activity of *porA* was also measured. In contrast to the effect on the activity of the *ciaB* promoter, DOC did not significantly alter *porA* promoter activity over time.

We also measured the activities of the *ciaB* and *porA* promoters in response to different concentrations of DOC. Here, MH and MH agar plates supplemented with various concentrations of DOC were inoculated with a log-phase culture of *C. jejuni* F38011 harboring P*ciaB*-pMW10 and P*porA*-pMW10. The β-galactosidase activity was measured at 15 h postinoculation. In contrast to the activity of the *porA* promoter, the activity of the *ciaB* promoter was influenced directly by the concentration of DOC. Specifically, the maximum increase in *ciaB* promoter activity observed when the *C. jejuni* F38011 strain was cultured on an MH agar plate supplemented with 0.2% DOC was twofold (Fig. 4B). Similar to the results of previous experiments, the *ciaB* promoter activity was also found to be maximal at 15 h postinoculation with 0.2% DOC (not shown). These results indicate that DOC stimulates *ciaB* promoter activity in a dose- and time-dependent manner.

Exposure to DOC stimulates the expression of *ciaB*. To identify genes that are differentially expressed in response to DOC, we performed microarray experiments with RNA extracted from *C. jejuni* cultured in the presence and absence of DOC. Real-time RT-PCR was performed using RNA isolated at 3, 6, 9, 12, and 15 h to determine the difference in the *ciaB*

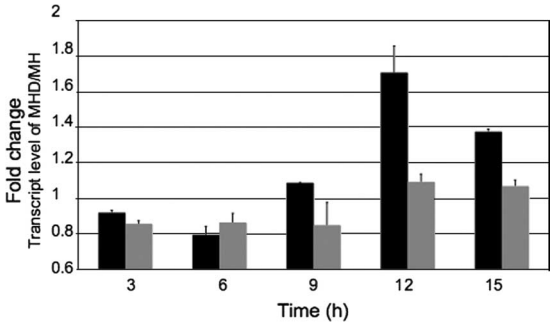

FIG. 5. Temporal expression of *ciaB* and *porA* in *C. jejuni* F38011 cultured in the presence of DOC. Real-time RT-PCR analysis was performed with total RNA extracted from the *C. jejuni* F38011 strain grown on MH and MHD agar plates for 3, 6, 9, 12, and 15 h. The changes in the *ciaB* (black bars) and *porA* (gray bars) transcript levels were measured using the comparative threshold cycle method, and *glyA* was used as the internal control.

transcript levels in *C. jejuni* when it was cultured with DOC. The maximum increase in the *ciaB* transcript level was observed when *C. jejuni* was cultured with 0.1% DOC for 12 h (Fig. 5). Based on this result, we performed microarray experiments with RNA extracted from *C. jejuni* cultured with 0.1% DOC for 12 h to determine if genes were coexpressed with *ciaB*. A total of 202 genes were differentially expressed in response to culture of *C. jejuni* F38011 with 0.1% DOC for 12 h. A total of 156 genes, including *ciaB*, were upregulated ≥1.5-fold (Table 2), while 46 genes were downregulated ≥1.5-fold (Table 3) in the presence of DOC. To confirm the microarray data, genes representing each functional class (Fig. 6) were selected, and real-time RT-PCR was performed (Table 4). The genes found to be upregulated in the presence of DOC by microarray analyses were also found to be upregulated by real-time RT-PCR analyses.

Not surprisingly, the *cmeABC* operon, which encodes an efflux pump that participates in the resistance of *C. jejuni* to the deleterious effects of bile salts (21–24), was upregulated in response to culture with DOC. Moreover, approximately 20% of the genes upregulated in the presence of DOC were genes whose products were associated with the cell envelope of *C. jejuni*. Twelve percent of the genes upregulated in the presence of DOC were involved in synthesis and modification of RNA and DNA molecules. Ten percent of the genes upregulated in the presence of DOC were genes that encode components of transport systems (*ceuE*, *chuA*, *exbB1*, *modB*, and *tonB*). Perhaps most relevant to this study, genes known to play an important role in *C. jejuni* pathogenesis were upregulated (*ciaB*, *dccR*, *flgS*, and *tlyA*). Finally, a large number of genes upregulated in response to DOC were conserved hypothetical protein genes (~20%) and genes with unknown functions (11%). Overall, the results of the microarray experiments indicated that expression of virulence genes in *C. jejuni* is increased in the presence of DOC.

DISCUSSION

Invasion is an important virulence determinant in *C. jejuni* pathogenesis. Newell et al. (30) reported that clinical isolates are more invasive than environmental isolates. Further, Everest et al. (8) observed that *C. jejuni* isolates recovered from individuals with colitis were significantly more invasive than isolates recovered from individuals with noninflammatory diarrhea. Evidence from a number of animal studies also supports the hypothesis that disease development is related to the invasive potential of the *C. jejuni* strain (3, 9, 13, 48, 51). Here we report that culturing *C. jejuni* with the bile acid DOC triggers its invasive potential by stimulating the expression and synthesis of the Cia proteins. Specifically, we monitored the effect of DOC on the expression of *ciaB* and found that the expression was upregulated by three independent assays, the β-galactosidase reporter assay, the real-time RT-PCR assay, and microarrays.

The ability of *C. jejuni* to invade epithelial cells is dependent on several bacterial properties, including motility, adherence, and protein secretion. Thus, the effect of DOC on all three of these virulence factors was investigated. We found that culturing *C. jejuni* in the presence of DOC did not alter the organism's motility or adherence potential. The zones of migration measured for the *C. jejuni* F38011 strain on semisolid MH and MHD agar were comparable. In addition, the abilities of bacteria harvested from MH and MHD agar plates to bind to epithelial cells did not differ; this finding was reproducible regardless of the multiplicity of infection used. However, there was a marked difference in the amount of the Cia proteins secreted by *C. jejuni* when it was cultured in the presence of DOC.

To determine if DOC plays a role in Cia synthesis, the bacteria were labeled with [^{35}S]methionine and incubated in the presence of Cm prior to induction of Cia secretion. Addition of Cm helped distinguish the proteins synthesized in the presence of DOC from the proteins synthesized subsequently in presence of FBS, which was added to stimulate Cia protein secretion. While the Cia proteins were detected in the supernatants of *C. jejuni* harvested from MHD agar plates, we did not detect any secreted proteins in the supernatants of *C. jejuni* harvested from MH agar plates. This finding indicated that presynthesized Cia proteins were present in the bacteria cultured in the presence of DOC.

The Cia proteins are required for maximal invasion of host cells. Thus, bacteria cultured in the presence of DOC are "primed" to invade epithelial cells as they harbor presynthesized Cia proteins. This conclusion is supported by the difference in the invasion kinetics of bacteria harvested from MH and MHD agar plates. While bacteria harvested from MH agar plates require 3 h to achieve maximal invasion, bacteria harvested from MHD agar plates are able to invade INT 407 cells within 15 min. Taken together, the data indicate that culturing *C. jejuni* in the presence of a physiological concentration of DOC results in global changes in gene expression and alteration of the bacterium's phenotype which significantly enhances its invasive potential.

A number of in vitro studies have been performed to better understand how bacteria modulate gene expression when they encounter hostlike conditions and to determine the growth conditions that alter an organism's invasive behavior (4, 11, 14, 37, 47, 49, 50). We hypothesized that we could use the expression of the *ciaB* gene as a marker to better define the kinetics of *cia* induction by DOC and use the data to identify when the other *cia* genes are maximally expressed. Using a *ciaB* promoter-*lacZ* fu-

TABLE 2. Transcripts upregulated in the *C. jejuni* F38011 strain in the presence of 0.1% sodium DOC

Gene	Common name	Change (fold)	Functional classification
Cj0002	*dnaN*	1.6	DNA replication
Cj0008	Cj0008	1.6	Conserved hypothetical proteins
Cj0037c	Cj0037c	1.9	Electron transport
Cj0040	Cj0040	2.9	Unknown
Cj0073c	Cj0073c	1.7	Conserved hypothetical proteins
Cj0080	Cj0080	5.1	Cell envelope (membranes, lipoproteins, and porins)
Cj0100	Cj0100	2.2	Cell division
Cj0114	Cj0114	1.6	Cell envelope (miscellaneous periplasmic proteins)
Cj0155c	*rpmE*	1.5	Ribosomal protein synthesis and modification
Cj0179	*exbB1*	1.6	Transport/binding proteins (others)
Cj0181	*tonB1*	5.1	Transport/binding proteins (others)
Cj0188c	Cj0188c	1.5	Conserved hypothetical proteins
Cj0189c	Cj0189c	1.6	Conserved hypothetical proteins
Cj0190c	Cj0190c	2.2	Miscellaneous
Cj0193c	*tig*	1.9	Chaperones, chaperonins, heat shock
Cj0199c	Cj0199c	7.5	Cell envelope (miscellaneous periplasmic proteins)
Cj0207	*infC*	1.9	Protein translation and modification
Cj0237	*cynT*	1.5	Central intermediary metabolism
Cj0246c	Cj0246c	2.0	Signal transduction
Cj0247c	Cj0247c	3.0	Unknown
Cj0295	Cj0295	2.0	Miscellaneous
Cj0301c	*modB*	1.8	Transport/binding proteins (anions)
Cj0309c	Cj0309c	3.3	Drug sensitivity
Cj0323	Cj0323	1.6	Unknown
Cj0324	*ubiE*	1.8	Biosynthesis of cofactors, prosthetic groups, and carriers
Cj0346	*trpD*	1.7	Amino acid biosynthesis (aromatic amino acid family)
Cj0352	Cj0352	1.6	Cell envelope (membranes, lipoproteins, and porins)
Cj0356c	Cj0356c	2.0	Conserved hypothetical proteins
Cj0365c	*cmeA*	1.9	Antibiotic resistance
Cj0366c	*cmeB*	1.6	Antibiotic resistance
Cj0367c	*cmeC*	2.1	Antibiotic resistance
Cj0381c	*pyrF*	2.1	Pyrimidine biosynthesis
Cj0382c	*nusB*	1.5	RNA synthesis, RNA modification, and DNA transcription
Cj0395c	Cj0395c	2.2	Unknown
Cj0397c	Cj0397c	1.9	Conserved hypothetical proteins
Cj0413	Cj0413	2.8	Cell envelope (miscellaneous periplasmic proteins)
Cj0484	Cj0484	1.9	Transport/binding proteins (others)
Cj0512	*purC*	1.5	Purine biosynthesis
Cj0526c	*fliE*	2.0	Cell envelope (surface structures)
Cj0539	Cj0539	1.7	Unknown
Cj0561c	Cj0561c	3.5	Cell envelope (miscellaneous periplasmic proteins)
Cj0573	Cj0573	3.9	Conserved hypothetical proteins
Cj0579c	Cj0579c	1.6	Conserved hypothetical proteins
Cj0580c	Cj0580c	1.5	Miscellaneous
Cj0588	*tlyA*	1.7	Pathogenicity
Cj0609c	Cj0609c	1.6	Cell envelope (miscellaneous periplasmic proteins)
Cj0618	Cj0618	2.2	Unknown
Cj0659c	Cj0659c	2.4	Cell envelope (miscellaneous periplasmic proteins)
Cj0667	Cj0667	1.6	Conserved hypothetical proteins
Cj0679	*kdpD′*	2.2	Transport/binding proteins (cations)
Cj0683	Cj0683	2.7	Cell envelope (miscellaneous periplasmic proteins)
Cj0690c	Cj0690c	2.1	DNA replication
Cj0692c	Cj0692c	1.5	Cell envelope (membranes, lipoproteins, and porins)
Cj0705	Cj0705	1.8	Conserved hypothetical proteins
Cj0706	Cj0706	1.9	Conserved hypothetical proteins
Cj0707	*kdtA*	1.5	Cell envelope (surface polysaccharides, lipopolysaccharides, and antigens)
Cj0713	*trmD*	1.7	Aminoacyl tRNA synthetases and their modification
Cj0724	Cj0724	1.5	Unknown
Cj0729	Cj0729	2.7	Unknown
Cj0733	Cj0733	1.7	Conserved hypothetical proteins
Cj0734c	*hisJ*	2.5	Transport/binding proteins (amino acids and amines)
Cj0742	Cj0742	1.9	Cell envelope (membranes, lipoproteins, and porins)
Cj0777	Cj0777	1.6	DNA replication
Cj0778	*peb2*	1.9	Cell envelope (miscellaneous periplasmic proteins)
Cj0784	Cj0784	1.5	Cell envelope (miscellaneous periplasmic proteins)
Cj0785	*napD*	2.3	Electron transport
Cj0786	Cj0786	14.0	Unknown

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TABLE 2—*Continued*

Gene	Common name	Change (fold)	Functional classification
Cj0787	Cj0787	1.6	Conserved hypothetical proteins
Cj0788	Cj0788	1.8	Conserved hypothetical proteins
Cj0789	Cj0789	1.5	RNA synthesis, RNA modification, and DNA transcription
Cj0793	*flgS*	1.5	Signal transduction
Cj0798c	*ddlA*	1.7	Cell envelope (murein sacculus and peptidoglycan)
Cj0837c	Cj0837c	2.0	Unknown
Cj0838c	*metS*	1.5	Aminoacyl tRNA synthetases and their modification
Cj0842	Cj0842	2.6	Cell envelope (membranes, lipoproteins, and porins)
Cj0843c	Cj0843c	1.6	Protein degradation
Cj0848c	Cj0848c	3.3	Conserved hypothetical proteins
Cj0852c	Cj0852c	1.7	Cell envelope (membranes, lipoproteins, and porins)
Cj0862c	*pabB*	1.5	Biosynthesis of cofactors, prosthetic groups and carriers
Cj0863c	*xerD*	2.0	DNA replication
Cj0881c	Cj0881c	1.5	Conserved hypothetical proteins
Cj0884	*rpsO*	1.6	Ribosomal protein synthesis and modification
Cj0914c	*ciaB*	1.5	Pathogenicity
Cj0926	Cj0926	1.5	Cell envelope (membranes, lipoproteins, and porins)
Cj0960c	*rnpA*	1.6	RNA synthesis, RNA modification, and DNA transcription
Cj0962	Cj0962	1.5	Miscellaneous
Cj0963	Cj0963	1.7	Conserved hypothetical proteins
Cj0967	Cj0967	1.8	Cell envelope (miscellaneous periplasmic proteins)
Cj0983	Cj0983	1.7	Cell envelope (membranes, lipoproteins, and porins)
Cj0987c	Cj0987c	1.6	Cell envelope (membranes, lipoproteins, and porins)
Cj0989	Cj0989	4.4	Cell envelope (membranes, lipoproteins, and porins)
Cj1006c	Cj1006c	3.0	Conserved hypothetical proteins
Cj1028c	Cj1028c	1.8	Miscellaneous nucleoside/nucleotide reactions
Cj1038	*ftsW*	2.4	Cell division
Cj1053c	Cj1053c	6.3	Cell envelope (membranes, lipoproteins, and porins)
Cj1056c	Cj1056c	1.5	Conserved hypothetical proteins
Cj1070	*rpsF*	1.7	Ribosomal protein synthesis and modification
Cj1071	*ssb*	1.5	DNA replication
Cj1079	Cj1079	2.1	Cell envelope (miscellaneous periplasmic proteins)
Cj1103	*csrA*	2.0	Broad regulatory function
Cj1180c	Cj1180c	2.0	Transport/binding proteins (others)
Cj1181c	*tsf*	1.7	Protein translation and modification
Cj1191c	Cj1191c	1.5	Signal transduction
Cj1201	*metE*	2.3	Amino acid biosynthesis (aspartate family)
Cj1204c	*atpB*	1.7	ATP-proton motive force
Cj1212c	*rbn*	1.8	Aminoacyl tRNA synthetases and their modification
Cj1217c	Cj1217c	2.5	Conserved hypothetical proteins
Cj1223c	Cj1223c	1.5	Signal transduction
Cj1224	Cj1224	5.3	Transport/binding proteins (cations)
Cj1242	Cj1242	5.2	Unknown
Cj1289	Cj1289	1.8	Cell envelope (miscellaneous periplasmic proteins)
Cj1349c	Cj1349c	2.2	Pathogenicity
Cj1355	*ceuE*	1.5	Transport/binding proteins (cations)
Cj1384c	Cj1384c	1.6	Unknown
Cj1385	*katA*	1.6	Detoxification
Cj1388	Cj1388	1.5	Conserved hypothetical proteins
Cj1416c	Cj1416c	1.7	Cell envelope (surface polysaccharides, lipopolysaccharides, and antigens)
Cj1417c	Cj1417c	1.5	Miscellaneous
Cj1418c	Cj1418c	1.8	Miscellaneous
Cj1442c	Cj1442c	1.6	Unknown
Cj1450	Cj1450	1.7	Unknown
Cj1457c	Cj1457c	1.6	Conserved hypothetical proteins
Cj1458c	*thiL*	1.5	Biosynthesis of cofactors, prosthetic groups, and carriers
Cj1463	Cj1463	2.4	Conserved hypothetical proteins
Cj1472c	Cj1472c	2.3	Cell envelope (membranes, lipoproteins, and porins)
Cj1473c	Cj1473c	1.7	Unknown
Cj1475c	Cj1475c	2.2	Unknown
Cj1484c	Cj1484c	1.5	Cell envelope (membranes, lipoproteins, and porins)
Cj1492c	Cj1492c	1.7	Signal transduction
Cj1495c	Cj1495c	1.5	Conserved hypothetical proteins
Cj1530	Cj1530	1.6	Conserved hypothetical proteins
Cj1531	*dapF*	1.8	Amino acid biosynthesis (aspartate family)
Cj1540	Cj1540	1.5	Cell envelope (miscellaneous periplasmic proteins)

Continued on following page

TABLE 2—*Continued*

Gene	Common name	Change (fold)	Functional classification
Cj1547	Cj1547	2.3	Conserved hypothetical proteins
Cj1556	Cj1556	2.8	Conserved hypothetical proteins
Cj1581c	*dppD*	1.8	Transport/binding proteins (others)
Cj1589	Cj1589	2.8	Conserved hypothetical proteins
Cj1603	*hisF*	6.9	Amino acid biosynthesis (histidine)
Cj1611	*rpsT*	1.6	Ribosomal protein synthesis and modification
Cj1614	*chuA*	3.0	Transport/binding proteins (cations)
Cj1621	Cj1621	5.9	Cell envelope (miscellaneous periplasmic proteins)
Cj1640	Cj1640	2.0	Conserved hypothetical proteins
Cj1646	*iamB*	1.9	Transport/binding proteins (others)
Cj1675	*fliQ*	2.4	Cell envelope (surface structures)
Cj1679	Cj1679	1.8	Unknown
Cj1695c	*rplE*	1.5	Ribosomal protein synthesis and modification
Cj1724c	Cj1724c	1.7	Conserved hypothetical proteins
CJE1111	CJE1111	2.7	Miscellaneous
CJE1278	CJE1278	1.5	Miscellaneous
CJE1470	CJE1470	2.5	Miscellaneous
CJE1472	CJE1472	1.7	Miscellaneous
ORF00215	CJE0220	2.0	Miscellaneous
ORF00225	CJE0230	1.5	Miscellaneous
ORF00226	CJE0231	1.9	Miscellaneous
ORF00236	CJE0241	3.4	Miscellaneous
ORF00237	CJE0243	3.8	Miscellaneous

sion construct, we found that DOC was able to induce *ciaB* promoter activity. The *ciaB* promoter activity increased moderately over time; however, higher levels of induction were observed with increasing concentrations of bile. We observed maximal induction of the *ciaB* promoter in the presence of 0.2% DOC at 15 h. It is noteworthy that bile has been reported to have a similar effect on the promoter that drives the expression of the genes encoding the multidrug efflux pump CmeABC (21), which is inducible by bile acids in a dose- and time-dependent manner.

Based on the data from the β-galactosidase assays, we performed a real-time RT-PCR analysis with the *C. jejuni* F38011 strain cultured with DOC for 3, 6, 9, 12, and 15 h to determine when the *ciaB* gene was maximally expressed. The RT-PCR analysis revealed that there was maximal expression of *ciaB* following 12 h of incubation with DOC, results which are complementary to the results of the β-galactosidase assay. Although the temporal expression of the *ciaB* gene with DOC was slower than one might predict, the induction kinetics are similar to the induction kinetics of the *cmeABC* operon in response to bile (21). The kinetics of *ciaB* induction in vivo are not known, nor are the other factors that contribute to *ciaB* expression.

Because the genes coregulated with *ciaB* might code for Cia proteins and other unidentified virulence factors, RNA was extracted from *C. jejuni* cultured with DOC, and microarray experiments were performed to determine the entire transcriptome. DOC differentially regulated a total of 202 genes in the *C. jejuni* F38011 strain, 150 of which were upregulated and 46 of which were downregulated. A number of *C. jejuni* genes predicted to play a role in signal transduction were upregulated by DOC, including *flgS* and *dccR*. The FlgS sensor kinase is a part of the FlgSR two-component system known to play an important role in *C. jejuni* motility (53). The *dccRS* two-component system has been shown to play a role in the in vivo colonization of immunocompetent limited flora (I-LF) mice, severe combined immunodeficient limited flora (SCID-LF) mice, and 1-day-old chicks (25). Taken together, the evidence indicated that DOC acts as a stimulus to trigger a global regulatory response. Whether DOC acts directly or indirectly to induce the signal transduction pathways requires additional study.

Since bile salts are surface-active, amphipathic molecules that act as detergents, exerting their effect primarily on cell membranes (5), it is not surprising that 31 genes encoding cell envelope-associated proteins were significantly upregulated when *C. jejuni* was cultured with DOC. In addition, genes encoding members of the ABC transporter family, including *exbB1* and *modB*, along with genes involved in iron transport, including *chuA* and *ceuE* (42, 43), were upregulated. The gene encoding hemolysin A (TlyA), a member of a family of contact-dependent hemolysins found in *Helicobacter*, *Serpulina*, and *Mycobacterium*, was also found to be upregulated. In *Helicobacter pylori*, TlyA is homologous to a pore-forming cytolysin, and a *tlyA* defined mutant showed reduced in vitro hemolytic activity and reduced adherence to human gastric adenocarcinoma cells and did not colonize the gastric mucosa of mice (27). Also of interest was the finding that culturing *C. jejuni* with DOC resulted in upregulation of a number of conserved hypothetical protein genes and genes with unknown functions. Some of these genes may code for virulence factors, including the other Cia proteins.

It is noteworthy that Fox et al. (10) studied the effect of culturing *C. jejuni* with a concentration of bile that exceeds the concentration normally found in human and chicken intestinal tracts. Using a proteomic approach, these workers found that culturing *C. jejuni* with 2.5% ox bile for 18 h increased the synthesis of GroEL, GalU, and bacterioferritin proteins (10). These proteins are indicative of a bacterial stress response. We did not observe that these genes were upregulated in *C. jejuni* in response to DOC, as judged by the microarray analysis,

TABLE 3. Transcripts downregulated in the *C. jejuni* F38011 strain in the presence of 0.1% sodium DOC

Gene	Common name	Change (fold)	Functional classification
Cj0056c	Cj0056c	1.6	Unknown
Cj0069	Cj0069	1.6	Unknown
Cj0076c	*lctP*	1.8	Transport/binding proteins
Cj0105	*atpA*	1.6	Respiration
Cj0107	*atpD*	1.5	Respiration
Cj0136	*infB*	1.5	Synthesis and modification of macromolecules
Cj0146c	*trxB*	2.1	Biosynthesis of cofactors, prosthetic groups, and carriers
Cj0153c	Cj0153c	1.7	Synthesis and modification of macromolecules
Cj0453	*thiC*	3.5	Biosynthesis of cofactors, prosthetic groups, and carriers
Cj0471	*rpmG*	2.3	Synthesis and modification of macromolecules
Cj0472	*secE*	2.4	Protein and peptide secretion
Cj0473	*nusG*	2.9	Synthesis and modification of macromolecules
Cj0475	*rplA*	1.7	Synthesis and modification of macromolecules
Cj0499	Cj0499	1.6	Conserved hypothetical proteins
Cj0537	*oorB*	1.7	Respiration
Cj0698	*flgG*	1.7	Cell envelope
Cj0720c	*flaC*	2.0	Cell envelope
Cj0855	*folD*	1.5	Biosynthesis of cofactors, prosthetic groups, and carriers
Cj0864	*dsbA*	1.5	Cell envelope
Cj0913c	*hup*	1.8	Synthesis and modification of macromolecules
Cj0982c	Cj0982	1.5	Transport/binding proteins
Cj0998c	Cj0998c	2.2	Cell envelope
Cj1014c	*livF*	1.6	Transport/binding proteins
Cj1015c	*livG*	1.6	Transport/binding proteins
Cj1060c	Cj1060c	1.5	Unknown
Cj1068	Cj1068	1.5	Cell envelope
Cj1163c	Cj1163c	1.7	Transport/binding proteins
Cj1168c	Cj1168c	1.7	Cell envelope
Cj1198	*luxS*	1.7	Conserved hypothetical proteins
Cj1229	*cbpA*	1.5	Chaperones, chaperonins, heat shock
Cj1274c	*pyrH*	1.6	Purines, pyrimidines, nucleosides, and nucleotides
Cj1290c	*accC*	1.5	Fatty acid biosynthesis
Cj1291c	*accB*	1.8	Fatty acid biosynthesis
Cj1293	*flmA*	1.8	Cell envelope
Cj1309c	Cj1309c	1.5	Unknown
Cj1364c	*fumC*	1.5	Energy metabolism
Cj1400c	*fabI*	1.5	Fatty acid biosynthesis
Cj1403c	*gapA*	1.6	Energy metabolism
Cj1502c	*putP*	1.8	Transport/binding proteins
Cj1548c	Cj1548c	1.8	Miscellaneous
Cj1567c	*nuoM*	1.6	Respiration
Cj1628	*exbB2*	1.8	Transport/binding proteins
Cj1658	Cj1658	1.6	Cell envelope
Cj1659	Cj1659	2.0	Cell envelope
Cj1682c	*gltA*	1.7	Energy metabolism
Cj1719c	*leuA*	1.8	Amino acid biosynthesis

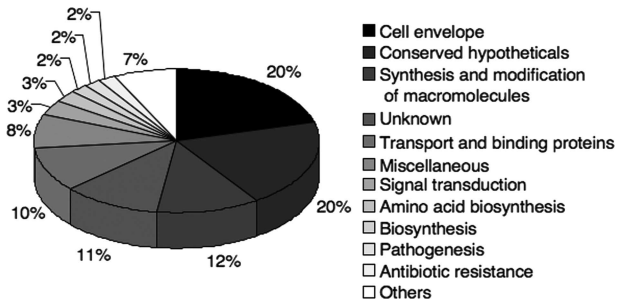

FIG. 6. Functional classification of the *C. jejuni* F38011 genes upregulated ≥1.5-fold in the presence of DOC. The chart shows the percentages of genes belonging to functional classes based on the total number of genes upregulated in the presence of DOC. The percentages are the values for functional categories containing more than 3% of the total number of genes upregulated. Functional categories with two or fewer genes were grouped together as "Others".

indicating that the conditions used in this study did not induce a bacterial stress response.

We show here that the bile acid DOC acts as a signal for *C. jejuni*, triggering its pathogenic behavior, as indicated by its ability to invade epithelial cells. More specifically, we show that culture with DOC "primes" *C. jejuni* to invade epithelial cells by stimulating the synthesis of the Cia proteins. This is a significant finding as it highlights the effect of in vivo-like culture conditions on virulence factors of *C. jejuni*. Harvesting *C. jejuni* from MHD agar plates should help shorten the time required for invasion assays currently performed from 3 h to 15 min. It should also allow researchers to synchronize the infection and to dissect the early events in *Campylobacter* invasion of host cells. Studies are currently being performed to characterize the proteins encoded by the genes whose expression mirrors that of *ciaB*, which encodes a known virulence factor. These genes may code for unidentified virulence factors that

TABLE 4. Real-time RT-PCR analysis of transcripts upregulated in the *C. jejuni* F38011 strain in the presence of 0.1% sodium DOC

Gene	Increase (fold)
Pathogenesis	
ciaB	3.4
tlyA	2.2
Antibiotic resistance	
cmeA	4.0
Amino acid biosynthesis	
dapF	1.7
hisF	2.0
Biosynthesis of cofactors, prosthetic groups, and carriers	
pabB	2.0
thiL	1.7
Cell envelope	
Cj0989	1.9
Cj0561	4.5
Conserved hypothetical proteins	
coaA	3.2
Cj0706	2.9
Signal transduction	
Cj0246	2.5
dccR	1.7
Synthesis and modification of macromolecules	
xerD	1.7
rbn	2.1
Transport/binding proteins	
Cj1224	2.4
tonB1	1.8
Unknown	
Cj0786	3.3
Cj0323	3.8

may play a role in bile resistance and/or invasion of the intestinal tract.

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REFERENCES

1. **Allos, B. M.** 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. Clin. Infect. Dis. **32:**1201–1206.
2. **Altekruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow.** 1999. *Campylobacter jejuni*—an emerging foodborne pathogen. Emerg. Infect. Dis. **5:**28–35.
3. **Babakhani, F. K., G. A. Bradley, and L. A. Joens.** 1993. Newborn piglet model for campylobacteriosis. Infect. Immun. **61:**3466–3475.
4. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
5. **Begley, M., C. G. Gahan, and C. Hill.** 2005. The interaction between bacteria and bile. FEMS Microbiol. Rev. **29:**625–651.
6. **Crushell, E., S. Harty, F. Sharif, and B. Bourke.** 2004. Enteric campylobacter: purging its secrets? Pediatr. Res. **55:**3–12.
7. **Elliot, W. H.** 1985. Metabolism of bile acids in liver and extrahepatic tissues, p. 3003–3329. *In* H. Danielsson and J. Sjovall (ed.), Sterols and bile acids. Elsevier Science Publishers, Amsterdam, The Netherlands.
8. **Everest, P. H., H. Goossens, J. P. Butzler, D. Lloyd, S. Knutton, 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.
9. **Field, L. H., V. L. Headley, J. L. Underwood, S. M. Payne, and L. J. Berry.** 1986. The chicken embryo as a model for campylobacter invasion: comparative virulence of human isolates of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. **54:**118–125.
10. **Fox, E. M., M. Raftery, A. Goodchild, and G. L. Mendz.** 2007. *Campylobacter jejuni* response to ox-bile stress. FEMS Immunol. Med. Microbiol. **49:**165–172.
11. **Galan, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879–1885.
12. **Gupta, S., and R. Chowdhury.** 1997. Bile affects production of virulence factors and motility of *Vibrio cholerae*. Infect. Immun. **65:**1131–1134.
13. **Humphrey, C. D., D. M. Montag, and F. E. Pittman.** 1985. Experimental infection of hamsters with *Campylobacter jejuni*. J. Infect. Dis. **151:**485–493.
14. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
15. **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.
16. **Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis.** 1999. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. Mol. Microbiol. **32:**691–701.
17. **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.
18. **Konkel, M. E., D. J. Mead, and W. Cieplak, Jr.** 1993. Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. J. Infect. Dis. **168:**948–954.
19. **Konkel, M. E., M. R. Monteville, V. Rivera-Amill, and L. A. Joens.** 2001. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. Curr. Issues Intest. Microbiol. **2:**55–71.
20. **Lin, J., M. Akiba, O. Sahin, and Q. Zhang.** 2005. CmeR functions as a transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. Antimicrob. Agents Chemother. **49:**1067–1075.
21. **Lin, J., C. Cagliero, B. Guo, Y. W. Barton, M. C. Maurel, S. Payot, and Q. Zhang.** 2005. Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. J. Bacteriol. **187:**7417–7424.
22. **Lin, J., and A. Martinez.** 2006. Effect of efflux pump inhibitors on bile resistance and *in vivo* colonization of *Campylobacter jejuni*. J. Antimicrob. Chemother. **58:**966–972.
23. **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.
24. **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.
25. **MacKichan, J. K., E. C. Gaynor, C. Chang, S. Cawthraw, D. G. Newell, J. F. Miller, and S. Falkow.** 2004. The *Campylobacter jejuni dccRS* two-component system is required for optimal *in vivo* colonization but is dispensable for *in vitro* growth. Mol. Microbiol. **54:**1269–1286.
26. **Malik-Kale, P., B. H. Raphael, C. T. Parker, L. A. Joens, J. D. Klena, B. Quinones, A. M. Keech, and M. E. Konkel.** 2007. Characterization of genetically matched isolates of *Campylobacter jejuni* reveals that mutations in genes involved in flagellar biosynthesis alter the organism's virulence potential. Appl. Environ. Microbiol. **73:**3123–3136.
27. **Martino, M. C., R. A. Stabler, Z. W. Zhang, M. J. Farthing, B. W. Wren, and N. Dorrell.** 2001. *Helicobacter pylori* pore-forming cytolysin orthologue TlyA possesses in vitro hemolytic activity and has a role in colonization of the gastric mucosa. Infect. Immun. **69:**1697–1703.
28. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. **Monteville, M. R., and M. E. Konkel.** 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. Infect. Immun. **70:**6665–6671.
30. **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.
31. **Olive, A. J., R. Kenjale, M. Espina, D. S. Moore, W. L. Picking, and W. D. Picking.** 2007. Bile salts stimulate recruitment of IpaB to the *Shigella flexneri* surface, where it colocalizes with IpaD at the tip of the type III secretion needle. Infect. Immun. **75:**2626–2629.
32. **Osawa, R., E. Arakawa, T. Okitsu, S. Yamai, and H. Watanabe.** 2002. Levels

of thermostable direct hemolysin produced by *Vibrio parahaemolyticus* O3:K6 and other serovars grown anaerobically with the presence of a bile acid. Curr. Microbiol. **44:**302–305.
33. **Osawa, R., and S. Yamai.** 1996. Production of thermostable direct hemolysin by *Vibrio parahaemolyticus* enhanced by conjugated bile acids. Appl. Environ. Microbiol. **62:**3023–3025.
34. **Pace, J. L., T. J. Chai, H. A. Rossi, and X. Jiang.** 1997. Effect of bile on *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **63:**2372–2377.
35. **Parker, C. T., B. Quinones, W. G. Miller, S. T. Horn, and R. E. Mandrell.** 2006. Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. J. Clin. Microbiol. **44:**4125–4135.
36. **Pope, L. M., K. E. Reed, and S. M. Payne.** 1995. Increased protein secretion and adherence to HeLa cells by *Shigella* spp. following growth in the presence of bile salts. Infect. Immun. **63:**3642–3648.
37. **Porter, M. E., and C. J. Dorman.** 1994. A role for H-NS in the thermo-osmotic regulation of virulence gene expression in *Shigella flexneri*. J. Bacteriol. **176:**4187–4191.
38. **Prouty, A. M., I. E. Brodsky, S. Falkow, and J. S. Gunn.** 2004. Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. Microbiology **150:**775–783.
39. **Prouty, A. M., I. E. Brodsky, J. Manos, R. Belas, S. Falkow, and J. S. Gunn.** 2004. Transcriptional regulation of *Salmonella enterica* serovar Typhimurium genes by bile. FEMS Immunol. Med. Microbiol. **41:**177–185.
40. **Prouty, A. M., and J. S. Gunn.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infect. Immun. **68:** 6763–6769.
41. **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.
42. **Richardson, P. T., and S. F. Park.** 1995. Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system. Microbiology **141:**3181–3191.
43. **Ridley, K. A., J. D. Rock, Y. Li, and J. M. Ketley.** 2006. Heme utilization in *Campylobacter jejuni*. J. Bacteriol. **188:**7862–7875.
44. **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.
45. **Rivera-Amill, V., and M. E. Konkel.** 1999. Secretion of *Campylobacter jejuni* Cia proteins is contact dependent. Adv. Exp. Med. Biol. **473:**225–229.
46. **Schuhmacher, D. A., and K. E. Klose.** 1999. Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae*. J. Bacteriol. **181:**1508–1514.
47. **Sen, A., M. A. Leon, and S. Palchaudhuri.** 1991. Environmental signals induce major changes in virulence of *Shigella* spp. FEMS Microbiol. Lett. **68:**231–236.
48. **Sosula, L., E. M. Nicholls, and M. Skeen.** 1988. Ultrastructure of *Campylobacter jejuni* in gamma-irradiated mouse jejunum. Am. J. Pathol. **131:**125–131.
49. **Tobe, T., S. Nagai, N. Okada, B. Adler, M. Yoshikawa, and C. Sasakawa.** 1991. Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. Mol. Microbiol. **5:**887–893.
50. **Tobe, T., M. Yoshikawa, and C. Sasakawa.** 1994. Deregulation of temperature-dependent transcription of the invasion regulatory gene, virB, in *Shigella* by *rho* mutation. Mol. Microbiol. **12:**267–276.
51. **Welkos, S. L.** 1984. Experimental gastroenteritis in newly-hatched chicks infected with *Campylobacter jejuni*. J. Med. Microbiol. **18:**233–248.
52. **Wosten, M. M., M. Boeve, M. G. Koot, A. C. van Nuene, and B. A. van der Zeijst.** 1998. Identification of *Campylobacter jejuni* promoter sequences. J. Bacteriol. **180:**594–599.
53. **Wosten, M. 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.