

## Role of the DksA-Like Protein in the Pathogenesis and Diverse Metabolic Activity of *Campylobacter jejuni*<sup>∇</sup>

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Received 22 January 2008/Accepted 22 April 2008

**DksA is well known for its regulatory role in the transcription of rRNA and genes involved in amino acid synthesis in many bacteria. DksA has also been reported to control expression of virulence genes in pathogenic bacteria. Here, we elucidated the roles of a DksA-like protein (CJJ81176\_0160, Cj0125c) in the pathogenesis of *Campylobacter jejuni*. As in other bacteria, transcription of stable rRNA was repressed by the DksA-like protein under stress conditions in *C. jejuni*. Transcriptomic and proteomic analyses of *C. jejuni* 81-176 and an isogenic mutant lacking the DksA-like protein showed differential expression of many genes involved in amino acid metabolism, iron-related metabolism, and other metabolic reactions. Also, the *C. jejuni* DksA-like protein mutant exhibited a decreased ability to invade intestinal cells and induce release of interleukin-8 from intestinal cells. These results suggest that the DksA-like protein plays an important regulatory role in diverse metabolic events and the virulence of *C. jejuni*.**

When exposed to nutrient depletion, many bacteria show global changes in gene expression called the stringent response (15, 38). The effector molecule of the stringent response is guanosine tetraphosphate (ppGpp). Upon amino acid starvation, the abundance of uncharged tRNA results in ribosome stalling, activating ribosome-associated RelA or SpoT and producing ppGpp (15, 38). ppGpp binds to the  $\beta$  and  $\beta'$  subunits of the RNA polymerase (RNAP) core enzyme and affects transcription of genes primarily at the stage of transcription initiation during the formation of the open promoter complex (4, 15, 38, 47). In many bacteria, ppGpp-dependent regulation affects bacterial physiology, including the functionality of some sigma factors and the virulence of pathogenic bacteria (15, 18, 58). The best-known types of regulation by ppGpp include negative regulation of rRNA transcription and positive regulation of amino acid biosynthesis, in which ppGpp potentiates direct activation or repression in concert with an RNAP-associated protein designated DksA (6, 7, 20, 48).

DksA was originally identified in *Escherichia coli* as a multicopy suppressor of the temperature-sensitive phenotype of *dnaKJ* mutants in 1990 (30). Analysis of bacterial genomes revealed the ubiquitous distribution of *dksA* open reading frames, which encode highly conserved amino acid sequences (50). DksA is reported to have pleiotropic effects, such as defects in chaperonin function, gene expression, cell division, amino acid biosynthesis, phage sensitivity, and quorum sensing (9, 13, 14, 27, 30, 40, 66). The involvement of DksA in bacterial virulence has also been reported for many pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Shigella flexneri*, and enterohemorrhagic *E.*

*coli* (29, 41, 43, 56, 65). Unlike most transcriptional regulators, DksA does not bind directly to DNA but binds to the secondary channel of RNAP (47, 50). *E. coli* DksA is composed of a globular domain and a coiled coil with two highly conserved aspartate (Asp) residues at its tip (50). DksA binds directly to RNAP, positioning the Asp residues near the active site to coordinate a ppGpp-bound Mg<sup>2+</sup> ion with the Asp residues, thereby stabilizing the ppGpp-RNAP complex (50). In many papers, DksA has been reported to play a role as a cofactor of ppGpp regulation (50). However, Magnusson and colleagues recently demonstrated that DksA regulates gene expression without ppGpp, suggesting that DksA may have another mechanism of gene regulation which is independent of ppGpp (39).

*Campylobacter jejuni* is a microaerophilic, spiral-shaped, gram-negative bacterium belonging to the epsilon group of the *Proteobacteria*. *C. jejuni* is a major causative agent of human bacterial gastroenteritis throughout the world (17). *C. jejuni* is a commensal gastrointestinal bacterium in birds, and consumption of undercooked poultry meat may result in campylobacteriosis ranging from mild diarrhea to bloody enteritis (61). *Campylobacter* infection is also associated with Guillain-Barré syndrome, a form of neuromuscular paralysis (57). However, how *C. jejuni* causes campylobacteriosis or how *C. jejuni* can survive in hostile environments is not clearly understood. Factors known to be associated with the virulence of *C. jejuni* include motility, colonization, adhesion to intestinal cells, invasion of intestinal cells, N-linked protein glycosylation, and production of cytolethal distending toxin (33, 60, 64). Like many other bacteria, *C. jejuni* mounts a SpoT (Cj1272c)-mediated stringent response upon nutrient deprivation (18). A *C. jejuni* mutant with *spoT* deleted showed defects in aerotolerance, rifampin resistance, stationary-phase survival, and adherence to, invasion of, and survival in epithelial intestinal cells (18). The stringent response mediated by SpoT in *C. jejuni* is considered to be important for the survival and virulence of this bacterium, because *C. jejuni* lacks the stationary-phase

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<sup>∇</sup> Published ahead of print on 2 May 2008.

sigma factor *rpoS* (46), which is essential for stress and stationary-phase responses in many gram-negative bacteria (26). Since *C. jejuni* is able to use ppGpp as its stringent signal, DksA is expected to play a regulatory role in concert with ppGpp and to affect the physiology and virulence of *C. jejuni*. To examine this possibility, we characterized a DksA-like protein in *C. jejuni*. It was found that the DksA-like protein in *C. jejuni* modulates gene expression and affects the virulence, as well as the physiology, of this bacterium.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, cell lines, and growth conditions.** *C. jejuni* 81-176 (11), a pathogenic strain, and derivatives of this strain were used in this study. *E. coli* DH5 $\alpha$  was used as a cloning host. *C. jejuni* 81-176 and an isogenic mutant lacking the DksA-like protein (FMB1104) were cultured using Mueller-Hinton (MH) broth or agar at 37°C under microaerobic conditions generated with MART (Anoxomat). Mannitol-free morpholinepropanesulfonic acid (MOPS)-MGS medium (50 mM MOPS [pH 7.4], 2 mM MgSO<sub>4</sub>, 0.25 mM CaCl<sub>2</sub>, 19 mM glutamic acid, 0.004 mM biotin) was used as a minimal medium for *C. jejuni* to induce the stringent response (18). A human intestinal epithelial cell line, INT-407, was used for the invasion assay and was grown in minimal essential medium supplemented with 10% fetal bovine serum (MEM-FBS) containing penicillin (100 U/ml) and streptomycin (50  $\mu$ g/ml) in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air.

**DNA manipulations.** All DNA manipulations, including cloning, transformation into *E. coli*, isolation of DNA from agarose gels, PCRs, and DNA sequencing, were performed by using standard techniques (55) and the manufacturers' instructions. The oligonucleotide primers used in PCRs are described elsewhere (<http://plaza.snu.ac.kr/~fmb/main/pu-01.htm>). DNA sequencing was performed using a BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI Prism 3700 DNA analyzer (Perkin-Elmer) at the National Instrumentation Center for Environmental Management (Seoul, Korea).

**Construction of a mutant with *cat* inserted in CJJ81176\_0160.** *C. jejuni* 81-176 was used as a parental strain. The chloramphenicol resistance cassette (*cat*) was amplified from pRY112 (69) with Vent polymerase (New England Biolabs) using primers catF and catR. To construct the mutant with a mutation in the DksA-like protein (CJJ81176\_0160), a DNA fragment containing CJJ81176\_0160 and a flanking region was amplified using primers Cj0125cF1 and Cj0125cR1, digested with PstI and EcoRI, and then ligated into pUC19 digested with the same enzymes. The resultant plasmid was digested with SspI, and then the *cat* cassette was inserted into the SspI site. The orientation of the *cat* cassette was confirmed by sequencing. The plasmid with the *cat* cassette inserted in the same orientation as CJJ81176\_0160 was designated pFMB1104, which was electroporated into *C. jejuni* 81-176. The mutant with *cat* inserted into CJJ81176\_0160 was screened by growing it on MH agar plates containing chloramphenicol (12.5  $\mu$ g/ml). The mutant was confirmed by PCR and designated *C. jejuni* FMB1104.

**RNA manipulation.** For reverse transcription (RT)-PCR, total RNA was isolated from *C. jejuni* cultures using an RNeasy mini kit (Qiagen). After DNase treatment of the total RNA solution using a Turbo DNA-free kit (Ambion), cDNA was synthesized using an Omniscript RT kit (Qiagen) and random hexamers (Invitrogen) according to the manufacturers' instructions. A negative control for RT-PCR was prepared by omitting reverse transcriptase at the cDNA synthesis step. For RNA quantification, total RNA was prepared from 5 ml of each *C. jejuni* culture using Trizol reagent (Invitrogen) according to the manufacturer's instructions. We changed the RNA purification method to obtain total RNA, because the other RNA preparation method using columns resulted in partial removal of rRNA. Then the RNA concentration was determined by measuring the optical densities at 260 and 280 nm of the RNA solution using GeneQuant *pro* (Amersham Pharmacia Biotech).

**Proteomic analyses. (i) Protein preparation and two-dimensional gel electrophoresis (2DGE).** The wild type and the mutant were grown to mid-log phase in 50 ml MH broth in Erlenmeyer flasks (500 ml) at 37°C with rotation at 130 rpm for 6 h. Protein was prepared from the wild type and the mutant as described by Yoon et al. (72). Protein concentrations were determined by the Bradford assay (12). For first-dimension separation, 400 or 900  $\mu$ g of protein from each sample was separated by isoelectric focusing using 13-cm IPG strips (GE Healthcare). For the second-dimension separation, proteins were separated based on molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10 or 12% acrylamide gels. Gels were visualized by colloidal Coomassie blue staining (Invitrogen).

**(ii) Image analysis and protein identification.** Quantitative data were obtained from multiple gel runs using ImageMaster 2D Platinum version 6.0 (GE Healthcare). A twofold difference in expression was used as a threshold during image analysis to detect proteins that were differentially expressed. Protein spots showing different levels were excised from the stained gels, which was followed by in-gel digestion using the method of Jensen et al. (28). Peptide mass fingerprinting was done with a Voyager-DETM STR biospectrometry workstation (PerSeptive Biosystems, Framingham, MA), and proteins were identified by peptide mass fingerprinting with MASCOT (<http://www.matrixscience.com>) supplemented with the option for *Campylobacter* in the NCBI database.

**Transcriptomic analyses. (i) Preparation of total RNA for a DNA microarray.** Total RNA was extracted from six independent cultures of the wild type and the mutant, which were cultured under the same conditions that were used for 2DGE. *C. jejuni* strains were inoculated into MH broth to an optical density at 600 nm of 0.05. Bacterial cultures were grown at 37°C with shaking at 130 rpm for 6 h. Total RNA was isolated using RNeasy bacterial reagent (Qiagen) and an RNeasy kit (Qiagen) according to the manufacturer's protocol with optional on-column DNase treatment with RNase-free DNase (Qiagen). The RNA concentration was determined with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE).

**(ii) cDNA synthesis and microarray hybridization.** cDNA was synthesized using aminoallyl deoxynucleoside triphosphates with the ChipShot indirect cDNA labeling system (Promega) according to the manufacturer's instructions. After purification, aminoallyl-labeled cDNA was fluorescently labeled with a CyDye postlabeling reactive pack (Amersham Bioscience, Piscataway, NJ). After purification, the fluorescent labeling of the cDNA was quantified, and equal amounts of labeled cDNA of the wild type and the mutant were mixed and dried in a speed vacuum concentrator. After resuspension with hybridization buffer provided by the slide manufacturer, the mixture was denatured by heating at 95°C for 3 min. The hybridization mixture was loaded onto a microarray slide (OciChip; Ocimum Biosolutions, Indianapolis, IN) and hybridized in a shaking incubator at 42°C for 24 h. The slides were washed using the manufacturer's recommendations and dried by centrifugation. In total, six slides were hybridized using RNA extracted from six independent cultures. For three slides, the wild-type strain was labeled with Cy3 and the mutant strain was labeled with Cy5. For the remaining three slides, the dye-sample combination was reversed to control for differences in dye labeling of the cDNA samples. Further control for dye bias was achieved by adjusting the volume of cDNA hybridized based on the actual concentration of Cy dye incorporated as measured by a NanoDrop ND-1000 (NanoDrop Technologies).

**(iii) Image acquisition and analysis.** The data analysis was performed essentially as described by Madsen et al. (37). Using a ScanArray Express laser scanner (Applied Biosystems Inc., Foster City, CA), each dye channel of each slide was scanned at 10- $\mu$ m resolution with three predetermined laser power settings (high, medium, and low) to prevent saturation of high signals while ensuring adequate sensitivity for genes with low signals. ImaGene software (BioDiscovery, El Segundo, CA) was used to measure the spot-specific mean signal strength and the spot-specific background median of each spot. Using the R statistical package (version 2.0.1; The R Foundation for Statistical Computing), the spot-specific background median was subtracted from the spot-specific signal mean to obtain a background-subtracted value for each spot. The natural logarithm of each background-subtracted value was obtained, and median centering was performed as previously described (37). The median of the individual spot signals for each gene obtained at different power settings was determined and used for all further calculations. Data obtained from the two dye channels were subjected to locally weighted scatterplot smoother (LOWESS) normalization (68) to balance the fluorescence intensities of the two dyes (Cy3 and Cy5) used to label cDNA. This normalization process was used to rescale the fluorescence intensity of each transcript, preventing the dye bias problem (16, 35).

**(iv) Statistical analysis.** The normalized data generated for individual probes of each array by dye combination were subjected to an independent mixed linear model using the SAS statistical software package (67). The model included fixed effects for treatment (wild type versus mutant) and dye (Cy3 versus Cy5) in addition to the random effect for slides. A *t* test was performed using the null hypothesis that there was no difference in the signal intensities of the treatment groups. The resulting *P* values for each of the probes were sorted in ascending order and converted to a *Q* value by the method of Storey and Tibshirani (59). *Q* values are used to estimate a false discovery rate for a subset of gene targets. Complete results of these experiments are available at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE9866.

**Quantitative real-time RT-PCR (qRT-PCR).** Total RNA was isolated from *C. jejuni* cultures using an RNeasy mini kit (Qiagen). After DNase treatment of the total RNA solution with a Turbo DNA-free kit (Ambion), cDNA was synthe-

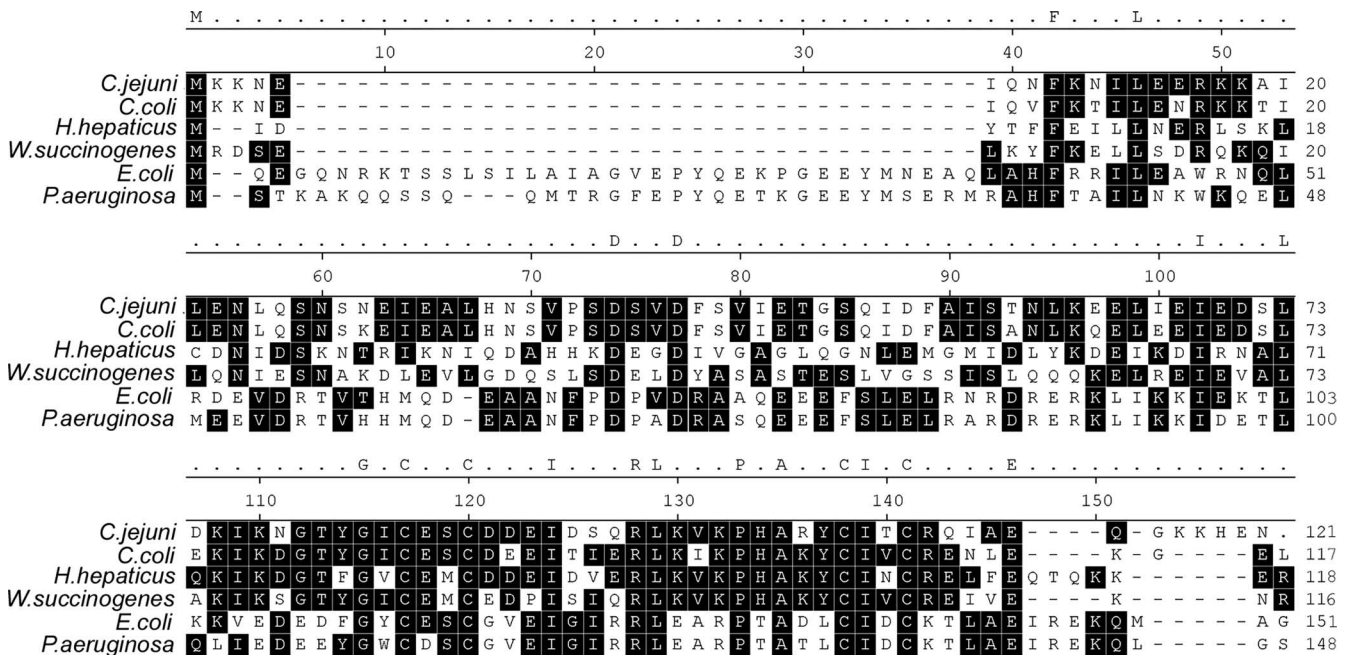


FIG. 1. Amino acid sequence alignment of DksA homologs. The DksA-like protein of *C. jejuni* (CJJ81176\_0160, YP\_999849.1) was aligned with DksA homologs of *Campylobacter coli* RM2228 (ZP\_00367836.1), *H. hepaticus* ATCC 51449 (NP\_860767.1), *W. succinogenes* DSM 1740 (NP\_906418.1) *E. coli* K-12 (NP\_414687.1), and *P. aeruginosa* PAO1 (NP\_253411.1). A black background indicates dominant amino acid residues in the aligned sequences. Two Asp residues that are known to be critical for fixing the position of the ppGpp bound to Mg<sup>2+</sup> (54) are indicated by asterisks. Four Cys residues that are required for a canonical C<sub>4</sub> Zn finger motif (54) are indicated by plus signs.

sized using an Omniscript RT kit (Qiagen) and random hexamers (Invitrogen). Quantification of cDNA was carried out using IQ SYBR green PCR Supermix (Bio-Rad), and real-time amplification of PCR products was analyzed using the iCycler optical module (Bio-Rad). The amplification program consisted of one cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The mRNA expression levels for target genes were normalized to the 16S rRNA gene (*rrsA*) expression level. The relative amount of cDNA was calculated using the 2<sup>-ΔΔC<sub>t</sub></sup> method (36). The primers used for real-time PCR are described elsewhere (<http://plaza.snu.ac.kr/~fmb/main/pu-01.htm>).

**Invasion assay.** Approximately 1 × 10<sup>5</sup> cells of INT-407 were seeded in each well of a 24-well plate and grown to semiconfluence in antibiotic-free MEM-FBS for 16 h. Then each strain of *C. jejuni* in exponential phase was added at a multiplicity of infection of 100. The plates were centrifuged at 200 × g for 5 min to maximize bacterium-host cell contact and were incubated for 4 h at 37°C in the presence of 5% CO<sub>2</sub>. After three washes with phosphate-buffered saline, the monolayers were incubated in MEM-FBS containing gentamicin (100 μg/ml) for 2 h to kill extracellular bacteria. After three additional washes with phosphate-buffered saline, infected epithelial cells were lysed with 1% Triton X-100 for 5 min. Intracellular bacteria were enumerated by plating serial dilutions on MH agar plates.

**Interleukin-8 secretion assay.** As in the invasion assay, approximately 1 × 10<sup>5</sup> INT-407 cells were infected with *C. jejuni* at a multiplicity of infection of 100. The plates were centrifuged at 200 × g for 5 min to maximize bacterium-host cell contact and incubated for 4 h at 37°C in the presence of 5% CO<sub>2</sub>. The culture medium was harvested and centrifuged at 15,000 × g for 10 min to pellet the residual bacteria. The supernatant was collected and stored at -80°C until analysis. The secreted interleukin-8 (IL-8) levels were determined using a BD OptEIA human IL-8 enzyme-linked immunosorbent assay II kit (BD Biosciences).

**Ferrous iron uptake assay.** The wild type and the mutant were grown to mid-log phase under microaerobic conditions in MEM<sub>α</sub> (Invitrogen) at 37°C with rotation at 130 rpm for 6 h. The uptake of ferrous iron into *C. jejuni* was measured using <sup>55</sup>FeCl<sub>3</sub> (Perkin-Elmer) as described by Naikare et al. (42). The activity, expressed in cpm, was determined using a Wallac 1400 liquid scintillation counter (Turku, Finland), and the values were converted to picomoles of <sup>55</sup>Fe using a previously determined standard curve.

**Other computer programs.** Sequence manipulation and sequence alignment were performed with DNASTAR software (DNASTAR Inc.). Primer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) was used to design primers

used in qRT-PCR. GraphPad Prism 4 (GraphPad Inc.) was used to plot the charts.

RESULTS AND DISCUSSION

**Identification of a DksA homolog in *C. jejuni*.** Analysis of the genomic sequence revealed that *Campylobacter* possesses DksA-like proteins, which are designated CJJ81176\_0160 and Cj0125c in *C. jejuni* 81-176 and *C. jejuni* NCTC 11168 (46), respectively. All the *C. jejuni* strains whose genome sequences were available by March 2008 also possess DksA-like proteins (data not shown). The DksA-like protein is composed of 120 amino acids with a calculated molecular mass of 13.5 kDa. A BLASTP search showed that CJJ81176\_160 (Cj0125c) was similar to *E. coli* DksA (26% identity and 53% similarity). CJJ81176\_160 showed high levels of identity (ca. 63 to 89%) to the DksA-like proteins of bacteria belonging to the epsilon group of the *Proteobacteria*, such as other species of *Campylobacter*, *Helicobacter hepaticus*, and *Wolinella succinogenes* (2). However, *Helicobacter pylori* does not have a DksA homolog (1, 61). The DksA-like protein of *C. jejuni* contains conserved sequences of DksA proteins (Fig. 1).

In *C. jejuni* 81-176, CJJ81176\_0160 is located in a cluster containing seven genes, CJJ81176\_0164 to *dusB* (Fig. 2A). There are four genes upstream of CJJ81176\_0160 and two genes downstream. Among the six flanking genes, only *accD* (CJJ81176\_0162) and *dusB* (CJJ81176\_0158) are annotated, as genes encoding acetyl-coenzyme A carboxylase and tRNA dihydrouridine synthase B. The other genes encode proteins with unknown functions. RT-PCR generated an RT-PCR product spanning two ad-

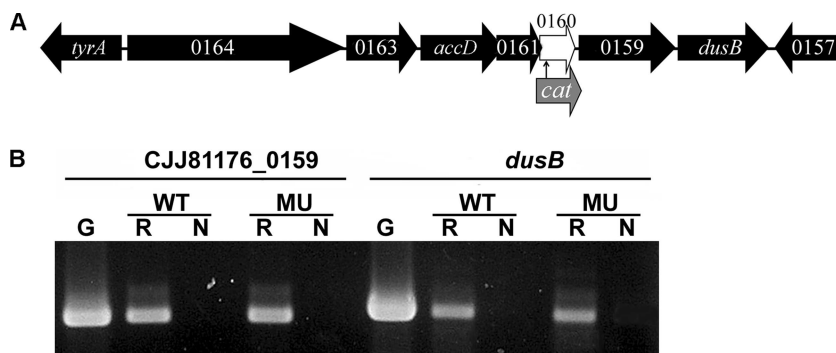


FIG. 2. Operon structure of CJJ81176\_0160 and flanking genes. (A) Genomic structure of CJJ81176\_0160 and flanking genes and site of nonpolar insertion of a *cat* cassette. (B) RT-PCR results obtained with mRNA isolated from the wild type (WT) and the mutant (FMB1104) (MU), confirming the transcription of CJJ81176\_0159 and *dusB*, which are downstream of CJJ81176\_0160. G, PCR performed with genomic DNA of the wild type; R, PCR performed with reverse transcriptase and *C. jejuni* mRNA; N, PCR performed without reverse transcriptase but with *C. jejuni* mRNA (negative control).

adjacent open reading frames with all seven genes in the cluster (data not shown), suggesting that CJJ81176\_0160 belongs to an operon composed of CJJ81176\_0164 to *dusB* (CJJ81176\_0158).

To define the functions of the DksA-like protein (CJJ81176\_0160), we disrupted CJJ81176\_0160 in *C. jejuni* 81-176. To minimize the polar effect of a *cat* insertion, the *cat* gene was cloned in the same orientation as CJJ81176\_0160, which was confirmed by sequencing. RT-PCR demonstrated that the transcription of CJJ81176\_0159 and *dusB* (CJJ81176\_0158) was not affected (Fig. 2B). Also, qRT-PCR revealed that the levels of expression of CJJ81176\_0161, CJJ81176\_0159, and CJJ81176\_0158 in *C. jejuni* FMB1104 are approximately 102, 74, and 82% of those in wild-type *C. jejuni*, respectively (data were not shown). Therefore, the insertion in CJJ81176\_0160 did not have a polar effect on the downstream genes. In addition, the mutant did not show growth defects in rich medium (MH medium) compared with the wild type in 24 h (data not shown).

The best-known regulatory mechanism of DksA is regulation of the transcription of stable RNA, such as rRNA in *E. coli* (47, 53). In concert with ppGpp, DksA regulates rRNA transcription negatively by reducing the open complex lifetime and enhancing the effect of ppGpp (47). The effect of DksA on rRNA transcription has also been demonstrated in other bacteria, such as *P. aeruginosa* and *S. flexneri* (51, 56). Cessation of stable RNA synthesis results in a net decrease in the total RNA, because more than 90% of the total RNA is stable RNA. Total RNA was prepared from *C. jejuni* 81-176 and FMB1104 grown to the mid-log phase, and then the cultures were either maintained in rich medium (MH broth) or shifted to minimal medium (MOPS-MGS medium). The nutrient downshift from rich medium to minimal medium was reported previously to induce stringent responses by producing ppGpp (18). Viability (CFU) and RNA levels were monitored for 3 h under microaerobic conditions. As expected, neither strain of *C. jejuni* grew in the minimal medium, but there was no loss of viability for 3 h (data not shown). The total amount of RNA in the mutant ( $282.1 \pm 85.9$  fg/CFU) was similar to that in the wild type ( $244.1 \pm 58.2$  fg/CFU) in rich medium ( $P > 0.05$ ). Following the nutrient downshift, the total amount of RNA was greatly decreased in the wild type ( $158.2 \pm 33.7$  fg/CFU), whereas the total amount of RNA was only slightly reduced in

the mutant ( $241.4 \pm 22.9$  fg/CFU), which resulted in a 1.4-fold-higher level of total RNA in the mutant ( $P < 0.05$ ). These results support the hypothesis that the DksA-like protein functions as a negative regulator of stable RNA transcription under stringent conditions, which is consistent with the findings for other bacteria (47, 51).

**Mutation of the DksA-like protein affected the expression of multiple genes in *C. jejuni*.** The DksA-like protein was expected to be a transcriptional regulator in *C. jejuni*, as it is in other bacteria. Transcriptomic profiles of the wild type (*C. jejuni* 81-176) and the isogenic mutant lacking the DksA-like protein were compared by using a DNA microarray. Since a microarray for *C. jejuni* 81-176 was not commercially available, we used a commercial oligomer chip (OciChip; Ocimum Biosolutions, Indianapolis, IN) that was designed based on the genome sequence of *C. jejuni* NCTC 11168. Thus, the genetic differences between *C. jejuni* NCTC 11168 and *C. jejuni* 81-176 had to be taken into consideration when the results were interpreted, because the 38 genes unique to the *C. jejuni* 81-176 chromosome and the genes of two plasmids in *C. jejuni* 81-176, pVir and pTet, were not present in the microarray slide (5, 10, 24, 44). A total of 51 of 1,633 genes on the array were determined to be differentially regulated by more than 1.5-fold ( $P < 0.05$ ) based on the results of six microarray slides. Twenty-one genes were down-regulated, and 30 genes were up-regulated in the mutant. These genes are described elsewhere (<http://plaza.snu.ac.kr/~fmb/main/pu-01.htm>).

Profiles of protein expression for the wild type and the mutant were compared by using 2DGE. As shown in Fig. 3, many protein spots that showed differences in expression between the wild type and the mutant were detected, and protein spots with more-than-twofold differences were selected and identified by matrix-assisted laser desorption ionization–time of flight analyses after digestion with trypsin. Fourteen proteins were down-regulated in the mutant, whereas nine proteins were up-regulated in the mutant. The identified proteins are indicated in Fig. 3 and are described elsewhere (<http://plaza.snu.ac.kr/~fmb/main/pu-01.htm>). The proteins showing differential expression in the wild type and the mutant identified by 2DGE did not agree with the results obtained from the transcriptome analyses. Correlations between DNA microar-

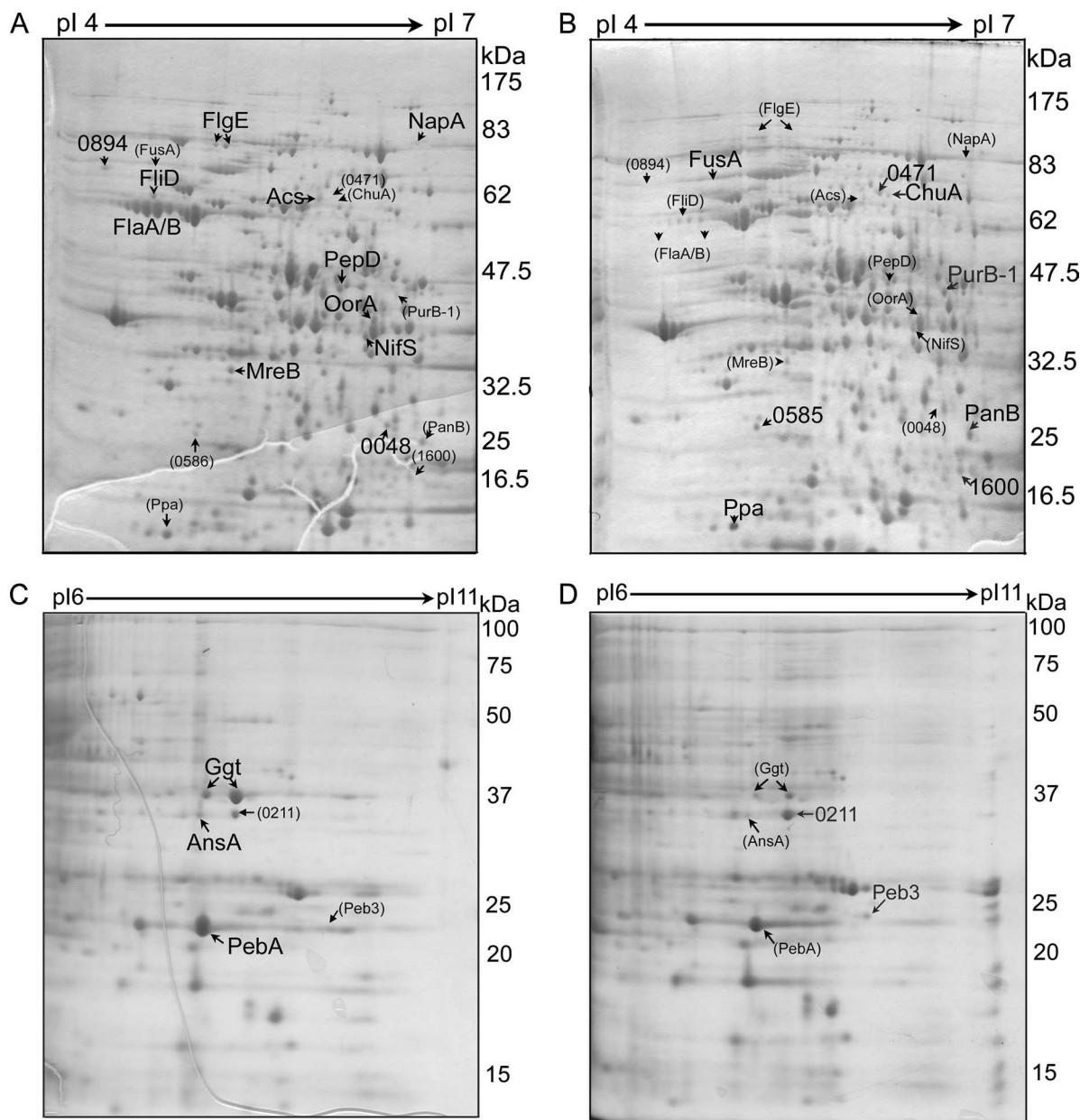


FIG. 3. 2DGE images of whole-cell lysates from the wild type (A and C) and the mutant lacking the DksA-like protein (B and D). Proteins were separated by 2DGE using two ranges, pH 4 to 7 (A and B) and pH 6 to 11 (C and D). Protein spots with increased expression (more than twofold greater) in one gel are indicated by large letters, while the corresponding spots in the other gel are indicated by small letters and numbers in parentheses. A full list of the identified proteins is given elsewhere.

ray and proteomic results are known to be highly variable, and inconsistency in findings between the two methods has been reported in many studies (16, 21). The discrepancy between the transcriptomic and proteomic data is ascribed to the intrinsic differences between these two methods and the biological differences between the transcription and translation processes, such as posttranscriptional regulation, posttranslational modification, and proteolysis (16, 21). Despite the discrepancy, genes or proteins involved in amino acid metabolism and iron-related metabolism were determined to be expressed differently in the wild type and the mutant by both methods. These genes and proteins are categorized and discussed below.

**The DksA-like protein is involved in amino acid metabolism in *C. jejuni*.** The expression of several genes involved in amino acid synthesis and transport was affected by mutation of the DksA-like protein in *C. jejuni*, which is consistent with findings for other bacteria (43, 52). The results of 2DGE demonstrated that there were reductions in the expression of Ggt ( $\gamma$ -glutamyltransferase [GGT]), PebA (periplasmic ABC transporter of amino acids), AnsA (cytoplasmic L-asparaginase), PepD (aminoacyl-histidine dipeptidase), and CjJ81176\_0265 (Cj0240c, cysteine desulfurase). Also, the DNA microarray results revealed that the transcriptional levels of the *tpABDEF* (tryptophan synthesis) operon, *pepA* (leucyl amino transferase), and Cj1258 (CjJ81176\_1274, possible phosphotyrosine pro-

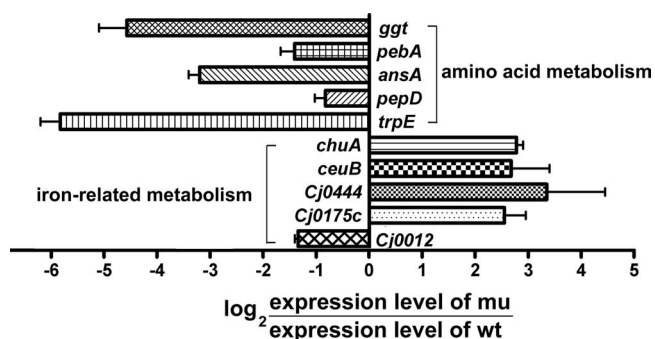


FIG. 4. Confirmation of expression levels by qRT-PCR. The transcription levels of genes that exhibited differential expression in the wild type (wt) and the mutant lacking the DksA-like protein (mu) as determined by transcriptomic or proteomic analyses were confirmed by qRT-PCR. Negative values indicate down-regulation in the mutant, while positive values indicate up-regulation in the mutant.

tein phosphatase) were reduced in the mutant. The down-regulation of these genes was confirmed by real-time PCR (Fig. 4).

Some of these genes have previously been reported to be involved in bacterial virulence. The GGT (EC.2.3.2.2) catalyzes transpeptidation and hydrolysis of the  $\gamma$ -glutamyl group of glutathione and related compounds and hence is known to be involved in glutathione metabolism in eukaryotes and prokaryotes (74). GGT is not present in *C. jejuni* NCTC 11168 but is present in *C. jejuni* 81-176 (24). Barnes et al. recently reported that the *ggt* gene is present in approximately 19.4% of *C. jejuni* isolates (8). Inactivation of *ggt* in *C. jejuni* reduced colonization in mice (24) and chickens (8). *C. jejuni* GGT also influenced the invasiveness of this bacterium and is involved in apoptosis of colon epithelial cells (8). Mutation of the DksA-like protein in *C. jejuni* also reduced the expression of *PebA* (or *Peb1A* as annotated in *C. jejuni* NCTC 11168). *PebA* is reported to be involved in amino acid transport as a component of an ABC transporter of aspartate and glutamate (34). *PebA* was originally identified as cell-binding factor 1, and inactivation of this protein in *C. jejuni* reduces interactions with epithelial cells and intestinal colonization of mice (49). Although other genes have not been functionally characterized in terms of amino acid metabolism, the multiple changes caused by mutation of the DksA-like protein strongly suggest

that the DksA-like protein influences amino acid utilization and metabolism in *C. jejuni*.

**The DksA-like protein is involved in iron-related metabolism in *C. jejuni*.** Like other bacteria, *Campylobacter* has an absolute requirement for iron as a nutrient. However, iron also participates in the formation of reactive oxygen species, and bacteria must maintain intracellular iron homeostasis to cope with oxidative stress (62). To achieve successful iron homeostasis, many bacteria have a Fur (ferric uptake regulator) system regulating ferric ion transport proteins (3). Fur with its corepressor Fe<sup>2+</sup> binds to a consensus sequence in the promoters of Fur-regulated genes. Thus, the transcription of Fur-regulated genes is repressed when the Fe<sup>2+</sup> concentration is high. *C. jejuni* possesses several mechanisms to obtain iron from the environment, although there is diversity in the iron uptake system among *C. jejuni* strains (24). *C. jejuni* 81-176 contains genes encoding *FeoAB* for ferrous iron uptake, *ChuABCD* for heme/hemoglobin uptake, *CeuBCDE* for enterochelin transport, a putative iron uptake transport system (*Cfbp*, CJJ81176\_0209 to CJJ81176\_0211), and a TonB-dependent siderophore receptor (CJJ81176\_0471) (24). Expression of all of these genes except *feoAB* is dependent on Fur (25, 45, 54). In *C. jejuni*, there is another Fur-like regulator, *PerR* (63). *PerR* is known to form a *PerR-Fe<sup>2+</sup>* complex like Fur and to act as a repressor of genes involved in oxidative stress responses (63).

Transcriptomic and proteomic analyses showed that mutation of the DksA-like protein in *C. jejuni* caused changes in the expression levels of several genes involved in iron-related metabolism. Proteomic analysis showed that the expression of iron transporter proteins, including *ChuA*, CJJ81176\_0211 (*Cj0175c*, ferric iron ABC transporter periplasmic iron-binding protein), and CJJ81176\_0471 (*Cj0444*, putative TonB-dependent iron uptake protein) was higher in the mutant than in the wild type. *ChuZ* (heme oxygenase, CJJ81176\_1600 or *Cj0163c*) also was up-regulated in the mutant and was reported to be a Fur regulon (54). Transcriptomic analysis also demonstrated that there was up-regulation of genes associated with iron uptake (*Cj0175c* and *Cj0173c*) and *chuZ* and down-regulation of *Cj0012c* (*rbr*) encoding a nonheme iron protein, which was reported to be activated upon iron addition (25, 45). Changes in the transcriptional levels of these iron-related genes were also confirmed by qRT-PCR (Fig. 4 and 5A). The expression

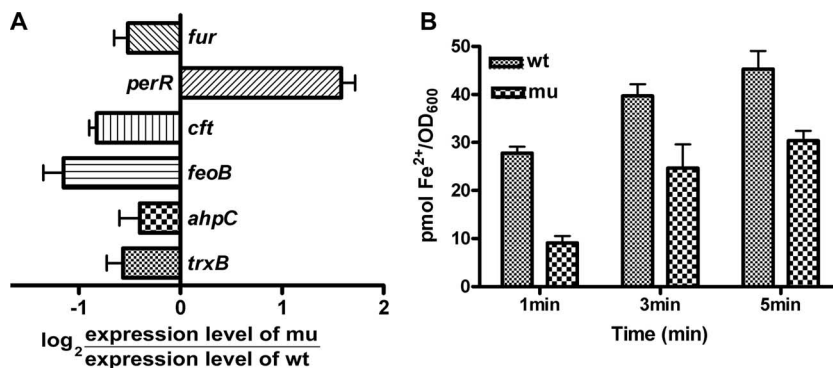


FIG. 5. Involvement of the DksA-like protein in iron-related metabolism of *C. jejuni*. (A) Comparison of transcription levels in the wild type (wt) and the mutant lacking the DksA-like protein (mu) for iron metabolism-related genes other than the Fur regulons. (B) Ferrous iron uptake was reduced in the mutant compared with the wild type. OD<sub>600</sub>, optical density at 600 nm.

levels of other proteins that may be important for iron-related metabolism were also investigated by qRT-PCR (Fig. 5A). The expression of *fur* was reduced approximately 50% in the mutant compared to the wild type. Interestingly, the expression of *perR* was increased in *C. jejuni* FMB1104, unlike the expression of *fur* (Fig. 5A). The expression levels of *perR*-regulated genes, such as *ahpC* and *trxB*, were reduced, which is consistent with the finding that *perR* expression was increased by the mutation (Fig. 5A).

The expression levels of a ferrous iron uptake gene (*feoB*) and the gene encoding ferritin (*cft*), an iron storage protein, were also down-regulated in the mutant. The down-regulation of *feoB* transcription in the mutant was verified by a ferrous iron uptake assay, in which less ferrous iron was taken up by the mutant with the DksA-like mutant protein than by the wild type (Fig. 5B). Based on all the findings, we concluded that mutation of the DksA-like protein causes down-regulation of *feoAB*, which results in a reduction in ferrous iron uptake and a concomitant decrease in the concentration of the Fur-Fe<sup>2+</sup> complex. Therefore, Fur regulons are up-regulated in the mutant.

There has been only one report concerning the relationship between DksA and iron-related metabolism, and this report indicated that the transcription of *fur* in *S. flexneri* was reduced twofold in the absence of *dksA* (56). The relationship between iron-related metabolism and DksA in other bacteria has not been reported. Also, there have been no reports about ppGpp-dependent iron metabolism, except for one report for *Geobacter sulfurreducens*, which showed that genome-wide expression profiling of a ppGpp-negative mutant (*rel<sub>Gsu</sub>* deletion mutant) of *G. sulfurreducens* identified genes that possess Fur-regulated motifs (32). The ppGpp-negative strain of *C. jejuni* (the *spoT* mutant) did not show any changes in iron-related metabolism in *C. jejuni* (18). Thus, the DksA regulation of iron-related genes is likely mediated in a ppGpp-independent manner. However, it is not certain whether the regulation of iron-related metabolism by the DksA-like protein in *C. jejuni* results directly from the regulation by the DksA-like protein or indirectly from other changes in the mutant, such as changes in amino acid metabolism.

#### Other metabolic activities affected by the DksA-like protein.

Other genes exhibiting differential expression in the wild type and the mutant included genes involved in flagellar synthesis, N-linked protein glycosylation, energy metabolism, and small-molecule metabolism.

Proteomic analysis of mutations in the DksA-like protein also demonstrated that some flagellar genes were down-regulated in the mutant. These genes encode flagellar hook protein (FlgE), flagellar capping protein (FliD), flagellin family protein (CJJ81176\_0894, FlaD), and flagellin (CJJ81176\_1338 and CJJ81176\_1339, FlaAB); however, the results of the DNA microarray analysis did not show any noticeable changes in flagellar genes. Also, some of the genes, such as *wlaGH* (*pglAC*), which are involved in N-linked protein glycosylation (60), showed up-regulation in *C. jejuni* FMB1104. Of the 30 putative glycoproteins (73), 5 (Cj0175c, PEB3, Cj0420, FusA, and Ppa) were up-regulated in *C. jejuni* FMB1104 according to the results of 2DGE and microarray analyses.

Some genes involved in energy metabolism showed differential expression in the wild type and *C. jejuni* FMB1104. In

the mutant, putative cytochrome *c* genes (Cj0037c and Cj1163), *napA* (periplasmic nitrate reductase), and *oorA* (2-oxoglutarate ferredoxin oxidoreductase) were down-regulated, whereas the *nuoD* operon (NADH dehydrogenase) was up-regulated. Genes that are involved in small-molecule metabolism were also influenced by the mutation in the DksA-like protein. In the mutant, *acs* (acetyl-coenzyme A synthetase) was down-regulated, whereas *panB* (3-methyl-2-oxobutanoate hydroxymethyltransferase), *ppa* (inorganic pyrophosphatase), and *purB* (adenylosuccinate lyase) were up-regulated.

**Invasion and IL-8 secretion were impaired by mutation of the DksA-like protein.** *C. jejuni* 81-176 is known to be a highly invasive strain among campylobacters. Human intestinal INT-407 cells were infected with *C. jejuni* 81-176 or the isogenic mutant lacking the DksA-like protein, and the total numbers of bacteria that invaded the INT-407 cells were determined. For the wild type the invasion level was 0.22% ± 0.03% of the input bacteria, while for the mutant the invasion level was 0.0018% ± 0.0006% of the input bacteria, which represents a 120-fold reduction in invasiveness. The differences were statistically significant ( $P < 0.001$ ). Previous studies of the DksA protein of *S. flexneri* showed that DksA was involved in the intercellular spread of this bacterium but not in invasion (41).

The invasion of epithelial cells by *C. jejuni* is associated with its virulence in an animal model (71). Many factors have been reported to affect the invasiveness of *C. jejuni*. Motility is one factor associated with the invasion of host cells by *C. jejuni* (31, 70). Other factors affecting the invasiveness of *C. jejuni* include FlaC, Cia proteins, FspA, and Cj0977, and all of these proteins except Cj0977 require a flagellar export apparatus for secretion (19, 31, 52, 58). This is associated with the finding that mutation in the DksA-like protein of *C. jejuni* resulted in down-regulation of some flagellar genes at the protein level. In addition, the *spoT* gene of *C. jejuni* was up-regulated during bacterial infection of the INT-407 cells, and the *spoT* mutant of *C. jejuni* was defective in invasion and intracellular survival (18), suggesting that the stringent response of *C. jejuni* is associated with the invasiveness of this bacterium. Based on all the findings, the defect in invasiveness caused by mutation of the DksA-like protein in *C. jejuni* is likely associated with the stringent response.

*Campylobacter* enterocolitis is typically associated with a local acute inflammatory response, inducing inflammatory cytokine responses. IL-8 is a proinflammatory cytokine and a mediator of localized inflammatory responses. It has been reported that the invasiveness of *C. jejuni* is associated with IL-8 secretion (22). To determine if a mutation in the DksA-like protein influences IL-8 secretion, INT-407 cells were infected with *C. jejuni* 81-176 and FMB1104, and IL-8 secretion into the culture medium was determined. The level of IL-8 secretion induced by the wild type was 83.54 ± 7.61 pg/ml after 4 h of infection, while the level of IL-8 secretion induced by the mutant was 54.88 ± 3.91 pg/ml, which is approximately 65% of the wild-type value. The difference in IL-8 secretion was statistically significant ( $P < 0.05$ ). The reduced secretion of IL-8 was likely due to the significant decrease in the invasiveness of the mutant. However, the reduction was modest considering the remarkable invasion defect, probably because the transcription of cytolethal distending toxin genes, another factor

that affects IL-8 secretion (23), was not influenced by mutation of the DksA-like protein (data not shown).

**Concluding remarks.** The DksA-like protein of *C. jejuni* was involved in expression of genes for amino acid metabolism, iron-related metabolism, and other metabolic activities. In addition, mutation of the DksA-like protein in *C. jejuni* resulted in reductions in invasiveness and IL-8 secretion. The results of this study suggest that the DksA-like protein plays a regulatory role in *C. jejuni*. The mechanism of these changes was not explained; this mechanism may be dependent on or independent of the stringent response and warrants further investigation.

#### ACKNOWLEDGMENTS

We thank M. Blaser for providing *C. jejuni* 81-176 and P. Guerry for providing pRY112.

This work was supported by the Korea Research Foundation (grant KRF-2006-005-J04702) and by a National Research Initiative competitive grant (2007-35201-18278) from the USDA Cooperative State Research, Education, and Extension Service. J. Yun was a recipient of a graduate fellowship provided by the Ministry of Education through the Brain Korea 21 Project.

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