JOURNAL OF BACTERIOLOGY, Oct. 2007, p. 7399–7407 Vol. 189, No. 20
0021-9193/07/$08.00+0 doi:10.1128/JB.01012-07

Cj0011c, a Periplasmic Single- and Double-Stranded DNA-Binding Protein, Contributes to Natural Transformation in *Campylobacter jejuni*[▽]

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Received 26 June 2007/Accepted 6 August 2007

Campylobacter jejuni **is an important bacterial pathogen causing gastroenteritis in humans. *C. jejuni* is capable of natural transformation, which is considered a major mechanism mediating horizontal gene transfer and generating genetic diversity. Despite recent efforts to elucidate the transformation mechanisms of *C. jejuni*, the process of DNA binding and uptake in this organism is still not well understood. In this study, we report a previously unrecognized DNA-binding protein (Cj0011c) in *C. jejuni* that contributes to natural transformation. Cj0011c is a small protein (79 amino acids) with a partial sequence homology to the C-terminal region of ComEA in *Bacillus subtilis*. Cj0011c bound to both single- and double-stranded DNA. The DNA-binding activity of Cj0011c was demonstrated with a variety of DNAs prepared from *C. jejuni* or *Escherichia coli*, suggesting that the DNA binding of Cj0011c is not sequence dependent. Deletion of the cj0011c gene from *C. jejuni* resulted in 10- to 50-fold reductions in the natural transformation frequency. Different from the *B. subtilis* ComEA, which is an integral membrane protein, Cj0011c is localized in the periplasmic space of *C. jejuni*. These results indicate that Cj0011c functions as a periplasmic DNA receptor contributing to the natural transformation of *C. jejuni*.**

Natural transformation is a process by which bacteria take up exogenous DNA under natural growth conditions and is considered an important mechanism for horizontal gene transfer among bacterial organisms (16). The molecular mechanisms of natural transformation have been documented in some bacteria, such as *Bacillus subtilis*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* (7). The process of natural transformation necessitates the function of multiple competence proteins involved in DNA binding, uptake, and recombination. As an early step in the transformation process, DNA binding occurs via DNA receptor proteins and, possibly, other bacterial surface structures (7, 16). In *B. subtilis*, an integral membrane protein named ComEA is identified as a DNA receptor and is essential for the natural competence of this bacterium (24). In *N. gonorrhoeae*, the ComE protein (a ComEA ortholog) has been recognized as a DNA receptor involved in natural transformation and is predicted to be a periplasmic protein, but its cellular location has not been experimentally determined (8).

Campylobacter jejuni is a gram-negative bacterium and a significant cause of food-borne diseases in humans in industrialized countries (20). More than two million cases of human campylobacteriosis occur each year in the United States (34). Recently, the increased resistance of *Campylobacter* to antibiotics, especially fluoroquinolones and macrolides, has become a major public health concern (18, 21). One of the striking characteristics of *C. jejuni* is its enormous population diversity, reflected by both genotypic and phenotypic variability among different strains/isolates (15, 19, 23, 37, 40). Although *C. jejuni* may have multiple means for the exchange of genetic materials that potentially encode antibiotic resistance or virulence factors, natural transformation is considered to be a major mechanism mediating horizontal genetic transfer among individual organisms or different strains in *Campylobacter* (13, 26, 51).

C. jejuni is naturally competent for DNA uptake, with a high selectivity for *Campylobacter* DNA (48). The natural competence of *C. jejuni* varies among different strains (49), is affected by growth phase (highest in the early log phase) (48), and is influenced by the CO_2 concentration in liquid culture, with higher transformation frequencies in a low (0.7%) CO_2 atmosphere than in a high (10%) CO_2 atmosphere (51). Several genes in *C. jejuni* have been identified as factors involved in natural competence. An early study reported that natural transformation in *C. jejuni* depends on *recA* (22). A recent work using transposon mutagenesis identified 11 genes contributing to natural transformation in *C. jejuni*, and several of them encode products involved in type II secretion and the biogenesis of type IV pili (50). The VirB10 protein encoded by a gene carried on a virulent plasmid in strain 81-176 also contributes to natural transformation in *C. jejuni* (2, 3). A recent study further showed that VirB10 is glycosylated, and mutagenesis of the N-linked protein glycosylation system significantly reduced the natural transformation of *C. jejuni* (29).

Despite the recent efforts in understanding the genetic basis of natural transformation in *C. jejuni*, the detailed mechanism involved in DNA uptake by *Campylobacter* is still unclear. Particularly, the molecular basis of DNA binding, a key event in natural transformation, has not been well characterized in *C. jejuni*. Here we report the identification of a previously unrecognized DNA-binding competence protein, Cj0011c, in *C. jejuni*. We demonstrate that Cj0011c is localized in the periplasm, binds to both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), and contributes to natural transformation in *C. jejuni*.

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▽ Published ahead of print on 10 August 2007.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* NCTC 11168 was used in this study. Bacteria were grown at 42°C on Mueller-Hinton (MH) agar plates (Difco) under microaerobic conditions (85% N_2, 5% O_2, and 10% CO_2). The mutant strain (Δcj0011c::*aphA3*) was cultured on MH agar plates supplemented with kanamycin at a concentration of 50 μg ml^{-1}. For the strains harboring the pRY112 plasmid (54) and its derivative, chloramphenicol (10 μg ml^{-1}) was added to MH agar plates.

Cloning and purification of recombinant Cj0011c (rCj0011c). rCj0011c was produced in *Escherichia coli* JM109 (Promega, Madison, WI) with the pQE-30 vector (QIAGEN, Valencia, CA). The cj0011c gene was PCR amplified using primers 11pQE-F (5′-TTCTCGGATCCGCTGTAA ATATCAACACTGCAAC AC-3′; restriction site is underlined) and 11pQE-R (5′-GGCAAAACTGCAGT TTTATTCTAT TGTGATATC-3′), which were designed to amplify the cj0011c gene without the N-terminal signal peptide (17 residues). After digestion with BamHI and PstI, the PCR product was cloned into pQE-30, which had been digested with the same enzymes. The rCj0011c was purified under native conditions according to the protocol supplied by the manufacturer (QIAGEN). Rabbit polyclonal antisera against rCj0011c were prepared by Pacific Immunology Corp. (Ramona, CA) using the purified rCj0011c.

dsDNA-binding assays. Southwestern blotting was performed as described previously (43), with some modifications. rCj0011c was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.2-μm polyvinylidene difluoride (PVDF) membrane (Immun-Blot; Bio-Rad, Hercules, CA). The membrane was then blocked and soaked in the renaturation buffer (50 mM NaCl, 10 mM Tris-hydrochloride, pH 7.4, 1 mM EDTA, 5% low-fat dry milk) at room temperature for 4 h and then incubated overnight in the binding buffer (50 mM NaCl, 10 mM Tris-hydrochloride, pH 7.4, 1 mM EDTA) containing the DNA probe, which was labeled at the 3′ end with digoxigenin-11–ddUTP (DIG-11–ddUTP) by using a DIG oligonucleotide 3′-end labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Two DNA probes, including a 274-bp PCR product of the upstream region of *mobA* (*cj1350*) and a 170-bp internal fragment of *cmeA* (31), were used in the binding assays. These two probes were chosen because they have different sequences and are readily available in our laboratory. The *mobA* probe was amplified from *C. jejuni* NCTC 11168 using primers 5′-GTATAAATCGGATCCATTGCACGAGTAAGA-3′ and 5′-CCATACGTCTAGATTTACCACCACATAAAA-3′. The PCR product was purified from the agarose gel with a gel purification kit (QIAGEN) before being labeled with DIG. The membrane was washed three times for 30 min in the binding buffer. DIG-labeled DNA was detected and visualized by using alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals). Southwestern dot blotting was done by transferring rCj0011c to a PVDF membrane using a vacuum blotter. The blots were incubated with the same DIG-labeled DNA probe used for Southwestern blotting and visualized as described above.

Polyacrylamide gel retardation assays were performed using the same DIG-labeled PCR products described above. The DIG-11–ddUTP-labeled DNA (0.2 pmol) was incubated with purified rCj0011c in 20 μl of binding buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 5 mM dithiothreitol, 0.2% Tween 20, and 30 mM KCl. For the competition assay, poly(dI-dC) (Amersham Biosciences, Piscataway, NJ) was added to the reaction mixture. The reaction mixtures were incubated at room temperature for 15 min and then subjected to electrophoresis on a nondenaturing 6% (wt/vol) polyacrylamide gel in 0.25× TBE (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA [pH 8.0]) at 200 V for 45 min. The DNA in the gel was transferred to a nylon membrane with a vacuum blotter. DIG-labeled DNA was detected and visualized as described above.

Agarose gel retardation assays were performed using several different plasmids, including pUC19 (Invitrogen, Carlsbad, CA), pQE30, pET-20b(+) (Novagen, San Diego, CA), pRSET-mCherry (45), pMW10 (52), pWM1007 (36), and pRY112 (54). pRY112, an *E. coli*-*C. jejuni* shuttle plasmid, was purified from *E. coli* DH5α or *C. jejuni* NCTC 11168. Other plasmids were prepared from *E. coli*. Plasmid DNA (300 ng) was incubated with rCj0011c in binding buffer for 15 min at room temperature. Then, the mixture was separated by an 0.8% agarose gel and the DNA on the gel was visualized by ethidium bromide staining.

ssDNA-binding assays. The agarose gel retardation assay was performed by mixing 100 ng of the positive-strand DNA of bacteriophage M13mp18 (Sigma, St. Louis, MO) with various amounts of rCj0011c in binding buffer for 15 min at room temperature. Samples were run in an 0.8% agarose gel and visualized by ethidium bromide staining. Polyacrylamide gel retardation assays were performed using 8% (wt/vol) nondenaturing polyacrylamide gel as described above. A 33-mer oligonucleotide (5′-GCAACAAACAAACAAGATATTGCCACTAG TAAA-3′) which was arbitrarily chosen from the sequence of *cj1211* (41) was labeled with a DIG oligonucleotide 3′-end labeling kit (Roche Molecular Biochemicals). The DIG-labeled oligonucleotide was boiled for 5 min and rapidly cooled down on ice before use.

Preparation of cell fractions and measurement of cytochrome *c* oxidoreductase activity. Cell fractions were prepared with a PeriPreps Periplasting kit (Epicenter, Madison, WI). *C. jejuni* cultures were microaerobically grown in 200 ml of MH broth overnight. Cells were collected by centrifugation at 6,000 × *g* for 10 min and were resuspended in 200 μl of PeriPreps Periplasting buffer (200 mM Tris-HCl, pH 7.5, 20% sucrose, 1 mM EDTA, 30 U/ml ready-lyse lysozyme) supplemented with 30 μg ml^{-1} of DNase I. The bacterial suspension was incubated for 5 min at room temperature. An amount of 200 μl of cold water was added to the suspension and mixed by inversion. After being incubated on ice for 10 min, the lysed cells were pelleted by centrifugation for 2 min at 16,000 × *g*. The supernatant contained the periplasmic fraction. The pellet was resuspended in 2 ml of sterilized distilled water and sonicated with a sonicator (Virsonic 600; VirTis). The sonicated suspension was centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant was collected and then ultracentrifuged at 100,000 × *g* for 100 min at 4°C. The resultant supernatant contained the cytoplasmic fraction, and the pellet was the membrane fraction. The membrane pellet was washed seven times and resuspended in 10 mM HEPES buffer (pH 7.4). The protein concentration of each fraction was determined using bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL). The sulfite/cytochrome *c* oxidoreductase (SOR) activity, which is a periplasmic indicator, was measured for each fraction as described by Myers and Kelly (39). Briefly, the enzymatic reaction was initiated by adding sodium sulfite to a final concentration of 2.5 mM to the reaction mixture containing 900 μl 10 mM Tris-HCl (pH 8), 100 μl horse heart cytochrome *c* (10 mg ml^{-1}), and 10 to 30 μl cell fraction. The increase in absorbance at 550 nm was measured by using a spectrophotometer (SmartSpec 3000; Bio-Rad).

Proteinase K treatment. *C. jejuni* whole cells were treated with proteinase K as described previously (25). Overnight *C. jejuni* cultures were collected, washed with distilled water, and resuspended in distilled water at a concentration of 5 × 10^8 cells ml^{-1}. Aliquots (100 μl) of the bacterial suspension were treated with different concentrations of proteinase K at 37°C for 15 min. After the proteinase K treatment, cells were pelleted and subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. Protein samples were boiled for 5 min in the SDS-PAGE sample buffer and centrifuged at 10,000 × *g* for 1 min to remove undissolved proteins. The samples were fractionated by SDS-PAGE on a 12.5% polyacrylamide gel in Tris-Tricine buffer and blotted to PVDF membrane (Immun-blot; Bio-Rad). The membrane was incubated in blocking buffer (phosphate-buffered saline containing 5% skim milk and 0.05% Tween 20) for 1 h and was probed with the primary antibody (rabbit anti-rCj0011c; 1:500 dilution) for 1 h in the blocking buffer. After three washings with the washing buffer (phosphate-buffered saline containing 0.05% Tween 20), the membrane was incubated with the secondary antibody (1:1,000 dilution of peroxidase-labeled goat anti-rabbit immunoglobulin G; Kirkegaard & Perry Laboratories). After three washings, the blots were developed with a 4 CN membrane peroxidase substrate system (Kirkegaard & Perry Laboratories).

Primer extension assay. A primer extension assay was used to determine the transcriptional start site of cj0011c using a 5′-FAM (6-carboxyfluorescein)-labeled primer (5′-CTTCTTTGCGGTATTCTAAAATCGCTTTA-3′) as described elsewhere (33, 44). Briefly, the FAM-labeled primer (final concentration, 10 nM) was ethanol precipitated with 30 μg of total bacterial RNA that was purified from *C. jejuni* 11168 using TRIzol reagent (Invitrogen). The pellet was resuspended in 20 μl of 250 mM KCl, 2 mM Tris (pH 7.9), and 0.2 mM EDTA. The mixture was heated to 57°C and then allowed to cool down to room temperature for 1 h. After annealing, 50 μl of the reaction solution containing 5 μg of actinomycin D, 700 μM deoxynucleoside triphosphates, 10 mM $MgCl_2$, 5 mM dithiothreitol, 20 mM Tris (pH 8.3), and 100 U of SuperScript III reverse transcriptase (Invitrogen) was added. The mixture was incubated at 50°C for 70 min and treated with RNaseA (QIAGEN). The cDNA was precipitated and then washed with 70% ethanol. The size of the FAM-labeled cDNA was analyzed with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Construction of a deletion mutant of cj0011c. A 226-bp region was deleted from cj0011c and replaced with the kanamycin resistance gene (*aphA3*). For this purpose, a 659-bp region upstream of cj0011c was PCR amplified with primer pair 11UpX_F (5′-GAAATGTATCTCTAGAGATTTAACTGCAGTAA-3′) and 11UpB_R (5′-CGTTAAAGCAAAAAATAAAAATAGTAATTGGATCC TTTTCTT-3′). Also, a 756-bp region downstream of cj0011c was prepared by PCR using primers 11DownK_F (5′-CAAAAATGGTACCACACAATAGA ATAA AGGGCATT-3′) and 11DownE_R (5′-CAAACCACTCATAAAGAAT

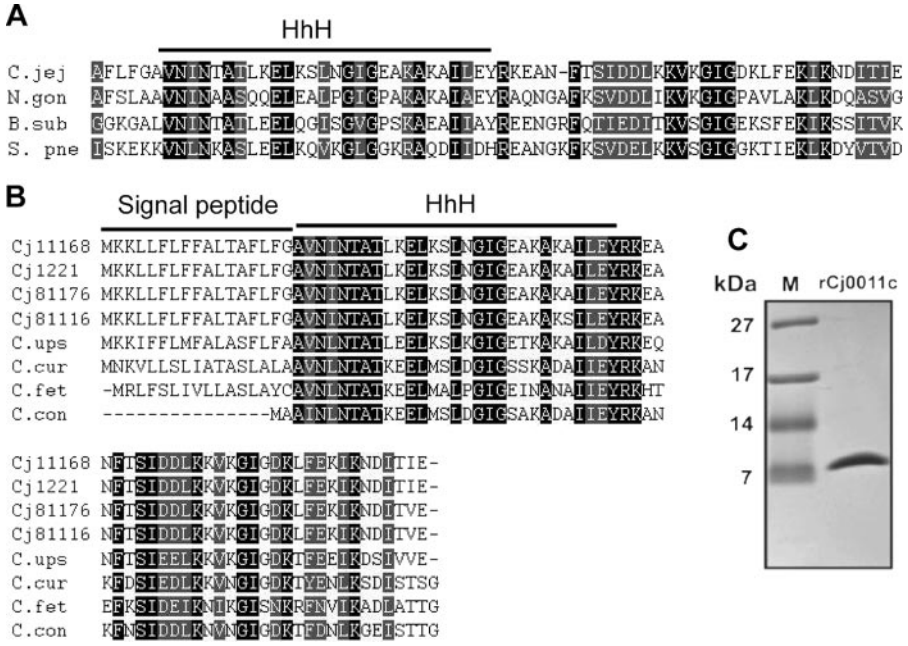

FIG. 1. Sequence features of Cj0011c and the production of rCj0011c of *C. jejuni*. Source sequences for alignments are all from GenBank, and their accession numbers are indicated in parentheses. (A) Alignment of amino acid sequences of Cj0011c and other ComEA proteins from different bacterial species: C.jej, *C. jejuni* (F81416); N.gon, *N. gonorrhoeae* (AAG18334.1); B.sub, *B. subtilis* (BAA12452.1); and S.pne, *S. pneumoniae* (AAC23741.1). The predicted HhH motif is overlined; black and gray backgrounds indicate identical and similar amino acids, respectively. (B) Sequence alignment of Cj0011c homologs in different *C. jejuni* strains and *Campylobacter* species: Cj11168, *C. jejuni* NCTC 11168 (F81416); Cj1221, *C. jejuni* RM1221 (YP_178039.1); Cj81176, *C. jejuni* 81-176 (ZP_01088238.1); Cj81116, *C. jejuni* 81116 (ABA77536.1); C.ups, *C. upsaliensis* (ZP_00369920.1); C.cur, C. *curvus* (EAU00437.1); C.fet, *C. fetus* (ZP_01409997.1); and C.con, *C. concisus* (ZP_01374313.1). The predicted signal peptide and HhH motif are marked above the sequences. (C) SDS-PAGE analysis of rCj0011c (His_6-Cj0011c). The gel was stained with Coomassie brilliant blue R-250. Lane M contains protein size markers (Bio-Rad).

TCTTTGCTTGTG-3′). The unique restriction site in each primer is underlined. Each product was digested with the corresponding restriction enzymes and cloned into pUC19 that had been digested with the same enzymes. The construct was designated pUC19-UD. The *aphA3* gene was amplified from pMW10 (52) by primer pair Kan_F (5′-CTTATCAATATATCCATGGAATGGGCAAAGCAT-3′) and Kan_R (5′-GATAGAACCATGGATAATGCTAAGACAATCACTAA A-3′) using $Vent_R$ DNA polymerase (New England Biolab, Beverly, MA), which generates blunt-ended PCR products. The *aphA3* gene was cloned into a SmaI site of pUC19-UD, which allowed the insertion of the *aphA3* gene between the cloned upstream and downstream sequences of cj0011c. The orientation of the inserted kanamycin resistance cassette was confirmed by PCR. This plasmid construct was introduced into *C. jejuni* NCTC 11168 by electroporation. Homologous recombination in *C. jejuni* resulted in the deletion of cj0011c and the simultaneous insertion of *aphA3*. Transformants were selected on MH agar plates containing kanamycin and confirmed by PCR.

Complementation of the cj0011c mutant in *trans*. The cj0011c gene and its promoter sequence, which was identified in this study, were PCR amplified by using $Vent_R$ DNA polymerase from strain 11168. The primers used for the PCR were 11comp_F (5′-GAGTATTTTAAACGCGAATTTTTGGGTTAAG-3′) and 11comp_R (5′-CTGCTTCATCAATACCGATTAAATTTATATCAA-3′). The PCR product containing cj0011c and its promoter was cloned into the EcoRV site of pRY112. The cj0011c-containing pRY112 plasmid was transferred from *E. coli* DH5α to *C. jejuni* by triparental mating as described elsewhere (36).

Natural transformation. The biphasic natural transformation method was used in this study as described previously (48). *C. jejuni* strains were grown overnight on MH agar and resuspended in MH broth to an optical density at 600 nm of 0.5. Aliquots of 500 μl were transferred to biphasic culture tubes and incubated for 2 h at 42°C. One microgram of DNA was then added to each culture. Genomic DNA of a fluoroquinolone-resistant isolate of *C. jejuni* NCTC 11168 which has a C257T mutation (leading to the Thr-86-Ile change) in *gyrA* (53) was used as the donor DNA. Negative transformation controls were prepared by adding an equal volume of sterilized distilled water into the culture. After incubation with DNA for 3 h, the cultures were serially diluted and plated onto MH agar plates supplemented with ciprofloxacin (2 μg ml^{-1}). The total bacterial number was counted by plating on MH without antibiotics. Each experiment was done in quadruplicate, and the transformation experiment was repeated three times. The transformation frequency represents the number of transformants from 1 μg of donor DNA per total number of bacteria. The natural transformation experiment was also performed with donor DNA prepared from the *cmeF* mutant (*cmeF*::*cat*) of *C. jejuni* that contains the *cat* gene encoding chloramphenicol resistance (1).

RESULTS

Identification of Cj0011c as a DNA-binding protein in *C. jejuni*. In the process of searching for proteins interacting with the promoter DNA of the *cmeDEF* operon (1), a few proteins were captured with promoter DNA-coated magnetic beads from the whole-cell lysate of *C. jejuni* NCTC 11168 (Y.-W. Barton and Q. Zhang, unpublished data). The N-terminal sequences of these proteins were determined by Edman degradation. One of the amino acid sequences obtained matched to Cj0011c in the genome of *C. jejuni* NCTC 11168 (41). In fact, this obtained sequence (AVNINTATLKEL) represented the first 12 amino acids (aa) following the predicted signal peptide of Cj0011c. This finding suggested that Cj0011c was potentially a DNA-binding protein and that its signal peptide was cleaved from the mature product in *C. jejuni*.

According to the annotation of the genomic sequence of *C. jejuni* NCTC 11168 (41), Cj0011c is predicted to be a putative nonspecific DNA-binding protein. Cj0011c consists of 79 aa and shows 56% and 44% identity to the C-terminal regions of the ComEA proteins in *B. subtilis* (205 aa) and *S. pneumoniae*

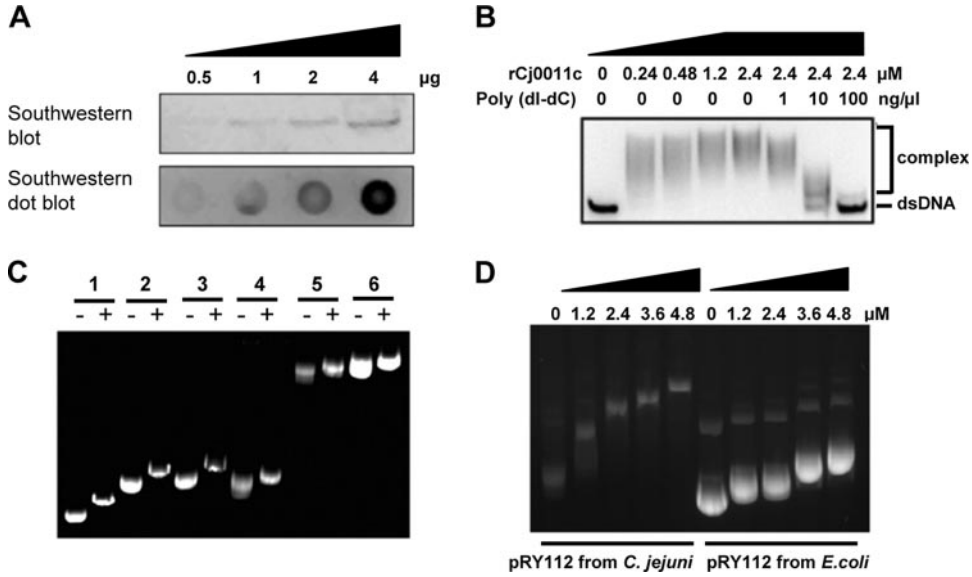

FIG. 2. Binding of rCj0011c to dsDNA. (A) Results of Southwestern blotting and dot blotting show the binding of rCj0011c to a representative DIG-labeled 274-bp PCR product (see Materials and Methods for details). The protein amounts loaded in each lane or spot are indicated above the panel. (B) Results of polyacrylamide gel retardation assay using rCj0011c and the DIG-labeled 274-bp PCR product. Samples were run in a 6% polyacrylamide gel under native conditions. The concentrations of rCj0011c and poly(dI-dC) used in the binding reaction mixtures are indicated above the panel. The positions of the dsDNA and the DNA-protein complexes are indicated on the right. (C) Results of agarose gel retardation assay showing the binding of rCj0011c to various plasmids: 1, pUC19; 2, pQE30; 3, pRSET-mCherry; 4, pET-20b(+); 5, pMW10; and 6, pWM1007. Plus and minus indicate the presence and absence, respectively, of rCj0011c in the binding reaction mixtures. The concentration of rCj0011c used in all reaction mixtures was 2.4 μM. Samples were run in an 0.8% agarose gel. (D) Results of agarose gel retardation assay using plasmid pRY112 purified from either *E. coli* DH5α or *C. jejuni* NCTC 11168. The rCj0011c concentrations used in the reaction mixtures are indicated above the gel. The samples were run in an 0.8% agarose gel.

(216 aa), respectively (Fig. 1A). Cj0011c also shares 57% identity to ComE (99 aa; ComEA ortholog) in *N. gonorrhoeae*. The partial sequence homology to the ComEA proteins in other bacteria suggests that Cj0011c is a DNA-binding protein. According to the prediction with SignalP 3.0 (4), Cj0011c has an N-terminal signal peptide and three potential cleavage sites between the 17th and 18th, 20th and 21st, and 23rd and 24th residues. The obtained N-terminal sequence of the mature Cj0011c captured by the DNA-coated magnetic beads confirmed that the cleavage occurred between the 17th and 18th aa. After cleavage of the signal peptide, the mature Cj0011c has only 62 aa. The Cj0011c sequence is highly conserved in different *Campylobacter* species, except in *C. concisus*, where the Cj0011c homolog lacks the predicted signal peptide (Fig. 1B). Cj0011c is predicted to contain a putative helix-hairpin-helix (HhH) motif (Fig. 1A and B), which is a motif known to be associated with non-sequence-specific DNA binding (14). To define the DNA-binding function of Cj0011c, we produced rCj0011c of *C. jejuni* NCTC 11168 in *E. coli*. The rCj0011c protein (without the predicted signal peptide) migrated as an 8-kDa band on the SDS-PAGE gel (Fig. 1C), which is comparable to the calculated molecular mass of the processed Cj0011c protein (8.3 kDa).

Binding to dsDNA. To confirm that Cj0011c of *C. jejuni* functions as a DNA-binding protein, we tested the binding of rCj0011c to DNA of various sources, including PCR products amplified from the genomic DNA of *C. jejuni* and plasmids isolated from *E. coli* or *C. jejuni*, such as pUC19, pQE30, pET-20b(+), pRSET-mCherry, pWM1007, pMW10, and pRY112 (Fig. 2). In a series of DNA-binding assays, including Southwestern blotting (Fig. 2A), polyacrylamide gel retardation (Fig. 2B), and agarose gel retardation assays (Fig. 2C and D), rCj0011c bound to all of the dsDNA examined in this study. The competition binding assay was performed by adding poly(dI-dC) to the binding reaction mixtures. Poly(dI-dC) is a synthetic polymer composed of inosine and cytosine residues and is often included in gel mobility shift assays as a nonspecific DNA competitor (28, 30). The binding of rCj0011c to dsDNA was reduced by 1 to 10 ng μl^{-1} of poly(dI-dC) and was totally inhibited by 100 ng $\mu l^{-1}$of poly(dI-dC) in the binding reaction mixture (Fig. 2B). The binding of rCj0011c to various DNAs and the inhibition by poly(dI-dC) suggest that it binds to dsDNA in a non-sequence-specific manner.

Binding to ssDNA. We also investigated the ssDNA-binding activity of Cj0011c. As shown in Fig. 3A, rCj0011c was able to bind to the positive strand of bacteriophage M13. In addition, we performed a polyacrylamide gel retardation assay using a 33-mer oligonucleotide, which also showed the binding of rCj0011c to ssDNA (Fig. 3B). According to the genomic sequence, *C. jejuni* NCTC 11168 contains only one ssDNA-binding protein, which is encoded by *cj1071* (41). Cj0011c was found to be 37% identical and 53% similar to the N-terminal 99 residues of Cj1071 (data not shown). This partial sequence homology further supports the findings shown in Fig. 3A and B.

Identification of the putative promoter sequence of cj0011c. To facilitate the characterization of the cj0011c gene in *C. jejuni*, we located its transcription start site using a primer extension assay. The cj0011c gene is flanked by cj0012c and *rnhB*, which encode putative homologs of rubrerythrin and

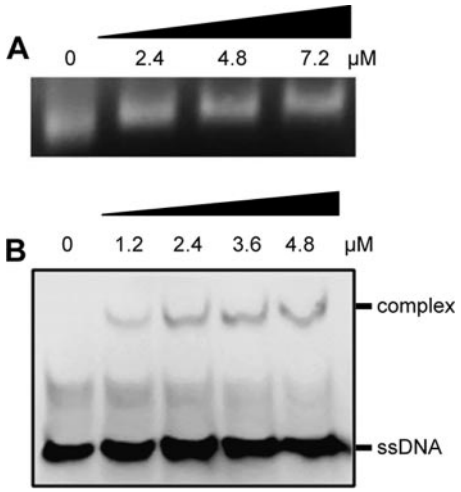

FIG. 3. Binding of rCj0011c to ssDNA. (A) Results of agarose gel retardation assay using the positive strand of bacteriophage M13. Samples were run in an 0.8% agarose gel. The concentrations of rCj0011c used in the reaction mixtures are indicated above the panel. (B) Polyacrylamide gel retardation assay using a DIG-labeled 33-mer oligonucleotide. The concentrations of rCj0011c used in the reaction mixtures are indicated above the figure, and the positions of the ssDNA and the DNA-protein complex are indicated on the right.

RNase HII, respectively (Fig. 4A). cj0011c is separated from cj0012c and *rnhB* by 64 bp and 32 bp, respectively. The primer extension assay indicated that the transcription of cj0011c starts 23 nucleotides upstream of its start codon (Fig. 4B and C). Based on the identified transcriptional start site and sequence homology to the consensus *rpoD* promoter sequence in *C. jejuni* (52), a putative cj0011c promoter was identified (Fig. 4B). This identified promoter was highly homologous to the consensus *rpoD* promoter sequence of *C. jejuni* (52).

Contribution of Cj0011c to natural transformation. The DNA-binding activity of Cj0011c and its sequence homology to the ComEA proteins in other bacteria suggested that Cj0011c might be a potential competence protein in *Campylobacter*. To determine if Cj0011c contributes to natural transformation in *C. jejuni*, we generated a deletion mutant of cj0011c. This mutation specifically knocked out Cj0011c, as determined by immunoblotting (Fig. 4D, lane 2). Complementation of the mutant in *trans* restored the production of Cj0011c (Fig. 4D, lane 3). Based on the densitometric analysis of the band intensity, it appeared that the level of Cj0011c expression was approximately 3.4 times higher in the complemented mutant strain than in the wild-type strain. Using these constructs, we performed natural transformation experiments with donor DNA that confers resistance to ciprofloxacin. In three independent experiments, the cj0011c mutant showed 10- to 50-fold reductions in transformation frequencies compared with the frequencies in the wild-type strain (Fig. 5), although the actual transformation frequencies varied in each experiment. The numbers of ciprofloxacin-resistant colonies observed on the plates spread with the wild-type strain were in the range of hundreds, while the numbers of transformants detected on the plates spread with the cj0011c mutant

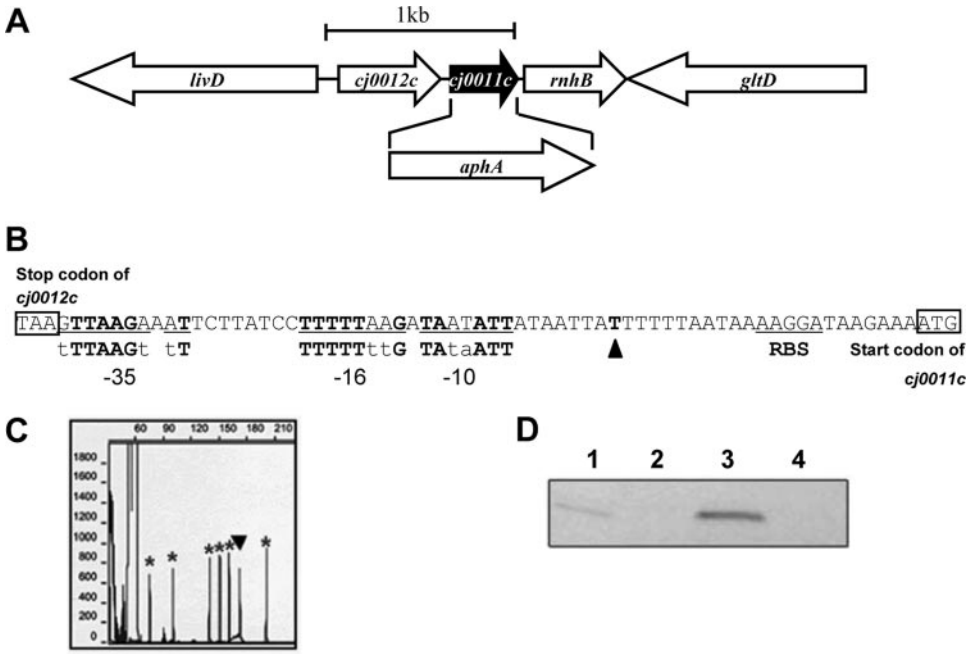

FIG. 4. Identification of the putative promoter of cj0011c in *C. jejuni*, and expression of cj0011c in various constructs. (A) Genomic organization of cj0011c and its flanking regions. The position of the inserted *aphA3* gene in the deletion mutant is indicated by a bracketed arrow. (B) Predicted promoter sequence for cj0011c. Putative −10, −16, and −35 regions are underlined. The consensus *rpoD* promoter sequence is listed below the predicted promoter, and the conserved nucleotides are shown in bold. The identified transcriptional start site is marked with an arrowhead. The ribosomal binding site (RBS) is underlined. (C) Electropherogram showing the results of the primer extension assay. The arrowhead indicates the transcriptional start site, and asterisks indicate the DNA size markers. (D) Immunoblotting analysis of cj0011c expression in wild-type *C. jejuni* 11168 (lane 1), the Δcj0011c::*aphA* mutant (lane 2), the Δcj0011c::*aphA* mutant complemented with pRY112 carrying cj0011c (lane 3), and the Δcj0011c::*aphA* mutant carrying the empty vector pRY112 (lane 4). The same amount (4 μg) of whole-cell proteins was loaded in each well, and the blotting was done using the anti-rCj0011c antibody.

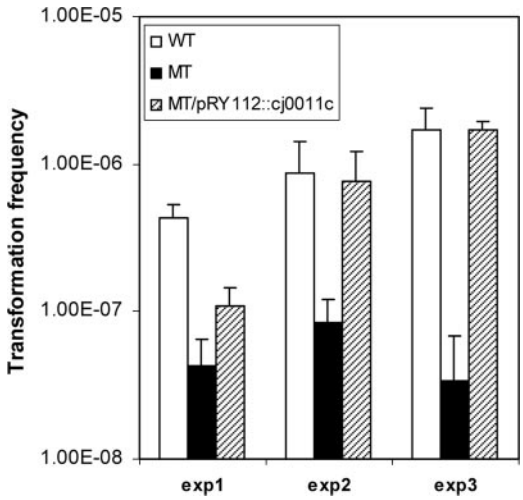

FIG. 5. Natural transformation frequencies of wild-type *C. jejuni* 11168 (WT), the Δcj0011c::*aphA* mutant (MT), and the Δcj0011c::*aphA* mutant complemented with pRY112::cj0011c (MT/pRY112::cj0011c). Each bar represents the mean ± standard deviation of the results for quadruplicate samples in a single experiment. The experiment was repeated three times (exp1, exp2, and exp3). The genomic DNA from a ciprofloxacin-resistant mutant of *C. jejuni* 11168 was used as the donor DNA.

strain were in the range of tens. The difference in transformation frequency between the wild-type and the mutant strains was statistically significant as determined by Student's *t* test ($P < 0.05$). Complementation of the cj0011c mutant with a plasmid-bearing cj0011c gene in the three experiments either partially or fully restored the transformation frequencies to the wild-type levels (Fig. 5). The rates of spontaneous mutation for ciprofloxacin resistance, as measured in the natural transformation reactions without added donor DNA, were lower than 2.0×10^{-8} in both the wild-type and the mutant strain when 2 μg ml^{-1} of ciprofloxacin was used in the selective plates (data not shown). Additionally, insertional mutagenesis of *rnhB*, the downstream gene of cj0011c (Fig. 4A), did not affect the natural transformation frequency of *C. jejuni* NCTC 11168 (data not shown). We also conducted transformation experiments using donor DNA from the *cmeF* mutant (*cmeF*::*cat*) that contains the *cat* gene encoding chloramphenicol resistance (1). With this donor DNA, an approximately 10-fold reduction in the natural transformation frequency was observed in the cj0011c mutant in comparison with the frequency in the wild-type strain (data not shown). Together, these results indicated that Cj0011c contributes to natural transformation in *C. jejuni*.

Localization of Cj0011c in the periplasm. The presence of a signal peptide in Cj0011c suggests that it is a secreted protein. To determine the cellular location of Cj0011c in *C. jejuni*, different cellular fractions were prepared. Immunoblotting of these fractions with the anti-Cj0011c antibody demonstrated that Cj0011c was predominantly associated with the periplasmic fraction (Fig. 6A). The CmeC and CmeR proteins of *C. jejuni* were used as indicators for the membrane and cytoplasmic fractions, respectively. CmeC is an outer membrane protein and showed as a doublet on the blot (32). CmeR is a cytoplasmic protein and functions as a transcriptional regulator modulating the expression of the CmeABC multidrug efflux pump (31). The presence of CmeC and CmeR in the corresponding fractions and their absence in the periplasmic fraction (Fig. 6A) validated the localization results. In addition, the periplasmic fraction was also confirmed by the presence of high SOR activity, which served as a periplasmic marker (Fig. 6B) (39). We also determined the sensitivity of Cj0011c to proteinase K treatment. When *C. jejuni* cells were exposed to increasing amounts of proteinase K, CmeC (outer membrane protein) was gradually degraded, whereas Cj0011c was protected from the proteinase K treatment (Fig. 6C), indicating that Cj0011c is not surface exposed in *C. jejuni*. Based on the sequence analysis, the mature product of Cj0011c lacks a transmembrane

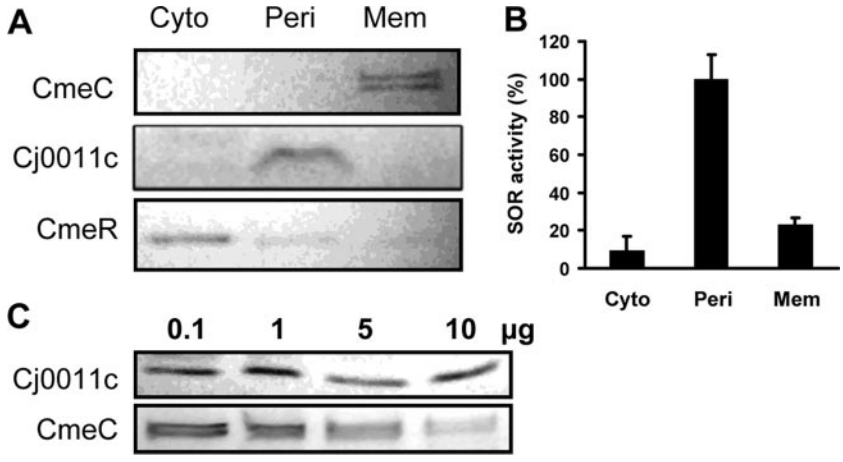

FIG. 6. Cellular location of Cj0011c within *C. jejuni*. (A) Results of SDS-PAGE and immunoblotting analysis of cytoplasmic (Cyto), periplasmic (Peri), and membrane (Mem) fractions with anti-CmeC, anti-Cj0011c, and anti-CmeR antibodies, respectively. (B) SOR activities measured in different cellular fractions. Abbreviations are as described for panel A. The SOR activity in each fraction was normalized using the activity in the periplasmic fraction (100%). Each bar represents the mean ± standard deviation of the results of three independent experiments. (C) Results of immunoblotting proteinase K-treated *C. jejuni* cells with the anti-Cj0011c and anti-CmeC antibodies, respectively. The amount of proteinase K added to each reaction mixture is indicated above the panel.

domain, suggesting that it is not inserted into the membrane. Taken together, these results indicate that Cj0011c is located in the periplasmic space of *C. jejuni*.

DISCUSSION

This study reports a previously unrecognized DNA-binding competence protein in *C. jejuni* which shares partial sequence homology with the ComEA proteins involved in natural transformation in other bacteria (Fig. 1A), such as *B. subtilis*, *S. pneumoniae*, and *N. gonorrhoeae* (6, 8, 24). Cj0011c was shown to be a periplasmic protein (Fig. 6A) which bound to both dsDNA and ssDNA (Fig. 2 and 3) and contributed to the natural transformation of *C. jejuni* (Fig. 5). These results establish that Cj0011c serves as a periplasmic DNA receptor involved in the natural transformation of *C. jejuni* and provide new insights into the mechanisms underlying the competence process in *Campylobacter*.

Cj0011c has 79 aa, including a signal peptide, and is smaller than the known ComEA proteins in other bacteria, such as *B. subtilis* ComEA (205 aa), *S. pneumoniae* ComEA (216 aa), and *N. gonorrhoeae* ComE (99 aa). Despite the size differences, these proteins commonly harbor an HhH motif (Fig. 1A) to which the non-sequence-specific DNA-binding activity of ComEA proteins is attributable (14). Deletion of the C-terminal domain (containing the HhH motif) of the *B. subtilis* ComEA protein rendered it unable to bind to dsDNA and ssDNA, suggesting that the C-terminal region of the ComEA protein is solely responsible for DNA binding (43). The HhH motif of Cj0011c is in the N-terminal region (Fig. 1A and B) and is likely to serve as the DNA-binding domain of this protein.

One interesting finding of this study is that Cj0011c binds to both dsDNA and ssDNA. Although the dsDNA-binding activities of ComEA proteins have been well documented in *B. subtilis* and *N. gonorrhoeae* (8, 43), there has been only a single report on ssDNA binding of ComEA, where the *B. subtilis* ComEA bound to ssDNA as small as a 22-mer oligonucleotide, but with a significantly lower affinity than to dsDNA (43). Based on the gel-shift assay (Fig. 2B and 3B), the binding of Cj0011c to dsDNA occurred in the nM range, while the binding to ssDNA occurred in the μM range, suggesting that Cj0011c also has a higher affinity to dsDNA than to ssDNA. The biological significance of ssDNA binding by ComEA homologs is unknown, but it may facilitate the uptake of ssDNA from the environment. For example, it was shown that *Pseudomonas stutzeri*, *H. influenzae*, and *N. gonorrhoeae* were transformable with ssDNA (35, 42, 46), and *N. gonorrhoeae* was transformed with ssDNA generated by phage M13 at a level similar to that with dsDNA (46). Alternatively, ssDNA binding may be an essential function of ComEA proteins. Although the DNA transport process has not been formally demonstrated in *Campylobacter*, it is known that in other bacteria dsDNA is degraded during the uptake process and only a single strand is transported to the cytosol (16). Thus, the binding of ssDNA by DNA receptors on the cell membrane (gram-positive bacteria) or in the periplasmic space (gram-negative bacteria) may protect the ssDNA from further degradation by nucleases and ensure its transfer to the cytosol, where cytoplasmic proteins (such as DprA and RecA) protect the incoming ssDNA from degradation by DNase (5, 47).

The level of contribution of Cj0011c to natural transformation in *Campylobacter* was different from the levels contributed by ComEA in other bacteria. *B. subtilis* contains a single copy of *comEA*, and mutagenesis of this gene resulted in a 10^7-fold reduction in natural transformation (24). *N. gonorrhoeae* harbors four copies of *comE*, a *comEA* ortholog in this bacterium. Deletion of all copies of *comE* decreased the natural transformation frequency significantly (4×10^4-fold) in *N. gonorrhoeae*, whereas deletion of a single copy had little effect on natural transformation (8). *C. jejuni* contains only one copy of cj0011c, according to the published genomic sequence (41). Deletion of cj0011c reduced but did not abolish the transformability of *C. jejuni* (Fig. 5), suggesting that Cj0011c contributes to but is not essential for natural transformation in *C. jejuni*. The level of contribution of Cj0011c to natural transformation is similar to that of VirB10 (29) but is significantly lower than that of the *cts* genes identified in the study by Wiesner et al. (50), in which the *cts* mutants showed approximately 1,000-fold reductions in transformation frequencies compared to the frequency in the wild-type strain. The nonessential nature of Cj0011c for natural transformation suggests that *C. jejuni* may have additional DNA receptors for transformation which overlap the function of Cj0011c.

Natural transformation in *Campylobacter* is mostly efficient with its own DNA and is extremely inefficient with foreign DNA, such as *E. coli* DNA (48, 51). The difference in natural transformation frequencies between self and nonself DNA may be partly explained by the presence of restriction-modification systems in *Campylobacter*, which restrict the transformation by nonself DNA (27, 38). In addition, DNA uptake in *Campylobacter* also appears to be highly selective for its own DNA, because previous studies showed that *Campylobacter* takes up *E. coli* DNA very poorly (48, 50). Some gram-negative bacteria, such as *N. gonorrhoeae* and *H. influenza*, recognize specific DNA uptake sequences (DUS) and distinguish between self DNA and nonself DNA based on the presence of DUS (12, 17). It has not been shown that *C. jejuni* possesses DUS, but the preferential uptake of its own DNA suggests that *C. jejuni* has a mechanism for differentiating self DNA from foreign DNA in the uptake process. This DNA selection step is unlikely to be associated with Cj0011c, since Cj0011c binds to DNA nonspecifically (Fig. 2). It is plausible to speculate that the specificity of DNA binding and uptake in *C. jejuni* is determined by a selective step involving factors located in the outer membrane, which remains to be defined in future studies.

The cellular location of Cj0011c was experimentally determined in this study. In gram-positive *B. subtilis*, ComEA is located in the cell membrane (24), while in *N. gonorrhoeae*, ComE (ortholog of ComEA) is speculated to be a periplasmic protein, but experimental evidence showing the location has not been reported (8). In this work, we showed that Cj0011c is secreted to the periplasmic space in *C. jejuni* (Fig. 6A). The localization of Cj0011c in the periplasmic space suggests that Cj0011c is not involved in the initial binding of foreign DNA to the bacterial surface of *C. jejuni*. Recently, transposon mutagenesis by Wiesner et al. (50) identified 11 genes involved in natural transformation, and 9 of them affected DNA uptake.

Several of the identified genes encode proteins that are similar to the ComG proteins required for DNA binding and uptake in *B. subtilis* (10, 11). In *B. subtilis*, ComGC forms a type II secretion system pseudopilus which is named competence pseudopilus (9). The pseudopilus traverses the cell wall and helps foreign DNA to access the ComEA receptor that is located in the membrane (9). The formation of the competence pseudopilus requires ComGC and six other ComG proteins (9). *C. jejuni* has some ComG homologs, but it is unknown if they form a transformation machinery similar to that seen in *B. subtilis* (50). As a periplasmic DNA receptor, Cj0011c potentially interacts with some other competence protein(s), such as the ComG homologs or other unidentified inner membrane transporters, in the process of DNA binding and transport. This possibility remains to be determined in future work.

ACKNOWLEDGMENTS

We thank Gregory J. Phillips for his helpful comments on the experimental design.

This study was supported by National Research Initiative competitive grant 2003-35212-13316 from the USDA Cooperative State Research, Education, and Extension Service.

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