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

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Abstract
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.

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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 CO2 concentration in liquid culture, with higher transformation frequencies in a low (0.7%) CO2 atmosphere than in a high (10%) CO2 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.
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% N₂, 5% O₂, and 10% CO₂). The mutant strain (∆cj0011c:aphA43) 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 programmed into plasmid pIM09 (Promega, Madison, WI) with the NdeI and HindIII vector (QIAGEN, Valencia, CA). The cj0011c gene in pIM09 was PCR amplified using primers 11pOE-F (5'-TTTCTGCGATCTGGTAAAATACCACTGAC-3'; restriction site underlined) and 11pOE-R (5'-GCGAAGAACCTGCGT TTTAATCTGTTGATAC-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 antiserum 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). DNA was visualized by immersing the membrane in Tris-borate-EDTA (TBE) containing the DNA probe, which was labeled at the 3'-end with digoxigenin (DIG)-11-ddUTP (Roche Molecular Biochemicals, Indianapolis, IN). The membrane was washed with 0.1 M sodium phosphate buffer containing 0.1% Tween 20 and soaked in the reaction buffer. DIG-labeled DNA was detected and visualized by using an enhanced chemiluminescence detection system (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. 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 rcj0011c in 20 μl of binding buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 0.1 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.

Agar gel retardation assays. Agar retardation assays were performed using several different plasmids, including pUC19 (Invitrogen, Carlsbad, CA), pQE30, pET-20b (Invitrogen, Madison, WI) with the bl-1 vector (QIAGEN, Valencia, CA), and pET-20b (E. coli DH5α) (No-Nox press, Madison, WI) with the pl-1 vector (QIAGEN, Valencia, CA). The cj0011c gene was PCR amplified using primers 11DownK_F (5'-CAAAAATGGTACCACACAATAGA-3') and 11UpB_R (5'-CAAAAATGGTACCACACAATAGA-3') and the reaction product was purified under native conditions using QIAquick PCR purification kit (QIAGEN). Rabbit polyclonal antisera against rcj0011c were prepared by Pacific Immunology Corp. (Ramona, CA) using the purified rcj0011c.

TAAA-3') which was arbitrarily chosen from the sequence of cj121f (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 Periplasm 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 Periplasm buffer (200 mM Tris-HCl, pH 7.5, 20% sucrose, 1 mM EDTA, 30 μM ready-lyse bozyme) 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 (Vinsonic 600; Vitris). 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 bichinchoninic 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 μM horse heart cytochrome c (10 μg ml⁻¹) added to 30 μl of membrane fraction. The mixture was incubated in absorbance at 550 nm was measured 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 × 10⁸ cells ml⁻¹. Aliquots (100 μl) of the bacterial suspension were treated with different concentrations of proteinase K at 37°C for 30 min. After 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 0.05% Tween 20) for 1 h and was probed with the primary antibody (rabbit anti-cj0011c; 1:50 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'-CTTCTTGGGATTTACAAAATGCGTTT-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 deoxyxynucleoside triphosphates, 10 mM MgCl₂, 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 5′-EcoRI upstream of cj0011c was PCR amplified with primer 11UpX_F (5′-GGAATGTACATGCTAGATGATACGCAAT-3') and 11Upb_R (5′-CGTTAAAGCAAAAAATATAAGTTGAGTACCTTGTCCTTCTTT-3'). Also, a 756-bp downstream of cj0011c was PCR amplified with primer 11DownK_F (5′-CGTAAAGCCAAAAATAAACATGATCACGGAAGCTATAG-3') and 11DownE_R (5′-CAACACTCCTAAAGGAAAT-3')
onto MH agar plates supplemented with ciprofloxacin (2 mg/ml) after incubation with DNA for 3 h, the cultures were serially diluted and plated. Genomic DNA of a fluoroquinolone-resistant isolate of C. jejuni used in this study as described previously (48). The cj0011c gene and its promoter were PCR amplified by using VentR 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 cj0011c gene between the aphA3 gene and H11032. The unique restriction site in each primer is underlined. The cj0011c gene and its promoter sequence, which was identified in this study, were PCR amplified by using Vent 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 cj0011c 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.

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 the cat gene encoding chloramphenicol resistance (1).

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 C2571T mutation (leading to the Thr-86-Ile change) in gyrB (53) was used as the donor DNA. Negative transformation controls were prepared by adding an equal volume of sterile 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 mg/ml). 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).
Cj0011c used in all reaction mixtures was 2.4, pWM1007. Plus and minus indicate the presence and absence, respectively, of Cj0011c in the binding reaction mixtures. The concentration of N. gonorrhoeae to ComE (99 aa; ComEA ortholog) in (216 aa), respectively (Fig. 1A). Cj0011c also shares 57% identity 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 8.3 kDa. To confirm that Cj0011c of C. jejuni NCTC 11168 contains only one ssDNA-binding site, 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.

**Binding to dsDNA.** To confirm that Cj0011c 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 μl⁻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.

**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
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...
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 × 10⁻³ 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 mhb, 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...
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 *comE*, and mutagenesis of this gene resulted in a 10^2-fold reduction in natural transformation (24). *N. gonorrhoeae* harbors four copies of *comE*, a comE 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. influenzae*, 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.

**REFERENCES**


