

## Nucleases Encoded by the Integrated Elements CJIE2 and CJIE4 Inhibit Natural Transformation of *Campylobacter jejuni*<sup>∇†</sup>

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**The species *Campylobacter jejuni* is naturally competent for DNA uptake; nevertheless, nonnaturally transformable strains do exist. For a subset of strains we previously showed that a periplasmic DNase, encoded by *dns*, inhibits natural transformation in *C. jejuni*. In the present study, genetic factors coding for DNase activity in the absence of *dns* were identified. DNA arrays indicated that nonnaturally transformable *dns*-negative strains contain putative DNA/RNA nonspecific endonucleases encoded by CJE0566 and CJE1441 of strain RM1221. These genes are located on *C. jejuni* integrated elements 2 and 4. Expression of CJE0566 and CJE1441 from strain RM1221 and a homologous gene from strain 07479 in DNase-negative *Escherichia coli* and *C. jejuni* strains indicated that these genes code for DNases. Genetic transfer of the genes to a naturally transformable *C. jejuni* strain resulted in a decreased efficiency of natural transformation. Modeling suggests that the *C. jejuni* DNases belong to the *Serratia* nuclease family. Overall, the data indicate that the acquisition of prophage-encoded DNA/RNA nonspecific endonucleases inhibits the natural transformability of *C. jejuni* through hydrolysis of DNA.**

Bacterial species display genetic diversity that can contribute to their capacity to adapt and survive in changing environments. One of the processes contributing to genetic diversity is horizontal gene transfer. This involves the acquisition of genetic material, ultimately resulting in insertion of the acquired DNA and/or deletion of existing genetic material (9). The ability to take up exogenous DNA is present in many bacteria (4, 30). In general, uptake of DNA during natural transformation involves binding of double-stranded DNA to bacterial surface components, followed by transport through the cytoplasmic membrane (5). Upon transport, one of the DNA strands is degraded into nucleotides, whereas the other strand enters the cytoplasm and may provide new characteristics to the host genome.

One of the bacterial species naturally competent for DNA uptake is the human pathogen *Campylobacter jejuni* (32). Worldwide, *C. jejuni* is one of the most frequent causes of human bacterial gastroenteritis (3). In *C. jejuni* horizontal gene transfer can occur within a host, as witnessed in chickens infected with two *C. jejuni* strains carrying distinct genetic markers (7). The ability to acquire exogenous DNA contributes to the generation of genetic diversity in *C. jejuni*, which is reflected by the genotypic variation seen among strains (8, 29, 33).

Thus far, several genes have been implicated in the process of natural transformation of *C. jejuni* (35). Yet not all *C. jejuni* strains are equally competent, since differences in natural transformation frequencies have been noted, and even nonnaturally transformable strains do exist (32, 34). Previously, we demonstrated that DNA-hydrolyzing activity inhibits natural transformation of *C. jejuni* and identified Dns as one of the responsible nucleases (13). Dns is encoded by a putative prophage present in *C. jejuni* strain RM1221, namely, *C. jejuni* integrated element 1 (CJIE1).

The presence of DNase activity in *C. jejuni* has long been known and was in fact one of the criteria for Lior's extended biotyping scheme for thermophilic campylobacters (21). Through identification of *dns* a genetic basis for DNase activity by a subset of *C. jejuni* strains has been provided, but the genetic factors responsible for DNase activity in *dns*-negative nonnaturally transformable *C. jejuni* strains are not yet known.

In this study, we attempted to identify and functionally characterize an additional DNase-encoding gene(s) present in a subset of nonnaturally transformable DNase<sup>+</sup> *C. jejuni* strains. Comparative genomic hybridization for DNase<sup>+</sup>/*dns*<sup>+</sup> and DNase<sup>+</sup>/*dns*-negative *C. jejuni* strains revealed three highly homologous genes encoded by the putative phage-related integrated elements CJIE2 and CJIE4. Functional analysis showed that these genes encode DNases that reduce the natural transformability of *C. jejuni* through hydrolysis of exogenous DNA.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. *C. jejuni* strains were grown on heart infusion (HI)

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TABLE 1. Bacterial strains used in the study

Strain	Relevant genotype and/or phenotype	Source or reference
<i>C. jejuni</i> wild-type strains		
C011300	NT, <sup>a</sup> DNase <sup>+</sup> /dns <sup>+</sup>	J. A. Frost (13)
C019165	NT, DNase <sup>+</sup> /dns <sup>+</sup>	J. A. Frost (13)
C012446	NT, DNase <sup>+</sup> /dns <sup>+</sup>	J. A. Frost (13)
C012599	NT, DNase <sup>+</sup> /dns <sup>+</sup>	J. A. Frost (13)
C356 (CNET076)	NT, DNase <sup>+</sup> /dns <sup>+</sup>	W. F. Jacobs-Reitsma (13)
CCUG10950	NT, DNase <sup>+</sup> /dns <sup>+</sup>	Culture Collection, University of Gothenburg, Gothenburg, Sweden (13)
RM1221	NT, DNase <sup>+</sup> /dns <sup>+</sup>	13, 22
D3141	NT, DNase <sup>+</sup> /dns negative	13, 23
D3226	NT, DNase <sup>+</sup> /dns negative	13, 23
40707L (CNET007)	NT, DNase <sup>+</sup> /dns negative	13, 16
07479	NT, DNase <sup>+</sup> /dns negative	13, 16
41239B (CNET005)	NT, DNase <sup>+</sup> /dns negative	13, 16
C019168	Naturally transformable, DNase <sup>-</sup>	J. A. Frost (13)
<i>C. jejuni</i> transformants		
C019168(pWM1007Pr1492)		This study
C019168(pWM1007Pr1492CJE0566 <sub>RM1221</sub> )		This study
C019168(pWM1007Pr1492CJE1441 <sub>RM1221</sub> )		This study
C019168(pWM1007Pr1492CJE0566 <sub>07479</sub> )		This study
C019168hipO::cat	Contains chloramphenicol resistance cassette ( <i>cat</i> ) in <i>hipO</i>	This study
<i>E. coli</i> DH5α	F <sup>-</sup> φ80dlacZΔM15 Δ( <i>lacZYA-argF</i> )U169 <i>recA1 endA1 hsdR17</i> (r <sub>K</sub> m <sub>K</sub> <sup>+</sup> ) <i>phoA supE44 λ<sup>-</sup> thi-1 gyrA96 relA1</i>	Invitrogen, Breda, The Netherlands

<sup>a</sup> NT, nonnaturally transformable.

agar plates (Difco) containing 5% sheep blood (HIS plates), at 37°C for 48 h under microaerobic conditions (6% O<sub>2</sub>, 7% CO<sub>2</sub>, 7% H<sub>2</sub>, and 80% N<sub>2</sub>). For electrotransformation and natural transformation, saponin plates were used, consisting of blood agar base no. 2 medium (Oxoid) containing 4% sheep blood lysed with 0.7% saponin. *E. coli* was cultured on Luria-Bertani agar or in Luria-Bertani broth at 37°C under aerobic conditions. When appropriate, media were supplemented with ampicillin (100 μg ml<sup>-1</sup>), chloramphenicol (12.5 μg ml<sup>-1</sup>), or kanamycin (30 μg ml<sup>-1</sup>).

**Microarray data analysis.** The comparative genomic hybridization data used were obtained in a previous study (13). Briefly, a whole *C. jejuni* genome open reading frame (ORF)-based DNA array, consisting of 1,530 ORFs (94%) from the naturally transformable strain NCTC 11168 and 227 unique ORFs from the nonnaturally transformable strain RM1221, was hybridized with chromosomal DNA isolated from six naturally transformable *C. jejuni* strains and 20 nonnaturally transformable strains. The microarrays were scanned and analyzed as described previously, and the status of a gene (present, divergent, or absent) was defined using comparative genomic indexing (13, 25).

**In silico analysis.** The candidate genes obtained via comparative genomic indexing were used to search for homologues with Basic BLAST or BLAST Assembled Genomes (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The Blastn algorithm (nucleotide-nucleotide BLAST) was used to search for homologues based on nucleotide sequences, and the Blastp algorithm (protein-protein BLAST) was used to find homologues based on amino acid sequences. Alignments were assumed to be significant when the probability values obtained were <1. By performing pairwise alignments with EMBOSS-Align (<http://www.ebi.ac.uk/Tools/emboss/align>; covering whole length sequences) the overall similarities were determined (19, 24). The presence and location of a signal peptide cleavage site in the amino acid sequence were predicted by SignalP 3.0 (2).

**Confirmation of the hybridization data by PCR.** The status (presence/absence) of CJE0566 and CJE1441 homologues was confirmed by PCR. To this end, chromosomal DNA was isolated from *C. jejuni* strains with the Gentra Puregene DNA isolation kit (Qiagen) according to the manufacturer's protocol for Gram-negative bacteria and used as a template in the PCR. The primer combinations used (CJE0566-Fint/CJE0566-Rint and CJE1441-Fint/CJE1441-Rint [Table 2]) were based on the coding sequences of CJE0566 and CJE1441 of strain RM1221 (NCBI reference sequence number NC\_003912) (10).

**Electrotransformation of *C. jejuni*.** *C. jejuni* mutant strains were constructed via electrotransformation as described previously (13). After recovery on non-selective saponin plates (5 h, 37°C, microaerobic conditions) the bacteria were

harvested and plated onto selective saponin plates (48 h, 37°C, microaerobic conditions) to select for transformants.

**Construction of DNase-encoding expression plasmids.** For gene expression in *C. jejuni*, the genes CJE0566 and CJE1441 from strain RM1221 and a CJE0566/CJE1441 homologue from strain 07479 were amplified by PCR using *Taq* polymerase with proofreading and primer sets CJE0566-F/CJE0566-R and CJE1441-F/CJE1441-R, respectively (Table 2). Obtained PCR fragments (706 bp with CJE0566 primers and 704 bp with CJE1441 primers) were cloned into pCR2.1 (Invitrogen, Breda, The Netherlands), resulting in pCRCJE0566<sub>RM1221</sub>, pCRCJE1441<sub>RM1221</sub>, and pCRCJE0566<sub>07479</sub> (4.6 kb). Verification of the inserts was done by sequence analysis (BaseClear, Leiden, The Netherlands). Subsequently, these three constructs were used as templates in PCRs to introduce a ribosomal binding site (AGGAG) and XbaI and MfeI restriction sites, using a different primer combination for each plasmid (CJE0566-Fexp/CJE0566-Rexp, CJE0566-1441-Fexp/CJE1441-Rexp, and CJE0566-1441-Fexp/CJE0566-Rexp [Table 2]). To introduce the same restriction sites into the kanamycin-resistant *C. jejuni* expression plasmid pWM1007Pr1492 (9.5 kb) (36), a PCR product was generated using the primers Pr1492XbaI and Pr1492MfeI (Table 2). After digestion with XbaI and MfeI, the resulting 671-bp fragments with CJE0566<sub>RM1221</sub>, CJE1441<sub>RM1221</sub>, and CJE0566<sub>07479</sub> were ligated into pWM1007Pr1492 to form pWM1007Pr1492CJE0566<sub>RM1221</sub>, pWM1007Pr1492CJE1441<sub>RM1221</sub>, and pWM1007Pr1492CJE0566<sub>07479</sub>, respectively (10.2 kb). Verification of the size of the insert was done by PCR, using primers pWM1007Pr1492-F and pWM1007-R (Table 2) located on pWM1007Pr1492. The resulting *C. jejuni* expression plasmids were transferred into *C. jejuni* strain C019168 via electrotransformation (Table 1).

**DNA hydrolysis assay.** DNase activity was detected using DNase agar (Oxoid) supplemented with 0.005% toluidine blue (15, 31). Cultures of *E. coli* grown in Luria-Bertani broth and of *C. jejuni* grown in HI broth, for 16 h at 37°C, were spun down (1.5 and 3 ml, respectively). The pelleted bacteria were suspended in 50 μl of 500 mM Tris-HCl (pH 7.5), incubated with 5 μl of polymyxin B (50 μg μl<sup>-1</sup>; 4°C, 15 min), followed by inoculation of the suspension into a well (5 mm in diameter) cut in the DNase agar plate after gelling. DNase I (2 μg; Roche Diagnostics, Indianapolis, IN) was included as a positive control, and 50 μl of 500 mM Tris-HCl (pH 7.5) was used as a negative control. The plate was incubated aerobically at 37°C for 24 h. Positive results for DNase activity were visible as colorless zones around the wells.

**Natural transformation of *C. jejuni*.** For determination of the natural transformation frequencies of *C. jejuni* C019168 derivatives the biphasic method was used (32). Homologous chromosomal DNA (2 μg) isolated from *C. jejuni* strain

TABLE 2. Primers and plasmids used in the study

Primer or plasmid	Sequence (5'-3') or relevant characteristic(s) <sup>a,b</sup>	Source or reference
<b>Primers</b>		
CJE0566-Fint	CAAAGTTGTTTCGCAAGTTTTAG	
CJE0566-Rint	CCTTTAAGATTTTAATATAAGAG	
CJE1441-Fint	CAAACTGCTCACAAGTTTTGG	
CJE1441-Rint	CCTTTAAAATCTTAGTGTAAGAG	
CJE0566-F	GAAATGATTAAGCAACAAGTTAAAC	
CJE0566-R	GTAAGCAAAAAGCACCCCATC	
CJE1441-F	CAAATTGAAAGGGTATGAAAC	
CJE1441-R	TGCAATTTCCATTTTTACTCC	
CJE0566-Fexp	GATCTTCTAGAAAGGAGAATAAATGAAAAAATCATAC	
CJE0566-Rexp	CCATGGCAATTGTTATATTTTATCACAAATC	
CJE0566-1441-Fexp	AGATCTTCTAGAAAGGAGAATAAATGAAAAAATTTATAATC	
CJE1441-Rexp	CCATGGCAATTGTTAAAAATGTCACATTTTAC	
Pr1492XbaI	AGATCTTCTAGACTCATTTAACGGTTGTCTCC	
Pr1492MfeI	ACTAGTCAATTGGCGATGGCCCTG	
pWM1007Pr1492-F	GCATATGAAGAGATTTTG	
pWM1007-R	CCAACCTGGTAATGGTAG	
<b>Plasmids</b>		
pCR2.1	TA cloning vector, Amp <sup>r</sup>	Invitrogen
pCRCJE0566 <sub>RM1221</sub>	pCR2.1 containing CJE0566 of RM1221	This study
pCRCJE1441 <sub>RM1221</sub>	pCR2.1 containing CJE1441 of RM1221	This study
pCRCJE0566 <sub>07479</sub>	pCR2.1 containing a CJE0566/CJE1441 homologue of 07479	This study
pWM1007Pr1492	<i>C. jejuni</i> expression plasmid containing the CJ1492 promoter, Km <sup>r</sup>	36
pWM1007Pr1492CJE0566 <sub>RM1221</sub>	<i>C. jejuni</i> expression plasmid with CJE0566 of RM1221	This study
pWM1007Pr1492CJE1441 <sub>RM1221</sub>	<i>C. jejuni</i> expression plasmid with CJE1441 of RM1221	This study
pWM1007Pr1492CJE0566 <sub>07479</sub>	<i>C. jejuni</i> expression plasmid with CJE0566/CJE1441 homologue of 07479	This study
pHipO::cat	<i>C. jejuni</i> suicide vector with <i>cat</i> inserted in <i>hipO</i> , Cm <sup>r</sup>	7

<sup>a</sup> Endonuclease restriction sites introduced into the sequences are underlined.

<sup>b</sup> Ribosomal binding sites are shown in bold.

C019168*hipO::cat* (Table 1) (7) served as donor DNA in the transformation (13). To determine the number of transformants per milliliter, 200- $\mu$ l aliquots of the appropriate serial dilutions ( $10^0$  to  $10^{-5}$ ) were plated onto selective saponin plates. The CFU per milliliter was quantified using track dilution (18), whereby 10- $\mu$ l aliquots of serial dilutions ( $10^{-4}$  to  $10^{-8}$ ) were spotted onto nonselective saponin plates. For calculation of the natural transformation frequencies, the number of transformants per milliliter (selective plates) was divided by the total number of bacteria per milliliter (nonselective plates). All tests were performed in triplicate. Student's *t* test was used for statistical analysis.

**Microarray series accession number.** The microarray data have been deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) with the series accession number GSE18399.

## RESULTS

**Comparative genomic hybridization.** In our search for differences in genome content between *dns*<sup>+</sup> and *dns*-negative *C. jejuni* strains that are nonnaturally transformable and display DNase activity, previously obtained comparative genomic hybridization data were used (13). To identify genes responsible for the DNase<sup>+</sup> phenotype in *dns*-negative strains, the hybridization data were screened for genes absent in the majority of six nonnaturally transformable DNase<sup>+</sup>/*dns*<sup>+</sup> *C. jejuni* strains but present in the majority of five nonnaturally transformable DNase<sup>+</sup>/*dns*-negative strains (see Table S1 in the supplemental material). A total of 34 genes were absent in almost all of the DNase<sup>+</sup>/*dns*<sup>+</sup> strains (83% to 100%) and present in the majority of DNase<sup>+</sup>/*dns*-negative strains (60% to 100%), suggesting that one of these genes may be responsible for DNase activity in the DNase<sup>+</sup>/*dns*-negative strains. One DNase<sup>+</sup>/*dns*<sup>+</sup> strain (C01965), however, exhibited hybridization patterns sim-

ilar to the DNase<sup>+</sup>/*dns*-negative strains, i.e., it contained the majority of the 34 genes in addition to *dns*.

The majority of the identified genes (24 genes) hybridized with genes of CJIE2. Nine genes hybridized with genes of CJIE4; seven of these genes are located on a module also present in CJIE2 and are indistinguishable by comparative genomic hybridization (25).

**Identification of nuclease-encoding genes.** *In silico* analysis of the 34 identified genes present in the majority of DNase<sup>+</sup>/*dns*-negative strains using Blastn analysis confirmed the annotation of two genes (CJE0566 and CJE1441) from strain RM1221 as DNA/RNA nonspecific endonucleases. A homologue with the same annotation was found in *Campylobacter lari* strain RM2100 (Cla\_0934). The analysis did not indicate a putative function of the remaining 32 genes in relation to natural transformability. Using Blastp and SignalP 3.0 (2), four putative nucleases were identified in *C. jejuni* strain RM1221 (CJE0556, CJE0566, CJE0575, and CJE1441), of which only CJE0566 and CJE1441 contained a functional signal peptide cleavage site, indicative of possible periplasmic localization. Both the CJE0566 and CJE1441 protein sequences contained a conserved domain of the NUC superfamily (SMART accession no. SM00477) characteristic for DNA/RNA nonspecific endonucleases (11, 12). Moreover, the translated sequences contained a DRGH motif (see Fig. S1 in the supplemental material) characteristic for the active site of members of the *Serratia* nuclease subfamily (26). Predicted structural models of CJE0566 and CJE1441 showed similarities with the crystal



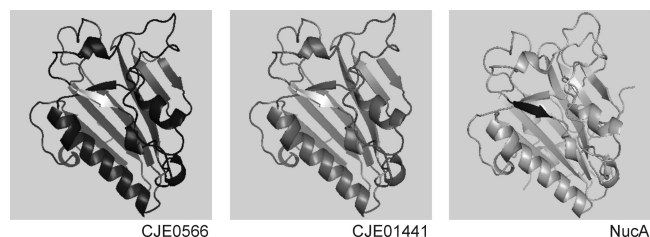


FIG. 1. Comparison of predicted structural models of proteins encoded by CJE0566 and CJE1441 from *C. jejuni* strain RM1221 with the structural model of NucA from *Anabaena* sp. The structures were generated using Swiss-Model (1). The residues DRGH are shown in white (CJE0566 and CJE1441) or black (NucA).

structure of NucA from *Anabaena* sp. (Fig. 1), which is also an extracellular nuclease of the *Serratia* nuclease family.

Taken together, the analysis suggests that CJE0566 and CJE1441 may code for secreted nucleases that belong to the *Serratia* nuclease family.

**DNA/RNA nonspecific endonucleases in *C. jejuni*.** Genome database analysis indicated CJE0566 and CJE1441 homologues in 11 out of the 12 available *C. jejuni* genome sequences, which included the DNase<sup>-</sup> strains NCTC 11168 (Cj0594c) and 260.94 (CJJ26094\_0651) (13). In addition to CJE0566 and CJE1441, a third nuclease was found in strain RM1221 (CJE0697). Closer inspection of the putative nucleases indicated that they all code for proteins of 216 amino acids with a decreased probability of signal peptide cleavage (SignalP 3.0 [2]) and a mutation in the DRGH motif (D→T; see Fig. S1 in the supplemental material). These differences may explain the absence of DNase activity in the strains NCTC 11168 and 260.94. Overall, the results indicate that homologues of the putative nucleases encoded by CJE0566 and CJE1441 are widely spread throughout the *C. jejuni* species but that perhaps only a subset of these homologues is functional.

**Cloning and sequencing of three *C. jejuni* DNA/RNA nonspecific endonucleases.** The presence of CJE0566 and CJE1441 homologues in the nonnaturally transformable DNase<sup>+</sup>/dns-negative *C. jejuni* strains was confirmed by PCR using primer sets based on the coding sequences of both genes from strain RM1221 (data not shown). Detailed genomic indexing of all CJIE2 and CJIE4 genes of strain RM1221 in all nonnaturally transformable DNase<sup>+</sup>/dns-negative *C. jejuni* strains revealed that the genes flanking CJE0566 and CJE1441 in RM1221 were not present in these strains, with the exception of CJE0565 and CJE0567 in strain 07479 (see Table S2 in the supplemental material). From this strain, gene CJE0566 was successfully amplified by PCR. This gene codes for a protein of 217 amino acids and contains a DRGH motif and a signal peptide with a high cleavage probability (see Fig. S1 in the supplemental material).

To functionally characterize CJE0566 from strain 07479 and the homologues from strain RM1221, the three genes were cloned and introduced into *E. coli* strain DH5α. DNA hydrolysis assays using DNase agar with toluidine blue demonstrated that each of the plasmids converted the *E. coli* phenotype from DNase<sup>-</sup> into DNase<sup>+</sup> (data not shown), indicating that CJE0566 and CJE1441 from strain RM1221 and CJE0566 from strain 07479 indeed code for functional DNases.

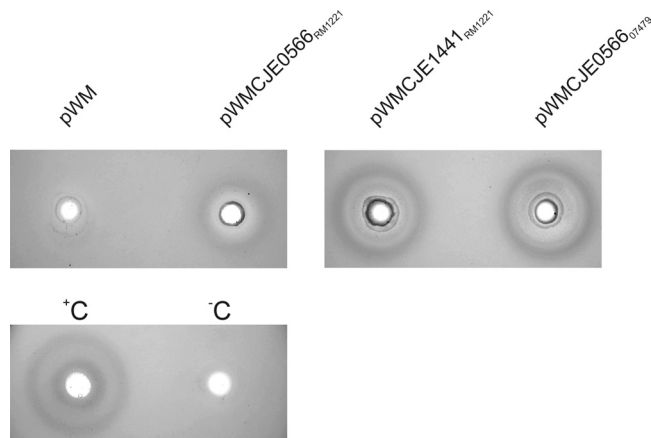


FIG. 2. Effects of CJE0566<sub>RM1221</sub>, CJE1441<sub>RM1221</sub>, and CJE0566<sub>07479</sub> on the DNase activity of *C. jejuni*. The three genes were placed on expression plasmid pWM1007Pr1492 (pWM) and introduced into *C. jejuni* strain C019168. DNase activity of polymyxin B-treated *C. jejuni* transformants was detected using DNase agar supplemented with toluidine blue. +C, with DNase I; -C, with 500 mM Tris-HCl (pH 7.5).

**Inhibition of natural transformation by DNA/RNA nonspecific endonucleases.** To investigate the effect of different DNases on the process of natural transformation in *C. jejuni*, the three genes encoding the proteins were introduced via electrotransformation into the naturally transformable DNase<sup>-</sup> *C. jejuni* strain C019168 using the expression plasmid pWM1007Pr1492 (36). As a control, the same vector without an insert was introduced. DNA hydrolysis assays demonstrated the presence of DNA-hydrolyzing activities for the transformants carrying the CJE0566<sub>RM1221</sub>, CJE1441<sub>RM1221</sub>, and CJE0566<sub>07479</sub> plasmids, but not for the control strain carrying the empty vector (Fig. 2).

To determine the effect of the presence of DNase activity on natural transformation, the derivatives of *C. jejuni* strain C019168 were transformed with homologous chromosomal DNA with a chloramphenicol resistance cassette in *hipO* (C019168*hipO::cat*). This construct demonstrated a 35- to 40-fold decrease in the natural transformation frequencies for the DNase-positive strains compared to the control strain carrying the empty plasmid ( $P < 0.01$ ) (Table 3). These results indicate that the nucleases encoded by CJE0566 and CJE1441 from *C. jejuni* strain RM1221 and their homologue from strain 07479

TABLE 3. Effects of CJE0566<sub>RM1221</sub>, CJE1441<sub>RM1221</sub>, and CJE0566<sub>07479</sub> on the natural transformation frequency of *C. jejuni*

Strain	Natural transformation frequency <sup>a</sup>
C019168(pWM1007Pr1492) .....	$(1.1 \pm 0.1) \times 10^{-4}$
C019168(pWM1007Pr1492CJE0566 <sub>RM1221</sub> ) .....	$(3.1 \pm 1.0) \times 10^{-6b}$
C019168(pWM1007Pr1492CJE1441 <sub>RM1221</sub> ) .....	$(2.8 \pm 2.0) \times 10^{-6b}$
C019168(pWM1007Pr1492CJE0566 <sub>07479</sub> ) .....	$(2.6 \pm 3.3) \times 10^{-6b}$

<sup>a</sup> The natural transformation frequency was determined by dividing the number of transformants per milliliter by the total number of CFU per milliliter. The values are the averages of three experiments.

<sup>b</sup>  $P < 0.01$  by Student's *t* test for the comparison with the control strain carrying the empty expression plasmid (pWM1007Pr1492).

impair natural transformation in DNase<sup>+</sup>/*dns*-negative *C. jejuni* strains.

## DISCUSSION

The species *C. jejuni* is naturally transformable, but the efficiency of transformation is variable, and even nontransformable strains exist (32, 34). Many nonnaturally transformable *C. jejuni* strains express DNase activity. For a subset of strains this is caused by a periplasmic DNase encoded by *dns* (13). A second subset of nonnaturally transformable *C. jejuni* strains, however, contains DNase activity in the absence of *dns* (13). The present study provides evidence that these DNase<sup>+</sup>/*dns*-negative strains contain genes coding for DNA/RNA non-specific endonucleases that are homologous to CJE0566 and CJE1441 from *C. jejuni* strain RM1221. These genes confer DNase activity to *E. coli* and inhibit natural transformation of a naturally transformable *C. jejuni* strain. These results demonstrate that these genes code for DNases and that besides *Dns*, which is a member of the endonuclease I family, also DNases of the DNA/RNA nonspecific endonuclease family influence the natural transformability of *C. jejuni*.

The novel *C. jejuni* DNases were identified by analysis of comparative genomic hybridization of *dns*-positive and *dns*-negative nonnaturally transformable DNase<sup>+</sup> strains. This showed that the integrated elements CJIE2 and CJIE4 contained genes that are present in the *dns*-negative strains but absent in the *dns*<sup>+</sup> strains. CJIE2 and CJIE4 are two of the three phage-related integrated elements present in *C. jejuni* strain RM1221 but absent in strain NCTC 11168 (10). The presence of these integrated elements in other *C. jejuni* strains and their diversity between strains (see Table S2 in the supplemental material) are in agreement with earlier reports (6, 25). The majority of CJIE2 and CJIE4 genes present in DNase<sup>+</sup>/*dns*-negative *C. jejuni* strains encode hypothetical proteins, but the homologues of CJE0566 (CJIE2) and CJE1441 (CJIE4) are annotated as DNA/RNA nonspecific endonucleases, a family of endonucleases that is widely distributed among prokaryotic and eukaryotic species (26). Indeed, the predicted protein structures of CJE0566 and CJE1441 resembled the three-dimensional (3D) structure of NucA from *Anabaena* sp. (Fig. 1), a member of the *Serratia* nuclease family which belongs to the group of DNA/RNA nonspecific endonucleases (14, 28). CJE0566 and CJE1441 are very similar at the amino acid level and display considerable similarity to Cla\_0934 from *C. lari* RM2100. In contrast to CJE0566 and CJE1441, Cla\_0934 is not located on integrated element CLIE1 in *C. lari*, although CLIE1 is very similar to CJIE4 of *C. jejuni* (10).

Members of the *Serratia* nuclease family contain a so-called DRGH motif {DRGH[QLIM]-X(3)-[AG]; accession no. PDOC00821} in which histidine is the active site residue (11, 12). Although the amino acid sequences and the predicted 3D structures of CJE0566 and CJE1441 share similarity with members of this family, the sequences deviate from the DRGH motif in their fifth residue (Q→T). Evidently, the presence of a threonine at this position in the gene products of CJE0566 and CJE1441 does not affect activity, as expression of these genes in DNase<sup>-</sup> *E. coli* renders the cells DNase positive. The observed inhibition of natural transformation after introduc-

tion of the DNase-encoding genes into the DNase-negative *C. jejuni* provides functional evidence that, besides *dns* (13), additional DNases may impair genetic transfer via natural transformation (Table 3). It should be noted that although functionally similar, *Dns* does not belong to the *Serratia* nuclease protein family, as evidenced by the absence of the *Serratia* nuclease-specific DRGH motif.

*In silico* analysis of *C. jejuni* genomes revealed that 11 out of 12 *C. jejuni* genomes available in the public databases contain homologues of CJE0566/CJE1441, including the DNase<sup>-</sup> strains NCTC 11168 and 260.94 (13). The DNase<sup>-</sup> phenotype of these strains may be caused by differences in protein expression levels but more likely can be attributed to amino acid changes. We expect the nucleases from DNase<sup>-</sup> strains to be inactive, as the first amino acid of the conserved DRGH motif is missing. *Serratia* nuclease mutagenesis of this aspartic acid results in reduced enzyme activity (11, 12). In fact, when searching the SMART nonredundant database (<http://smart.embl-heidelberg.de>) (20, 27) for proteins with NUC domains (accession no. SM00477), it appeared that among 183 entries of bacterial origin approximately 20% are predicted to be inactive due to amino acid substitutions in the catalytic site. Besides the incomplete DRGH motif, the inactive *C. jejuni* proteins also appear to contain a degenerated signal peptide sequence which may hamper transport to the periplasm. Changes in protein localization (from cytoplasmic to periplasmic) can be beneficial to a host for some properties (17). However, we consider it unlikely that nucleases from DNase<sup>-</sup> strains are functionally expressed in the cytoplasm, as induced high-level expression in *E. coli* resulted in the cessation of growth (unpublished observation). The apparent degeneration of DNase-encoding genes in some *C. jejuni* strains may indicate that the presence of this trait brings no selective advantage to these strains.

Finally, our findings indicate that *C. jejuni* strains have acquired phage-like integrated elements that code for different types of periplasmic DNases. These elements are genetically variable and widely spread through the *C. jejuni* population, thereby contributing to genetic diversity of the species. On the other hand, the encoded DNases limit horizontal gene transfer in *C. jejuni* through inhibition of natural transformation. This suggests that bacteriophages may both create and restrain genetic diversity of the species *C. jejuni*.

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