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A DNase Encoded by Integrated Element CJIE1 Inhibits Natural Transformation of *Campylobacter jejuni*▽†

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The species *Campylobacter jejuni* is considered naturally competent for DNA uptake and displays strong genetic diversity. Nevertheless, nonnaturally transformable strains and several relatively stable clonal lineages exist. In the present study, the molecular mechanism responsible for the nonnatural transformability of a subset of *C. jejuni* strains was investigated. Comparative genome hybridization indicated that *C. jejuni* Mu-like prophage integrated element 1 (CJIE1) was more abundant in nonnaturally transformable *C. jejuni* strains than in naturally transformable strains. Analysis of CJIE1 indicated the presence of *dns* (CJE0256), which is annotated as a gene encoding an extracellular DNase. DNase assays using a defined *dns* mutant and a *dns*-negative strain expressing Dns from a plasmid indicated that Dns is an endogenous DNase. The DNA-hydrolyzing activity directly correlated with the natural transformability of the knockout mutant and the *dns*-negative strain expressing Dns from a plasmid. Analysis of a broader set of strains indicated that the majority of nonnaturally transformable strains expressed DNase activity, while all naturally competent strains lacked this activity. The inhibition of natural transformation in *C. jejuni* via endogenous DNase activity may contribute to the formation of stable lineages in the *C. jejuni* population.

Bacterial populations of a single species often display considerable genetic diversity that supposedly aids their adaptation and survival in changing environments. One mechanism that contributes to the genetic diversity is horizontal gene transfer (HGT) (12). This process involves the assimilation of acquired genetic material. Many bacterial species have evolved sophisticated systems to enable the uptake of exogenous DNA, allowing natural transformation (6, 45). The mechanism of uptake of DNA differs among species but typically involves binding of double-stranded DNA to components of the cell surface, processing, and transport through the cytoplasmic membrane (6, 7, 11). For formation of the DNA uptake apparatus, different species use related proteins. These proteins have similarity with proteins involved in pilus assembly and secretion system formation. On arrival at the cytoplasmic membrane, only one of the DNA strands is transported into the cytoplasm and can be integrated into the bacterial genome, whereas the complementary strand is degraded into nucleotides (7).

One bacterial species that is naturally competent for DNA uptake is *Campylobacter jejuni*, the most common cause of bacterial gastroenteritis in humans in industrialized countries (4). Genetic typing indicates that there is great genetic diversity among *C. jejuni* strains (10, 47, 50). Multilocus sequence typing suggests that *C. jejuni* has a weakly clonal population structure and that HGT is common in this species (9, 41). Direct experimental evidence of the occurrence of HGT and the generation of genetic diversity in vivo was obtained from experiments with chickens infected with two *C. jejuni* strains carrying distinct genetic markers (8). However, genetic typing also indicates that stable lineages of *C. jejuni* exist and thus that not every strain in the *C. jejuni* population is able to acquire foreign DNA (20, 29, 34, 48).

The molecular basis for the apparent existence of clonal lineages of *C. jejuni* is not known. Thus far, the *C. jejuni* genes *galE*, *dprA*, and Cj0011c have been implicated in the process of natural transformation, particularly in the binding of DNA (16, 23, 43). For DNA uptake, the type II secretion system is important as type II secretion system-deficient mutants show a drastic decrease in the frequency of natural transformation (51). In some *C. jejuni* strains, a type IV secretion system may contribute to this process (2, 26). After arrival in the cytoplasm, the acquired DNA is integrated into the genome of *C. jejuni* via RecA-dependent homologous recombination (18). Lastly, it has been shown that N-linked protein glycosylation is required for full competence of *C. jejuni* (26). Thus far, the many steps involved in the natural transformation of *C. jejuni* and the variation in this process between strains have made it difficult to decipher the cause of the preservation of stable lineages in the *C. jejuni* population.

In the present study, we attempted to unravel the basis of the clonal behavior of distinct *C. jejuni* strains using a comparative

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TABLE 1. Bacterial strains used in this study

Strain	Penner serotype	Genotype	Natural transformability[a]	Source or reference
C. jejuni wild-type strains				
C019165	HS:1		Nontransformable	J. A. Frost
C013199	HS:1		Transformable	J. A. Frost
C011338	HS:1		Transformable	J. A. Frost
C017289	HS:1		Nontransformable	J. A. Frost
C011672	HS:1		Nontransformable	J. A. Frost
C019168	HS:1		transformable	J. A. Frost
C011300	HS:1		Nontransformable	J. A. Frost
C013500	HS:2		Transformable	J. A. Frost
C356 (CNET076)	HS:2		Nontransformable	W. F. Jacobs-Reitsma
C012599	HS:2		Nontransformable	J. A. Frost
5003 (CNET002)	HS:2		Transformable	S. L. On
C012446	HS:2		Nontransformable	J. A. Frost
D3468	HS:19		Nontransformable	31
D3141	HS:19		Nontransformable	31
CCUG10950	HS:19		Nontransformable	Culture Collection, University of Göteborg, Göteborg, Sweden
GB18	HS:19		Transformable	13
D3226	HS:19		Nontransformable	31
233.95	HS:41		Nontransformable	48
308.95	HS:41		Nontransformable	A. J. Lastovica
21.97	HS:41		Nontransformable	A. J. Lastovica
386.96	HS:41		Nontransformable	48
260.94	HS:41		Nontransformable	48
41239B (CNET005)	HS:55		Nontransformable	20
07479	HS:55		Nontransformable	20
12795850312	HS:55		Nontransformable	D. L. Baggesen
40707L (CNET007)	HS:55		Nontransformable	20
NCTC 11168-O	HS:2		Transformable	17
RM1221	HS:53		Nontransformable	30
C. jejuni mutant strain and transformants				
C356*dns*::*cat*				This study
CNET002(pWM1007Pr1492*dns*)				This study
CNET002(pWM1007Pr1492)				This study
E. coli strains				
DH5α		F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*		Invitrogen, Breda, The Netherlands
K12 ER2925		*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R*(*zgb210*::Tn*10*)Tet^s *endA1 rpsL136 dam13*::Tn*9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2*		NEB, Ipswich, MA

[a] The lowest level of detection for the natural transformation frequency is 10^{-10}. For natural transformation frequencies see Table S1 in the supplemental material.

whole-genomic approach. Systematic analysis of DNA microarrays probed with DNA of naturally transformable and nonnaturally transformable *C. jejuni* strains resulted in the identification of a gene (*dns*) whose presence was strongly linked to a lack of natural competence. Inactivation of this gene in a clonal, nonnaturally transformable strain restored competence, while introduction of an intact copy of the gene into a transformable strain resulted in reduced transformability. Functional analysis revealed that the *dns* gene encodes a DNase that inhibits natural transformation through hydrolysis of exogenous DNA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study and their natural transformability are shown in Table 1. The natural transformation frequencies of these strains were analyzed using homologous pUOA13 plasmid DNA (see Table S1 in the supplemental material). The lowest detectable level of the natural transformation frequency (10^{-10}) is based on a minimum of one colony per plate. *C. jejuni* strains were cultured on heart infusion (HI) agar (Difco) supplemented with 5% sheep blood (HIS plates) or on blood agar base no. 2 medium (Oxoid) containing 4% sheep blood lysed with 0.7% saponin (Sigma) (saponin plates). *C. jejuni* was incubated at 37°C for 48 h under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, and 80% N_2) created with an Anoxomat system (Mart Microbiology, Drachten, The Netherlands). *Escherichia coli* was grown on Luria-Bertani agar or in Luria-Bertani broth at 37°C under aerobic conditions. When appropriate, media were supplemented with chloramphenicol (12.5 μg ml^{-1}), kanamycin (30 μg ml^{-1}), or ampicillin (100 μg ml^{-1}).

Construction of the *C. jejuni* microarray. DNA fragments of individual open reading frames (ORFs) were amplified by using the Sigma-7 Genosys (The Woodlands, TX) *C. jejuni* ORFmer primer set specific for strain NCTC 11168 coding sequences and by using primers from Operon Technologies (Alameda, CA) specific for unique sequences of strain RM1221, as previously described

(35). A total of 1,530 and 227 PCR products were amplified from strain NCTC 11168 and strain RM1221, respectively. The PCR products were purified with a Qiagen 8000 robot by using a QIAquick 96-well Biorobot kit (Qiagen, Valencia, CA) and spotted in duplicate onto Ultra-GAPS glass slides (Corning Inc., Corning, NY) by using an OmniGrid Accent 17 (GeneMachines, Ann Arbor, MI), as described previously (35). Immediately after printing, the microarrays were UV cross-linked at 300 mJ by using a Stratalinker 1800 UV cross-linker (Stratagene, La Jolla, CA) and stored in a desiccator. Before use, microarrays were blocked with Pronto! prehybridization solution (Corning Inc.) used according to the manufacturer's specifications.

Isolation and fluorescent labeling of chromosomal DNA. Chromosomal DNA was isolated from *C. jejuni* using a Puregene DNA isolation kit (Gentra Systems, BIOzymTC, Landgraaf, The Netherlands) according to the manufacturer's protocol for gram-negative bacteria. For each hybridization reaction reference DNA (equal amounts of chromosomal DNA isolated from *C. jejuni* strains NCTC 11168-O and RM1221) was labeled with indodicarbocyanine (Cy5)-dUTP (GE Healthcare, Piscataway, NJ), and DNA isolated from the test strain was labeled with indocarbocyanine (Cy3)-dUTP (GE Healthcare). For each labeling reaction 2 μg of chromosomal DNA was mixed with 5 μl of 10× NEBlot labeling buffer (New England Biolabs [NEB], Ipswich, MA) and water to obtain a final volume of 41 μl. This mixture was incubated at 95°C for 5 min and then at 4°C for 5 min. When the mixture was cool, the other components of the labeling reaction were added, namely, 5 μl of a 10× deoxynucleoside triphosphate mixture (1.2 mM each of dATP, dCTP, and dGTP and 0.5 mM dTTP; Promega Corporation, Madison, WI), 3 μl of Cy3-dUTP (1 mM) or Cy5-dUTP (1 mM), and 1 μl Klenow fragment (5 U; NEB). After 16 h of incubation at 37°C, the labeled DNA was purified using a Qiagen QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. After purification, the labeled reference DNA and test DNA were mixed and dried with a vacuum.

Microarray hybridization. Hybridization reactions were performed in duplicate for each test strain. Labeled DNA was dissolved in 47 μl of Pronto! cDNA hybridization solution (Corning Inc.) and incubated at 95°C for 5 min. For each hybridization reaction 15 μl of this mixture was put onto a microarray slide. The slide was sealed with a coverslip and placed in a hybridization chamber (Corning Inc.). After incubation at 42°C for 16 h, the slide was rinsed twice with wash solution I (2× saline sodium citrate [SSC], 0.1% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) at 42°C for 10 min, which was followed by two rinses with wash solution II (1× SSC) at room temperature for 10 min and two rinses with wash solution III (0.1× SSC) at room temperature for 10 min. The microarray slide was dried by centrifugation (10 min, 300 × *g*).

Microarray data analysis. Two independent microarray analyses were performed for each strain. Microarrays were scanned and analyzed as previously described (35). Generally, DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Inc., Union City, CA). Features and the local background intensities were detected and quantified with GenePix 4.0 software (Axon Instruments, Inc.). Poor features were excluded from further analysis if they contained abnormalities or were within regions with nonspecific fluorescent background. The data were filtered so that spots with a reference signal less than the background signal plus three standard deviations of the background signal were discarded. Signal intensities were corrected by subtracting the local background, and then the Cy5/Cy3 (test/reference) ratios were calculated. To compensate for unequal dye incorporation, data normalization was performed as described previously (1, 35). The NCTC 11168 and RM1221 strain-specific spots hybridized to only-half of the reference DNA (the Cy5-labeled mixture of NCTC 11168-O and RM1221 DNA), increasing the Cy3/Cy5 ratio twofold. The ratios for these spots were therefore divided by two before the status of the gene was determined. The ratios for spots for each individual gene were then averaged. As previously described (35), the comparative genomic indexing analysis defined the status of a gene as present when the Cy3/Cy5 intensity ratio was >0.6, as divergent when the Cy3/Cy5 intensity ratio was between 0.6 and 0.3, and as absent when the Cy3/Cy5 intensity ratio was <0.3. The presence, divergence, and absence status data for all genes were converted into trinary scores (2, present; 1, divergent; 0, absent). The trinary gene scores for each replicate for all strains were analyzed further with GeneSpring microarray analysis software, version 7.3 (Agilent Technologies, Redwood City, CA), and subjected to average-linkage hierarchical clustering with the standard correlation and bootstrapping. The comparative genomic indexing analysis to assign present and absent genes of the *C. jejuni* strains was further verified using the GENCOM software (36, 37). For each of these hybridizations, the Cy5 and Cy3 signal intensities were corrected by subtracting the local background before submission to the GENCOM program for determination of the presence or absence assignments for each gene.

Electrotransformation of *C. jejuni*. *C. jejuni* strains that are not naturally competent can be forced to accept DNA by using electrotransformation. This approach allowed us to generate transformants using plasmid (or chromosomal) DNA from different sources and for various purposes, including introduction of resistance markers, generation of knockout mutants, or introduction of resistance markers on replicating plasmids. Electrotransformation of *C. jejuni* was carried out essentially as described previously (49). In brief, electrocompetent bacteria were prepared from *C. jejuni* grown on HIS plates for 16 h. The bacteria were harvested and washed four times with 15% glycerol–272 mM sucrose (4°C). Finally, the suspension was adjusted to an optical density at 600 nm of 80. Aliquots (50 μl) were stored at −80°C. For electrotransformation 5 μl of isolated plasmid DNA was added to 50 μl of electrocompetent bacteria. The mixture was transferred to a 0.2-cm Gene Pulser cuvette (Bio-Rad, Hercules, CA) and pulsed (2.48 kV, 25 μF, 600 Ω) with a Gene Pulser (Bio-Rad). After electroporation the bacteria were allowed to recover on nonselective saponin plates for 4 h at 37°C under microaerobic conditions. After recovery, the bacteria were harvested and plated onto selective saponin plates.

Construction of a *dns* knockout strain. The *dns* gene (CJE0256) was amplified from the chromosome of *C. jejuni* RM1221 using primers dns-F and dns-R (Table 2) and a *Taq* polymerase with proofreading. The PCR product (672 bp) was cloned into the cloning vector pCR2.1 (Invitrogen, Breda, The Netherlands), resulting in plasmid p*dns*. Subsequently, the complete p*dns* plasmid (4.6 kb) was amplified with primers dnsSacII and dnsMfeI (Table 2) to introduce SacII and MfeI restriction sites in the *dns* coding sequence. Next, primers catSacII and catMfeI (Table 2) were used to generate a 1,038-bp PCR product containing the *Campylobacter coli cat* gene (chloramphenicol acetyltransferase; Cm^r) from pUOA23. Both PCR products were digested with SacII and MfeI to allow insertion of the *cat* gene into *dns*. The resulting knockout construct, p*dns*::*cat* (5.6 kb), was introduced into *dns*$^+$ *C. jejuni* strain C356 via electrotransformation, resulting in C356*dns*::*cat*. Integration into the chromosome was verified by PCR using chromosomal DNA as the template with primers dnscontr-F and dnscontr-R. These primers are complementary to sequences of the disrupted *dns* gene upstream and downstream of the *cat* insert (Table 2).

Construction of the expression plasmid carrying Dns. To construct a *dns* expression plasmid, the *dns* gene (CJE0256) along with 22 bp of its 5′ flanking region was amplified from chromosomal DNA of *C. jejuni* RM1221 using primers dnsexpr-F and dnsexpr-R (Table 2), which introduced XbaI and MfeI restriction sites. To introduce the same restriction sites into the kanamycin-resistant expression plasmid pWM1007Pr1492 (52), a PCR product was generated using primers Pr1492MfeI and Pr1492XbaI (Table 2). After digestion with XbaI and MfeI, *dns* (704 bp) was ligated into pWM1007Pr1492 (10.6 kb) to form pWM1007Pr1492*dns* (11.3 kb). The resulting *C. jejuni* Dns expression plasmid was electrotransformed into the *dns*-negative *C. jejuni* strain CNET002, resulting in CNET002(pWM1007Pr1492*dns*).

Natural transformation frequency of *C. jejuni*. For natural transformation of *C. jejuni*, the biphasic method was used (46). In brief, *C. jejuni* grown for 16 h on HIS plates was harvested in HI broth (optical density at 600 nm, 1.0), and 200 μl of the suspension was added to a 5-ml polystyrene tube containing 2 ml of HI agar. After 3 h of incubation at 37°C under microaerobic conditions, 2 μg of a homologous plasmid (pUOA13; Km^r) or homologous chromosomal DNA isolated from strains containing a chloramphenicol resistance cassette in *hipO* (i.e., homologous to the recipient strain) was added. For each strain the type of DNA used was dependent on the availability and combination of antibiotic resistance markers. After additional incubation (3 h, 37°C) under microaerobic conditions, the bacteria were harvested from the polystyrene tube and collected by centrifugation (3,300 × *g*, 8 min). The pellet was resuspended in 400 μl of HI broth. For determination of natural transformation frequencies, the number of CFU ml^{-1} was calculated using the track dilution technique (24), in which 10-μl portions of serial dilutions (10^{-5} to 10^{-8}) were spotted onto nonselective saponin plates. To determine the number of transformants per ml, 200-μl portions of the appropriate serial dilutions were plated onto selective saponin plates. After incubation at 37°C, the numbers of colonies present on the plates were determined. The natural transformation frequency was calculated by dividing the number of transformants ml^{-1} (selective plates) by the total number of bacteria ml^{-1} (nonselective plates). The resulting natural transformation frequency specified the fraction of bacteria that acquired a resistance marker.

DNase assay. For measurement of DNase activity, *C. jejuni* was grown in 200 ml of HI broth under microaerobic conditions (16 h, 37°C, 160 rpm) and used to isolate fractions enriched for periplasmic proteins, as described previously (25). Protein concentrations of the fractions were determined using Coomassie Plus protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. For the DNase assay a 30-μl reaction mixture containing 500 ng of lambda DNA (500 μg ml^{-1}; NEB), 2 μl of 100 mM $MgCl_2$, and 1 μg of protein

TABLE 2. Primers and plasmids used in this study

Primer or plasmid	Sequence (5′–3′) or relevant characteristics[a]	Source or reference
Primers		
dns-F	ATGAAAAAAATAATAAG CGTTTTAATAC	
dns-R	TTAGAGTAATGCTCTAAT TCTTTTTTC	
dnsSacII	CATATGCCGCGGGGCTC CTACTAATTTAAAAT ATAC	
dnsMfeI	GTCGACCAATTGTCAGC ATATCTAAAATTGCTTC TATC	
catSacII	CATATGCCGCGGCACAA CGCCGGAAACAAG	
catMfeI	GTCGACCAATTGCCGCA GGACGCACTACTCT	
dnscontr-F	CTTTTAAGATTTCTCGTT TGTCG	
dnscontr-R	CAAGCCTTGAAATAATG CATAATG	
dnsexpr-F	AGATCTTCTAGATAAAA AATAAAAAGGAGAATA AATG	
dnsexpr-R	CCATGGCAATTGTTAGA GTAATGCTCTAATTC	
Pr1492MfeI	ACTAGTCAATTGGCGAT GGCCCTG	
Pr1492XbaI	AGATCTTCTAGACTCATT TAACGGTTGTCTCC	
Plasmids		
pCR2.1	TA cloning vector, Amp^r	Invitrogen
p*dns*	pCR2.1 containing the *dns* gene of *C. jejuni* strain RM1221	This study
pUOA23	*E. coli*-*C. jejuni* shuttle vector containing the *cat* gene of *C. coli*, Cm^r	
p*dns*::*cat*	p*dns* with *cat* inserted in *dns*, Cm^r	This study
pWM1007Pr1492	*C. jejuni* expression plasmid containing the Cj1492 promoter, Km^r	52
pWM1007Pr1492*dns*	*C. jejuni* expression plasmid with *dns*	This study
pUOA13	*E. coli*-*C. jejuni* shuttle vector, Km^r	46

[a] The endonuclease restriction sites introduced into the sequences are underlined.

from enriched fractions was assembled. After incubation for 1 h at 37°C, 6 μl of 6× blue/orange loading dye (Promega Benelux BV, Leiden, The Netherlands) was added, and DNase activity was analyzed by electrophoresis using a 0.8% agarose gel.

RESULTS

Hierarchical clustering of *C. jejuni* strains by comparative genome hybridization. In the search for differences in genome content between naturally transformable and nonnaturally transformable *C. jejuni* strains, comparative genome hybridization was used. A multistrain *C. jejuni* microarray was designed that comprised PCR fragments of 1,530 ORFs (94%) of the naturally transformable strain NCTC 11168 and 227 unique ORFs of the nonnaturally transformable strain RM1221. During validation of this microarray by Parker et al. (35), the chromosomal DNA of strains NCTC 11168 and RM1221 hybridized equally well to the ORFs common to both strains, and the results also correlated with the results of sequence analysis. In the present study, the array was hybridized with chromosomal DNA isolated from six naturally transformable and 20 nonnaturally transformable *C. jejuni* strains, as well as control strains NCTC 11168-O (naturally transformable) and RM1221 (nonnaturally transformable). Of the naturally transformable strains, five were Penner serotype HS:1 or HS:2 strains, while one was an HS:19 strain. The 20 nonnaturally transformable strains belonged to Penner serotypes HS:1, HS:2, HS:19, HS:41, and HS:55 (Table 1). The hybridization data obtained were analyzed, and the status of a gene (present, absent, or divergent) was defined using comparative genomic indexing. This served as a basis for hierarchical clustering of the strains.

As shown in Fig. 1, distinct clusters were formed by strains belonging to Penner serotypes HS:55, HS:19, HS:41, and HS:2, while the HS:1 strains were not located in a single cluster. Based on the distance scores, the genetic diversity within the Penner HS:41 cluster (all South African isolates) was less than that within the clusters formed by the HS:55, HS:19, and HS:2 strains. No difference in the level of diversity between these clusters was noted. The HS:1 strains were the most diverse subset of strains used in this study. As in this analysis the naturally transformable and nonnaturally transformable strains did not appear to be distinct clusters, no indication of genes that are restricted to one of these phenotypes was obtained.

***C. jejuni* competence genes.** Closer inspection of the hybridization data indicated that all the chromosomal genes thus far implicated in the process of natural transformation in *C. jejuni* were present in the 26 strains analyzed (Table 3). To identify novel genes thus far not associated with natural transformation, the hybridization data were screened for genes present in ≥50% of the naturally transformable strains and absent in ≥50% of the nonnaturally transformable strains. A total of 64 genes were present in the majority (67% to 100%) of the naturally transformable strains and absent in more than one-half (50% to 80%) of the nonnaturally transformable strains (see Table S2 in the supplemental material). Fifty-nine of these genes are located in intraspecies hypervariable regions 1, 6 to 14, and 16 of the *C. jejuni* genome (35, 42). In a second analysis the hybridization data were screened for genes absent in ≥50% of the naturally transformable strains and present in ≥50% of the nonnaturally transformable strains. A total of 37 genes were absent in most (67% to 83%) of the naturally transformable strains and present in more than one-half (55% to 90%) of the nonnaturally transformable strains (Table 4). Five of these genes are located in intraspecies hypervariable regions 13, 14, and 17 of the *C. jejuni* genome (35), but the majority (32 genes) hybridized with genes of *C. jejuni* integrated element 1 (CJIE1) present in *C. jejuni* strain RM1221 (15).

Identification of a DNase-encoding gene. Because of the remarkable difference in occurrence of CJIE1 genes between naturally transformable and nonnaturally transformable *C. jejuni* strains, CJIE1 was studied in more detail. CJIE1 encodes several proteins with similarity to bacteriophage Mu and Mu-like prophage proteins (15, 32). Of the 32 CJIE1 genes of interest for this

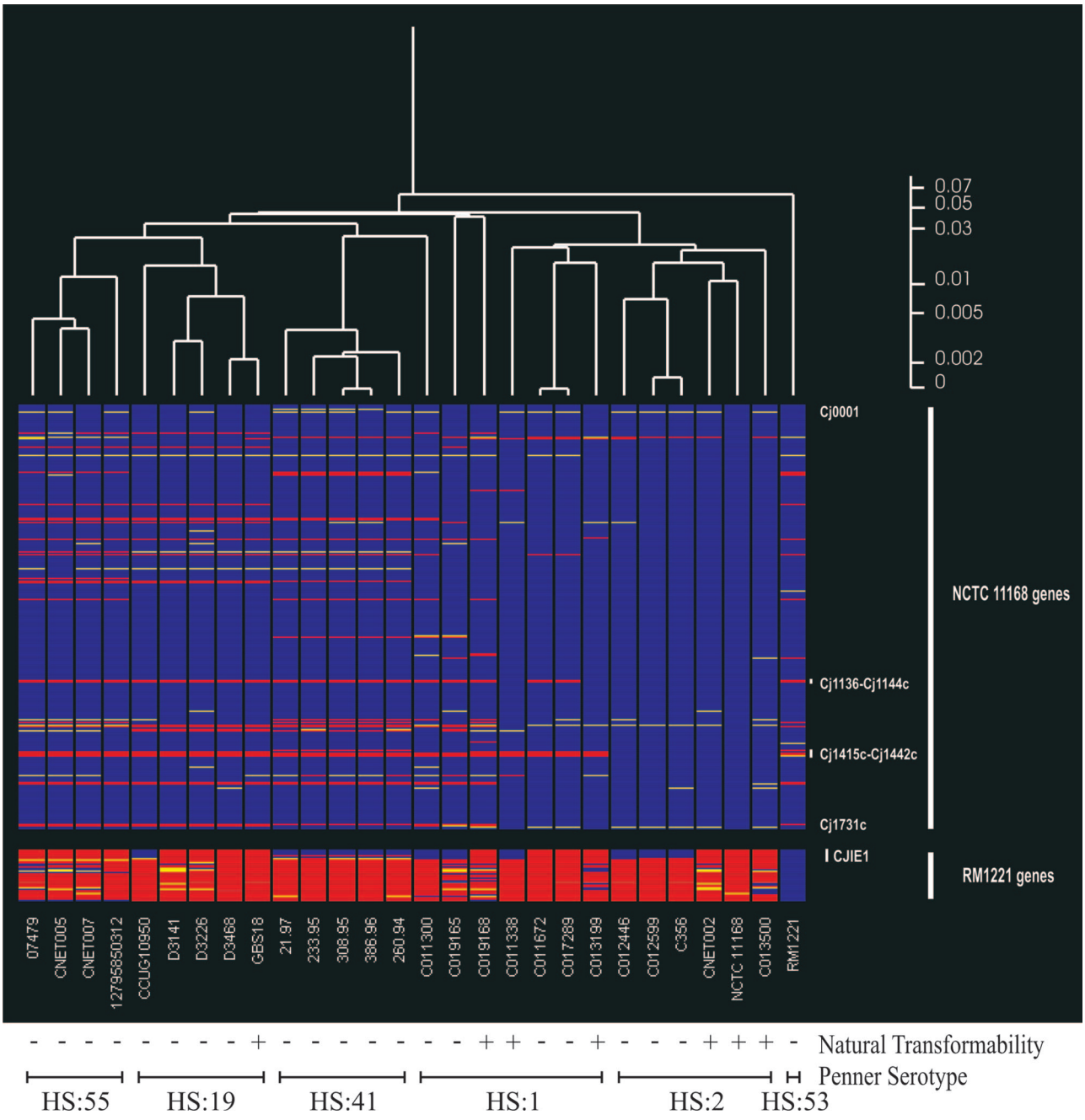

FIG. 1. Comparison of *C. jejuni* strains by cluster analysis of comparative genomic indexing results. The genes are presented in the order of their positions on the genome of *C. jejuni* NCTC 11168 and are followed by the genes of *C. jejuni* RM1221 that are absent from *C. jejuni* NCTC 11168. For frames of reference, the lipooligosaccharide biosynthesis locus (Cj1136-Cj1144c) and the capsular biosynthesis locus (Cj1415c-Cj1442c) from *C. jejuni* NCTC 11168 are indicated. The Mu-like prophage insertion element (CJIE1) from *C. jejuni* RM1221 is also indicated. The gene status is color coded as follows: blue, present; yellow, variable or unknown; red, absent; gray, no data. For cutoffs for absence and presence predictions, see Materials and Methods. An average linkage hierarchical clustering of the *C. jejuni* strains was compiled in GeneSpring, version 7.3, from the comparative genome hybridization data for each element with the standard correlation and bootstrapping. A scale for distance scores is on the right.

study, 16 are annotated as genes encoding phage-related proteins, 12 are annotated as genes encoding hypothetical proteins, and 3 are annotated as genes encoding conserved domain proteins (Table 4). The remaining gene has been annotated as a gene encoding an extracellular DNase (CJE0256; *dns*) on the basis of its similarity at the protein level with *dns* from *Aeromonas hydrophila*. The translated sequence of *dns* from *C. jejuni* strain RM1221 contains a domain typical of endonuclease I proteins, which are bacterial secreted or periplasmic endonucleases. In agreement with this is the prediction of a signal peptide cleavage site in the amino acid sequence of Dns (SignalP 3.0) (3).

To investigate the function of Dns in more detail, several

TABLE 3. *C. jejuni* natural competence and transformation genes

Locus tag	Gene	Protein	Reference
Cj0011c		Putative nonspecific DNA binding protein	23
Cj0634	*dprA*	SMF family protein	43
Cj0825		Putative processing peptidase	51
Cj1028c		Possible purine/pyrimidine phosphoribosyltransferase (*ctsW*)	51
Cj1076	*proC*	Pyrroline-5-carboxylate reductase	51
Cj1077	*ctsT*	Putative periplasmic protein	51
Cj1131c	*galE*	UDP-glucose 4-epimerase	16
Cj1343c		Putative periplasmic protein (*ctsG*)	51
Cj1352	*ceuB*	Enterochelin uptake permease	51
Cj1470c		Pseudogene (type II protein secretion system F protein) (*ctsF*)	51
Cj1471c	*ctsE*	Putative type II protein secretion system E protein	51
Cj1472c		Hypothetical protein (*ctsX*)	51
Cj1473c	*ctsP*	Putative ATP/GTP-binding protein	51
Cj1474c	*ctsD*	Putative type II protein secretion system D protein	51
Cj1475c	*ctsR*	Hypothetical protein	51

mutant strains were constructed using electrotransformation (see Materials and Methods). The *dns* gene was inactivated in the nonnaturally transformable *C. jejuni* strain C356 via insertion of a chloramphenicol resistance cassette. The inactivated gene located on a suicide plasmid was introduced into strain C356 by electrotransformation, yielding the *dns* knockout mutant C356*dns*::*cat*. Transformants carried the inactivated gene in their genomes, indicating that the process of recombination was functional in this nonnaturally transformable strain. The function of Dns was then assessed by a DNase assay in which the ability of the strains to hydrolyze phage lambda DNA was determined. As shown in Fig. 2, the *dns*$^+$ C356 wild-type strain hydrolyzed DNA (lane 1). This activity was not observed for C356*dns*::*cat* (Fig. 2, lane 2). These results suggest that Dns indeed is critical for *C. jejuni* endonuclease activity.

Since the availability and possible combinations of antibiotic resistance markers did not allow us to complement inactivated *dns* in strain C356*dns*::*cat*, we introduced *dns* of RM1221 into the naturally transformable *dns*-negative *C. jejuni* strain CNET002. This gene was placed on expression plasmid pWM1007Pr1492, which was introduced by electrotransformation into strain CNET002. As a control, the same vector but without the insert was introduced into this strain. Analysis of isolated fractions of the different strains for DNase activity indicated the presence of DNA-hydrolyzing activity for the strain with the *dns*$^+$ plasmid (Fig. 2, lane 4) but not for the parental strain or the control strain possessing the expression plasmid without the insert (Fig. 2, lanes 3 and 5).

Inhibition of natural transformation by Dns. To investigate the effect of Dns activity on the process of natural transformation, we determined the natural transformation frequencies for the *dns*$^+$ strain C356 and its *dns* mutant derivative C356*dns*::*cat*, using the biphasic method of natural transformation (Table 5). The potential effect of restriction-modification systems was avoided by using homologous plasmid DNA which conferred kanamycin resistance. While parental strain C356 yielded no kanamycin-resistant transformants, inactivation of *dns* in C356*dns*::*cat* resulted in a change of phenotype from nonnaturally transformable to naturally transformable with a natural transformation frequency of $(2.4 \pm 0.6) \times 10^{-6}$ (Table 5).

Natural transformation frequencies were also determined for the *dns*-negative *C. jejuni* wild-type strain CNET002 and its derivatives carrying the expression plasmid pWM1007Pr1492 with or without the *dns* gene, using homologous chromosomal DNA as a donor (CNET002*hipO*::*cat*). The donor DNA used contains a chloramphenicol resistance marker obtained through electrotransformation with the suicide plasmid pHipO::cat (8). In this experiment, expression of Dns in the naturally transformable strain CNET002 caused a 3-log reduction in the natural transformation frequency compared to that of the parental control strains (Table 5). No reduction in natural transformation frequency was observed for strain CNET002 that contained the empty expression vector. Altogether, these results indicate that the presence of Dns impairs natural transformation in *C. jejuni*.

Prevalence of DNase activity in naturally transformable and nonnaturally transformable *C. jejuni* strains. To assess the broader importance of Dns as a determining factor of natural transformation in *C. jejuni*, we determined the DNase activity for a set of naturally transformable strains. None of the naturally transformable strains hydrolyzed DNA (Fig. 3, lanes 1 to 5). One of the strains (C011338) does contain *dns*, but sequence analysis and comparison with the RM1221 *dns* sequence revealed a point mutation in the start codon (ATG → ATA), which is probably responsible for the absence of DNase activity in C011338.

All of the tested nonnaturally transformable *dns*$^+$ strains displayed DNase activity (Fig. 3, lanes 6 to 10), except the five strains belonging to Penner serotype HS:41 (lanes 11 to 15). Reverse transcription-PCR analysis indicated that *dns* is transcribed in these strains (data not shown). However, alignment of the *dns* sequence (locus tag CJJ26094_0510) from Penner HS:41 strain 260.94 with the *dns* sequence from strain RM1221 revealed nine point mutations, resulting in four amino acid substitutions (e.g., a C → Y substitution), which may explain the lack of DNase activity.

Analysis of the nonnaturally transformable *dns*-negative strains for the presence of DNA-hydrolyzing activity revealed DNase activity for five of the nine nonnaturally transformable *dns*-negative strains (Fig,. 4, lanes 3, 4, 6, 7, and 9). This indicates that a different, as-yet-undefined DNase is present in a subset of the *dns*-negative strains, which may account for the nonnatural transformability of these strains. These results underpin the importance of DNase activity as a major determinant of natural transformation of *C. jejuni*.

DISCUSSION

C. jejuni is one of the bacterial species which are known to be naturally competent (28). Naturally transformable *C. jejuni* strains are able to acquire DNA from the environment and integrate this genetic material into their genomes. A subset of *C. jejuni* strains, however, appears to be deficient in natural transformation. The present study provides evidence that 11 of 20 strains that are naturally transformation deficient express DNase activity. Genetic transfer of the identified *dns* gene, which encodes DNase activity, to a naturally transformable strain results in strongly reduced natural transformability. Con-

TABLE 4. Genes absent in at least 67% of the naturally transformable *C. jejuni* strains and present in the majority (≥55%) of nonnaturally transformable strains

Region	Locus tag	Product	Locus	Presence in *C. jejuni* strains									
				Naturally transformable						Nonnaturally transformable			
				Penner serotype HS:1			Penner serotype HS:2		Penner serotype HS:19	Penner serotype HS:1			
				C013199	C011338	C019168	C013500	5003 (CNET002)	GB18	C011300	C017289	C019165	C011672
CJIE1[a]	CJE0215	Phage repressor protein, putative		−	+	−	−	−	+	+	−	+	−
	CJE0220	Adenine-specific DNA methyltransferase	*dam*	−	+	−	−	−	+	+	−	+	−
	CJE0221	Phage virion morphogenesis protein, putative		−	+	−	−	−	−	+	−	+	−
	CJE0226	Phage-related tail protein		−	+	−	−	−	−	+	−	+	−
	CJE0227	Tail sheath protein		−	+	−	−	−	−	+	−	+	−
	CJE0228	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0230	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0231	Putative phage tail fiber protein H		−	+	−	−	−	−	+	−	+	−
	CJE0232	Putative phage tail protein		−	+	−	−	−	−	+	−	+	−
	CJE0233	Baseplate assembly protein J, putative		−	+	−	−	−	−	+	−	+	−
	CJE0234	Baseplate assembly protein W, putative		−	+	−	−	−	−	+	−	+	−
	CJE0236	Baseplate assembly protein V, putative		−	+	−	−	−	−	+	−	+	−
	CJE0237	Conserved hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0241	Conserved hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0243	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0244	Mu-like prophage I protein, putative		−	+	−	−	−	−	+	−	+	−
	CJE0245	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0246	Conserved hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0247	Conserved domain protein		−	+	−	−	−	−	+	−	+	−
	CJE0248	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0249	Phage uncharacterized protein, C-terminal domain, putative		−	+	−	−	−	−	+	−	+	−
	CJE0250	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0252	Putative tail-related phage protein		−	+	−	−	−	−	+	−	+	−
	CJE0254	Tail protein D, putative		−	+	−	−	−	−	+	−	+	−
	CJE0256	Extracellular DNase	*dns*	−	+	−	−	−	−	+	−	+	−
	CJE0257	Conserved domain protein		−	+	−	−	−	−	+	−	+	−
	CJE0258	Conserved domain protein		−	+	−	−	−	−	+	−	+	−
	CJE0259	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0261	Hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0265	Host nuclease inhibitor protein Gam, putative		−	+	−	−	−	−	+	−	+	−
	CJE0266	Conserved hypothetical protein		−	+	−	−	−	−	+	−	+	−
	CJE0269	Bacteriophage DNA transposition protein B, putative		−	+	−	−	−	−	+	−	+	−
Variable region 17[b]	CJE0310	Adenine-specific DNA methyltransferase		−	+	−	−	−	−	+	−	+	−
Variable region 14[c]	CJE1725	4-Carboxymuconolactone decarboxylase, putative		−	−	+	−	−	+	+	−	+	−
	CJE1727	Conserved hypothetical protein		−	−	+	−	−	+	+	−	+	−
	CJE1728	Transporter, putative		−	−	+	−	−	+	+	−	+	−
Variable region 13[d]	Cj1442c			−	−	−	+	+	−	−	−	+	−

[a] CJIE1/CMLP1 (CJE0213 to CJE0273) (15).
[b] Cj0258 to Cj0263 and CJE0308 to CJE0313 (35).
[c] Cj1543c to Cj1563c and CJE1714 to CJE1734 (restriction-modification locus) (35).
[d] Cj1414c to Cj1449c and CJE1601 to CJE1622 (capsular locus) (35).

versely, inactivation of the gene in a nonnaturally transformable *C. jejuni* strain renders the strain competent for natural transformation. These results, for the first time, provide a genetic basis for the lack of natural transformation of *C. jejuni* strains and provide the opportunity to manipulate the system to our benefit. The presence of DNase activity may contribute to the appearance of subsets of *C. jejuni* strains as stable lineages in genetic typing studies.

TABLE 4—*Continued*

Presence in *C. jejuni* strains															
Nonnaturally transformable															
Penner serotype HS:2			Penner serotype HS:19				Penner serotype HS:41					Penner serotype HS:55			
C012446	C012599	C356 (CNET076)	D3141	D3226	CCUG10950	D3468	21.97	233.95	260.94	308.95	386.96	40707L (CNET007)	07479	12795850312	41239B (CNET005)
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
+	+	+	−	−	+	−	+	+	+	+	+	−	−	−	−
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+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+

Our strategy to discover determinants of natural transformation involved comparative genome hybridization using whole-genome ORF-based DNA arrays. This approach initially resulted in genomic clustering of the investigated strains only according to Penner serotype rather than according to competence for natural transformation (Fig. 1). The formation of clusters for the Penner HS:19, HS:41, and HS:55 strains is in agreement with the results obtained by other workers (20, 34, 48). Specific analysis of the hybridization data for the absence or presence of previously identified genes involved in natural transformation of *C. jejuni* (16, 23, 43, 51) also did not discriminate between the tested naturally transformable and nonnaturally transformable strains; all chromosomally located competence genes identified so far (Table 3) appeared to be present

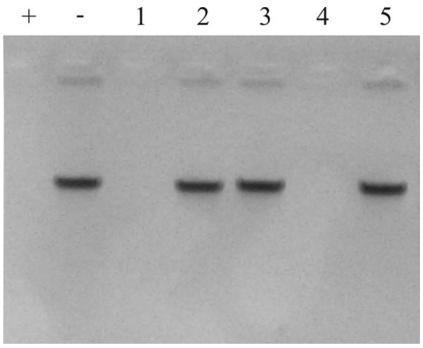

FIG. 2. Determination of the DNase activity of a *C. jejuni dns* knockout mutant (C356*dns*::*cat*) and a Dns expression construct [CNET002-(pWM1007Pr1492*dns*)]. Phage lambda DNA (500 ng) was incubated (1 h, 37°C) with fractions isolated from *C. jejuni* and analyzed by agarose gel electrophoresis. Lane 1, nonnaturally transformable *dns*-positive strain C356; lane 2, *dns* knockout strain C356*dns*::*cat*; lane 3, naturally transformable strain CNET002; lane 4, CNET002 with Dns expression construct [CNET002-(pWM1007Pr1492*dns*)]; lane 5, CNET002 containing the empty expression plasmid [CNET002(pWM1007Pr1492)]; lane +, positive control (DNA treated with 1 μg DNase I); lane −, negative control (mock-treated DNA).

in all strains tested. The functionality of these genes could obviously not be established from the results of the DNA array analysis.

Clear genetic differences between the sets of naturally transformable and nonnaturally transformable strains were observed when the hybridization data sets were analyzed on the basis of genes present in ≥50% of the naturally transformable strains and absent in ≥50% of the nonnaturally transformable strains and vice versa (Table 4; see Table S2 in the supplemental material). These settings yielded a remarkable distinction for the presence of CJIE1-encoded genes in nonnaturally transformable *C. jejuni* strains compared to naturally transformable strains. CJIE1 is one of the four large integrated elements present in strain RM1221 but absent in strain NCTC 11168 (15). The majority of genes located on CJIE1 encode proteins with similarity to bacteriophage Mu and Mu-like prophage proteins (15, 32) or code for hypothetical proteins. However, one gene (*dns*; CJE0256) is annotated as a gene encoding an extracellular DNase. In our data sets *dns* was always found in combination with other CJIE1 genes.

At the protein level *C. jejuni* Dns displays considerable sequence similarity with other bacterial nucleases, e.g., Dns of *Vibrio cholerae* (14), EndA of *E. coli* (22), Dns of *A. hydrophila* (5), NucM of *Erwinia chrysanthemi* (33), and Vvn of *Vibrio vulnificus* (53). In addition, Dns of *C. jejuni* is predicted to contain a domain typical of endonuclease I proteins. In general, members of this class of enzymes hydrolyze DNA and are located in the periplasm or secreted into the environment. This corresponds with the prediction of a signal peptide cleavage site in Dns and the observation by Hänninen (19) that disruption of the *Campylobacter* cell envelope by polymyxin B treatment accelerates DNA hydrolysis. On the basis of these characteristics, we hypothesized that the Dns protein may act as an inhibitor of natural transformation in *C. jejuni*.

Functional evidence that the identified *dns* gene encodes DNase activity was obtained from DNase assays performed with a genetically defined *dns* knockout mutant and a *C. jejuni* strain in which Dns of RM1221 was expressed from a plasmid (Fig. 2). Natural transformation assays with these strains further demonstrated that inactivation of *dns* in a nonnaturally transformable strain changed the phenotype into a naturally transformable phenotype and that the acquisition of DNase activity results in a strong reduction in natural transformability (Table 5). Homologous plasmid DNA and homologous chromosomal DNA were used to determine the natural transformability of these strains, depending on the availability of antibiotic resistance markers. In our hands naturally transformable strains can be naturally transformed with both homologous plasmid and chromosomal DNA, in contrast to nonnaturally transformable strains, suggesting that the system for natural transformation does not differentiate between plasmid and chromosomal DNA.

The significance of DNase activity as a cause of a lack of natural competence was further underpinned by the absence of DNase activity in all of the naturally transformable strains tested and the presence of DNase activity in more than one-half of the nonnaturally transformable *C. jejuni* strains, including a subset of strains that do not contain the *dns* gene (Fig. 3 and 4). The latter finding indicates that some strains contain another (unidentified) nuclease that may account for their apparent defect in natural transformation.

A special group of strains in our data set is the five Penner serotype HS:41 strains that were analyzed. These strains, all of which originate from South Africa, carry the CJIE1 element, including *dns* (Table 4). These strains are nonnaturally transformable but lack DNase activity (Fig. 3). This suggests that this group of strains may carry a defect elsewhere in the DNA transformation mechanism, similar to the group of nonnaturally transformable strains that show no DNase activity. Activity of restriction-modification systems is unlikely to be the cause of the lack of transformation as we used donor DNA derived from the homologous *C. jejuni* strain in the natural transformation assays. The cause of inactivity of Dns in these strains is not known. For Vvn, the Dns homologue of *V. vulnificus*, it is known that disulfide bridges are required for activity (53). The noted change of one cysteine residue to tyrosine in

TABLE 5. Effect of Dns on natural transformation frequencies of *C. jejuni*

Strain[a]	Natural transformation frequency[b]
C356	–
C356*dns*::*cat*	$(2.4 \pm 0.6) \times 10^{-06}$[c]
CNET002	$(2.5 \pm 0.2) \times 10^{-05}$
CNET002(pWM1007Pr1492)	$(2.7 \pm 1.4) \times 10^{-05}$
CNET002(pWM1007Pr1492*dns*)	$(2.1 \pm 1.8) \times 10^{-08}$[c]

[a] To determine the natural transformation frequency of *C. jejuni* strains C356 and C356*dns*::*cat*, homologous pUOA13 plasmid DNA (kanamycin resistance marker) was used; for strains CNET002, CNET002(pWM1007Pr1492), and CNET002(pWM1007Pr1492*dns*) homologous chromosomal DNA was used (chloramphenicol resistance marker).

[b] The natural transformation frequency was determined by dividing the number of transformants ml^{-1} by the total number of CFU per ml^{-1}. The values are the averages of three experiments. −, below the detection limit ($5.6 \times 10^{-10} \pm 5.0 \times 10^{-10}$), which is based on a minimum of one colony per plate, adapted to the volumes used in the procedure.

[c] $P < 0.05$ (Student's t test) for a comparison with the corresponding parental strain.

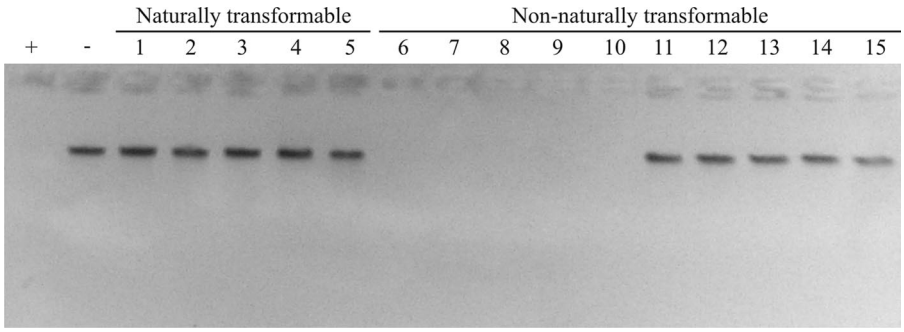

FIG. 3. Determination of the DNase activity of naturally transformable *C. jejuni* strains and *dns*-positive nonnaturally transformable strains. DNase activity was assayed as described in the legend to Fig. 2. Fractions were isolated from naturally transformable *C. jejuni* strains C013199, C011338, C019168, C013500, and GB18 (lanes 1 to 5, respectively) and from *dns*-positive nonnaturally transformable *C. jejuni* strains C011300, C019165, C012446, C012599, CCUG10950, 21.97, 233.95, 260.94, 308.95, and 386.95 (lanes 6 to 15, respectively). Lane +, positive control (DNA treated with 1 μg DNase I); lane −, negative control (mock-treated DNA).

Dns of *C. jejuni* strain 260.94 could be responsible for the lack of DNase activity.

The presence of DNA-hydrolyzing activity in *C. jejuni* has previously been observed (21, 38) and is in fact one of the criteria for Lior's extended biotyping scheme for thermophilic campylobacters (27). Indeed, analysis of our set of strains with the Lior DNA hydrolysis biotyping method, using agar with 0.005% toluidine blue and a polymyxin B treatment (19), yielded results similar to those of our DNase assays (data not shown). The present identification of Dns in a subset of these strains provides for the first time a genetic basis for the DNA hydrolysis component of Lior's extended biotyping method. The presence of Dns may also explain the previously observed DNA hydrolysis activity present in some *C. jejuni* strains, although in that study no clear correlation was found between DNase activity and competence (46).

Although Dns inhibits natural transformation in *C. jejuni*, Dns^+ strains could be transformed via electrotransformation. During electrotransformation bacteria are exposed to an electric field, resulting in the formation of transient pores in the membranes, through which DNA enters the cytoplasm (40). The pores are only short lived, implying that DNA that has reached the cytoplasm is briefly exposed to the periplasm and its nucleases. If DNA uptake takes more time during natural transformation, this may explain why nonnaturally transformable Dns^+ strains can be electrotransformed.

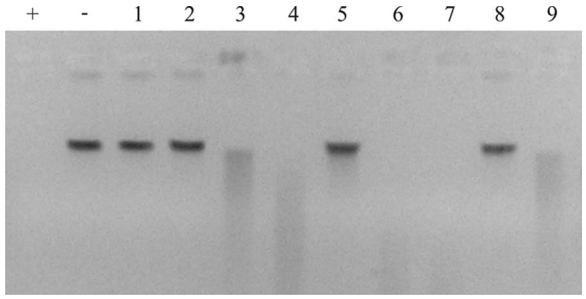

FIG. 4. Determination of the DNase activity of nonnaturally transformable *dns*-negative *C. jejuni* strains. DNase activity was assayed as described in the legend to Fig. 2. The nonnaturally transformable *dns*-negative strains tested were C017289, C011672, D3141, D3226, D3468, CNET007, 07479, 12795850312, and CNET005 (lanes 1 to 9, respectively). Lane +, positive control (DNA treated with 1 μg DNase I); lane −, negative control (mock-treated DNA).

The inhibition of natural transformation of *C. jejuni* through Dns likely affects the frequency of HGT within the species *C. jejuni*. The finding that the *dns* gene is located in the integration element CJIE1 may imply that insertion of this element may have contributed to the development of relatively stable clonal lineages. The strains may still acquire genes via conjugative plasmids that are present in some *C. jejuni* strains (39, 44). However, the presence of relatively stable genetic lineages suggests that under natural conditions this mode of gene transfer may be limited. In the laboratory, induction of loss of CJIE1 by, e.g., mitomycin C treatment (15) or targeted inactivation of *dns*, as used in the present study, may be a valuable new asset for restoring natural transformation in genetically poorly accessible strains, such as strain RM1221.

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