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Role of the *Campylobacter jejuni* Cj1461 DNA Methyltransferase in Regulating Virulence Characteristics▽†

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Mutation of the cj1461 predicted methyltransferase gene reduced the motility of *Campylobacter jejuni* 81-176. Electron microscopy revealed that the mutant strain had flagella but with aberrant structure. The Δcj1461 mutant was sevenfold more adherent to but 50-fold less invasive of INT-407 human epithelial cells than the wild type.

Campylobacter jejuni is a gram-negative bacterium and one of the most common bacterial causes of gastroenteritis worldwide (38). Major symptoms of *Campylobacter* infection include diarrhea, abdominal cramping, and fever, although most infections are self-limiting (27). Sources of infection include undercooked poultry meat, raw milk, and environmental water (9).

In bacteria, the N^6 position of adenine and C^5 or N^4 position of cytosine can be methylated by DNA methyltransferases (MTases) (35). While DNA MTases are often involved in restriction-modification (R-M) systems (4), certain MTases, such as DNA adenine methylase (Dam), affect bacterial housekeeping functions, such as DNA replication and mismatch repair (23). Furthermore, Dam can also positively or negatively regulate the expression of numerous genes, including those involved in virulence phenotypes, such as motility, adherence to and invasion of host cells, M-cell cytotoxicity, and host colonization (1, 8, 11, 15, 19, 20, 22, 23). Dam affects gene expression by methylating GATC sites in promoter and operator regions, thus modifying interactions with the transcription apparatus and regulatory proteins (23).

In *C. jejuni*, elucidation of the mechanisms of gene regulation and virulence remains incomplete. In particular, a role for gene regulation by a DNA MTase (epigenetic regulation) has not been demonstrated. In this study, we report that mutation of the predicted MTase Cj1461 affects several phenotypes related to virulence, suggesting that epigenetic regulation may play a role in *C. jejuni* pathogenesis.

Cj1461 is a DNA MTase but is not a Dam ortholog. The cj1461 gene is annotated as encoding a "possible DNA methylase" in the genome of *C. jejuni* NCTC 11168 (32), and orthologs are found in all sequenced *Campylobacter* genomes. Cj1461 has the conserved domain FGG (*S*-adenosyl methionine [SAM] binding site) and the DNA MTase active-site motif LYLDPPF (Prosite motif PS00092), which are signatures of N^6-adenine-specific DNA methylases. No endonuclease-encoding gene is found adjacent to the cj1461 gene, suggesting that it is not part of an R-M system (24).

Because Cj1461 is predicted to be an N^6-adenine-specific DNA MTase, we assayed Cj1461 for DNA MTase activity. In *E. coli*, we first overproduced Cj1461 containing an N-terminal His_6 tag and affinity purified Cj1461-His_6 on a nickel column. Purified Cj1461-His_6 was used in an MTase assay (6) using chromosomal DNA from the Δcj1461 mutant (Table 1; also, see below) as a nonmethylated substrate (Fig. 1). Commercially purchased Dam (New England Biolabs, Ipswich, MA) was used as a positive control, with JM110 (*dam* mutant) chromosomal DNA as the substrate. This assay measures the ability of an enzyme to transfer the radiolabeled [^{3}H]methyl group from SAM to substrate DNA. Both Dam and Cj1461 showed MTase activity, confirming sequence predictions that Cj1461 is a DNA MTase (Fig. 1). Control reactions (data not shown) using purified recombinant Cj1253 or Cj0355 failed to detect such activity, indicating that the observed activity was specific for Cj1461. The amount of Cj1461 MTase activity was somewhat lower than that of commercially purchased Dam. This could be due to a variety of factors, including a smaller number of target DNA sequences in the *C. jejuni* chromosome (Dam has >15,000 GATC targets per *Escherichia coli* chromosome), nonoptimized in vitro reaction conditions for Cj1461 activity, or partial Cj1461 enzymatic activity due to the His_6 tag.

Because Cj1461 shares very limited homology with Dam, wild-type *C. jejuni* 81-176 (Table 1) was tested for Dam activity using the GATC-recognizing restriction endonucleases Sau3AI (insensitive to Dam methylation), DpnI (requires Dam methylation), and MboI (inhibited by Dam methylation). 81-176 chromosomal DNA was completely digested with Sau3AI and MboI but was resistant to DpnI digestion (see Fig. S1 in the supplemental material), showing that Dam methylation does not exist in 81-176 and that the sequence specificity of Cj1461 is different from that of Dam.

Generation of cj1461 mutant and complemented strains. To generate a cj1461 mutant, the cj1461 gene was amplified from *C. jejuni* 81-176 genomic DNA using PCR with the primers

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Resistance[a]	Source
Strains			
C. jejuni 81-176	Wild type		21
C. jejuni 81-176 Δcj1461	cj1461 mutant of 81-176	Kan	This study
C. jejuni 81-176 Δcj1461+cj1461	Δcj1461 mutant complemented with cj1461	Kan Cm	This study
C. jejuni 81-176 Δcj1461+*flgI*-cj1463	Δcj1461 mutant complemented with *flgI*-cj1463	Kan Cm	This study
E. coli JM110	*dam* strain		New England Biolabs
Plasmids			
pRY111	*C. jejuni-E. coli* shuttle vector	Cm	36
pBluescript SKII(−)	*E. coli* cloning vector	Amp	Stratagene
pBS-cj1461	cj1461 gene in pBluescript SKII(−)	Amp	This study
pBS-cj1461K	pBS-cj1461 with transposon insertion in cj1461	Amp Kan	This study
pBS-cj0046	Pseudogene cj0046 in pBluescript SKII(−)	Amp	This study
pSAT1000	pBS-cj0046 with *cat* insertion	Amp Cm	This study
pSAT1001	pSAT1000 containing cj1461 gene	Amp Cm	This study

[a] Amp, ampicillin; Kan, kanamycin; Cm, chloramphenicol.

cj1461-F1 and cj1461-R1 (Table 2), and cloned into pBluescript SKII(−) to yield pBS-cj1461. In vitro transposition was performed to inactivate the cj1461 gene using a kanamycin-resistant (Km^r) EZ::TN transposon (Epicentre, Madison, WI) as previously described (31). A plasmid (pBS-cj1461K) containing a transposon insertion after nucleotide 370 of the cj1461 gene was introduced into *C. jejuni* 81-176 via natural transformation-mediated allelic replacement. Numerous transformants were selected on kanamycin (60 μg/ml) and confirmed to have the cj1461 mutation. The ease with which cj1461 mutants were recovered further suggested that Cj1461 is not part of an R-M system, since mutation of the MTase component of an R-M system is expected to be lethal.

To complement the cj1461 mutation, we inserted a second copy of cj1461 (under the control of its native promoter) into the cj0046 pseudogene (Fig. 2). The cj0046 pseudogene was amplified from *C. jejuni* 81-176 using the primers If313-F and If314-R (Table 2) and was subcloned into pBluescript SKII(−) to yield pBS-cj0046. Inverse PCR with primers If320-IV-F and If319-IV-R (Table 2) was used to introduce an NsiI site in cj0046. A chloramphenicol resistance (Cm^r) gene, *cat*, was PCR amplified from pRY111 (36) using the primers If317-F and If318-R (Table 2) and ligated with the pBS-cj0046 inverse PCR product to yield pSAT1000.

The cj1461 gene, including its ribosome-binding site, was amplified from *C. jejuni* 81-176; this product was cloned into pSAT1000 to yield pSAT1001. A 172-bp fragment containing the cj1461 promoter (upstream of cj1459) (Fig. 2 and data not shown) was inserted upstream of cj1461 to direct its expression at native levels. Because the σ^{54}-regulated and motility-related cj1462 (*flgI*) and cj1463 genes were 59 bp downstream of cj1461, to determine any indirect effects of the cj1461 mutation

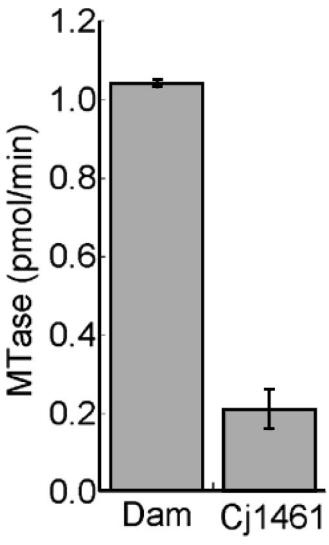

FIG. 1. DNA-MTase activity of 1 U Dam (New England Biolabs) or 1 μM Cj1461-His_6 was measured using in vitro reaction mixtures containing 100 μM SAM, with a substrate of 500 ng of chromosomal DNA (from *E. coli* JM110 *dam* or *C. jejuni* Δcj1461 cells, respectively). Both DAM and Cj1461 showed MTase activity.

TABLE 2. PCR primers used in this study

Primer	Sequence (5′→3′)[a]	Restriction site
cj1461-F1	TTGGAATTCAAACAAACTCGT AAAAGCAC	EcoRI
cj1461-R1	CCAGAATTCTTAAGAAGAGT AAAAACTTA	EcoRI
If313-F	CACTACCCAATTGAAAATC TAAG	NA[b]
If314-R	GTGAGTTAATGCCATCATAAC	NA
If320-IV-F	GAGATGCATGATCTTATAGCA CAAGCTAC	NsiI
If319-IV-R	GAGATGCATGAGCTGTAGCT GTAATAAAC	NsiI
If317-F	TGCTCGGCGGTGTTCCTTTC CAAG	NA
If318-R	TGCGCCCTTTAGTTCCTAAA GGGT	NA
JL15	TGAGCTCAATGATGAATTTAT AAGCGTTAAAG	SacI
JL16	GAGCTCTTAACCCCTTTCTTTT AAAAAATC	SacI

[a] Endonuclease restriction sites introduced for gene cloning purposes are underlined. Primers were based on the genome sequence from *C. jejuni* NCTC 11168.

[b] NA, not applicable.

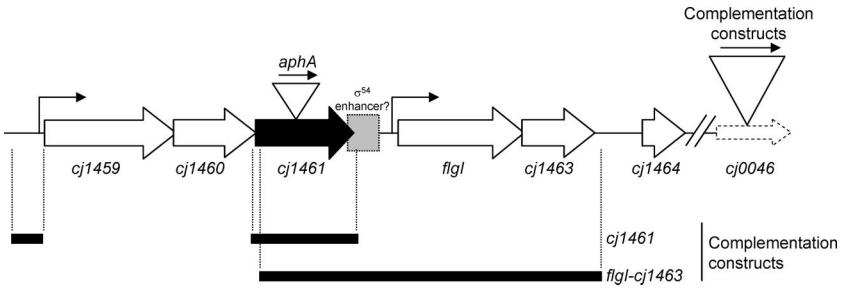

FIG. 2. Organization of cj1461, its surrounding genes, and complementation constructs in the cj0046 pseudogene. Gene designations are based on the *C. jejuni* NCTC 11168 genome (32); however, the organization of these genes is similar in *C. jejuni* 81-176. Arrows represent open reading frames and the direction of transcription. Arrows representing genes are not to scale for clarity of the figure. The solid arrow represents the cj1461 gene, and the dashed arrow indicates the cj0046 pseudogene. Triangles indicate the points of insertion of a kanamycin resistance transposon (*aphA*) in cj1461 and complementation constructs in cj0046. DNA included in the two complementation constructs is indicated by thick black lines below the gene map. Bent arrows represent promoters. The shaded box indicates the location of a putative σ^{54} enhancer for *flgI*. *flgI* encodes a flagellar structural component, the P ring. cj1459, cj1460, and cj1463 encode hypothetical proteins.

on *flgI* and cj1463 expression, we also expressed a second copy of *flgI* and cj1463 in the Δcj1461 mutant, along with 739 bp upstream of cj1462 containing the promoter and putative σ^{54} enhancer of these genes. This region was amplified using the primers JL15 and JL16 (Table 2) and inserted in the cj0046 pseudogene of the Δcj1461 mutant by using the methods described above.

Mutation of cj1461 causes a motility defect. Motility is a common bacterial virulence factor involved in colonization and invasion of host cells (30), including *C. jejuni* (3, 12, 17, 26, 28, 34, 37). Furthermore, Dam affects motility in several bacteria, including *E. coli* (29), *Salmonella enterica* serovar Typhimurium (1), *Aeromonas hydrophila* (5), and *Yersinia enterocolitica* (7). To investigate a role for cj1461 in *C. jejuni* motility, the Δcj1461 mutant was tested in a Mueller-Hinton soft-agar assay (Mueller-Hinton medium with 0.4% agar) (14), and the diameter of the area of motility was measured after 48 h incubation at 37°C. The cj1461 mutation caused a significant reduction in motility compared to the wild type (Fig. 3), and the motility defect was not related to a growth defect of the Δcj1461 mutant (data not shown). Complementation of the Δcj1461 mutant with the native cj1461 gene (Fig. 2) partially but significantly restored motility, confirming that cj1461 specifically affects the motility of 81-176 (Fig. 3). Wet-mount microscopy confirmed differences in motility and suggested that cj1461 mutation affected motility rather than chemotaxis, although a chemotactic defect cannot be excluded.

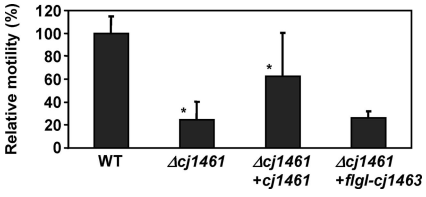

FIG. 3. Relative motility (percent of the value for the wild type) at 37°C of the complemented and uncomplemented Δcj1461 mutant cells. The cj1461 mutant showed impaired motility (*, $P < 0.01$), and complementation with cj1461 significantly restored motility (*, $P < 0.01$). In contrast, complementation with *flgI* and cj1463 did not restore motility. Data are the averages from at least three independent experiments. Bars represent standard deviation.

We considered the possibility that insertion of the Km^r transposon in cj1461 may have somehow disrupted the putative σ^{54} enhancer for the downstream *flgI* gene and that this could have indirectly affected the expression of *flgI* and motility of the mutant. To address this, we made a complementation construct containing nearly the entirety of cj1461 (but without its 5′ end to prevent cj1461 expression) and all of *flgI* and cj1463 (Fig. 2); this should contain all *flgI* and cj1463 transcriptional and translational initiation signals. Complementation of the Δcj1461 mutant with *flgI* and cj1463 together did not restore motility to the Δcj1461 mutant (Fig. 3). Together, these data suggest that the motility defect of the Δcj1461 mutant was not due to any indirect effect of the cj1461 mutation on *flgI* expression and that cj1461 is directly involved in the motility of 81-176.

Mutation of cj1461 causes a defect in flagellar appearance. Transmission electron microscopy was used to visualize flagella as previously described (33). Fifty cells each of the *C. jejuni* wild-type, the Δcj1461 mutant, and the cj1461-complemented mutant strains were randomly selected, and the length of flagella at both poles was measured for individual cells. There was no significant difference between any of the strains in flagellar length (Fig. 4A and data not shown). However, further investigation revealed a difference in flagellar morphology among the strains. The flagella in nearly all wild-type and cj1461-complemented cells had the appearance typical of normal *Campylobacter* flagella (Fig. 4B) (P. Guerry, personal communication) (10, 13, 14, 16, 18, 39). In contrast, while half of the Δcj1461 mutant cells had a wild-type morphology, half exhibited an aberrant phenotype, having a more dense appearance with poorly defined edges (Fig. 4B). The difference in flagellar appearance between the wild type and the Δcj1461 mutant suggests structural differences that may be related to the observed motility difference. We do not know the molecular basis for the change in flagellar appearance, but it could be related to Cj1461-regulated epigenetic changes in some aspect of flagellar biosynthesis. For example, glycosylation of flagellar subunits is important for their proper assembly (13). If Cj1461 is involved in regulating some aspect of flagellar glycosylation, it could yield an improperly assembled and partially defective flagellum. Alternatively, it is possible that expression of cellu-

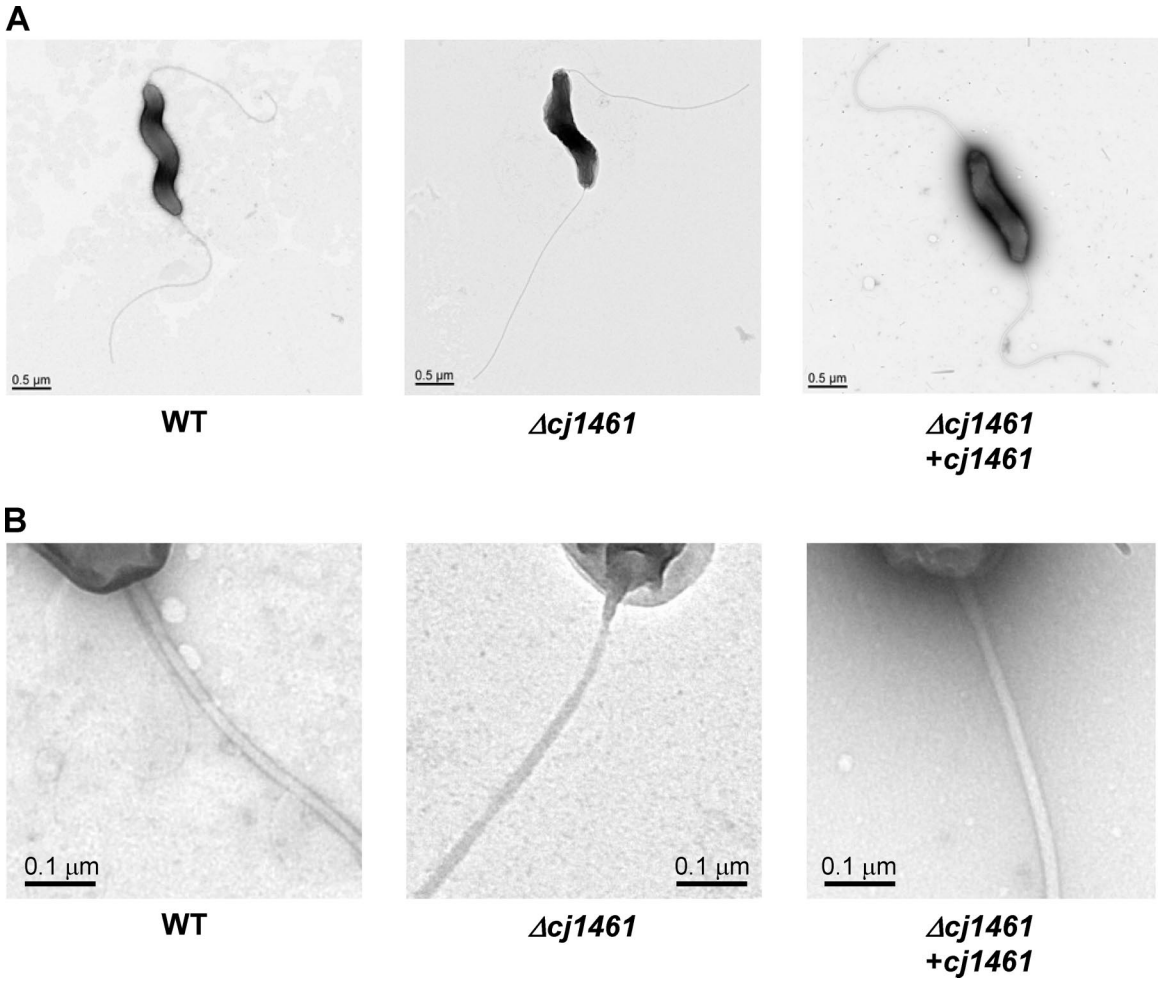

FIG. 4. Transmission electron micrographs reveal that the Δcj1461 mutant has normal flagellar length (A), but a large number of cells show altered flagellar structure (B). Fifty *Campylobacter* cells from two independent samples of each strain were analyzed. wt, wild type.

lar FlaA subunits could be decreased (but not absent) in the cj1461 mutant such that flagellum assembly kinetics are affected, resulting in flagella of normal length but aberrant structure. It is also possible that there are uncharacterized flagellar modifications (such as alternative covalent monomer modifications or variation in minor flagellar components) that could be changed in the cj1461 mutant. Elucidation of the molecular basis underlying the flagellar changes in the Δcj1461 mutant will be an interesting area of future research.

cj1461 mutation causes hyperadherence to but decreased invasion of INT-407 cells. Adherence and invasion assays were conducted as previously described (2, 25, 34). Briefly, *C. jejuni* cells were centrifuged onto semiconfluent INT-407 cells at a multiplicity of infection of about 20, and the number of adherent bacteria was quantified after 3 h. Gentamicin (250 μg/ml) was added to kill extracellular bacteria, and invasive bacteria were quantified after an additional 2 h incubation. Adherence was calculated as the number of adherent bacteria divided by the number of input bacteria. Invasion efficiency was calculated as the number of invasive bacteria divided by the number of adherent bacteria, to normalize for differences in adherence.

The Δcj1461 mutant was sevenfold more adherent to INT-407 cells than wild-type cells were (Fig. 5). Complementation with the cj1461 gene restored wild-type adherence, confirming that the absence of Cj1461 caused hyperadherence to INT-407 cells. No change in the mobility of lipooligosaccharide of the Δcj1461 mutant was found compared to the wild type on silver-stained gels (data not shown), indicating that gross changes in lipooligosaccharide did not account for the hyperadherence. The molecular basis for the hyperadherence of the Δcj1461 mutant is currently unknown but warrants further investigation.

In contrast to its hyperadherence, the Δcj1461 mutant had a severe defect (50-fold) in invasion of INT-407 cells compared to the wild type, and complementation with the cj1461 gene restored wild-type invasion efficiency (Fig. 5). All three strains had identical gentamicin MICs (data not shown), indicating that the invasion phenotypes of the strains were not due to differences in gentamicin susceptibilities. Alteration of the invasion efficiency in the Δcj1461 mutant indicates that cj1461 is involved in invasion of host cells by *C. jejuni* 81-176. Because motility is a prerequisite for invasion into eukaryotic cells, the motility defect of the mutant may be a significant contributor to the invasion deficiency of the mutant into INT-407 cells, although we cannot rule out possible contribution by other

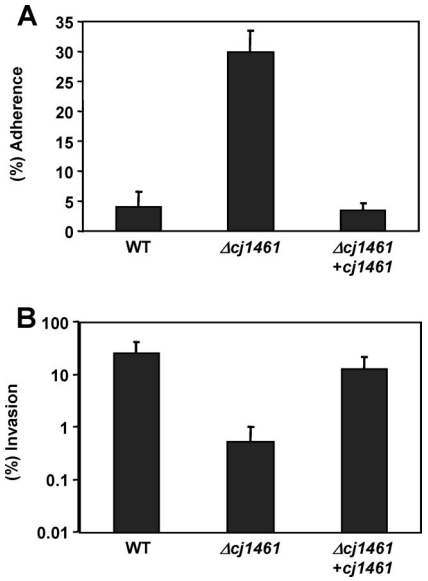

FIG. 5. Adherence to (A) and invasion of (B) INT-407 cells by wild-type (WT), Δcj1461 mutant, and complemented mutant strains. Mutation of cj1461 resulted in sevenfold increased adherence compared to the wild type (A). In contrast, the Δcj1461 mutant was 50-fold less invasive than the wild type (B). For both adherence and invasion, complementation with cj1461 restored wild-type phenotypes. Data are the averages from two independent experiments, each performed in triplicate. Bars represent standard deviations.

proteins that may be altered in the Δcj1461 mutant. The involvement of DNA MTases in host cell invasion was also demonstrated in *S. enterica* serovar Typhimurium and *Y. enterocolitica* expressing aberrant levels of Dam (15).

Conclusion. It has become evident that epigenetic regulation by DNA MTases can play a significant role in the virulence of pathogenic bacteria (15). This study shows that loss of the predicted *C. jejuni* Cj1461 DNA MTase affects virulence characteristics such as motility, adherence, and invasion in *C. jejuni* 81-176. These results suggest that epigenetic regulation by Cj1461 may control the expression of proteins involved in these processes and therefore could play a significant role in the pathogenesis of *C. jejuni*.

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