Journal of Animal and Veterinary Advances 9 (24): 3034-3038, 2010
ISSN: 1680-5593

Role of Flagella-Related *rPoN* and *fliA* Genes in *Campylobacter jejuni*

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Abstract: To investigate the role of the rpoN and fliA in the regulation of *C. jejuni* colonization, the *rpoN* and *fliA* gene were inactivated by allelic exchange with a defective copy of the gene carrying a tetracycline-resistance cassette. Phenotypically, the *C. jejuni* rpoN mutant exhibited aflagellar filament on transmission electron microscopy and non motility on MH medium supplemented with 0.4% Bacto agar while the fliA mutant possessed a truncated flagellar filament in most cells and strongly diminished motility. Adhesion and invasion ability tests of the rpoN and fliA mutants were performed to assess the role of the these genes in regulating *C. jejuni* infection. The rpoN and fliA mutants showed significantly less ($p<0.0005$) adherence and invasion compared to the wild type *C. jejuni*. To determine the colonization capacity of *C. jejuni* rpoN and fliA mutants, 1 day old chicks were inoculated with the defined *C. jejuni* mutants. The *C. jejuni* rpoN and fliA mutants demonstrated a marked impairment in their ability to colonize chicks as none of 10 chickens inoculated with rpoN and fliA mutants were colonized. In conclusion control of flagellin expression in *C. jejuni* involves the alternative sigma factor genes *rpoN* and *fliA*. The results of this study are consistent with the previous results that both *fliA* and *rpoN* genes regulate the process of binding and internalization of *C. jejuni* to epithelial cells *in vitro* and *in vivo*.

Key words: *Campylobacter jejuni*, rpoN, fliA, flagella, colonization, Republic of Korea

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) is a gram negative motile bacterium that emerged in the 1970s as an important zoonotic pathogen. They are considered to be a leading cause of diarrheal disease throughout the world (Harris *et al*., 1986; Tauxe *et al*., 1985; Van Vliet and Ketley, 2001).

The motility by the flagella of *C. jejuni* is critical for intestinal colonization and for invasion into intestinal epithelial cells (Aguero-Rosenfeld *et al*., 1990; Grant *et al*., 1993; Szymanksi *et al*., 1995; Van Vliet and Ketley, 2001; Wassenaar *et al*., 1991). The flagellum of *C. jejuni* is composed of a basal body, hook and filament. To produce functional flagella, bacteria must coordinate both the temporal expression of over 40 flagella genes and secretion of the encoded protein (Caldwell *et al*., 1985; Fernando *et al*., 2007). Flagella filament is comprised of two proteins termed FlaA and FlaB. Sigma factor σ^{28} encoded by *fliA* gene is involved in the expression of flaA, encoding the major flagellin that comprises a large proportion of the flagellar filament (Fernando *et al*., 2007).

Sigma factor σ^{54} encoded by *rpoN* gene is required for the expression of almost all genes that encode components of the flagella basal body, hook and filament (Hendrixson and Dirita, 2003; Jagannathan *et al*., 2001). Flagellin is the immunodominant antigen during human and animal infection and is absolutely required for colonization *in vivo* (Konkel *et al*., 2001; Nuijten *et al*., 1991). In this study, *C. jejuni* rpoN and fliA mutants were constructed by allelic exchange with genes inactivated by deletion and insertion. The mutants obtained were tested for the characteristics of flagella and invasiveness to the epithelial cell *in vitro* and their ability to colonize the chicken *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The *C. jejuni* 04011 (Cj04011) strain was isolated from a chicken carcass and virulence-associated properties in vasion (ciaB, iamA), lipopolysaccharide (wlaN and cgtB) adhesion (cadF, peblA, jlpA and porA), chemotaxis (docB and docC) and cytotoxin (cdtA, cdtB and cdtC) were

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confirmed by PCR (Muller *et al*., 2006). *C. jejuni* was cultured on Mueller Hinton (MH) agar (Difco) supplemented with 5% bovine blood under microaerobic conditions at 42°C. *Escherichia coli* DH5α used as hosts for the cloning experiments was grown in Luria-Bertani (LB) medium (Sigma) at 37°C. All strains were stored at -80°C in a 85% MH broth or LB medium with 15% glycerol solution.

Construction of *C. jejuni* rpoN and fliA mutant: For construction of the rpoN and fliA mutant, *C. jejuni* NCTC11168 sequences were obtained from the Sanger Center website (http://www.sanger.ac.uk/Projects *C. jejuni*) and primers for this study were shown in Table 1. Cj04011 chromosomal DNA was used for the amplification of DNA regions flanking rpoN and fliA with primer pairs of R1-R2 and F1-F2, respectively. The reaction conditions were 94°C for 2 min (1 cycle); 94°C for 45 sec, 65°C (-1°C per cycle) for 30 sec and 72°C for 3 min (5 cycles); 94°C for 15 sec, 60°C for 30 sec and 70°C for 4 min (30 cycles) and 72°C for 5 min (1 cycle). The PCR products were purified by using the QIAquick PCR Purification kit (Qiagen), digested with BamHI and then cloned into the BamHI site of pBS (SK+) (Invitrogen). Clones were selected on ampicillin-contaning blue-white plates and screened by restriction analysis.

The mutation construct was verified by DNA sequencing. Primer pairs R1I-R2I and F1I-F2I were designated to introduce a unique NheI restriction site and deletions of 998 and 509 bp within the cloned *rpoN* and *fliA* genes, respectively. The primers were oriented such that amplification of template DNA extended in opposite directions around the cloning vector with 20 ng of plasmid DNA as the template. The PCR cycling was the same with the condition for cloning of the *rpoN* and *fliA* genes. The products were digested with NheI enzyme, purified, ligated, transformed into competent *E. coli* DH5α cells and selected for ampicillin resistance. The 2.3 kbp Tet^r cassette was inserted into the NheI site of rpoN and fliA cloned into pBS (SK+) vector. Antibiotic-resistant colonies carrying the teracycline-resistance cassette in the same orientation with the cloned gene were identified by restriction analyses of the plasmid DNA and confirmed by DNA sequencing. These Tet^r modified constructs were electroporated into Cj04011. The target gene in Cj04011 was disrupted by allelic replacement via a double-crossover event between the target chromosomal gene and a plasmid-borne copy of the target gene containing an internal deletion.

Complementation of *C. jejuni* rpoN and fliA mutant: The rpoN and fliA complement strains were constructed for this study. Promoter genes for rpoN and fliA were amplified with primer pairs Rp1-Rp2 and Fp1-Fp2, respectively. The entire *rpoN* and *fliA* genes were amplified with primer pairs Rc1-Rc2 and Fc1-Rc2. Promoter and each target genes were amplified from Cj04011 by PCR. Following an intermediate cloning step into pCR2.1 (Invitrogen), the gel-purified insert was ligated into the NdeI site of pRY111. These plasmids were introduced into a Cj04011 rpoN and fliA mutant by conjugation.

Phenotypic analysis of the *C. jejuni* rpoN and fliA mutants: Motility assays were performed with Mueller Hinton broth supplemented with 0.4% Bacto agar (Difco, USA). A 10 μL suspension of each bacterial isolates was spotted on the surface of semisolid medium. Motility plates were incubated at 37°C under microaerophilic conditions for 48 h. For Transmission Electron Microscopy (TEM), bacterial suspensions were prepared from MH agar plates with phosphate-buffered saline and were added dropwise to Formvar coated copper

Table 1: Characteristics of the primers used in this study

Oligonucleotide	Sequences (5'-3')	Enzyme
R1	TTGGATCCGGAGGTGAAAGAAGGCGTTG	BamHI
R2	AAGGATCCAAACCAAGCGCTATGCCGCC	BamHI
F1	TTGGATCCTTGGAAGACATTTTAATAGAAG	BamHI
F2	AAGGATCCATTTCTTTGATTTCATCTTTATC	BamHI
R1I	AAGCTAGCGCTCTTGATGAAGAGGGAGAG	NheI
R2I	TTGCTAGCGCAACCAAGAGCGTAAAGTTT	NheI
R1I	AAGCTAGCCACAAGCTCATCTTGCTCTTTC	NheI
R2I	TTGCTAGCCACGAAGTGCTAGATGATCTTAAAG	NheI
Tet^r1	TTGCTAGCAGCTCTAGAGTCAACCGTGATATAG	NheI
Tet^r2	AAGCTAGCGTATAGAAGTGCGCCCTTTAGTTCC	NheI
Rp1	TTCCGCGG TAGAGCTCTCAAGGCGTCCTATACCGTG	SstII
Rp2	AACATATGTACATATGTTCAGAAATAGCTCTTC	NdeI
Fp1	AATGGATCCTATTAATTCTTCTTTGGCCTGTGC	BamHI
Fp2	AATCATATGGGATTTAACCTTAAAAATTTATTTTTAACC	NdeI
Rc1	TTCATATGATCATATGTTAAAGCAAAAAATCACCCAAG	NdeI
Rc2	TTCCGCGGATCCGCGGAATATTTAAAAACAGTTATTATTGTATCAC	SstII
Fc1	ATACATATGAATTCAAAGAAAGACGAAGAAATG	NdeI
Fc2	AATGGATCCTTCTTGGGAGAGTATCTCAGC	BamHI

grids. Bacteria were stained with 1% phosphotungstic acid. Samples were analyzed with a JOEL 1200 EX transmission electron microscope.

Adherence and invasion assay: Adherence and internalization assays were preformed with INT 407 as previously described (Grant *et al*., 1993). Briefly, approximately 1×10^7 cfu of bacteria was inoculated into a semi-confluent INT 407 cell monolayer (10^5 cells well^{-1}) on 24 well tissue culture tray. For binding, the infected monolayers were incubated for 3 h in a humidified, 5% CO_2 incubator at 37°C, rinsed 3 times with EMEM without FBS and lysed with a solution of 0.1% (v/v) Triton X-100 (Calbiochem, USA).

The suspensions were serially diluted and the number of adherent bacteria was determined by counting the colonies on MH-blood plates. The number of viable adherent bacteria was determined by counting the colonies. To measure bacterial internalization, the infected monolayer was washed three times with EMEM and reincubated for another 3 h in fresh EMEM containing 250 μg mL^{-1} of gentamicin (Sigma). The number of internalized bacteria was determined as described above. Significance of differences among samples was determined with Student's t-test following $\log_{10}$ transformation of the data. Two-tailed p-values were determined for each sample and a p-value of <0.0005 was considered significant. The experiments were conducted a minimum of three times.

Chick colonization assays: Colonization of day old chicks by *C. jejuni* was done as described by Carrillo *et al*. (2004). Briefly, sixty 1 day old Hyline brown commercial chicks were obtained and divided into six groups. Water and a commercial chick starter feed were provided *ad libitum*.

The chicks were orally inoculated with 0.5 mL of a bacterial suspension strains, 10^7 CFU cultured in Bolton's broth at 42°C for 16 h under microaerobic conditions prior to inoculation of the birds. One group of 10 chicks was kept as the uninoculated control group. The remaining groups of chicks were inoculated with the wild-type strain, rpoN mutant, fliA mutant, rpoN complemented strain and fliA complemented strain. After 7 days, chicks were euthanized and their ceca were removed. Cecal contents were weighed, diluted 1:10 (wt/vol) in Bolton's broth medium (Oxoid), thoroughly stomached and plated onto MH agar after serial dilution. *C. jejuni* colonies were counted after incubation in a microaerobic environment at 37°C for 72 h.

RESULTS AND DISCUSSION

Phenotypically, the *C. jejuni* rpoN mutant exhibited aflagellated filament on TEM and non-motily on MH medium supplemented with 0.4% Bacto agar while the fliA mutant possessed a truncated flagellar filament in most cells and strongly diminished motility (Fig. 1). Flagella sigma factor σ^{54} (rpoN) and sigma factor σ^{28} (fliA) regulate a large number of genes for the expression and function of *C. jejuni* flagella. Inactivation of the rpoN regulatory gene abolished flagellar function completely, otherwise inactivation of the *fliA* gene had a less detrimental effect on the flagella of *C. jejuni*.

A test of the ability of the rpoN and fliA mutant to invade INT 407 cells was performed to assess the role of these genes in *C. jejuni* pathogenesis and colonization *in vitro* (Grant *et al*., 1993). The rpoN and fliA mutants showed significantly less ($p<0.0005$) adherence and invasion compared to the wild type *C. jejuni*. While complemented mutants with entire *rpoN* and *fliA* genes showed similar values for adhesion and invasion as the corresponding wild-type strain but complement strains were not totally able to recover the ability of adhesion and invasion.

The investigators reported that *C. jejuni* flagellum is responsible for motility, protein secretion and invasion of host cells (Guerry *et al*., 1991; Wassenaar *et al*., 1991). *In vitro* adherence and invasion assays have been used extensively to characterize the interactions of *C. jejuni* with host cell and the binding of *C. jejuni* to INT 407 cell (a human intestinal epithelial cell) has been most extensively studied to be a more accurate reflection of *C. jejuni in vivo*.

Wassenaar *et al*. (1991) and Grant *et al*. (1993) reported that motility conferred by the expression of the *flaA*$^+$ gene was found necessary for the maximal invasion of eukaryotic cells and for the translocation of polarized cell monolyers by *C. jejuni* (Fig. 2). However, differences were noted in the invasive potential of the *C. jejuni* flA$^-$ flaB$^+$ and *C. jejuni* flaA$^-$ flaB$^-$ isolates with the former being more invasive (Wassenaar *et al*., 1991; Grant *et al*., 1993). Given the differences observed in invasive potential of the *C. jejuni* mutants, Grant *et al*. (1993) concluded that the flagella structure played a role in the internalization process of *C. jejuni* that was independent of motility.

In parallel with the results of *in vitro* experiments, the colonization abilities of the rpoN and fliA mutants were tested in a day old chicken model. In this study, both rpoN and fliA mutants were completely attenuated for cecal colonization. But the result of the cecal colonization

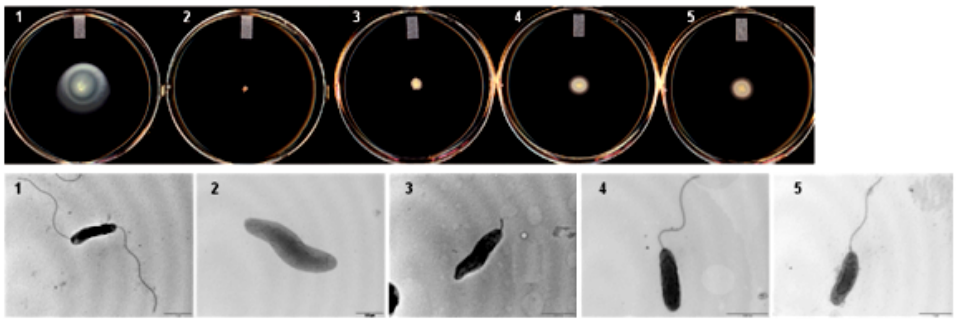

Fig. 1: Characterization of the rpoN and fliA mutants. (a) Motility phenotypes on MH medium supplemented with 0.4% Bacto agar after 48 h and (b) Transmission electron microscopy. In panels a and b, 1, 2, 3, 4 and 5 indicate wild-type *C. jejuni* strain 04011, Cj04011 rpoN mutant, Cj04011 fliA mutant, Cj04011 rpoN complemented strain and Cj04011 fliA complemented strain, respectively

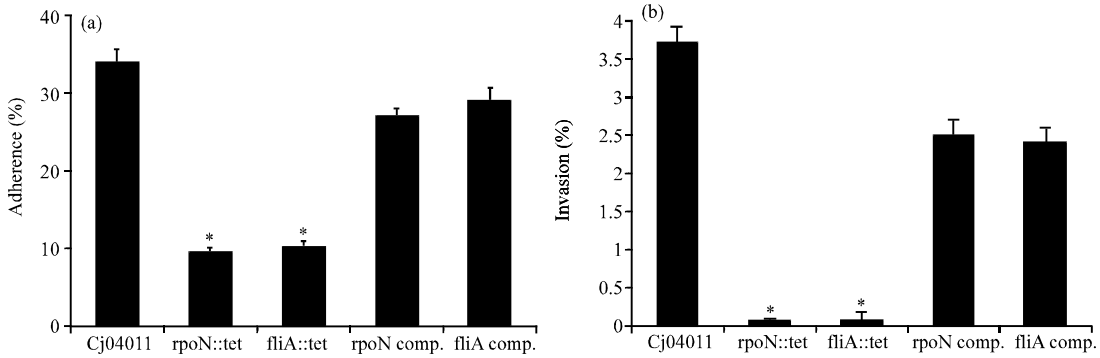

Fig. 2: Adherence and invasion of INT 407 cell with with-type *C. jejuni*, rpoN mutant, fliA mutant and complemented strains. The adherence and invasion data are averages of three independent experiments performed in duplicate. As asterisk indicates a statistically significant difference ($p<0.0005$) aginst the *C. jejuni* 04011 wild-type isolate as determined by Student's t-test

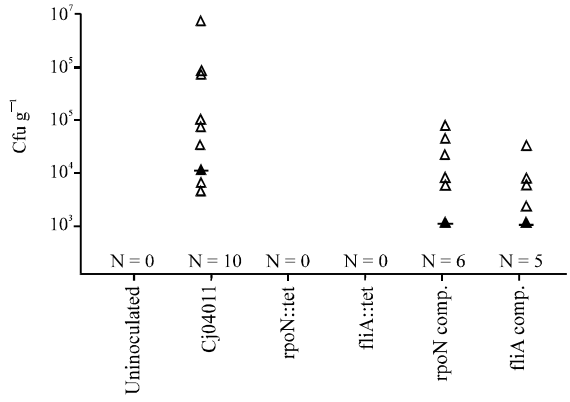

Fig. 3: Cecal colonization levels (cfu g^{-1}) of wild-type *C. jejuni*, rpoN mutant, fliA mutant and complemented strains. Ten birds were orally infected with 10^7 cfu of each strain at 1 day of hatch. Cecal samples were collected from at 7 dpi. The median values for each group are indicated by horizontal lines

level of wild type parent strain used in this study was lower than that used in previous studies and the defect in colonization capacity of the present rpoN and fliA mutants were much more severe than used in other studies (Biswas *et al*., 2005; Fernando *et al*., 2007). Moreover, the colonization ability of the wild type *C. jejuni* is a remarkable contrast between the strains. The relative ability of *C. jejuni* to invade cells appears to be strain-dependent (Newell *et al*., 1985) (Fig. 3). Newell *et al*. (1985) found that environmental isolates were much less invasive for HeLa cells than clinical isolates as determined by immunofluorescence and electron microscopy examination of *C. jejuni*-infected cells. Everest *et al*. (1992) observed a statistically significant difference in the level of invasion between *C. jejuni* strains isolated from individuals with colitis versus those isolated from individuals with noninflammatory diarrhea. It is also well documented that ability of *C. jejuni* to invade cells has been noted to decrease after extensive *in vitro* passage

(Konkel *et al*., 2001). In this study, despite the high prevalence of flagella in the wild type parent, cecal colonization shows the dissimilarity with previous reported wild type strains. These findings suggest the difference of unknown gene expression between strains *in vitro* and *in vivo* is related to its ability to invade the epithelial cells lining the intestinal tract.

CONCLUSION

In study control of flagellin expression in *C. jejuni* involves the alternative sigma factor genes *rpoN* and *fliA*. The results of this study are consistent with the previous results that both *fliA* and *rpoN* genes regulate the process of binding and internalization of *C. jejuni* to epithelial cells *in vitro* and *in vivo*.

ACKNOWLEDGEMENT

This research was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-1-E00077).

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