Original Article

Genetic and Phenotypic Variation of *Campylobacter jejuni* NCTC11168 Caused by *flhA* Mutation during Laboratory Passage*

CHEN Xiao Li[1], LIANG Hao[1,2], GUO Peng Bo[1,3], GU Yi Xin[1], WANG Jia Qi[1], WANG Hai Rui[1], ZHOU Gui Lan[1], SHAO Zhu Jun[1], ZHANG Jian Zhong[1], and ZHANG Mao Jun[1,#]

1. State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Department of Microbiology, School of Public Health, Cheeloo College of Medicine, Shandong University, Jinan 250100, Shandong, China; 3. Shandong First Medical University, Shandong Academy of Medical Sciences, Jinan 250000, Shandong, China

Abstract

Objective *Campylobacter jejuni* NCTC11168 is commonly used as a standard strain for flagellar biosynthesis research. In this report, two distinguished phenotypic isolates (CJ1Z, *flhA* mutant strain, lawn; CJ2S, *flhA* complemented strain, normal colony) appeared during laboratory passages for NCTC11168.

Methods Phenotypic assessments, including motility plates, transmission electron microscopy, biofilm formation assay, autoagglutination assay, and genome re-sequencing for these two isolates (CJ1Z, *flhA* mutant strain; CJ2S, *flhA* complemented strain) were carried out in this study.

Results Transmission electron microscopy revealed that the flagellum was lost in CJ1Z. Phenotypic assessments and genome sequencing of the two isolates were performed in this study. The capacity for biofilm formation, colony auto-agglutination, and isolate motility was reduced in the mutant CJ1Z. Comparative genomic analysis indicated a unique native nucleotide insertion in flhA (nt, 2154) that caused the I719Y and I720Y mutations and early truncation in flhA.

Conclusion FlhA has been found to influence the expression of flagella in *C. jejuni*. To the best of our knowledge, this is the first study to describe the function of the C-terminal of this protein.

Key words: *Campylobacter jejuni*; Phenotypic variation; Comparative genomics; *flhA*

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INTRODUCTION

Campylobacter jejuni, one of the most common causes of acute human gastroenteritis worldwide, is an important foodborne pathogen[1]. In addition, C. *jejuni* has received increasing attention for its association with Guillain-Barre and Miller-Fisher syndromes[2]. Although relatively little is known about the molecular mechanisms underlying *C.*

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#Correspondence should be addressed to Prof. ZHANG Mao Jun, Tel: 86-10-58900754, E-mail: zhangmaojun@icdc.cn

Biographical note of the first author: CHEN Xiao Li, female, born in 1995, PhD, majoring in prevention and control of infectious disease.

jejuni pathogenesis, it is widely accepted that flagellar motility is necessary for gastrointestinal tract colonization. Efficient colonization is a prerequisite for *C. jejuni* pathogenesis, and only motile strains have been recovered from human volunteers[3,4].

NCTC11168 has been extensively used in basic research on *Campylobacter* and represents a standard strain for studies on the assembly or conformation of flagella. A comprehensive analysis of flagellin glycosylation in *Campylobacter jejuni* NCTC 11168 revealed its importance in host colonization[5]. The genes *rpoN*, *flaA*, *fliA*, *flhA*, *flgA*, *motA*, and *flgR* have been shown to play important roles in flagellar biosynthesis in *Campylobacter jejuni*[6-10].

FlhA and flhB are the most well-characterized subunits of the membrane-inserted components of the flagellar export apparatus[11-16]. FlhA encodes a ~700 residue protein that is essential for flagellum biogenesis and contains an N-terminal integral membrane domain (amino acids 1–350), predicted to contain either six or eight membrane-spanning helices. In the absence of crystal structure experiments on the FlhAC protein from *C. jejuni*, the crystal structure of the cytoplasmic domain of FlhA from *Helicobacter pylori* was used[17].

Compared to other enteric pathogenic bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, *C. jejuni* does not utilize carbohydrates as a food source. *Campylobacter* have been labeled as asaccharalytic microorganisms since early biological investigations on substrate consumption demonstrated that *C. jejuni* is unable to utilize glucose or other six-carbon carbons as a carbon source[1,18].

The aim of the present study was to elucidate the genetic mechanism of flagellar loss in NCTC11168 using experimental laboratory passages and evaluate phenotypic variations in the mutant. The genomes of the mutant isolate CJ1Z (NCTC11168, *flhA* mutant strain, lawn colony) and wild-type isolate CJ2S (NCTC11168, *flhA* complemented strain, normal colony) were compared *ad hoc* in this study. We identified 15 single nucleotide polymorphisms (SNPs) between the two genomes as well as a motility-related gene *flhA* mutation. An insertion in the *flhA* gene was identified, which has been shown to affect the assembly or conformation of the flagella. Phenotypic variations in the FlhA mutant and their predicted functions are described in this study.

METHODS

Bacteria Strains and Growth Conditions

Campylobacter jejuni NCTC11168 is a well-characterized, sequenced strain that is widely used in infection studies. The frozen stock from which all the strains were derived was originally obtained from the American Type Culture Collection (ATCC 700819). Strain NCTC11168 was used for the laboratory evolution experiments in this study. *C. jejuni* cultures were grown from freezer stocks on *Campylobacter* agar base (Karmali) plates with 5% (v/v) sheep blood for 48 h at 37 °C in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2).

Colony variants emerged during serial passage by subculturing *Campylobacter* every 48 h at 37 °C in a microaerophilic environment for 30 d, named strain CJ1Z. We characterized the variant colonies and identified two distinct phenotypes: lawn colonies (CJ1Z, *flhA* mutant strain) and normal colonies (CJ2S, *flhA* complemented strain) (Figure 1A and 1B).

Motility Plates

The motility of *C. jejuni* strains was measured as described by Kalmokoff et al. with some modifications[19]. Bacteria were cultured on Mueller–Hinton agar (MH agar) plates for 48 h in PBS, and the optical density at 600 nm (OD_{600}) was adjusted to 0.5. Then, 10 mL of the bacterial suspension was inoculated into the center of a motility agar plate (MH broth containing 0.4% nutrient agar). Each plate was incubated upright for 48 h at 37 °C, after which it was photographed, and the halo diameter was recorded.

Transmission Electron Microscopy

C. jejuni grown on *Campylobacter* agar base (Karmali) was resuspended in Mueller-Hinton broth to an OD_{600} of 0.2 and grown for 4 h. The bacteria were diluted to an OD_{600} of 1 and collected by gentle centrifugation. The pellets were gently resuspended in a 2.5% (v/v) glutaraldehyde solution for fixation. Fixation was completed by incubating strains for 1 h at 4 °C. CJ1Z and CJ2S strains were stained with 1% (w/v) uranyl acetate and visualized using a JOEL 1,200× transmission electron microscope at 80 kV.

Growth Curves

CJ1Z and CJ2S strains cultured at 37 °C on *Campylobacter* agar base (Karmali) under

microaerophilic conditions for 48 h were inoculated into a 50 mL MH broth to a final OD_{600} of 0.02. The OD_{600} was recorded every 2 h for 30 h. At each time point, the turbidity of the medium was measured at 600 nm.

Biofilm-formation Assay

The ability of *C. jejuni* strains to form biofilms was measured using crystal violet staining[8]. Briefly, bacteria cultured for 20 h were harvested and diluted in MH broth to an OD_{600} of 0.025, after which CJ1Z and CJ2S strains were inoculated into a 96-well plate with 1 mL suspension at 37 °C under microaerobic conditions. After incubation for 24, 48, and 72 h, the plates were washed with PBS and dried at 55 °C for 30 min. Next, 1 mL of 1% crystal violet solution was added to each well to stain the formed biofilms. After staining at room temperature for 5 min, the plates were rinsed thoroughly with PBS thrice to remove unbound crystal violet. The plates were subsequently dried at 55 °C for 15 min, and the biofilms were quantified by determining the OD_{570} values after the remaining crystal violet was solubilized in a solution composed of 80% ethanol and 20% acetone.

Autoagglutination Assay

The autoagglutination assay was performed as previously described[20]. *C. jejuni* strains were grown for 20 h prior to use, after which they were suspended in PBS (pH7.5) and adjusted to an OD_{600} of 1.0. Then, 4 mL aliquots of the suspension were transferred to a series of sterile glass tubes. The bacterial solution was placed at room temperature (25 °C), 200 μL of the bacterial suspension was carefully removed from the top of each tube every 1 h, and the OD_{600} was measured to evaluate the degree of autoagglutination (AAG). Bacterial cells that strongly agglutinate do not remain in the upper part of the supernatant but rather fall to the bottom of the tube, leading to a decreased OD_{600} value.

Whole Genome Sequencing for Strains CJ1Z and CJ2S

DNA extraction was performed using the Qiagen QIAamp DNA Mini Kit according to the manufacturer's instructions. Genome sequences were obtained from the Beijing Genomics Institute. Here, 42 draft genomes were sequenced using the Illumina HiSeq 2500Xten platform (Illumina Inc., San Diego, CA, USA), generating

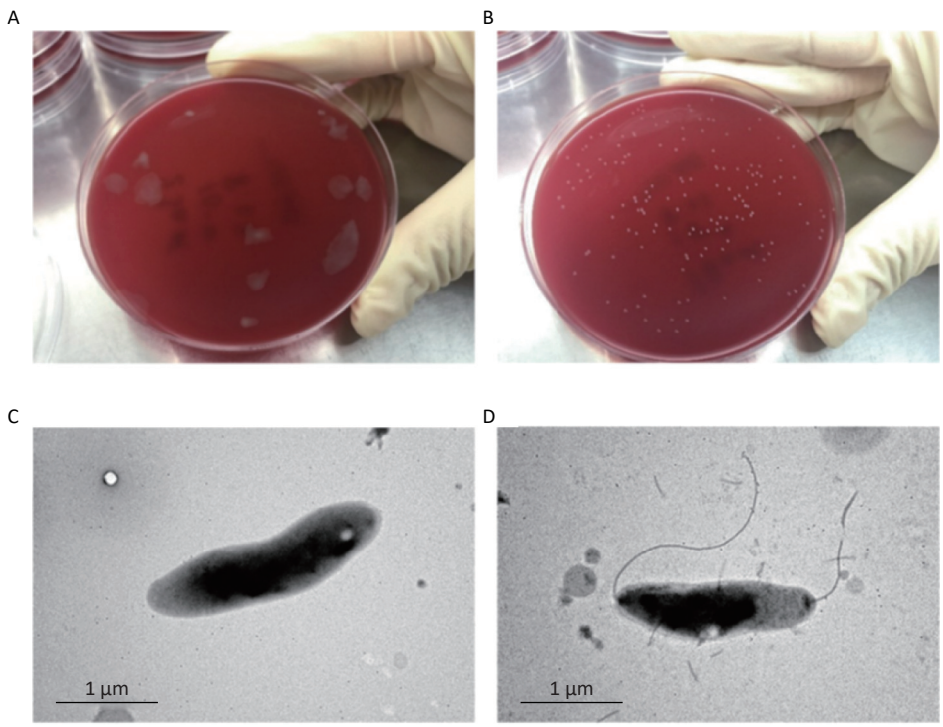

Figure 1. Difference in single colony and flagellation of *flhA* mutants. (A) CJ1Z in BHI agar following microaerobic growth; (B) CJ2S in BHI agar following microaerobic growth; (C, D) TEM was used to visualize the flagella of CJ1Z and CJ2S. The panels show representative TEM images for cells with: (C) no flagella, CJ1Z is represented; (D) bipolar flagella, CJ2S is represented.

reads of 300 bp in length. Complete sequencing was performed using PacBio, and long reads were analyzed using the pipelines provided by the SMRT Portal software. Clean reads were assembled using SOAPdenovo (SOAPdenovo 2.04, http://soap.genomics.org.cn/soapdenovo.html). The assembled genomes were annotated to predict the genes and their functions using Prokka V1.13.3. The genomes of strains CJ1Z and CJ2S were submitted to NMDC, and the accession numbers were assigned as NMDC60029075 and NMDC60029076. Whole genome sequencing data from the mutant and wild strains were compared by mapping against the reference strain *C. jejuni* NCTC11168 genome sequence (RefSeq: NC_002163.1) for all types of mutations using Mummer.

RESULTS

Variant Colony CJ1Z has no Flagellum by Transmission Electron Microscopy

In the present study, we investigated whether the flagellar structure of *C. jejuni* NCTC11168 is completely inhibited or affected in a more subtle manner. We examined two colony morphologies of the strains (CJ1Z and CJ2S) by transmission electron microscopy and found noticeable differences. The CJ1Z strain had no flagella but contained a spiral shape (Figure 1C). In contrast, the CJ2S strain had bipolar flagella (Figure 1D). Together, these observations show that the variant colony was accompanied by flagellar synthesis or assembly changes.

Strain CJ1Z has a Motility Defect with Decreasing Autoagglutination and Biofilm-formation Abilities but no Defect in Growth Kinetics

CJ1Z and CJ2S isolates were assessed for their swimming motility in MH agar broth. The data (Figure 2A) showed that the mutant CJ1Z strain had a motility defect compared to the CJ2S strain over the course of 48 h. This suggests that the loss of flagella may be a major contributor to the motility loss of *C. jejuni* NCTC11168.

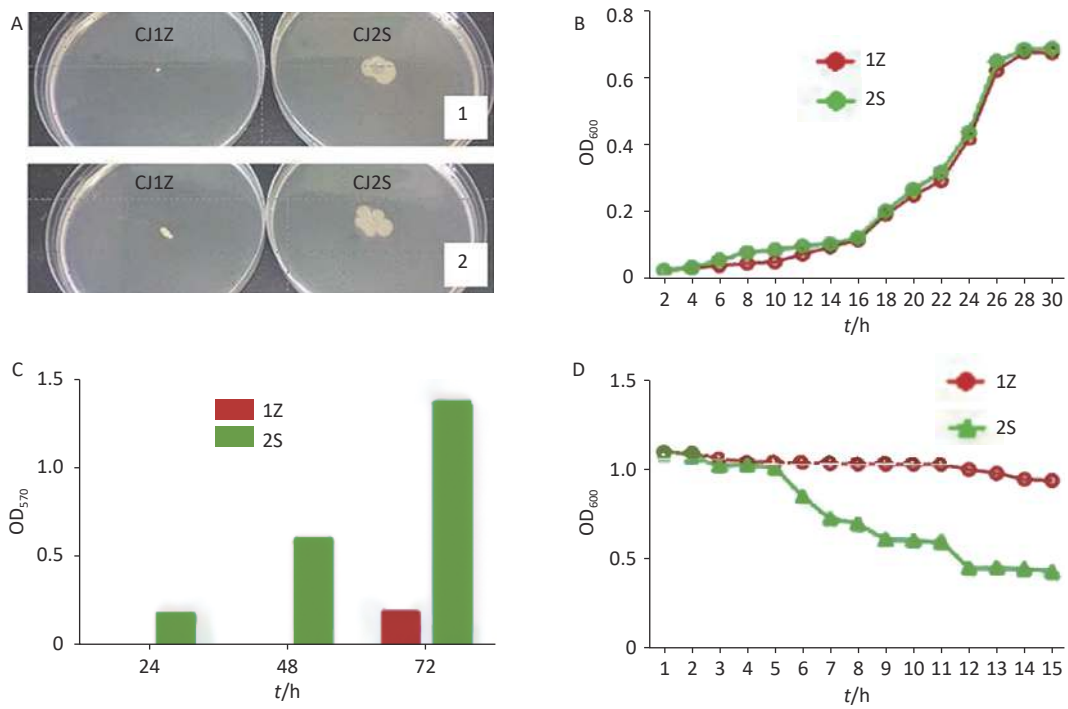

Figure 2. Motility, growth curves, biofilm formation, and autoagglutination of CJ1Z and CJ2S. (A) Motility haloes of *C. jejuni* CJ1Z (*flhA* mutant) and CJ2S on soft agar; (B) Growth curve of *C. jejuni* CJ1Z and CJ2S. *C. jejuni* CJ1Z and CJ2S were grown in MH broth, with 100 µL of samples withdrawn at designated time point to examine growth. The CJ1Z strain (red cycle) and CJ2S strain (green cycle) are represented by each sample; (C) Formed biofilms of *C. jejuni* CJ1Z and CJ2S. The formed biofilms were stained with crystal violet, and biofilm formation was quantitated by determining the OD_{570} value; (D) Autoagglutination abilities of the CJ1Z strain (hollow circle) and CJ2S strain (hollow triangle).

The observed growth curves indicated that mutation of the colony did not influence the normal growth of *C. jejuni*. When cultured in MH broth, the OD_{600} values for *C. jejuni* CJ1Z and CJZS strains were nearly the same at different times, with complemented differences observed, suggesting that these strains have a similar growth rate (Figure 2B).

The biofilms formed by the strains were quantified after static culture for 24, 48, and 72 h. As shown in Figure 2C, the biofilms formed by the CJ2S strain continuously increased over time; however, the *flhA* mutant CJ1Z strain showed a defect in biofilm formation, as the OD_{570} values of the tested strains across all assayed time points decreased compared to those observed for the CJ2S strain.

The AAG ability of *C. jejuni* strains was compared after incubation each hour (Figure 2D). In the first 5 h, there was no significant difference in the AAG ability between the CJ1Z and CJ2S strains. The influence of colony variants on the AAG of *C. jejuni* became noticeable after incubation for 5 h. The OD_{600} of *C. jejuni* CJ1Z was higher than that of the CJ2S strain, and this effect increased over time as the difference was more significant at 12–15 h.

Mutations were Detected in flhA Mutant and Complemented Strains

We identified 15 SNPs in the two genomes (5 nonsynonymous, 1 stop-gain, 1 synonymous, and 8 noncoding), among which 7 were located in the open reading frame (ORF) spacer region, and 2 INDELs were located in the ORF, which were distributed in 9 genes (Supplementary Table S1, available in www.besjournal.com). A total of one base pair insertion mutation (termination) was found in the motility-related gene *flhA* (Table 1). Additionally, eight distinct mutations were identified in seven non-motility-related genes (Figure 3 and Table 1). However, multiple other mutations were found in this strain and need to be acknowledged as potential influences on the overall phenotype of the bacterium.

Different flhA Alleles in Strains CJ1Z and CJ2S

As part of our efforts to investigate the reason for the phenotypic variation in the CJ1Z strain, we found that strains CJ1Z and CJ2S have different *flhA* alleles. Specifically, the *flhA* allele in strain CJ1Z had a one-base-pair insertion within the 3' end of the gene, which resulted in the formation of a premature stop codon (Figure 4A). FlhA consists of an N-terminal transmembrane (TM) domain with eight predicted TM α-helices and a large cytosolic domain at its C-terminal ($FlhA_C$) (Figure 4B). The truncated FlhA protein produced by the strain CJ1Z had two I719Y/I720Y missense mutations and residues 721 to 724 deleted, compared to 724 amino

Table 1. All mutations predicted in the *flhA* mutant and complemented wild strains

Position	CDS	Mutation	Mutation type
Motility-related			
Strain CJ1Z			
Scaffold1 (51967)	*flhA*	+G (R721*)	Insertion (Termination)
Non-motility-related			
Strain CJ1Z			
Scaffold4 (5943)	hypothetical protein	+C	Insertion
Scaffold4 (12612)	hypothetical protein	ΔTATCT	Deletion
Scaffold15 (2182)	*hisC_3*	+C	Insertion
Strain CJ2S			
Scaffold1 (79809)	hypothetical protein	G→A	Transition
Scaffold1 (471226)	hypothetical protein	+C	Insertion
Scaffold4 (117178)	hypothetical protein	+28 bp	Insertion
Scaffold5 (36939)	*kdpA_3*	ΔG	Deletion
Scaffold13 (6301)	hypothetical protein	+C	Insertion

Note. + indicates the insertion of the following base(s); for longer insertions, + is followed by the number of bases inserted. Δ indicates deletion of the following base(s); *symbolizes a stop codon.

acids for the full-length protein (Figure 4C). Thus, the sequence data suggest that strain CJ1Z has a null mutant *flhA* allele.

The crystal structures of the $FlhA_C$ protein from the CJ1Z and CJ2S strains were obtained using the SWISS-model (https://swissmodel.expasy.org/)[21-25]. In the absence of crystal structure experiments on the FlhAC protein from *C. jejuni*, the crystal structure of the cytoplasmic domain of FlhA from *Helicobacter pylori* was used (Supplementary Figure S1, available in www.besjournal.com)[17]. The *flhA* mutation (FlhA719) is located in the chaperone-binding site of $FlhA_C$ (Figure 5A), raising the question of whether the *flhA* mutation affects the interaction of $FlhA_C$ with chaperone/filament-type export substrate complexes. By searching for hydrogen bonds between amino acids using PyMOL software, we propose that the H-bonds of the last six amino acids (or less) of $FlhA_C$ may stop the export of flagellar biosynthesis protein and affect the expression of the flagella (Figure 5C). Several temperature-sensitive *Salmonella enterica serovar Typhimurium* $FlhA_C$

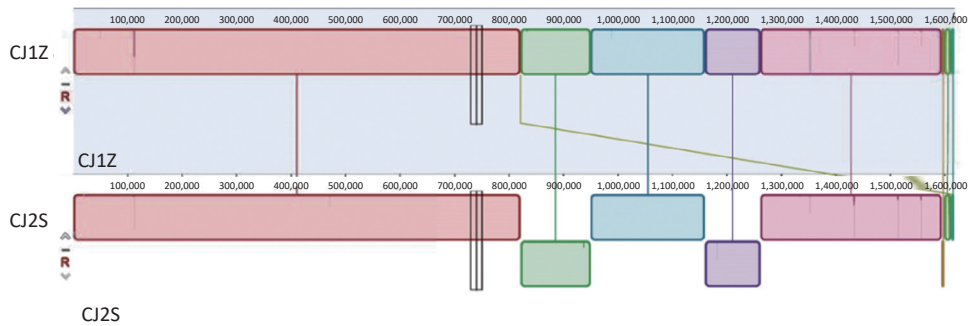

Figure 3. Comparison of complete genomes of *C. jejuni* using Mauve alignment tool. (A) Comparison of complete genomes of *C. jejuni* CJ1Z and CJ2S strains using strain CJ1Z as the reference genome.

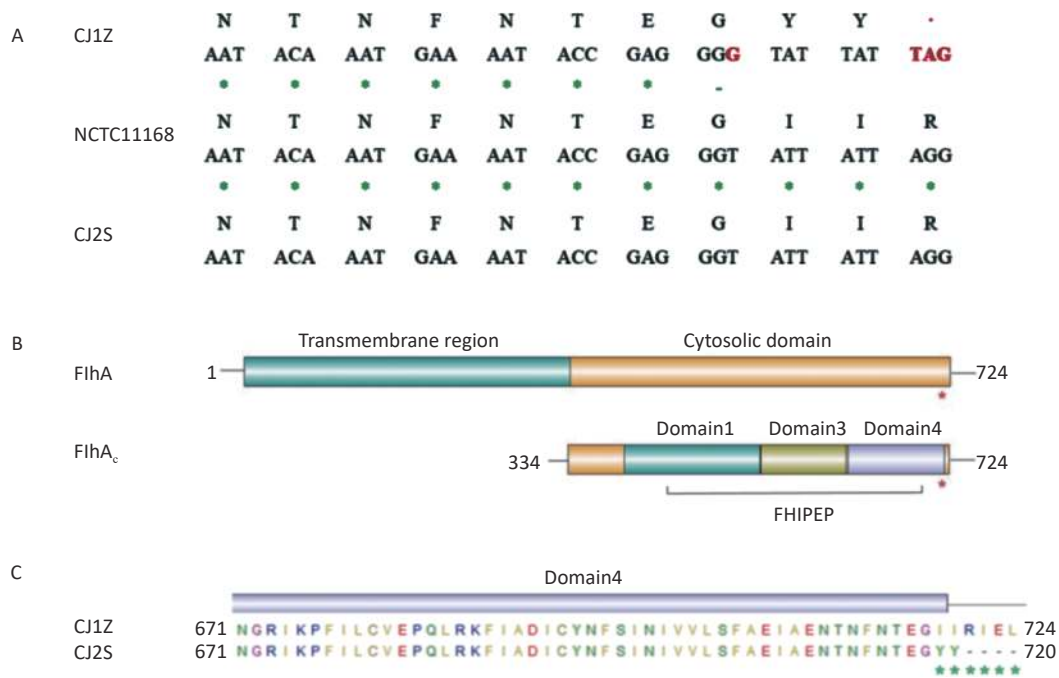

Figure 4. *FlhA* mutation in strain CJ1Z resulting in the early truncation of the protein. (A) Comparison of a section of the *flhA* gene and the translated product between the strains CJ1Z and CJ2S. The location of the 1-bp insert in strain CJ1Z is highlighted in red, as is the location of the stop codon that is subsequently introduced; (B) Domain structure of the FlhA protein. The location of the truncation in strain CJ1Z is highlighted with a red asterisk. The numbers correspond to amino acid positions; (C) Amino acid changes in strain CJ1Z. The numbers correspond to amino acid positions.

missense mutants that cannot regrow flagella at restrictive temperatures (42 °C) have been reported. These mutants have been extensively analyzed using the recently reported *S. Typhimurium* $FlhA_C$ structure. The positions of these mutations are shown in Supplementary Figure S2 (available in www.besjournal.com), and two of the mutations (green circles) have already been reported in relation to the conserved salt bridges at the interface between the helical and C-terminal domains[11,26]. *Campylobacter jejuni* FlhA I719Y mutation, marked with a red square, was located at the same site as *S. Typhimurium.*

DISCUSSION

NCTC11168 is a *C. jejuni* strain that was isolated from a human infection in 1977[27] and was the first *C. jejuni* to be genome-sequenced[1,28]. It has been used as a standard strain for basic research on flagellar biosynthesis genes[7,8,19].

The present study aimed to determine the reasons for the loss of flagellar motility of *C. jejuni* strain NCTC11168 by experimental laboratory evolution. According to the Sher study, at early time points, the motility defect was often reversible; however, after 35 days of serial culture, motility was irreversibly lost in most cells in five independently evolved populations[9]. By observing the phenotypic variation of the strains, we found that *C. jejuni* strain CJ1Z serially passaged in a rich medium exhibited a lack of flagella. This is accompanied by a loss of motility, decrease in the ability to form biofilms, and autoagglutination. Flagella is essential for motility[7,8] and plays a significant role in biofilm formation by *C. jejuni*[8,29]. AAG is often a preliminary step in the formation of microcolonies, and in *C. jejuni*, AAG has been shown to be highly associated with flagellar expression[30,31]. In addition, the role of flagella in the virulence of *Campylobacter spp.* is clearly far more complex and intricate than simple motility[32].

The phenotypes of the *flhA* mutants in *C. jejuni* strain CJ1Z indicate that the last six amino acids (or less) of FlhA are required for flagellar assembly. Sequence analysis of the *flhA* gene in strain CJ1Z revealed a native nucleotide insertion at amino acid position 719 (*flhA*719) that led to the loss of Ile-719, Ile-720, Arg-721, Ile-722, Glu-723, and Leu-724 at the end of the cytosolic domain at its C terminal ($FlhA_C$). This portion of FlhA was predicted to contain a stretch of 391 aa residues at the C-terminal of the protein (Figure 5). $FlhA_C$ belongs to the "flagellum/hypersensitive response/invasion" (FHIPEP) family of bacterial export proteins [33,34]. The

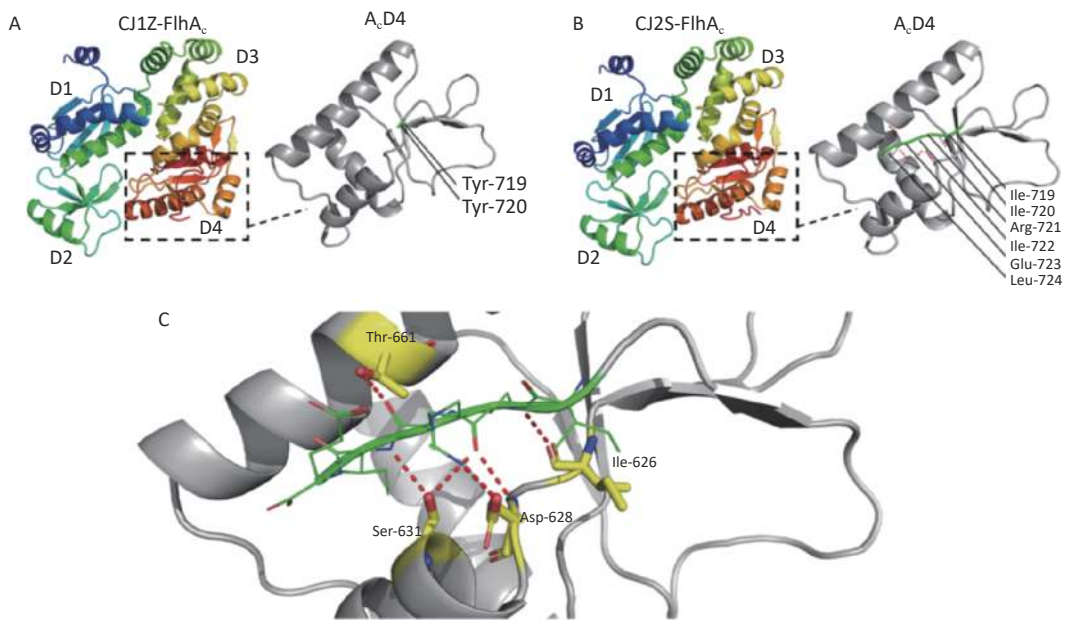

Figure 5. Location of the mutations in FlhA. (A, B) Crystal structure of *C. jejuni* $FlhA_C$, which consists of four domains, D1, D2, D3, and D4. Strain CJ1Z revealed a native nucleotide insertion at amino acid position 719 (*flhA*719) that leads to the substitution of (A) Ile-719, Ile-720, Arg-721, Ile-722, Glu-723, and Leu-724 to (B) Tyr-719 and Tyr-720. (C) The last six amino acids (or less) of FlhA in strain CJ1Z exhibited hydrogen bonds with amino acid residues of FlhA.

hydrophilic C-terminal domain of FlhA extends into the cytoplasm[35,36] and is involved in the energy-dependent secretion of flagellar protein[37]. $FlhA_C$ interacts with FliHIJ and FlhB and with the substrate-chaperone complex (hook–filament junction protein FlgK/FlgN, filament capping protein FliD/FliT, and flagellin FliC/FliS[34,38-40]). Disruption of the *flhA* gene in gram-positive or gram-negative bacteria leads to non-motile cells, which lack flagella and are unable to export flagellar proteins[41-43]. Hara identified a non-motile isolate containing a D208A mutation in FlhA and suggested that this charged residue may be directly involved in PMF-driven protein export[44]. A mutation in the FlhA-binding site for substrate–chaperone complexes (A489E) was found to reduce the binding affinity of these complexes. We speculate that the $FlhA^{719}$ mutation described in this study may influence the assembly or conformation of other components of the export apparatus, which in turn could modulate the expression of flagella.

Sher et al. demonstrated that the *C. jejuni* strain was rapidly attenuated by experimental laboratory evolution and *C. jejuni* in experimental laboratory evolution experiments undergo genomic instability with rapid gene attenuation, including insertions and deletions in the *flhA* gene. In addition, the loss of flagella observed in the present study in strain CJ1Z occurred in the *flhA* gene (FlhA I719Y mutation), which is inconsistent with the mutation described by Sher et al.[9] The binding site for the substrate–chaperone complex consists of α-helix and β-sheet structures and hydrogen bonds (H-bonds) between backbone N−H donors and CO acceptors[45]. H-bonds between the backbone N−H and CO moieties are ubiquitous in proteins, and their contribution to the structure is undisputed[46]. In this study, the last six amino acids (or less) of $FlhA_C$ in strain CJ1Z formed hydrogen bonds with the amino acid residues of FlhA. The absence of H-bonds may cause changes in the binding site that affect flagellar expression.

Carrillo[7] et al. provided evidence that the inactivation of *flhA* inhibits the transcription of both class II and class III flagellar genes and potential virulence factors regulated by σ^{28} and σ^{54} promoters, which are involved in virulence, flagellar biosynthesis, and carbohydrate modification. However, no differences were observed between the two variants in the gene sequence of *flhA* or other known regulators of flagellar biosynthesis. As reported in the literature, both the N-terminal and C-terminal domains of FlhA homologues have been conserved throughout evolution, and the cytoplasmic domains of FlhA and FlhB form complexes with FliH (regulator of FliI), FliI (ATPase), FliJ (general chaperone), and the substrate to be exported. Subsequently, through ATP hydrolysis by FliI, the substrate is translocated across the cytoplasmic membrane through the membrane-spanning domains of FlhA and FlhB, followed by the dissociation of FliH, FliI, and FliJ from the export apparatus[47]. In contrast, in this study, we compared the two variants of the genome-sequenced strains, CJ1Z and CJ2S, which indicated that the motility defect of CJ1Z was largely due to a change in the expression of *flhA*, the C-terminal domain of FlhA. Future work will focus on how the C-terminal domains of FlhA interact with other known regulators of flagellar biosynthesis to understand flhA regulation of flagellar assembly and virulence in *Campylobacter jejuni*. NCTC11168 is commonly used as a reference strain for studying the genes of the flagellar regulon and is accompanied by the loss of flagella after *in vitro* passage. The flagella-lacking strain had a truncated *flhA* gene and exhibited reduced biofilm formation, AGG, and different colony morphologies. In conclusion, *flhA* is more than a flagellar biosynthesis gene and plays an important role in controlling the vital functions of *C. jejuni*. Therefore, future evolutionary studies should be conducted to determine whether cell morphology is preserved. The effect of mutations on flagellar expression should be investigated to study the functions of genes related to flagellar regulation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

ZHANG Mao Jun, ZHANG Jian Zhong, and SHAO

Zhu Jun designed the study; GU Yi Xin, WANG Jia Qi, ZHOU Gui Lan, and WANG Hai Rui isolated and identified *C. jejuni* for strains CJ1Z and CJ2S; CHEN Xiao Li, LIANG Hao, and GUO Peng Bo analyzed the data and wrote the manuscript; and ZHANG Mao Jun supported this study. All authors have read and approved the final manuscript.

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