Research article

Involvement of SPI-2-encoded SpiC in flagellum synthesis in Salmonella enterica serovar Typhimurium

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Abstract

Background: SpiC encoded within Salmonella pathogenicity island 2 on the Salmonella enterica serovar Typhimurium chromosome is required for survival within macrophages and systemic infection in mice. Additionally, SpiC contributes to Salmonella-induced activation of the signal transduction pathways in macrophages by affecting the expression of FliC, a component of flagella filaments. Here, we show the contribution of SpiC in flagellum synthesis.

Results: Quantitative RT-PCR shows that the expression levels of the class 3 fliD and motA genes that encode for the flagella cap and motor torque proteins, respectively, were lower for a spiC mutant strain than for the wild-type Salmonella. Further, this mutant had lower expression levels of the class 2 genes including the fliA gene encoding the flagellar-specific alternative sigma factor. We also found differences in flagella assembly between the wild-type strain and the spiC mutant. Many flagella filaments were observed on the bacterial surface of the wild-type strain, whereas the spiC mutant had only few flagella. The absence of spiC led to reduced expression of the FlhD protein, which functions as the master regulator in flagella gene expression, although no significant difference at the transcription level of the flhDC operon was observed between the wild-type strain and the spiC mutant.

Conclusion: The data show that SpiC is involved in flagella assembly by affecting the post-transcription expression of flhDC.

Background

Salmonellae are gram-negative bacteria causing a variety of disease syndromes in humans and animals. For example, Salmonella enterica serovar Typhi causes a systemic disease in human known as typhoid fever, whereas S. enterica serovar Typhimurium is responsible for gastroenteritis in humans and a systemic disease in mice similar to human typhoid fever. The ability of Salmonellae to survive within macrophages is required for systemic disease [1]. Important virulence factors are introduced into the host environment including the host cell cytosol using two different type III secretion systems (TTSSs) encoded on the Salmonella pathogenicity islands, SPI-1 and SPI-2 [2]. SPI-1 TTSS mediates bacterial entry into non-phagocytic cells [3] and SPI-2 TTSS is required for survival and replication in the intracellular environment of host cells and contributes to systemic infection in animals [4-6].

The spiC gene is adjacent to spiR (srrA)/ssrB, a two-component regulatory gene, and is the initial gene for the oper-
ons encoding the structural and secretory components of SPI-2 [4]. Previous studies show that a strain carrying a mutation in the spiC gene is unable to survive within macrophages and has greatly reduced virulence in mice. The SpiC protein is necessary to inhibit the fusion of Salmonella-containing phagosomes with endosomal and lysosomal compartments [7]. SpiC is translocated by SPI-2 TTSS to the cytosol of the macrophages where it interacts with host proteins, i.e. TassC [8] or Hook3 [9], to alter intracellular trafficking. Further, several investigators report that SpiC is required for the translocation of SPI-2 effector proteins into the target cells by interacting with SsaM, a SPI-2 encoded protein [10-12]. In addition to these reports, we have shown that SpiC contributes to Salmonella-induced activation of the signal transduction pathways in macrophages, leading to the production of mediators such as interleukin-10, prostaglandin E2, and the expression of the suppressor in cytokine signaling 3 (SOCS-3) that are thought to have important roles in Salmonella virulence [13-15]. Additionally, our recent study shows that SpiC is involved in the expression of FliC, a component of the flagella filaments, where FliC plays a significant role in SpiC-dependent activation of the signal transduction pathways in macrophages following Salmonella infection [16]. However, the mechanism of how SpiC affects the expression of FliC remains unknown.

The flagellum is essential for bacterial motility. Its structure consists of a basal body, a hook, and a filament. In Salmonella, synthesis of the flagellum involves over 50 genes. The expression of these genes is organized into three hierarchies. At the top hierarchy is the class 1 flhDC operon and it is essential for transcription of all of the genes for the flagellar cascade. flhDC expression is influenced at the transcription or post-transcription level by a number of global regulatory factors. The class 2 operons contain genes encoding the hook-basal body-associated proteins, a few regulatory proteins, and a component of the flagellum-specific type III export pathway. The class 3 operons contain genes encoding the hook-basal body-associated proteins, a few regulatory proteins, and a component of the flagellum-specific type III export pathway. The class 3 operons are expressed in the stationary phase of growth.

Recent studies have shown that SpiC regulates flagellum synthesis in S. enterica serovar Typhimurium. It was recently shown to be recognized in the host cell cytosol by two different Nod (nucleotide-binding oligomerization domain)-like receptors, Ipaf and Naip5 (also known as Birc1e) [27,28].

Here, we investigate the mechanism of how SpiC regulates flagellum synthesis in S. enterica serovar Typhimurium. We found that SpiC is involved in flagella assembly by affecting the post-transcription expression of the fliDC operon.

Results and discussion

Transcription of the spiC gene is induced during the post-exponential phase of bacterial growth in LB medium

The spiC gene is adjacent to the spiR (ssrA)/ssrB gene set and is the initial gene for the operons encoding the structural and secretory components of SPI-2 [4]. Using primer extension analysis, we first examined the expression of the spiC gene in bacteria grown in LB because expression of SPI-2-encoded genes has been shown to be efficiently induced under limiting conditions such as in medium containing low concentrations of Mg2+ or Ca2+ [29,30]. The bacteria were grown in LB, and the total RNA was isolated when the bacterial culture had an optical density at 600 nm (OD600) of 0.3, 0.7, 1.1, and 1.5 (Fig. 1A). As shown in Fig. 1B, the extension product was only seen when the OD600 was 1.5, indicating that the spiC gene is expressed in the stationary phase of growth.

At the same time, we determined the transcription start site for spiC using a primer extension analysis (Fig. 1C). The size of the extension product showed that the transcription start site of spiC is an adenine that lies 18 nucleotides upstream of the spiC initiation codon (ATG) in agreement with the result of Walthers et al [31]. This indicates that the SpiC protein consists of 127 amino acids with a predicted molecular mass of 14.7 kDa.

Effect of the spiC mutation on the expression of class 3 flagellar genes

In a previous study, proteomic analysis using matrix-assisted desorption/ionization-time of flight mass spectrometry showed that the level of the FlIC protein, a component of the flagella filaments, was lower in the culture supernatant of a spiC mutant, which carries a non-polar mutation in the spiC gene, than in the supernatant from wild-type Salmonella. Further, SpiC is involved in the expression of the flIC gene at the transcription level [16]. These results suggest the possibility that SpiC participates in flagellar phase variation or the flIC gene expression directly. However, in addition to the FlIC protein, we newly identified a Flid flagella protein that was decreased in the spiC mutant using proteomic analysis with liquid chromatography-tandem mass spectrometry (K. Uchiya, unpublished result). Taken together, these results suggest that SpiC contributes to the flagellar system by mecha-
nisms other than phase variation or direct expression of the fliC gene in S. enterica serovar Typhimurium.

Flagella expression in S. enterica serovar Typhimurium is controlled in a hierarchical manner. At the top of the hierarchy is the class 1 flhDC operon that is essential for transcription of all of the genes in the flagellar cascade. The class 2 operons contain the genes encoding the hook-basal body-associated proteins, a few regulatory proteins, and a component of the type III export pathway. The class 3 operons contain genes involved in filament formation, flagella rotation and chemotaxis [17,18].

As described above, proteomic analysis showed that the spiC mutant had lower expression levels of FliC and FliD proteins, suggesting that SpiC is involved in the expression of the class 3 flagellar genes. Therefore, we first investigated the effect of the spiC mutation on the expression of the class 3 genes. The total RNA was isolated from bacteria grown to an OD<sub>600</sub> of 1.6 in LB to induce the expression of the spiC gene (Fig. 1B). We analyzed the transcript levels of the fliD and motA genes that encode the flagella cap and motor torque proteins [17], respectively, using quantitative real-time PCR (RT-PCR). The transcript levels of the fliD and motA genes in the spiC mutant were reduced

Figure 1
Expression of the spiC gene in LB. (A) Growth curve of wild-type Salmonella. An overnight culture in LB was inoculated into fresh LB at a 1:100 dilution. The cultures were grown at 37°C with aeration and monitored by measuring turbidity at an OD<sub>600</sub>. (B) Primer extension analysis of spiC transcription in LB. Bacteria were cultured in LB, and the total RNA was isolated when the OD<sub>600</sub> reached 0.3, 0.7, 1.1 and 1.5. Fifty micrograms of RNA was hybridized with a 5'-end-labelled DNA fragment specific for the spiC gene and subjected to 6% polyacrylamide-7 M urea gel electrophoresis. The GATC lane corresponds to dideoxy chain termination sequence reactions in the region encompassing the spiC promoter. A single extension product was seen only at an OD<sub>600</sub> of 1.5 corresponding to the stationary phase of growth. The asterisk indicates the transcription initiation site. (C) Nucleotide sequence of the spiC promoter region. The transcriptional start site for spiC is numbered as +1, and the hooked arrow indicates the direction of transcription. The proposed -10, -35, and Shine-Dalgarno (SD) sequences are underlined. The start codon is marked in bold. The double underline indicates the sequence of the designed primer for primer extension analysis.

Figure 2
Expression of the class 3 fliD and motA genes in the spiC mutant. Bacteria were cultured in LB to an OD<sub>600</sub> of 1.6, and the total RNA was extracted from the wild-type Salmonella (WT), spiC mutant strain, or spiC mutant strain carrying the spiC gene-containing plasmid pEG9127 (spiC<sup>+</sup>). Quantitative RT-PCR was conducted using a TaqMan probe. Levels of fliD (A) or motA (B) mRNA were normalized to 16S rRNA levels, and the results are shown relative to the expression in the wild-type strain. The expression levels of both genes in the spiC mutant were greatly reduced compared to the wild-type strain.
by approximately 15-fold and 6-fold compared to the wild-type strain, respectively (Fig. 2). Complementation of the spiC mutant with a plasmid carrying the wild-type spiC gene (pEG9127) restored the fliD and motA transcripts to about 80% of the level of the wild-type strain. Further, to confirm the contribution of SpiC in the regulation of class 3 flagellar gene transcription, we constructed a deletion mutant of the spiC gene by using the lambda Red mutagenesis technique and examined the motA mRNA level. The deletion mutant showed the same phenotype as the spiC mutant (EG10128) used in this study (data not shown). These data indicate that SpiC has an influence on the flagellar system.

The spiC mutant is defective in flagella filament formation

Because the flagella filament is made from the flagellin proteins FliC and FljB, we examined flagella of the respective Salmonella strains using electron microscopy. We found differences between the wild-type strain and the spiC mutant. Many flagella filaments were observed on the bacterial surface of the wild-type strain (Fig. 3A), whereas the spiC mutant had few flagella (Fig. 3B). Additionally, the defective flagella filament formation in the spiC mutant was restored by introducing pEG9127 (Fig. 3C). The data suggest that SpiC affects the formation of flagella filaments by controlling the expression of flagellar genes. We next examined the involvement of other SPI-2-encoded virulence factors in flagella assembly. As expected, a mutation in the spiR gene [4], a two-component regulatory gene involved in the expression of SPI-2-encoded genes, resulted in the defective formation of flagella filaments, similar to the spiC mutant (Fig. 3D); however, the defective phenotype was not seen in the ssaV mutant that lacks a putative component of the SPI-2 TTSS (Fig. 3E) [32]. This suggests the specific involvement of SpiC in the assembly of flagella filaments. Further, we examined the effect of SpiC on formation of flagella filaments using N-minimal medium containing low Mg²⁺ (pH 5.8) that is effective in inducing SPI-2 gene expression [29]. However, we did not observe flagella even in the wild-type strain (data not shown).

Because the absence of SpiC leads to the reduction of class 3 genes expression including the motA gene, which is necessary for motor rotation, we next investigated the motility of the respective Salmonella strains using LB semisolid plates (Fig. 3F). Like the results for flagella formation, the wild-type strain, the ssaV mutant, and the spiC mutant carrying pEG9127 made large swarming rings, whereas the spiC and spiR mutant had weak swarming abilities. And the flhD mutant was non-motile.

Expression of class 2 flagellar genes in the spiC mutant

To examine the mechanism by which SpiC is involved in the expression of the class 3 genes, we focused on the class 2 fliA gene encoding the flagellar-specific alternative sigma factor σ²⁸, which is required for transcription of the class 3 promoters [33,34]. The activity of the transcription factor σ²⁸ is negatively regulated by direct interaction with an anti-σ²⁸ factor, the FlgM in the cell [35,36]. FlgM is excreted out of the cell through the flagellum-specific type III export apparatus, leading to the induction of fliA gene transcription [37-39]. SpiC is reported to be required for secretion of some virulence factors from the cytoplasm using the SPI-2 TTSS [10,11], although the molecular mechanism is not known. Several genes encoding the SPI-2 TTSS and the flagellum-specific type III export system show sequence similarities [18,40]. Therefore, in addition to its role in SPI-2 TTSS, SpiC might participate in the export of FlgM proteins from the cytoplasm via the type III flagellar protein export system. To examine this possibility, cell lysates were prepared and the level of intracellular FlgM was assessed using Western blot with anti-FlgM antibody. Western blot analysis showed that the level of FlgM in the wild-type cell was higher than that in the spiC mutant (data not shown), indicating that a decrease in class 3 genes expression in the spiC mutant is due to an FlgM-independent mechanism.

In subsequent studies, we measured the expression level of the fliA gene by fusing the transcription regulatory
region of fliA to lacZ in pRL124, as described in the Materials and Methods (Fig. 4A), and quantitatively measured the expression level using RT-PCR (Fig. 4B). The expression level of the fliA gene in the spiC mutant was greatly reduced compared to the wild-type strain. In addition to the fliA gene, we further investigated the influence of SpiC on the expression of the class 2 fgbB and flif genes [17]. As shown in Fig. 4C and 4D, quantitative RT-PCR analysis showed that the transcript levels of the fgbB and flif genes in the spiC mutant were reduced approximately 7-fold and 3-fold in comparison to the wild-type strain, respectively. These results indicate that SpiC affects the regulation of class 2 genes transcription, and suggest the involvement of SpiC in the expression of the class 1 flhDC gene, which functions as the master regulator in flagellar genes expression [17].

** SpiC is required for the post-transcriptional expression of the master regulator, FlhDC**

The class 1 genes products FlhD and FlhC form a heterotetramer that activates the σ54 promoter in the class 2 genes by interacting with the RNA polymerase α subunit [41,42]. flhDC expression is influenced at the transcription or post-transcriptional level by a number of regulatory factors. For example, cyclic AMP-CRP [43-46], H-NS [46,47], QseBC [48], CsrA [49], and the heat shock-induced chaperones, DnaK, Dnak, and GacE [50], function as positive regulators, while negative regulation is mediated by OmpR [51], RcsCDB [52], LrhA [53], and ClpXP [54].

Because SpiC was found to affect the expression of the class 2 genes including the fliA gene, we examined the involvement of SpiC in the flhDC operon expression using an flhDC-lacZ fusion (Fig. 5A), and measured the level using quantitative RT-PCR (Fig. 5B). Although the spiC mutant showed a slight reduction in flhD expression compared to the wild-type strain, no significant difference in the flhD expression level was observed between the wild-type strain and the spiC mutant. Reports show that the flhD expression level is reduced approximately 2- to 3-fold by mutation to the regulatory genes affecting the flhD expression at the transcription level [46,48,51,53]. Together with these findings, we concluded that the reduced level of the class 2 gene expression in the spiC mutant is not dependent on flhDC transcription. To investigate whether SpiC participates in flhD expression at the post-transcriptional level, we performed Western blot analysis with anti-FlhD peptide antibody. Although the detection level of FlhD was low, we found significant differences between the wild-type strain and the spiC mutant (Fig. 5C and 5D). The absence of spiC led to the reduced expression of the FlhD protein, indicating that SpiC is involved in flhD expression at the post-transcriptional level.

Although the molecular mechanism by which SpiC contributes to the post-transcriptional regulation of the flhD expression remains unknown, it is thought that SpiC directly or indirectly participates in either flhD translation or in the stability of the FlhD protein. Almost all of the positive regulators that involved in flhDC expression regulate their expression at the transcription level [45-47,50], while CsrA, a RNA-binding protein, stimulates flhDC expression using a post-transcription mechanism [49]. CsrA is thought to allow flhDC translation by binding to the 5’ segment of the flhDC mRNA and stabilizing its mRNA. The Csr system consists of CsrA and the two small regulatory RNAs, csrB and csrC. The activity of CsrA is reported to be antagonized by csrB and csrC RNAs [55].

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**Figure 4**

Expression of the class 2 genes in the spiC mutant. (A) β-galactosidase activity from fliA-lacZ transcription fusion expressed by wild-type Salmonella (WT) and spiC mutant strain grown in LB to an OD600 of 1.6. β-galactosidase activity is expressed in Miller units. WT (pRL124) carries the vector with the promoterless lacZ. Quantitative analysis of fliA (B), fgbB (C), or flif (D) mRNA expression. Bacteria were cultured in LB, and the total RNA was extracted from the wild-type Salmonella, spiC mutant strain, or spiC mutant strain carrying pEG9127 (spiC+) when the OD600 was 1.6. Quantitative RT-PCR was conducted using a TaqMan probe. Levels of each mRNA were normalized to the 16S rRNA concentration, and the results are shown relative to the expression in the wild-type strain. The expression levels of the fliA, fgbB, or flif gene in the spiC mutant were greatly reduced compared to the wild-type strain.
where gene expression is controlled by the BarA/SirA two-component regulatory system that is involved in the expression of SPI-1-encoded genes [56-58]. One hypothesis is that SpiC affects FlhDC expression via a Csr post-transcriptional regulatory system. Therefore, we investigated the effect of SpiC on csrB and csrC expression using quantitative RT-PCR. However, no differences in the expression levels of these genes were observed between the wild-type strain and the spiC mutant (data not shown). More research is required to clarify the molecular mechanism in how SpiC regulates the post-transcriptional expression of the flhDC.

We next examined the expression of FlhD at bacterial growth phase of OD_{600} of 0.7 in LB, because the spiC expression is induced at over an OD_{600} of 1.5 when the bacteria are grown in LB. However, the expression level of FlhD in the spiC mutant was reduced compared to the wild-type strain even in the exponential growth phase (data not shown), indicating that the FlhD expression is not strictly growth phase-dependent. We cannot explain this phenomenon until the mechanism by which SpiC regulates the post-transcriptional expression of the flhDC or the molecular mechanism of SpiC become clear.

As described above, the BarA/SirA system is involved in not only the flagella gene expression but also the SPI-1 gene expression. Phosphorylated SirA directly interacts with promoters of the hiiA and hilC genes that are the SPI-1-encoded transcription regulator genes [58]. HilA, a member of the OmpR/ToxR family, directly activates transcription of the inv/spa and prg/org promoters on SPI-1 [59]. In addition to the BarA/SirA system, the AraC-like regulator RitA directly controls the hilA expression leading to SPI-1 gene expression, while RitB, a helix-turn-helix DNA binding protein, negatively regulates the expression of the flhDC [60]. Reports also show that the ATP-dependent ClpXP protease negatively regulates the expression of flagella and SPI-1 gene [54,61]. Interestingly, mutation in the SPI-2 genes also affects the expression of the SPI-1 gene [62]. And thus many reports show the relationship of flagella synthesis and SPI-1 gene expression.

Our recent studies show that the SpiC-dependent expression of FliC plays a significant role in activation of the signaling pathways leading to the induction of SOCS-3, which is involved in the inhibition of cytokine signaling, in Salmonella-infected macrophages [16]. Lyons et al. [63] also reported that infection of polarized epithelial cells by Salmonella leads to IL-8 expression by causing the SPI-2-dependent translocation of flagellin to a basolateral membrane domain expressing TLR5. Together with our previous results, these findings suggest the involvement of FliC in SPI-2-dependent events in the pathogenesis of Salmonella infection.

**Conclusion**

In conclusion, here we show that SpiC encoded within SPI-2 is required for flagella assembly in *S. enterica* serovar Typhimurium. We concluded that the mechanism is due to the involvement of SpiC in the post-transcriptional expression of FlhDC. The data indicate the possibility that SPI-2 plays a role in *Salmonella* virulence by making use of the flagellar system.
Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains used in this study were derived from the wild-type S. enterica serovar Typhimurium strain 14028s. The spiC::kan derivative EG10128 was described by Uchiya et al. [7]. The deletion mutant in the flhD gene was constructed using the Red recombination system by Uchiya et al. [7]. The deletion mutant in the flhD gene was confirmed using PCR with primer combinations are shown in Table 1. Threshold cycle values were calculated from the amplification plots, and the amount of each gene expression was determined relative to the level of the gene expression in wild-type Salmonella after both values were normalized to the 16S rRNA levels. Each sample was analyzed in triplicate.

Preparation of whole-cell proteins

An overnight culture in LB was inoculated into 15 ml of fresh LB at a 1:100 dilution. The cultures were grown at 37°C with mild aeration to an OD₆₀₀ of 1.6 (the spiC-inducing condition). After a 1-ml sample of the culture was centrifuged at 18,500 × g for 15 min, the bacterial pellet was suspended in 1 ml of cold water was mixed with trichloroacetic acid (final concentration 6%), placed on ice for 30 min, and centrifuged at 14,000 × g for 20 min. After drying, the pellets were dissolved in 100 μl of each fluorescent probe, and TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Amplification was performed in 96-well optical plates using the 7300 Real-Time PCR System (Applied Biosystems) with an initial incubation of 2 min at 50°C; followed by 10 min at 95°C; and then 40 cycles: 95°C for 15 s and 60°C for 1 min.

RNA preparation and primer extension analysis

Bacteria were grown in LB. When the OD₆₀₀ reached 0.3, 0.7, 1.1, and 1.5, the total RNA was isolated using an RNasy kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s protocol. The RNA (50 μg) was mixed with 32P-end-labeled synthetic oligonucleotide (5'-GCAGGATGCCCAATGATCT-3'), and 50 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) was added to 30-μl reaction mixtures containing 1 mM of deoxynucleotide triphosphates, 5 mM dithiothreitol, and 1 unit of RNasin/μl. The reaction was performed at 42°C for 1 h. The extension products were analyzed using electrophoresis on a 6% polyacrylamide-7 M urea gel and compared to sequence ladders initiated with the same primer.

Quantitative RT-PCR

Bacteria were grown in LB, and the total RNA was isolated when the OD₆₀₀ reached 1.6. The isolated RNA was treated with DNase I (Invitrogen) to remove contaminating DNA, and 2 μg of RNA was reverse-transcribed using SuperScript II reverse transcriptase with random primers. Real-time PCRs were performed in a 50-μl reaction mixture containing 1 μl cDNA, 0.9 μM each primer, 0.25 μM fluorescent probes are shown in italic.
sodium dodecyl sulfate (SDS)-sample buffer and boiled for 5 min.

Construction of the fliA or flhD-lacZ fusion on a plasmid
To construct the transcriptional fusion of the fliA or flhD promoter region to the promoterless lacZ gene using the promoter-probe vector pRL124 [65], a 0.51-kbp DNA fragment containing the fliA promoter region or a 0.73-kbp DNA fragment containing the flhD promoter region were amplified using PCR with the following primers: for fliA, 5'-ACGCCGTCGACTATGCGCCTGTAAGGCGC-3' and 5'-CGGGGTACCCGGATGTATGCATTGTGAC-3', and for flhD, 5'-ACGCCGTCGACGGCAGCAAATATGTAAGGGACAC-3' and 5'-CGGGGTACCCGCGGATGCAATCAGTGTAATGCTCCC-3'. The PCR products digested with SalI and KpnI were ligated into the same site in pRL124, producing pRL-fliA and -flhD.

Galactosidase assay
Bacteria were grown overnight in LB at 37 °C and diluted to 1:100 in fresh LB and grown with aeration to an OD 600 of 1.6. β-galactosidase activity was measured using the substrate o-nitrophenyl β-D-galactoside as described elsewhere [66]. Each sample was assayed in triplicate.

Transmission electron microscopy
Bacterial cells grown in LB for 20 h at 37 °C without shaking were deposited on carbon-ﬁlm grids, partially dried, and stained with 2.0% uranyl acetate. The negatively stained samples were observed using a 2000EX electron microscope (JEOL) at an acceleration voltage of 100 kV.

Western Blot Analysis
Whole-cell proteins (150 μg) from bacteria were fractionated in 16% Tricine-SDS-polyacrylamide gel, electrophoresed, and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as described previously [14]. The bands were detected using the ECL plus Western blot detection system (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s instructions. The peptide fragment, DHQUITRTLQDSRV, from the FlhD polypeptide was synthesized and an antiserum specific for the oligopeptide was obtained by immunization of rabbits with the peptide coupled to keyhole limpet hemocyanin using benzidine. The resulting anti-FlhD peptide antibody was used at a dilution of 1:300. DnaK was detected with a 1:1000 dilution of anti-DnaK antibody (Assay designs, Ann Arbor, MI). Bands were analyzed using a GS-800 calibrated densitometer (Bio-Rad).

Statistical analysis
Each experiment was performed at least three times. The results were expressed as means ± the standard deviations. The data were analyzed using analysis of variance with the Dunnett’s test. A value of p < 0.05 was considered statistically significant.

Authors’ contributions
AS performed experiments and analyses. TN helped to draft the manuscript. KU contributed to the experimental designs and drafted the manuscript. All authors read and approved the final manuscript.

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