**Expression and secretion pattern of FljB of *Salmonella enterica*
serovar Typhi in different culture conditions**

Xueming Zheng, Qisi Zhang, Ying Ji, Xin Zhao, Bin Ni, Shungao Xu,
Xinxiang Huang*

Department of Basic Medicine, School of Medicine, Jiangsu University,
Zhenjiang 212013, Jiangsu, China

* Corresponding author

Xinxiang Huang (huxinx@ujs.edu.cn)

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Introduction

Flagellin is the main structural protein of flagellum, which is the
motility apparatus of *Salmonella*. The variety of flagellin genes
contributes much to the diversity of the serovars of *Salmonella* [1, 2].
Most serovars of *Salmonella* are biphasic strains, which contain two
different flagellin genes, *fliC* and *fljB*, in different loci of the chromosome
[3, 4]. The biphasic strains can express and secrete one kind of flagellin at
any given time. In the process of infection, most biphasic strains can
alternately express these two flagellin genes through a process called
'phase variation', which is mediated by the recombinase Hin [5].

Salmonella enterica serovar Typhi (*S. Typhi*) is a human-specific and virulent pathogen which can cause typhoid fever or death in the worst case [6]. Most *S. Typhi* strains show monophasic because their genomes contain only *fliC* gene encoding either the full-length H:d antigen or the truncated H:j antigen [7, 8]. But in some *S. Typhi* strains collected from Indonesia, a new flagellar antigen z66 was discovered [9]. The H:z66 antigen is encoded by a *fljB*-like gene which is located on a novel 27-kb linear plasmid named pBSSB1. *S. enterica* serovar Typhi GIFU10007 (*S. Typhi* GIFU10007) is a z66+ strain which contains operon *fljBA* located on the linear plasmid pBSSB1 [8, 10, 11]. FljB is the only expressed flagellin gene although there is a *fliC* gene in the genome which is always suppressed by *fljA*. The z66+ strain was shown to undergo unidirectional flagellar phase change caused by the deletion of a 2.7-kb terminal region containing the majority of the *fljBA* gene cluster [12]. The molecular mechanism and biological significance of unidirectional flagellar phase change remains obscure.

In the process of infection, *Salmonella* encounters great challenges from host such as hyperosmolarity in the intestine, low pH and low Mg^{2+} in the *Salmonella*-containing vacuole (SCV) [13]. To facilitate successful invasion and replication, two kinds of type three secretion systems (T3SS) encoded in *Salmonella* pathogenicity island (SPI)-1 and SPI-2 are activated at different infection stages of *Salmonella* [14]. Many effector

proteins as well as flagellin are injected into host cells to facilitate successful infection of *Salmonella* [15, 16]. Activated type three secretion system 1 (T3SS1) is required for invasion of nonphagocytic cells, whereas type three secretion system 2 (T3SS2) is essential in intracellular survival and biogenesis of the SCV [17].

Flagellin monomers of *Salmonella* can be detected by surface-localized toll-like receptor 5 (TLR5) of host cells. Through the interaction of flagellin with TLR5, pathogens are sensed by host cells and trigger an inflammatory response that limits systemic spread of the bacteria [18, 19]. During the infection of *Salmonella*, flagellin can be injected into the host cells in a process depending on T3SS1 [16]. The intracellular flagellin can also be recognized by two different Nod-like receptors NLRC4 (also known as Ipaf) and Naip5 (also known as Bircle) [20]. The interaction activates NLRC4 inflammasome and downstream caspase-1, which promotes the secretion of the proinflammatory cytokines IL-1 β and IL-18 as well as pyroptosis [21, 22].

Since flagellin can arouse innate immune response in host cell, the regulation of flagellin is vital in the pathogenesis of *Salmonella*. Little work is done about the regulation of FljB in the infection process. We herein studied the expression and secretion pattern of FljB in *S. Typhi* GIFU10007 strain using in-vitro SPI-1 (hyperosmolarity) and SPI-2 (LPM, pH5.8) inducing conditions which mimic aspects of different

infection stages of *Salmonella*. The present study indicated the different expression and secretion pattern of FljB at different infection stages of *S. Typhi* GIFU10007.

Materials and Methods

Bacterial cultures

A z66-antigen-positive wild-type strain of *S. enterica* serovar Typhi GIFU10007 (*S. Typhi* GIFU10007) was used in this study. Bacteria were cultured at 37 °C in regular Luria–Bertani (LB) broth (170 mM NaCl) with shaking. For SPI-1 inducing condition (hyperosmolarity), regular LB broth was added additional NaCl to a final concentration of 340 mM. For SPI-2 inducing condition, a modified M9 medium with low-phosphate, low-magnesium medium (LPM) at pH5.8 is used. LPM consists of 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 38 mM glycerol (0.3% v/v), 0.1% Casamino acids, 8 μM MgCl₂, 337 μM PO₄⁻³, 80 mM MES, pH5.8 [23]. For changing the culture medium, *Salmonella* strains grown in LPM (pH 5.8) overnight were inoculated 1: 100 in SPI-1 inducing broth.

Preparation of the whole-cell lysates and the secretion proteins

When the bacteria in each medium were cultured overnight, the same amount of cells were taken out and centrifuged at 5,000 × g and 4 °C for

10 minutes. The cell pellets were resuspended in 200 μ l phosphate buffered saline (PBS) and were sonicated for 15 seconds three times with an interval of 30 seconds. The 20 μ l whole-cell lysates were taken out and subject to SDS-PAGE and Western blotting analysis. For the secretion proteins, bacteria were pelleted by centrifugation for 2 min at 10 000g (4 °C) and the supernatant was passed through a 0.22 mm filter and precipitated with trichloroacetic acid (10 %, v/v, final concentration) on ice for 15min and centrifuged at 10 000g for 10 min. The pellets were suspended with 0.3ml acetone and centrifuged at 10 000g for 5min twice to removing TCA completely [24]. The pellets were resuspended in phosphate buffered saline (PBS) and subjected to SDS-PAGE and Western blotting analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Immunoblotting Analysis

All the samples were subjected to SDS-PAGE with 15% acrylamide gel and visualized with Coomassie Blue staining. For Western blotting, the proteins on SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes (PerkinElmer). The blots were incubated with rabbit anti-FljB antiserum (National Institute of Infectious Disease, Japan), DnaK monoclonal antibody (Enzo Life Science) and horseradish peroxidase-conjugated anti-IgG secondary antibodies (Santa Cruz). The

proteins were visualized using an ECL detection kit (Amersham Pharmacia Biotech).

RT-PCR

RNA was purified using RNeasy RNA extraction kit (Qiagen) from *S. Typhi* cells following the manufacturer's instructions. The quality and quantity of purified RNA were examined on a ND-1000 spectrophotometer (Nanodrop). The recommended equal amount of RNA was prepared to generate cDNA using superscript II reverse transcriptase (Invitrogen). As template, cDNA from each culture medium was amplified with primers specific for *fljB* of *S. Typhi*. Sequences of primers are as following:

Forward Primer: 5'-GATTGCTAACCGCTTCAC-3'

Backward Primer: 5'-TTCTCAGCCAGCACTTTC-3'

Motility analysis

S. Typhi GIFU10007 cells were cultured overnight at 37 °C in regular LB, hyperosmolarity or LMP broth overnight. Equal amount of cells from each culture were spotted onto a motility plate (regular LB with 0.3% agar). The plates were incubated at 37 °C for 10 h and motility was measured by examining the diameter of circular swimming which was formed by the growing bacterial cells.

Construction of *fljA* and *phoP* deletion mutants

The *fljA* and *phoP* mutants were prepared by homologous recombination according to a previously described method with suicide plasmid pGMB15 [25]. Briefly, the gene-specific upstream and downstream fragments were amplified and cloned into suicide plasmid pGMB151. The suicide plasmid carrying the deleted gene fragment was transferred into wild-type *S. enterica* serovar Typhi GIFU10007 strain by electroporation, as described previously. After selective incubation on LB plates with ampicillin and streptomycin, the positive bacteria clones containing the recombinant suicide plasmid were selectively incubated on LB plates with 5% sucrose to drop the suicide plasmid and obtain the homologous mutant strain. The *fljA* and *phoP* mutants were confirmed by PCR using specific primers to the upstream and downstream regions of the respective deletion gene and DNA sequencing of the PCR product.

Results

Different expression and secretion pattern of FljB in SPI-1 and SPI-2 inducing conditions.

Since flagellin plays an important role in the interaction of pathogen with host, we are interested in the expression and secretion pattern of flagellin in the infection process of *Salmonella*. For successful infection,

two kinds of type three secretion systems (T3SS) encoded in SPI-1 and SPI-2 are activated at different stages of infection. We herein used SPI-1 and SPI-2 inducing conditions *in vitro* to mimic aspects of different infection stages of *S. Typhi* GIFU10007. As shown in Figure 1, compared with regular LB, hyperosmolality increased the secretion while there was no secretion of FljB in LPM condition. It was demonstrated that *Salmonella* expressed the same protein level of FljB in regular LB and hyperosmolality, but no detectable FljB in LPM by examining the whole cell lysates. The SDS-PAGE showed an obvious band around 51 kD (upper panel) which was confirmed by Western blotting using FljB antiserum (middle panel). In summary, SPI-1 inducing condition increases the secretion but not the expression level of FljB while LPM shuts down the expression and consequential secretion of FljB.

***S. Typhi* GIFU10007 can restore FljB expression and secretion after moving from SPI-2 to SPI-1 inducing condition.**

It is reported that there is rarely unidirectional flagellar phase change in z66+ strains [12]. Accordingly, we want to know whether *fljB* is deleted in the SPI-2 inducing condition. So, we examined the expression and secretion of FljB after transferring bacteria from SPI-2 to SPI-1 inducing condition. As shown in Figure 2A, *Salmonella* cultured in LPM can restore the expression and secretion of FljB in hyperosmolality medium,

which indicates that LPM leads to the silence but not the deletion of *fljB*. *S. Typhi* GIFU10007 seems to secrete more FljB although the expression of FljB is partially recovered after transferring culture (Figure 2A, middle panel). The reversible expression of *fljB* in SPI-2 inducing condition was also supported by the RT-PCR analysis of sample from different culture conditions with specific *fljB* primers (Figure 2B). In brief, we found that *S. Typhi* GIFU10007 can regulate the expression and secretion of FljB in different culture conditions. The molecular mechanism and biological significance of this phenomenon need to be further addressed.

The expression level of FljB in different culture conditions affects the motility of *S. Typhi* GIFU10007

Given the great difference in the expression and secretion of FljB in our in-vitro culture experiments, we wanted to know the motility of bacteria in SPI-1 and SPI-2 inducing conditions. As demonstrated on the motility plate, the *S. Typhi* GIFU10007 cells from LPM showed decreased motility while cells from transferring culture showed increased motility when compared with those from hyperosmolarity broth (Figure 3). The results of motility are consistent with the secretion of FljB in different culture conditions. The motility of cells from LPM culture may result from the restored expression of FljB during the culture on the plate.

No expression and secretion of FljB in SPI-2 inducing conditions is *phoP*-independent

Since the PhoP/Q two-component system has been implicated in response to SPI-2 inducing conditions, we investigated the expression and secretion of FljB with *phoP* mutant. As shown in Figure 4, there is no expression and secretion of FljB for *phoP* mutant in LPM condition, the same results with that of wild-type *S. Typhi* GIFU10007. But, the *phoP* mutant showed the same expression and secretion level of FljB in hyperosmolality and regular LB conditions, different from that of wild-type *S. Typhi* GIFU10007. The results indicated that hyperosmolality increased the secretion of FljB is dependent on PhoP, while the no expression and secretion of FljB in SPI-2 inducing conditions is *phoP*-independent.

The expression and secretion of FliC of *fljA* mutant are also shut down in SPI-2 inducing condition

In the genome of *S. Typhi* GIFU10007, there is another flagellin gene *fliC*, whose expression is suppressed by *fljA* in a post-transcriptional manner. To investigate the expression and secretion of FliC in different conditions, we constructed the *fljA* deletion mutant. As shown in Figure 5, *fljA* mutant expressed and secreted both FljB and FliC proteins. For *fljA* mutant, hyperosmolality leads to increased secretion while LPM results in

no expression of both FljB and FliC proteins compared with regular LB. The result indicated that *fliC* has the same regulation pattern with *fljB* in SPI-1 and SPI-2 inducing conditions although the promoters of the two flagellin genes showed some differences.

Discussion:

Previous work of our lab showed that transcription of *fljB* is dependent on *fliA* and *flhDC*, which are the transcriptional regulators of the *fliC* gene [26, 27]. However, the promoter sequence of *fljBA* operon from *S. Typhi* GIFU10007 is different from that of *fliC* and whether there are other regulators remain elusive. Our results showed that SPI-1 inducing condition (hyperosmolarity) increased the secretion but not the expression level of FljB, while the SPI-2 inducing condition (LPM, pH5.8) imitating the SCV niche completely suppressed the expression of fljB. Moreover we didn't find the secreted FliC protein which is putative 44 kD on the SDS-PAGE gel even when the expression of *fljB* is blocked in wild-type *S. Typhi* GIFU10007. After transferring from SPI-2 to SPI-1 inducing condition, the expression and secretion of FljB can be restored. The results demonstrate that FljB is the only flagellin protein to be expressed and secreted and there is no phase variation for *S. Typhi* GIFU10007 in our culture conditions. It was reported that flagellin was secreted into the cytosol of host cell by the T3SS1 [16]. The increased secretion of

flagellin in SPI-1 inducing condition may result from the activated T3SS1.

In response to SPI-2 inducing conditions, some two-component systems are activated to change the expression profile of *Salmonella*. Previous research showed that PhoP can be activated by low pH and low Mg^{2+} and the PhoP/Q regulon makes great contributions to survival and replication of *Salmonella* in macrophages [28]. We next investigated whether the expression is regulated by PhoP/Q two-component systems directly or indirectly. The *phoP* mutant also showed completely suppression of *fljB* in LPM condition, the same result as that of wild-type *S. Typhi* GIFU10007. So, *phoP* and its downstream genes are not responsible for the suppression of *fljB*. Whether SsrA/B or other two-component systems are involved and the regulators of *fljB* need to be addressed in the future.

Is the expression and secretion pattern restricted in FljB of *S. Typhi* GIFU10007? Since most *S. typhi* stains have only one flagellin gene *fliC* located in the genome [29], we are interested in regulation of FliC in the in-vitro culture conditions. The data from *fljA* mutant showed that FliC had the same pattern of expression and secretion with FljB. So, it seems a general strategy for *Salmonella enterica* Typhi to regulate the expression and secretion of flagellin in different stages of infection.

In SPI-1 inducing condition, Many effector proteins such as SipA, SopE, SopE2 and SopB are injected into host cells through the activated T3SS1. These SPI-1 effector proteins can activate the host innate immune response mainly through activating NF- κ B signal pathway [17]. Our data showed that SPI-1 inducing condition also increased the secretion of flagellin which can also activate the host innate immune response through inflammation pathway. But in SPI-2 inducing condition, the injected effector proteins through T3SS2 inhibit the NF- κ B signal pathway through a variety of mechanisms [30]. Our data showed the consistent result that the expression of *fljB* is completely shut down in SPI-2 inducing condition. So, our data indicate a new strategy for *Salmonella* to subvert the host's innate immune system through regulation of the expression and secretion of flagellin.

How do the different conditions induce the different expression and secretion of FljB and FliC? How does the regulated secretion of flagellin correlate with the successful infection of *Salmonella*? The questions should be intensely studied and addressed in the future.

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Figure legends:

Figure 1: Expression and secretion of FljB in vitro under different culture conditions. Wild-type *S. Typhi* GIFU10007 cells were cultured in regular LB, hyperosmolarity or LMP medium overnight, the secretion proteins and the whole cell lysates were analyzed by SDS-PAGE followed by Coomassie Staining (upper panel) and Western blotting with the indicated antibody (middle and lower panel). WCL: whole cell lysate; IB: immunoblot.

Figure 2: Expression and secretion of FljB can be restored after transferring *S. Typhi* GIFU10007 from SPI-2 to SPI-1 culture condition. A. Wild-type *S. Typhi* GIFU10007 cells were cultured in different conditions as indicated. The secretion proteins and the whole cell lysates were analyzed by SDS-PAGE followed by Coomassie Staining (upper panel) and Western blotting with FljB (middle panel) and DnaK as control (lower panel) antibodies. The band of FljB protein on the SDS-PAGE gel is indicated with an arrow. WCL: whole cell lysate; IB: immunoblot. B. RT-PCR of *fljB*. Cells were cultured in different conditions as indicated. Equal amount of cells in each culture were taken out and prepared for the cDNA. The expression of *fljB* is detected with a pair of primers specific for *fljB*. The positive band is indicated with an

arrow on the 2% agarose gel. The result is representative of three independent experiments

Figure 3: Motility of *S. Typhi* GIFU10007 after cultivation in SPI-1 or SPI-2 condition. Cells were cultured overnight in the conditions as indicated. The motility of cells from each culture was demonstrated on motility plate (A) and analysis by Student's t test (*, $P < 0.05$, $n=3$) (B).

Figure 4: Expression and secretion of FljB in *phoP* mutant under different culture conditions. *phoP* mutant cells of *S. Typhi* GIFU10007 ($\Delta phoP$) were cultured in regular LB, hyperosmolarity or LMP medium overnight, the secretion proteins and the whole cell lysates were analyzed by SDS-PAGE. The FljB band in the WCL was indicated with an arrow. WCL: whole cell lysate.

Figure 5: Expression and secretion of flagellin in *fljA* mutant under different culture conditions. *fljA* mutant cells of *S. Typhi* GIFU10007 ($\Delta fljA$) were cultured in regular LB, hyperosmolarity or LMP medium overnight, the secretion proteins and the whole cell lysates were analyzed by SDS-PAGE. The FljB and FliC protein were indicated with arrows. WCL: whole cell lysate.

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$\Delta phoP$

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