



Flagellar Basal Body Structural Proteins FlhB, FliM, and FliY Are Required for Flagellar-Associated Protein Expression in *Listeria monocytogenes*

Changyong Cheng^{1,2†}, Hang Wang^{1†}, Tiantian Ma¹, Xiao Han¹, Yongchun Yang¹, Jing Sun¹, Zhongwei Chen¹, Huifei Yu¹, Yi Hang¹, Fengdan Liu¹, Weihuan Fang^{1,2}, Lingli Jiang³, Chang Cai^{1,4} and Houhui Song^{1*}

OPEN ACCESS

Edited by:

Shihua Wang,
Fujian Agriculture and Forestry
University, China

Reviewed by:

Tohru Minamino,
Osaka University, Japan
Katsumi Imada,
Osaka University, Japan

*Correspondence:

Houhui Song
songhh@zafu.edu.cn

† These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Infectious Diseases,
a section of the journal
Frontiers in Microbiology

Received: 12 October 2017

Accepted: 30 January 2018

Published: 13 February 2018

Citation:

Cheng C, Wang H, Ma T, Han X,
Yang Y, Sun J, Chen Z, Yu H,
Hang Y, Liu F, Fang W, Jiang L,
Cai C and Song H (2018) Flagellar
Basal Body Structural Proteins FlhB,
FliM, and FliY Are Required
for Flagellar-Associated Protein
Expression in *Listeria
monocytogenes*.
Front. Microbiol. 9:208.
doi: 10.3389/fmicb.2018.00208

¹ China-Australian Joint Laboratory for Animal Health Big Data Analytics, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection and Internet Technology, College of Animal Science and Technology, Zhejiang A&F University, Lin'an, China, ² Zhejiang University Institute of Preventive Veterinary Medicine and Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, Hangzhou, China, ³ Department of Health Services and Management, Ningbo College of Health Sciences, Ningbo, China, ⁴ School of Veterinary and Life Sciences, Murdoch University, Perth, WA, Australia

Listeria monocytogenes is a food-associated bacterium that is responsible for food-related illnesses worldwide. In the *L. monocytogenes* EGD-e genome, FlhB, FliM, and FliY (encoded by *lmo0679*, *lmo0699*, and *lmo0700*, respectively) are annotated as putative flagella biosynthesis factors, but their functions remain unknown. To explore whether FlhB, FliM, and FliY are involved in *Listeria* flagella synthesis, we constructed *flhB*, *fliM*, *fliY*, and other flagellar-related gene deletion mutants using a homologous recombination strategy. Then, we analyzed the motility, flagella synthesis, and protein expression of these mutant strains. Motility and flagella synthesis were completely abolished in the absence of *flhB*, *fliM*, or *fliY*. These impaired phenotypes were fully restored in the complemented strains CΔ*flhB*, CΔ*fliM*, and CΔ*fliY*. The transcriptional levels of flagellar-related genes, including *flaA*, *fliM*, *fliY*, *lmo0695*, *lmo0698*, *fliI*, and *fliS*, were downregulated markedly in the absence of *flhB*, *fliM*, or *fliY*. Deletion of *flhB* resulted in the complete abolishment of FlaA expression, while it decreased FliM and FliY expression. The expression of FlaA was abolished completely in the absence of *fliM* or *fliY*. No significant changes were found in the expression of FlhF and two flagella synthesis regulatory factors, MogR and GmaR. We demonstrate for the first time that FlhB, FliM, and FliY not only mediate *Listeria* motility, but also are involved in regulating flagella synthesis. This study provides novel insights that increase our understanding of the roles played by FlhB, FliM, and FliY in the flagellar type III secretion system in *L. monocytogenes*.

Keywords: flagella, *Listeria monocytogenes*, motility, protein expression, type III secretion system

INTRODUCTION

The gram-positive bacterium *Listeria monocytogenes* is a ubiquitous intracellular pathogen, which has been implicated within the past decades as the causative organism in several outbreaks of foodborne disease. The bacterial flagellum is an ultra-large molecular complex composed of over 30 protein components (Morimoto and Minamino, 2014). In Gram-positive and Gram-negative bacteria, the flagellum is composed of three major parts, including the flagellar filament, the hook complex, and the basal body, which crosses the bacterial cell membrane, as well as a flagellar-associated cytoplasmic ring. Flagellar-related proteins are transported through the basal body to the outside of the cell, where they are assembled into the helical hook and filament complexes (Berg, 2003). Bacterial swimming is controlled by flagella in accordance with alternations of environment, through clockwise (CW) or counterclockwise (CCW) rotation of motors (Blair, 2003). For example, in *Escherichia coli* CCW rotation allows the several filaments to join in a bundle on a cell and drive it smoothly forward (a 'run'), while CW rotation destroys the filament bundle and causes rapid somersaulting (a 'tumble') (Blair, 2003), while binding of the response regulator phosphorylated CheY (CheY-P) to the rotor switch component FliM and FliN will change the direction of motor swimming (Delalez et al., 2010). FliG, FliM, and FliN belong to the cytoplasmic ring structure, while the MotA/MotB complex is a stator of the flagellar motor that acts as a H⁺ channel to couple the proton flow with torque generation (Hosking and Manson, 2008; Onoue et al., 2016). In the process of the bacterial flagella biosynthesis, the greatest challenge is the coordination of the complex flagellar assemble, which requires the timely transcription of the flagellar-associated genes, fine-tuning of intracellular processing, folding and export of flagellar proteins, and the final formation of a functional flagellar filament (Liu and Ochman, 2007). There are dozens of flagellar-related proteins in *L. monocytogenes*, which serve as regulators, structural proteins, cofactors, an ATPase, and proteins that are required protein transport and modification. These proteins play an important role in flagellar synthesis. Flagellar synthesis in *L. monocytogenes* is controlled mainly by the interaction of MogR and GmaR. The process of *Listeria* flagella biosynthesis is temperature dependent and finely regulated through an obviously different mechanism than the well-described hierarchical regulation of gram-negative bacteria (Grundling et al., 2004). Thus, at mammalian host physiologic temperature, 37°C, most *L. monocytogenes* strains do not produce flagella and are non-motile (Peel et al., 1988; Shen and Higgins, 2006). This is due to MogR repression of flagellar gene transcription at 37°C (Grundling et al., 2004). In contrast, at 30°C and below, *L. monocytogenes* is motile because MogR is inhibited by its anti-repressor GmaR, thus permitting flagellar gene transcription (Shen et al., 2006). DegU is also involved in regulating flagellar-related gene transcription, which in turn affects flagellar synthesis and bacterial motility (Williams et al., 2005). The flagellar-mediated transport and secretion system in most Gram-negative pathogenic bacteria is a type III secretion system (T3SS).

The flagellar T3SS is closely related to the effector toxin-exporting T3SSs of pathogenic bacteria. The pathogen-associated T3SSs are similar to the majority of the flagellar T3SSs, but the specific protein transport mechanism is not yet clear. FlhB, FliM, and FliY are involved mainly in the formation of the flagellum in Gram-negative bacteria, and they are flagellar T3SS component proteins that mediate the transport and of flagellar-related proteins (Liao et al., 2009; Meshcheryakov et al., 2013).

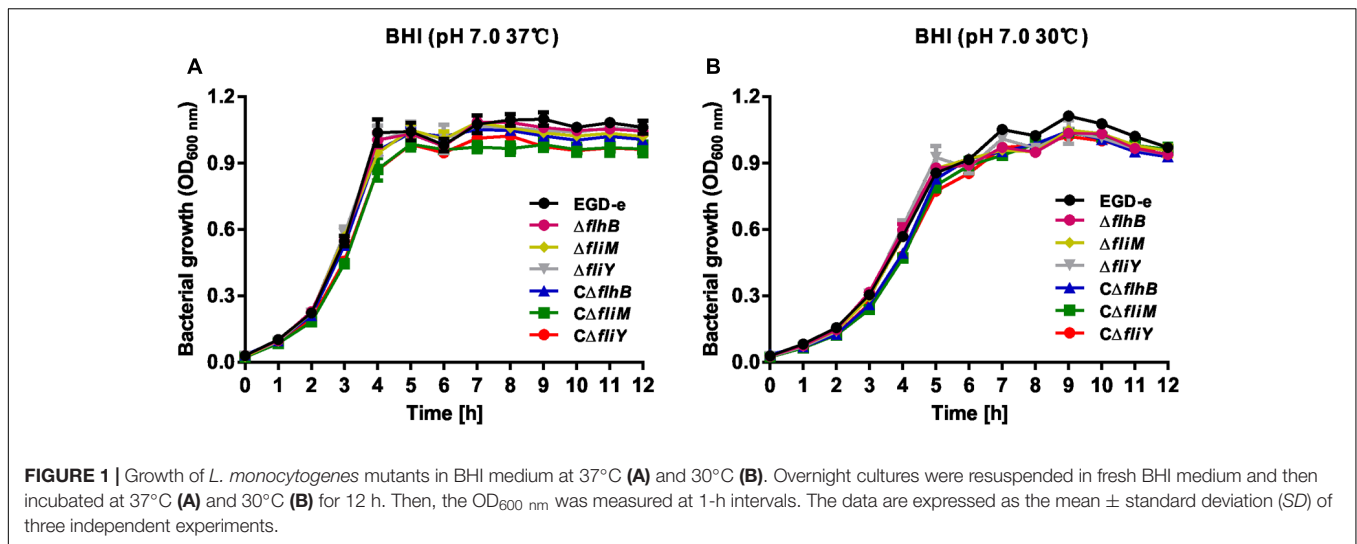
Among various bacteria, the pathogenic non-flagellar T3SS is merely found in Gram-negative bacteria, and in Gram-positive bacteria has not been reported. The bacterial flagellar-T3SS and the non-flagellar T3SS have a high degree of homology, both of the two systems can secrete virulence proteins, so there is the structural distinction as well functional similarity (Duan et al., 2013). For example, in Gram-positive *Bacillus thuringiensis*, secretion of some virulence determinants is dependent on a functional flagellar export apparatus (Young et al., 1999; Ghelardi et al., 2002). In Gram-negative *Campylobacter jejuni*, due to the lack of a classical T3SS, the flagellar export system therefore plays a major role in secreting the virulence factors during bacterial infection (Konkel et al., 2004).

The function of the FlhB, FliM, and FliY proteins in *L. monocytogenes* has not been reported. To better understand the relationship between flagellar gene expression and assembly of the flagella in *L. monocytogenes*, genes encoding various export apparatus components were disrupted, and bacterial motility, flagellar biosynthesis, and the transcript levels of the selected flagellar genes were studied. First, we used genetic recombination techniques to construct deletion mutants of *flhB*, *fliM*, and *fliY*. Second, the phenotypes, including motility, flagellar assembly, growth, flagellar-related gene transcription, and changes in flagellar protein levels, of the deletion mutants were analyzed. The results showed that FlhB, FliM, and FliY are involved in flagellar synthesis and motility, as well as the expression, transport of flagellar-related proteins. Our results show, for the first time, that FlhB, FliM, and FliY are required for flagella synthesis and motility in *L. monocytogenes*. These results lay the foundation for further exploration of the flagellar T3SS in *L. monocytogenes*.

RESULTS

The Flagellar Genes *flhB*, *fliM*, and *fliY* Are Not Required for Growth *in Vitro*

The *flhB*, *fliM*, and *fliY* deletion mutants were constructed successfully, and then their phenotypes were analyzed. The mutant strains were grown at different temperatures and there were virtually no significant growth differences among the strains at 30°C and 37°C, compared with the wild-type strain and the complemented strains CΔ*flhB*, CΔ*fliM*, and CΔ*fliY* (Figures 1A,B), suggesting that *flhB*, *fliM*, and *fliY* are not essential for growth *in vitro*.



The *L. monocytogenes* Mutant Strains $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ Lack Flagellar and Are Non-motile

The motility of the *flhB*, *fliM*, and *fliY* mutant strains was abolished completely in semisolid culture medium (tryptic soy agar) at 30°C, while the wild-type strain (EGD-e) and the complemented strains produced circular “swarms” (Figure 2A). All the strains were non-motile at 37°C, which inhibited flagellar formation in many *Listeria* strains (Figure 2B) (Grundling et al., 2004). Therefore, it can be seen that FlhB, FliM, and FliY are critical for the motility of *L. monocytogenes*, as any single mutant abolished motility completely. Because of the loss of motility in the mutants, we wondered whether the mutants adversely affected flagellar synthesis. Therefore, we used transmission electron microscopy (TEM) to examine whether these mutants produced flagella. The results showed that the $\Delta flhB$, $\Delta fliM$, $\Delta fliY$ mutants did not produce any flagellar filaments, while the EGD-e, $C\Delta flhB$, $C\Delta fliM$, and $C\Delta fliY$ strains did (Figure 2C). These results clearly show that the loss of *flhB*, *fliM*, and *fliY* abolished flagellar synthesis and motility in *L. monocytogenes*.

Changes in the Levels of Flagellar-Associated Gene Transcripts in the $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ Strains

Genes encoding the flagellar-associated genes from *L. monocytogenes* EGD-e are present in a 41-gene cluster encoding proteins related to flagellar and motility (Figure 3A) (Desvaux and Hebraud, 2006). We used a quantitative real-time PCR (qRT-PCR) to demonstrate that the deletion of *flhB* resulted in the downregulation of the transcription of most flagellar-associated genes (Figure 3B, the ratio of expression of the wild-type strain EGD-e to that of the $\Delta flhB$ strain was ≥ 1.5), and the transcription of *flaA*, *fliY*, *fliS*, *motA*, *lmo0695*, *lmo0698* was downregulated significantly (Figure 3B). The absence of *fliM* also resulted in the downregulation of the transcription of most flagellar-associated genes (Figure 3C, the ratio of expression of

the wild-type strain EGD-e to that of the $\Delta fliM$ strain was ≥ 1.5), and *flaA*, *lmo0698*, *lmo0695*, *flgK*, *fliS*, and *fliI* transcription was downregulated significantly (Figure 3C). Similarly, the deletion of *fliY* resulted in the downregulation of the transcription of most flagellar-associated genes (Figure 3D, the ratio of expression of the wild-type strain EGD-e to that of the $\Delta fliY$ strain was ≥ 1.5), and the transcription of *flaA*, *flhA*, *fliI*, and *fliNY* was downregulated significantly (Figure 3D). In these three mutants, the transcription of genes in the *fliN*, *flaA*, *lmo0696*, and *lmo0703* operons was mainly downregulated. The results confirm that *flhB*, *fliM*, and *fliY* are essential for the transcriptional regulation and synthesis of other genes related to bacterial flagella.

FlhB, FliM, and FliY Are Involved in the Expression of Flagellar-Associated Proteins

In the $\Delta flhB$ strain, the expression of FlaA was abolished completely, while FliS was expressed. Moreover, the expression of the cytoplasmic proteins FliY and FliM was reduced fractionally, while the expression of FlhF and the regulatory factors MogR and GmaR was not affected (Figure 4A). The results indicate that FlhB can adversely affect the expression of some flagellar-associated proteins. The absence of *fliM* resulted in the complete loss of expression of the secretory FlaA. Additionally, the expression of the cytoplasmic protein FliY was attenuated slightly as well, while the expression of FlhF and the transcriptional regulators MogR and GmaR was not affected (Figure 4B). The results show that FliM adversely affects the expression and secretion of bacterial flagellar-associated proteins. Furthermore, the results showed that the deletion of *fliY* resulted in the complete loss of expression of FliM and FlaA, the expression of FlhF and the transcriptional regulatory factors MogR and GmaR was not affected (Figure 4C). These results demonstrate that FliY adversely affects the expression and of flagellar-associated proteins. Collectively, these results are consistent with the previous motility assay, the results of the TEM analysis, and the aforementioned changes of the transcript levels of flagellar genes.

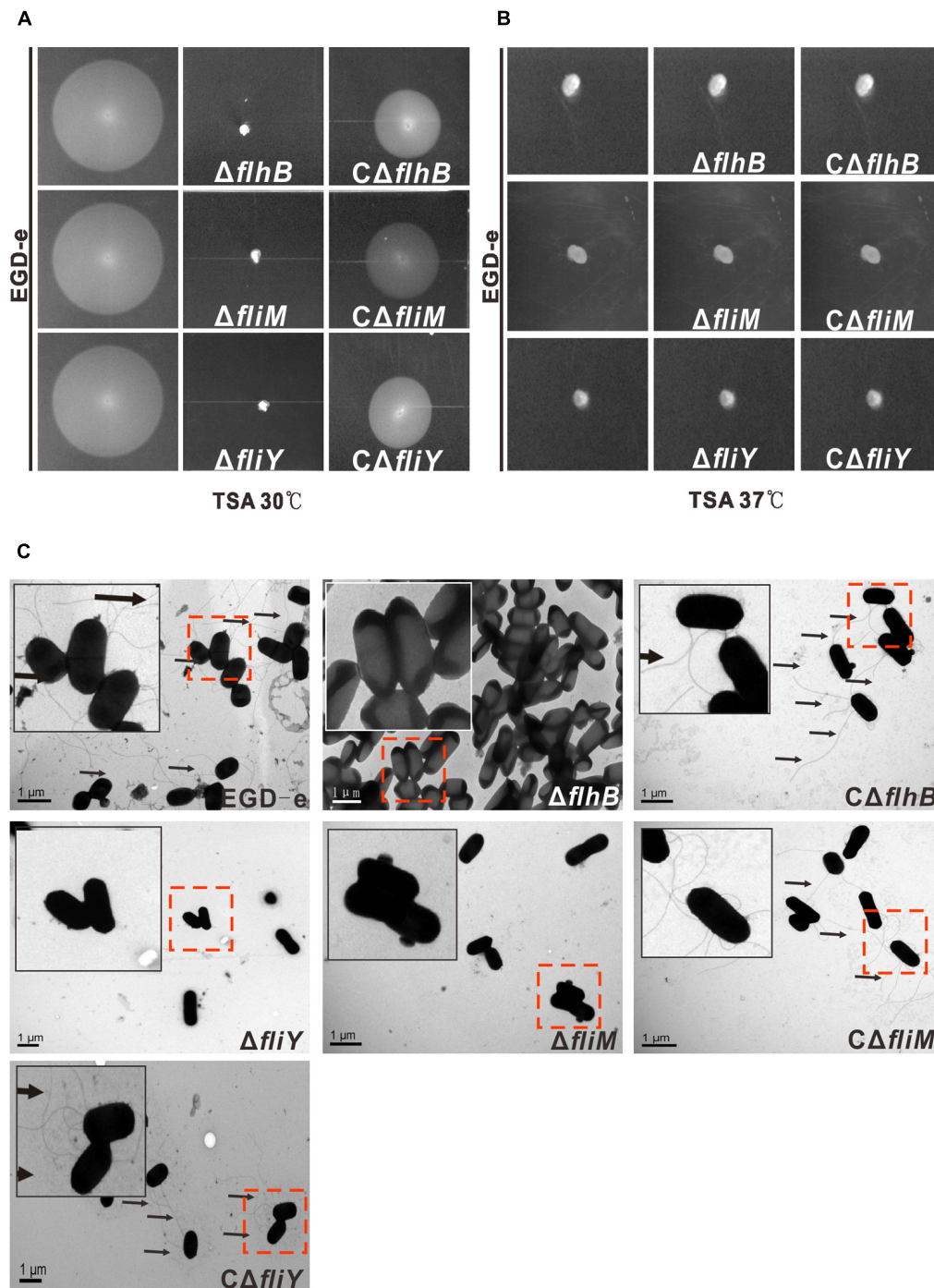


FIGURE 2 | FliB, FliM, and FliY contribute to *L. monocytogenes* motility and flagellar formation. A motility assay (A,B) and TEM (C) were performed by growing the *L. monocytogenes* wild-type strain EGD-e, the mutants $\Delta fliB$, $\Delta fliM$ and $\Delta fliY$, and the complemented strains $C\Delta fliB$, $C\Delta fliM$, and $C\Delta fliY$ on soft agar (0.25%) at 30°C or 37°C for 16 h. Scale: 1 μ m.

DISCUSSION

The bacterial flagellum has previously been shown to play a critical role as an export apparatus in mediating extracellular secretion of non-flagella virulence factors and other important

heterologous polypeptides (Haiko and Westerlund-Wikstrom, 2013). The effect of flagella on bacterial pathogenicity in Gram-positive bacteria has not been reported, but the effect of flagella on the pathogenicity of Gram-negative bacteria is important (Osterman et al., 2015). Flagellar not only mediate the movement

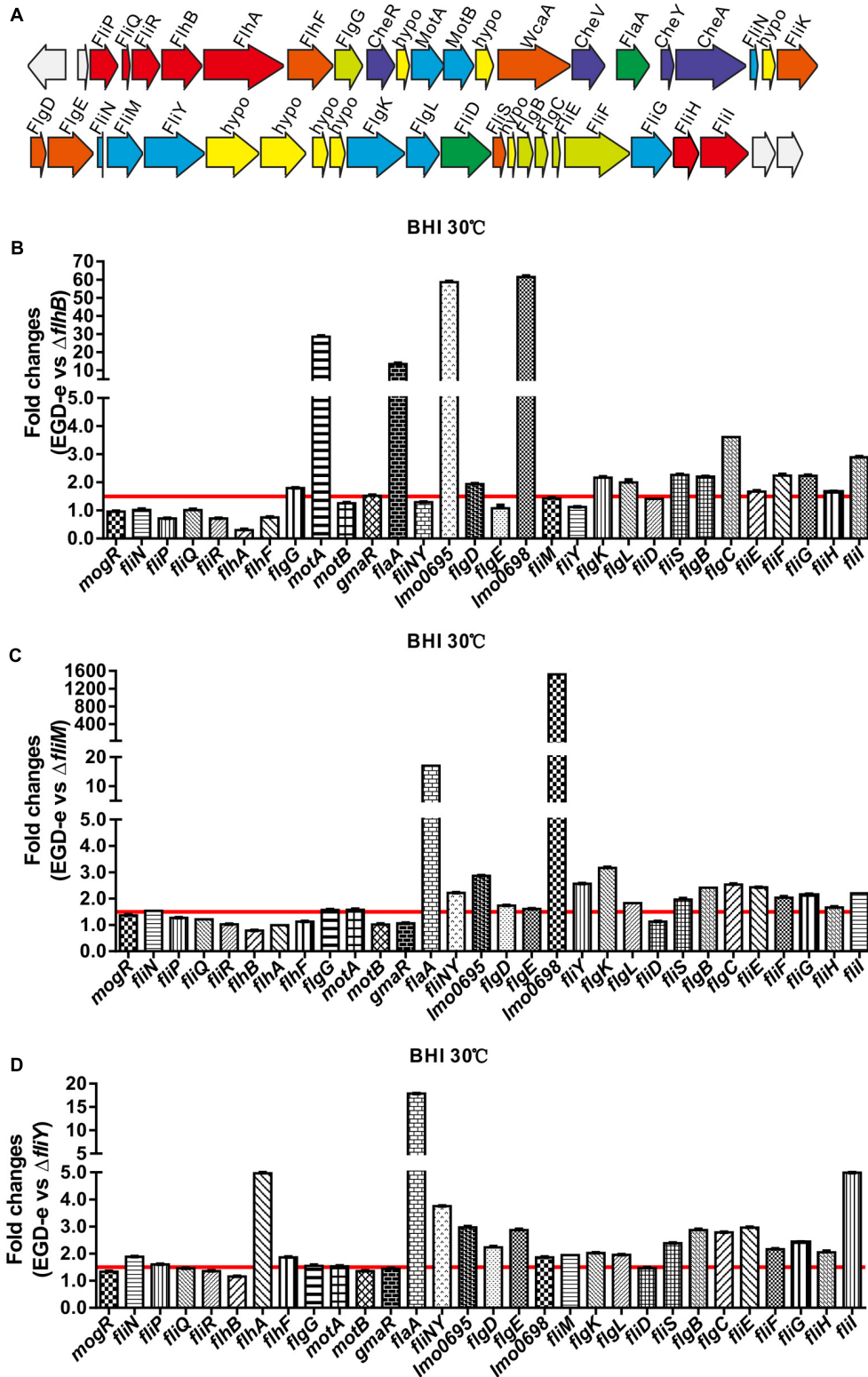


FIGURE 3 | The flagellar-motility gene cluster in *L. monocytogenes* EGD-e (**A**) and the changes of the transcriptional levels of flagellar-related genes in the absence of *flhB* (**B**), *flhM* (**C**), or *flhY* (**D**). Relative quantification of flagellar-related gene mRNA levels in the *L. monocytogenes* wild-type strain EGD-e, the mutants Δ flhB, Δ flhM, and Δ flhY, and the complemented strains C Δ flhB, C Δ flhM, and C Δ flhY at 30°C. Values are expressed as the mean \pm SD. Solid lines indicate a 1.5-fold change in transcription of the genes of interest.

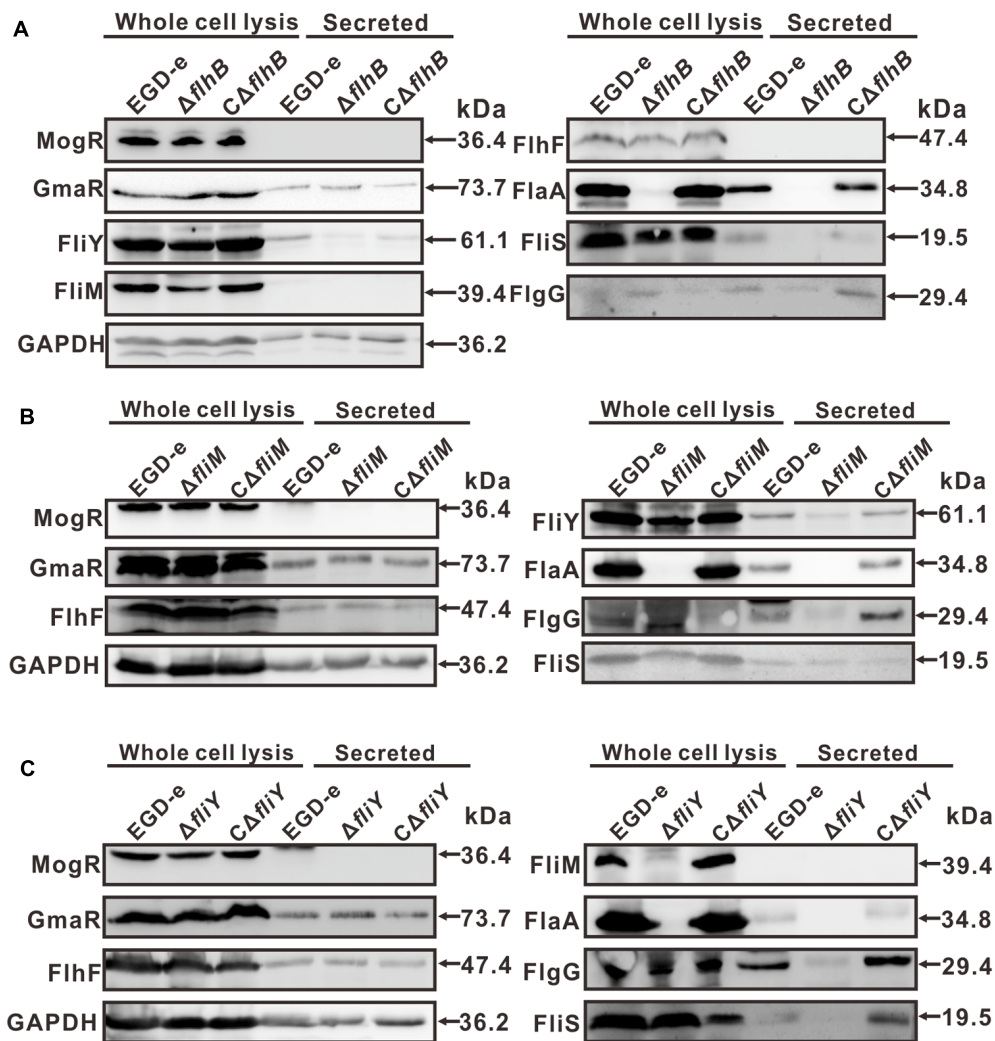


FIGURE 4 | Changes in the levels of flagellar-related proteins in the absence of *flhB* (A), *flhM* (B), or *flhY* (C). Bacterial overnight cultures of the *L. monocytogenes* wild-type strain EGD-e, the $\Delta flhB$, $\Delta flhM$, $\Delta flhY$ mutants, and the complemented strains $C\Delta flhB$, $C\Delta flhM$, and $C\Delta flhY$ were diluted 1:100 into 100 ml of fresh BHI broth and statically grown to stationary phase. Bacterial sediments and culture supernatants were collected to obtain the different cell fractions. Proteins were separated by 12% SDS-PAGE and immunoblotted with α -FlgG, α -MogR, α -FlhF, α -FliM, α -FliY, α -FlaA, α -FliS, or α -GmaR antisera. GAPDH was used as an internal control. The predicted molecular weight of each protein is indicated on the right.

of bacteria, but they are also involved in bacterial pathogenicity through the flagellar T3SS secretion system, which is mainly involved in effector transport (Bange et al., 2010; Osterman et al., 2015).

In Gram-negative bacteria, FlhB is a constituent protein of the basal body (Casey et al., 2014; Diepold and Armitage, 2015), and FlhB is encoded by *lmo0679* in *L. monocytogenes* strain EGD-e. In this study, the absence of *flhB* resulted in the inability to synthesize flagellar (Figure 2C), which resulted in a non-motile phenotype on tryptic soy agar semi-solid medium (Figure 2). This indicates that the $\Delta flhB$ mutant cannot form flagellar and that it is non-motile at a low temperature. The qRT-PCR results showed that the deletion of *flhB* led to the downregulation of the transcription of most flagellar-related genes (Figure 3B). The highly conserved membrane protein FlhB, as an important

component of the flagellar secretion system, plays an active role in regulating proteins export (Meshcheryakov et al., 2013). The analysis of expression of flagellar-related proteins in the deletion mutants showed that in the $\Delta flhB$ mutant, the expression of FlaA was abolished completely. Additionally, the expression of the cytoplasmic proteins FliY and FliM was reduced slightly, while the expression of FlhF and the regulatory factors MogR and GmaR was not affected (Figure 4B). These results indicate that FlhB affects the expression and secretion of flagellar-associated proteins. This study, for the first time, showed that FlhB is involved in regulating the flagellar synthesis of important flagellar proteins, and that it affects the secretion of other bacterial proteins in *L. monocytogenes*. The main function of FlhB in *Yersinia pseudotuberculosis* is to mediate the transport of T3SS effector molecules and mediate the transport of bacterial

toxins into host cells (Minamino and Macnab, 2000a; Macnab, 2004; Barker and Samatey, 2012; Login and Wolf-Watz, 2015; McMurry et al., 2015).

Flagellar-associated proteins in the majority of Gram-negative bacteria and only a few in Gram-positive bacteria are found involved in the synthesis and transport of effector molecules (Ghelardi et al., 2002; Diepold and Armitage, 2015). The main components of the flagellar T3SS are located mainly in the cytoplasmic membrane of bacteria, and they include FlhA, FlhB, FliP, FliQ, FliR, and FliO, which constitute the intramembrane protein complex (Minamino and Macnab, 1999). The function of the flagellar T3SS is to transport flagellar-related proteins out of the cell. Cytoplasmic flagellar proteins include FliI, FliH, and FliJ. FliI is ATPase (Fan and Macnab, 1996); FliH is a negative regulator of FliI (Minamino and MacNab, 2000b), whereas FliJ is a molecular chaperone that facilitates the transport of flagellar T3SS proteins (Minamino et al., 2000; Stafford et al., 2007). The key protein in the cytoplasmic membrane complex is FlhA, and the C-terminal domains of both FlhA and FlhB are predictively located in the cytoplasm and might be involved in substrate translocation and substrate specificity switching, respectively (Minamino and MacNab, 2000c). In *Bacillus thuringiensis*, FlhA mediates the transport of bacterial virulence factors into host cells, which is required for pathogenesis (Ghelardi et al., 2002), but its mechanism of action has not been demonstrated. The function of FlhB has not been reported in Gram-positive bacteria except *B. subtilis* (Calvo and Kearns, 2015). However, in Gram-negative bacteria and some thermophiles, FlhB and the hook-length control protein FliK form an export switching machinery that switches export specificity of the flagellar-T3SS upon completion of the hook structure, thereby coordinating flagellar gene expression with assembly (Kutsukake et al., 1994). FlhB has two structural domains: the amino (N)-terminal domain (FlhB_{TM}) and the carboxyl (C)-terminal domain (FlhB_C). The amino acids linking these domains are critical for the function of FlhB. The deletion or mutation of these amino acids adversely affects T3SS-mediated transport (Fraser et al., 2003; Zarivach et al., 2008). The presence of Asn-Pro-Thr-His sequence within the C-terminal domain in *Salmonella* FlhB is required for FlhB function, and cleavage of the Asn-Pro bond is required for substrate switching process. Collectively, our results show that the transport protein FlhB is involved in the operation of the flagellar export in *L. monocytogenes*.

In addition to FlhB, FliM, and FliY play important roles in the flagellar system of Gram-negative bacteria. FliM is a C-ring component, which plays a role in flagellar assembly and CW/CCW switching of the direction of flagellar rotation (Onoue et al., 2016), in Gram-positive bacteria, its function and mechanism of operation have not been studied and confirmed. FliY is a flagellar rotor protein of the CheC phosphatase family, and it is localized in the flagellar switch complex, which contains the stator-coupling protein FliG and the target of CheY-P, FliM. Regions of FliM that mediate contacts within the rotor compose the phosphatase active sites in FliY. Despite the similarity between FliY and FliM, FliY does not bind FliG and thus is unlikely to be a substitute for FliM in the center of the switch complex (Sircar et al., 2013). It is clear that FliM and FliY are

important flagellar-related proteins that are required for flagellar synthesis and export in *L. monocytogenes*.

In summation, FlhB, FliM, and FliY are involved in flagellum synthesis in *L. monocytogenes*, and they are required for motility and the secretion of other flagellar-related proteins. The expression and secretion of some other flagellar-related proteins, such as MogR, GmaR, the GTPase FlhF was not affected in the *flhB*, *fliM*, and *fliY* mutants, indicating that FlhB, FliM, and FliY partially affect flagellar function, and that there are other flagellar control mechanisms in *L. monocytogenes* (Figure 5). Studies of the functions of flagellar-related proteins in *L. monocytogenes* are in the preliminary stage, and we in the future aim to discover important effector proteins that are regulated by *flhB* and to determine whether there is a pathogen-associated T3SS in *L. monocytogenes* and, if so, whether its function is related closely to the flagellar T3SS.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

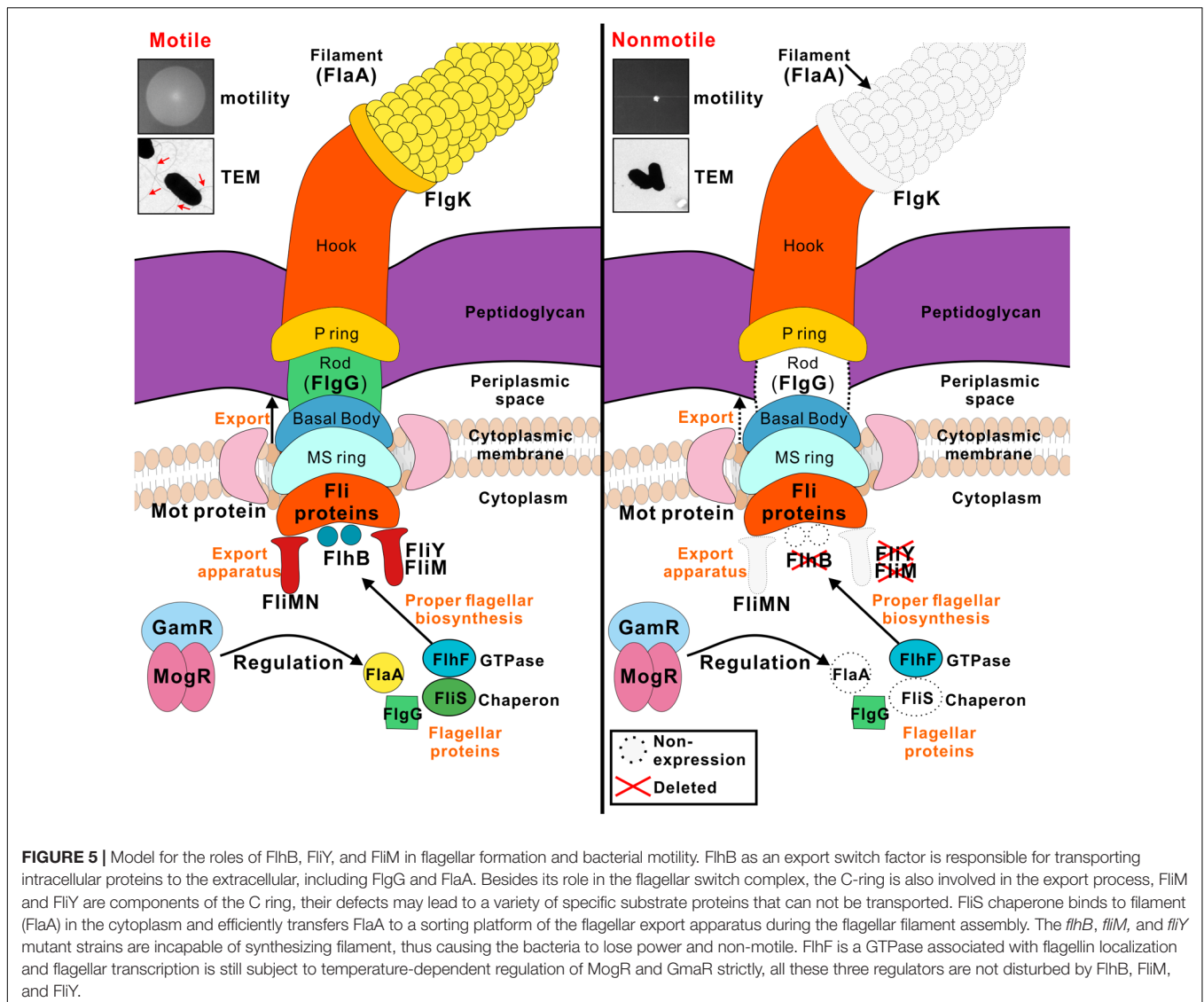
The *L. monocytogenes* EGD-e strain was used as the wild-type strain. *Escherichia coli* DH5 α was employed for cloning experiments and as the host strain for the plasmids pET30a(+) (Merck, Darmstadt, Germany), pIMK2, and pKSV7. The *E. coli* Rosetta (DE3) strain was used for protein expression. *L. monocytogenes* was cultured in brain-heart infusion (BHI) medium (Oxoid, Hampshire, England). The DH5 α and Rosetta (DE3) strains were grown at 37°C in Luria-Bertani (LB) broth (Oxoid). Stock solutions of ampicillin (50 mg/ml), erythromycin (50 mg/ml), kanamycin (50 mg/ml), or chloramphenicol (50 mg/ml) was added to the media when necessary. All chemicals were obtained from Sangon Biotech (Shanghai, China), Merck, or Sigma-Aldrich (St. Louis, MO, United States) at the highest purity available (Cheng et al., 2015).

Construction of Gene Deletion Mutants

The temperature-sensitive pKSV7 shuttle vector was used to create mutations in *L. monocytogenes* strain EGD-e. A homologous recombination strategy using the overlap extension (SOE) PCR procedure was used to construct in-frame deletions in the *flhB*, *fliM*, and *fliY* genes (Monk et al., 2008). DNA fragments containing homologous arms upstream and downstream of the genes of interest were obtained by PCR amplification of EGD-e genomic DNA using the SOE primer pairs listed in Supplementary Table S1. The resulting in-frame-deletion mutants were further verified by DNA sequencing (Sangon Biotech, Inc., Shanghai, China).

Complementation of the Gene Deletion Mutants

To complement the *L. monocytogenes* $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ strains, we constructed three complemented strains using the integrative plasmid pIMK2, which harbors the constitutive *Listeria* promoter P_{help} as performed previously (Cheng et al.,



2017b). We amplified the complete open reading frames of *flhB*, *fliM*, and *fliY* from EGD-e genomic DNA using the primer pairs listed in Supplementary Table S1 and inserted them downstream of P_{help} after digestion with appropriate restriction enzymes. Then, the resulting plasmids were electroporated into the corresponding *L. monocytogenes* $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ strains. The electroporated cells were plated on BHI agar containing kanamycin (50 $\mu\text{g/ml}$).

Expression and Purification of Recombinant Proteins

The recombinant proteins used in this study were expressed as fusion proteins with an N-terminal histidine-tag using pET30a(+) as the expression vector (Cheng et al., 2017a). The *E. coli* Rosetta (DE3) strain was used as the expression host. The full-length open reading frames of the genes of interest from the EGD-e genome were amplified with the primer pairs listed in Supplementary Table S1, inserted into the pET30a(+) vector,

and finally transformed into *Rosetta* competent cells. *Escherichia coli* cells harboring the recombinant plasmids were grown in 500 ml of LB medium supplemented with 50 $\mu\text{g/ml}$ kanamycin at 37°C until the cultures reached an optical density at 600 nm ($\text{OD}_{600 \text{ nm}}$) of 0.6–0.8. Then, IPTG was added to a final concentration of 0.2 mM to induce protein expression for an additional 3 h, and the proteins purified using nickel-chelated affinity column chromatography.

Preparation of Polyclonal Antibodies against the Recombinant Proteins

The purified recombinant proteins were used to raise polyclonal antibodies in New Zealand white rabbits according to a standard protocol as previously described (Cheng et al., 2015, 2017b). The rabbits were first immunized by sub-cutaneous injections of 500 μg of protein with an equal volume of Freund's complete adjuvant (Sigma-Aldrich). After 2 weeks, the rabbits were boosted subcutaneously three times with 250 μg of protein

in incomplete Freund's adjuvant (Sigma–Aldrich) at 10-day intervals. The rabbits were bled approximately 10 days after the last injection.

Growth Analysis in BHI Broth

The *L. monocytogenes* wild-type strain EGD-e, the $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ mutant strains, and the three complemented strains ($C\Delta flhB$, $C\Delta fliM$, and $C\Delta fliY$) were grown overnight at 37°C in BHI broth with shaking. The cultures were collected by centrifugation at $5,000 \times g$ at 4°C, washed once in phosphate-buffered saline (10 mM, pH 7.4), and the initial OD_{600 nm} was adjusted to 0.6. Then, the bacteria were diluted 1:100 into fresh BHI broth and incubated at 37°C and 30°C for 12 h. The OD_{600 nm} was measured at 1-h intervals.

Motility Assay and TEM

The motility assay was performed on soft LB agar (0.25%) as described (Paxman et al., 2009; Cheng et al., 2017b). Specifically, *L. monocytogenes* strains were grown overnight in BHI medium, and the cultures were adjusted to an OD_{600 nm} of 0.20 (approximately 2×10^8 CFU/ml). Bacterial samples (5 μ L) were dropped onto the soft LB agar and incubated at 30°C or 37°C for 48 h to allow for growth. Motility was assessed by examining the outward migration of the bacteria through the agar from the point of inoculation. TEM experiments were performed as described previously (Dingle et al., 2011), in the Institute of Agrobiological and Environmental Sciences of Zhejiang University. Single *L. monocytogenes* colonies that were grown overnight at 30°C from BHI agar plates were suspended in 50 μ L of monoethanolamine buffer (pH 10.0), and then 10 μ L of the suspension was applied to carbon-coated copper grids and incubated for 2 min at room temperature. Excess liquid was subsequently wicked away using filter paper, and the bacteria were stained with 10 μ L of 0.5% phosphotungstic acid (pH 7.0) for 10 s at room temperature. Excess stain was gently wicked away using filter paper, and the dried grids were examined using a Hitachi H-7650 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

qRT-PCR Assay

The *L. monocytogenes* wild-type strain EGD-e and the $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ mutant strains were grown to stationary phase (OD_{600 nm} of 0.6) in BHI broth at 30°C without shaking. Total RNA was extracted using the Trizol reagent, and cDNA was synthesized with reverse transcriptase (TOYOBO, Osaka, Japan). Then, quantitative PCRs were performed by using the SYBR qPCR mix (TOYOBO) to measure the transcriptional levels of *mogR*, *fliN*, *fliP*, *fliQ*, *fliR*, *flhA*, *flhF*, *flgG*, *motA*, *motB*, *gmaR*, *flaA*, *fliNY*, *lmo0698*, *fliM*, *fliY*, *flgK*, *flgL*, *fliD*, *fliS*, *flgB*, *flgC*, *fliE*, *fliF*, *fliG*, *fliH*, and *fliI* using an iCycler iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, United States) with specific primer pairs. Relative transcription levels were quantified by the $2^{-\Delta\Delta C_T}$ method and are shown as relative fold changes (Livak and Schmittgen, 2001). The transcriptional analysis was repeated three times for each test condition.

Changes in the Expression Levels of Flagellar-Associated Proteins in the Absence of *flhB*, *fliM*, and *fliY*

To further determine whether *flhB*, *fliM*, and *fliY* are involved in bacterial flagellar synthesis, western blotting was employed to analyze the changes in expression of major flagellar-associated factors. Bacterial overnight cultures of the *L. monocytogenes* wild-type EGD-e, the $\Delta flhB$, $\Delta fliM$, and $\Delta fliY$ mutant strains, and the three complemented strains ($C\Delta flhB$, $C\Delta fliM$, and $C\Delta fliY$) were diluted into 100 ml of fresh BHI broth, and then the bacteria were grown to stationary phase. We used the fractionation procedure described by Lenz and Portnoy (2002), with minor modifications, to isolate secreted proteins. Briefly, the bacteria were pelleted by centrifugation at $13,000 \times g$ for 20 min at 4°C, and the resulting culture supernatant was collected and then filtered through a 0.22- μ m polyethersulfone membrane filter (Merck). Trichloroacetic acid was added to the supernatant to a final concentration of 10%. Proteins were trichloroacetic acid-precipitated on ice overnight and washed with ice-cold acetone (Cheng et al., 2017b). The washed precipitates of the supernatant proteins were re-suspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (5% SDS, 10% glycerol, and 50 mM Tris-HCl, pH 6.8). The samples were boiled for 5 min and stored at –20°C before electrophoresis. The method for total cell protein isolation was performed as described previously (Cheng et al., 2017b). The protein samples were separated using 12% SDS-PAGE and immunoblotted with α -FlgG, α -MogR, α -FlhF, α -FliM, α -FliY, α -FlaA, α -FliS, α -GmaR, and α -glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antisera. GAPDH was used as an internal control.

Statistical Analysis

Data were analyzed using a two-tailed homoscedastic Student's *t*-test. Differences with *P* values < 0.05 were considered to be statistically significant.

ETHICS STATEMENT

All animal care and use protocols were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. The protocol was approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University (Permit Number: ZJAFU/IACUC_2011-10-25-02). All the *Listeria monocytogenes*-involved experiments in our study were conducted at Biosafety Level 2 (BSL-2) Laboratory.

AUTHOR CONTRIBUTIONS

CCh and HS conceived the study. CCh, HW, TM, JS, LJ, XH, YH, HY, and FL carried out the experiments. CCh, YY, LJ, and ZC analyzed the data. CCh, WF, and HS drafted the manuscript. All authors

contributed to this study, prepared the final version of the manuscript, and read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (Nos. 31402215, 31602062, 31770040, and 31470179), Zhejiang Provincial Natural Science Foundation (Nos. LY17C180001 and LY15C010003), Zhejiang Provincial Key Laboratory of Preventive Veterinary

Medicine (ZPKLPVM2017KFKT002), Zhejiang Department of Science and Technology (No. 2014C32047), and ZAFU Talents Starting Program (Nos. 2014FR073 and 2015FR007).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.00208/full#supplementary-material>

REFERENCES

- Bange, G., Kummerer, N., Engel, C., Bozkurt, G., Wild, K., and Sinning, I. (2010). FlhA provides the adaptor for coordinated delivery of late flagella building blocks to the type III secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11295–11300. doi: 10.1073/pnas.1001383107
- Barker, C. S., and Samatey, F. A. (2012). Cross-complementation study of the flagellar type III export apparatus membrane protein FlhB. *PLOS ONE* 7:e44030. doi: 10.1371/journal.pone.0044030
- Berg, H. C. (2003). The rotary motor of bacterial flagella. *Annu. Rev. Biochem.* 72, 19–54. doi: 10.1146/annurev.biochem.72.121801.161737
- Blair, D. F. (2003). Flagellar movement driven by proton translocation. *FEBS Lett.* 545, 86–95. doi: 10.1016/S0014-5793(03)00397-1
- Calvo, R. A., and Kearns, D. B. (2015). FlgM is secreted by the flagellar export apparatus in *Bacillus subtilis*. *J. Bacteriol.* 197, 81–91. doi: 10.1128/JB.02324-14
- Casey, A., Fox, E. M., Schmitz-Esser, S., Coffey, A., McAuliffe, O., and Jordan, K. (2014). Transcriptome analysis of *Listeria monocytogenes* exposed to biocide stress reveals a multi-system response involving cell wall synthesis, sugar uptake, and motility. *Front. Microbiol.* 5:68. doi: 10.3389/fmicb.2014.00068
- Cheng, C., Dong, Z., Han, X., Sun, J., Wang, H., Jiang, L., et al. (2017a). *Listeria monocytogenes* 10403S arginine repressor ArgR finely tunes arginine metabolism regulation under acidic conditions. *Front. Microbiol.* 8:145. doi: 10.3389/fmicb.2017.00145
- Cheng, C., Dong, Z., Han, X., Wang, H., Jiang, L., Sun, J., et al. (2017b). Thioredoxin a is essential for motility and contributes to host infection of *Listeria monocytogenes* via Redox interactions. *Front. Cell. Infect. Microbiol.* 7:287. doi: 10.3389/fcimb.2017.00287
- Cheng, C., Wang, X., Dong, Z., Shao, C., Yang, Y., Fang, W., et al. (2015). Aminopeptidase T of M29 family acts as a novel intracellular virulence factor for *Listeria monocytogenes* infection. *Sci. Rep.* 5:17370. doi: 10.1038/srep17370
- Delalez, N. J., Wadhams, G. H., Rosser, G., Xue, Q., Brown, M. T., Dobbie, I. M., et al. (2010). Signal-dependent turnover of the bacterial flagellar switch protein FlhM. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11347–11351. doi: 10.1073/pnas.1000284107
- Desvaux, M., and Hebraud, M. (2006). The protein secretion systems in *Listeria*: inside out bacterial virulence. *FEMS Microbiol. Rev.* 30, 774–805. doi: 10.1111/j.1574-6976.2006.00035.x
- Diepold, A., and Armitage, J. P. (2015). Type III secretion systems: the bacterial flagellum and the injectisome. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20150020. doi: 10.1098/rstb.2015.0020
- Dingle, T. C., Mulvey, G. L., and Armstrong, G. D. (2011). Mutagenic analysis of the *Clostridium difficile* flagellar proteins, FlhC and FlhD, and their contribution to virulence in hamsters. *Infect. Immun.* 79, 4061–4067. doi: 10.1128/IAI.05305-11
- Duan, Q., Zhou, M., Zhu, L., and Zhu, G. (2013). Flagella and bacterial pathogenicity. *J. Basic Microbiol.* 53, 1–8. doi: 10.1002/jobm.201100335
- Fan, F., and Macnab, R. M. (1996). Enzymatic characterization of FlhI. An ATPase involved in flagellar assembly in *Salmonella typhimurium*. *J. Biol. Chem.* 271, 31981–31988. doi: 10.1074/jbc.271.50.31981
- Fraser, G. M., Hirano, T., Ferris, H. U., Devgan, L. L., Kihara, M., and Macnab, R. M. (2003). Substrate specificity of type III flagellar protein export in *Salmonella* is controlled by subdomain interactions in FlhB. *Mol. Microbiol.* 48, 1043–1057. doi: 10.1046/j.1365-2958.2003.03487.x
- Ghelardi, E., Celandroni, F., Salvetti, S., Beecher, D. J., Gominet, M., Lereclus, D., et al. (2002). Requirement of flhA for swarming differentiation, flagellin export, and secretion of virulence-associated proteins in *Bacillus thuringiensis*. *J. Bacteriol.* 184, 6424–6433. doi: 10.1128/JB.184.23.6424-6433.2002
- Grundling, A., Burrack, L. S., Bouwer, H. G., and Higgins, D. E. (2004). *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12318–12323. doi: 10.1073/pnas.0404924101
- Haiko, J., and Westerlund-Wikstrom, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology* 2, 1242–1267. doi: 10.3390/biology2041242
- Hosking, E. R., and Manson, M. D. (2008). Clusters of charged residues at the C terminus of MotA and N terminus of MotB are important for function of the *Escherichia coli* flagellar motor. *J. Bacteriol.* 190, 5517–5521. doi: 10.1128/JB.00407-08
- Konkel, M. E., Klena, J. D., Rivera-Amill, V., Monteville, M. R., Biswas, D., Raphael, B., et al. (2004). Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J. Bacteriol.* 186, 3296–3303. doi: 10.1128/JB.186.11.3296-3303.2004
- Kutsukake, K., Minamino, T., and Yokoseki, T. (1994). Isolation and characterization of FlhK-independent flagellation mutants from *Salmonella typhimurium*. *J. Bacteriol.* 176, 7625–7629. doi: 10.1128/jb.176.24.7625-7629.1994
- Lenz, L. L., and Portnoy, D. A. (2002). Identification of a second *Listeria secA* gene associated with protein secretion and the rough phenotype. *Mol. Microbiol.* 45, 1043–1056. doi: 10.1046/j.1365-2958.2002.03072.x
- Liao, S., Sun, A., Ojcius, D. M., Wu, S., Zhao, J., and Yan, J. (2009). Inactivation of the flhY gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. *BMC Microbiol.* 9:253. doi: 10.1186/1471-2180-9-253
- Liu, R., and Ochman, H. (2007). Stepwise formation of the bacterial flagellar system. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7116–7121. doi: 10.1073/pnas.0700266104
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Login, F. H., and Wolf-Watz, H. (2015). YscU/FlhB of *Yersinia pseudotuberculosis* Harbors a C-terminal Type III Secretion Signal. *J. Biol. Chem.* 290, 26282–26291. doi: 10.1074/jbc.M114.633677
- Macnab, R. M. (2004). Type III flagellar protein export and flagellar assembly. *Biochim. Biophys. Acta* 1694, 207–217. doi: 10.1016/j.bbamcr.2004.04.005
- McMurry, J. L., Minamino, T., Furukawa, Y., Francis, J. W., Hill, S. A., Helms, K. A., et al. (2015). Weak interactions between *Salmonella enterica* FlhB and other flagellar export apparatus proteins govern type III secretion dynamics. *PLOS ONE* 10:e0134884. doi: 10.1371/journal.pone.0134884
- Meshcheryakov, V. A., Barker, C. S., Kostyukova, A. S., and Samatey, F. A. (2013). Function of FlhB, a membrane protein implicated in the bacterial flagellar type III secretion system. *PLOS ONE* 8:e68384. doi: 10.1371/journal.pone.0068384
- Minamino, T., Chu, R., Yamaguchi, S., and Macnab, R. M. (2000). Role of FlhJ in flagellar protein export in *Salmonella*. *J. Bacteriol.* 182, 4207–4215. doi: 10.1128/JB.182.15.4207-4215.2000
- Minamino, T., and Macnab, R. M. (1999). Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J. Bacteriol.* 181, 1388–1394.

- Minamino, T., and Macnab, R. M. (2000a). Domain structure of *Salmonella* FlhB, a flagellar export component responsible for substrate specificity switching. *J. Bacteriol.* 182, 4906–4914.
- Minamino, T., and MacNab, R. M. (2000b). FliH, a soluble component of the type III flagellar export apparatus of *Salmonella*, forms a complex with FliI and inhibits its ATPase activity. *Mol. Microbiol.* 37, 1494–1503.
- Minamino, T., and MacNab, R. M. (2000c). Interactions among components of the *Salmonella* flagellar export apparatus and its substrates. *Mol. Microbiol.* 35, 1052–1064.
- Monk, I. R., Gahan, C. G., and Hill, C. (2008). Tools for functional postgenomic analysis of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 74, 3921–3934. doi: 10.1128/AEM.00314-08
- Morimoto, Y. V., and Minamino, T. (2014). Structure and function of the bi-directional bacterial flagellar motor. *Biomolecules* 4, 217–234. doi: 10.3390/biom4010217
- Onoue, Y., Abe-Yoshizumi, R., Gohara, M., Nishino, Y., Kobayashi, S., Asami, Y., et al. (2016). Domain-based biophysical characterization of the structural and thermal stability of FliG, an essential rotor component of the Na⁺-driven flagellar motor. *Biophys. Physicobiol.* 13, 227–233. doi: 10.2142/biophysico.13.0_227
- Osterman, I. A., Dikhtyar, Y. Y., Bogdanov, A. A., Dontsova, O. A., and Sergiev, P. V. (2015). Regulation of flagellar gene expression in bacteria. *Biochemistry* 80, 1447–1456. doi: 10.1134/S000629791511005X
- Paxman, J. J., Borg, N. A., Horne, J., Thompson, P. E., Chin, Y., Sharma, P., et al. (2009). The structure of the bacterial oxidoreductase enzyme DsbA in complex with a peptide reveals a basis for substrate specificity in the catalytic cycle of DsbA enzymes. *J. Biol. Chem.* 284, 17835–17845. doi: 10.1074/jbc.M109.011502
- Peel, M., Donachie, W., and Shaw, A. (1988). Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and western blotting. *J. Gen. Microbiol.* 134, 2171–2178.
- Shen, A., and Higgins, D. E. (2006). The MogR transcriptional repressor regulates nonhierarchical expression of flagellar motility genes and virulence in *Listeria monocytogenes*. *PLOS Pathog.* 2:e30. doi: 10.1371/journal.ppat.0020030
- Shen, A., Kamp, H. D., Grundling, A., and Higgins, D. E. (2006). A bifunctional O-GlcNAc transferase governs flagellar motility through anti-repression. *Genes Dev.* 20, 3283–3295. doi: 10.1101/gad.1492606
- Sircar, R., Greenswag, A. R., Bilwes, A. M., Gonzalez-Bonet, G., and Crane, B. R. (2013). Structure and activity of the flagellar rotor protein FliY: a member of the CheC phosphatase family. *J. Biol. Chem.* 288, 13493–13502. doi: 10.1074/jbc.M112.445171
- Stafford, G. P., Evans, L. D., Krumscheid, R., Dhillon, P., Fraser, G. M., and Hughes, C. (2007). Sorting of early and late flagellar subunits after docking at the membrane ATPase of the type III export pathway. *J. Mol. Biol.* 374, 877–882. doi: 10.1016/j.jmb.2007.09.080
- Williams, T., Joseph, B., Beier, D., Goebel, W., and Kuhn, M. (2005). Response regulator DegU of *Listeria monocytogenes* regulates the expression of flagella-specific genes. *FEMS Microbiol. Lett.* 252, 287–298. doi: 10.1016/j.femsle.2005.09.011
- Young, G. M., Schmiel, D. H., and Miller, V. L. (1999). A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6456–6461. doi: 10.1073/pnas.96.11.6456
- Zarivach, R., Deng, W., Vuckovic, M., Felise, H. B., Nguyen, H. V., Miller, S. I., et al. (2008). Structural analysis of the essential self-cleaving type III secretion proteins EscU and SpaS. *Nature* 453, 124–127. doi: 10.1038/nature06832

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Cheng, Wang, Ma, Han, Yang, Sun, Chen, Yu, Hang, Liu, Fang, Jiang, Cai and Song. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.