Pakistan J. Zool., pp 1-7, 2021. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20190628120649

FliI Role in Flagellar Assembly of *Salmonella* Δ*fliI* Mutant Strain Determines Motility and Biofilm Formation

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Article Information
Received 28 June 2019
Revised 30 July 2019
Accepted 18 August 2020
Available online 10 December 2020

Authors' Contribution
IL conceived and designed the study. IL and SS performed the experiments and wrote the article. IL, SAM and SA, MFQ analyzed the data. IL and IUH helped in reviewing manuscript.

Key words
FliI, *Salmonella*, Alexa fluor staining, Δ *fliI* mutant, Flagellar motility, Complementation study.

ABSTRACT

Biofilms formation is a serious problem in both clinical and environmental settings. Various gram negative bacteria exhibit biofilm formation mediated by flagellum-mediated motility. Type III protein secretion systems of several gram-negative bacterial pathogens use flagella to invade foreign surfaces, host tissues and substrates. Flagellar biosynthesis and function in *Salmonella typhimurium* is regulated by >50 genes. Bioinformatics analysis of flagellar assembly in *S. typhimurium* identified several conserved structural elements. In this study, FliI a flagellar protein required for flagellar assembly and involved in a specialized protein export pathway was cloned and overexpressed. Δ*fliI* mutant *Salmonella* strain was used to transform *fliI* overproducing plasmid pTrc99A by electroporation. Using vital dyes (Alexaflour 488), visualization of motility was observed in wild type, Δ *fliI* mutant *and fliI* complemented strain which was further assessed by biofilm formation ability. Swimming, swarming motility alongwith significantly reduced biofilm formation was observed in Δ *fliI* mutant *compared to wild type and fliI* complemented strains. This study will extend initial evidence that FliI plays important role in flagellar export system and flagellum-mediated rotation is critical for swimming, swarming motility and biofilm formation. The flagellar basal body has an ancient and evolutionarily conserved macromolecular assembly and known architecture making it an ideal drug target. The knowledge obtained will help to elucidate mechanism and design principles necessary to understand protein secretion systems.

INTRODUCTION

Motility and biofilm formation plays important role in bacterial pathogenesis hence making this aspect ideal to understand bacterial physiology. Almost every microbe including *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella* and pathogenic *E. coli*, has ability to show motility but how this motility contribute to biofilm forming ability has not thoroughly studied (Chevance and Hughes, 2008).

Bacteria exhibit both swimming and surface swarming. In aqueous medium, microbes prefer swimming motility while on semi-solid surfaces, swarming, collective migration of bacteria occurs (Fraser and Hughes, 1999). For swarming motility, vegetative bacteria undergo a process of elongation and hyper flagellation which make them highly virulent (Harshey *et al*., 1994). Furthermore, in this mode bacteria also have the increased ability to form a biofilm thus enhancing their pathogenesis ability (Kearns, 2010; Murray *et al*., 2010). A biofilm means bacteria adhered to surface and encased in self-secreted exopolysaccharide (EPS) matrix (Branda *et al*., 2005; Hall-Stoodley and Stoodley, 2009). Biofilm formation enables bacteria to survive well in host by inactivating both innate and adaptive immune responses.

The flagellar contribution to pathogenicity has been studied in many bacteria including *Helicobacter pylori, Campylobacter* sp., *Legionella* sp., *Aeromonas* sp. and *Vibrio* sp. (Bigot *et al*., 2005). In a recent study by Chakroun *et al*. (2018), authors investigated the role of flagella in virulence and biofilm formation. *Salmonella typhimurium* uses variety of virulence factors, including flagella, fimbriae, adhesins, and invasins to exhibit motility leading to biofilm formation. The flagellar assembly requires approximately 50 genes (Chevance and Hughes, 2008). Structural and other proteins required for export are transported through a flagellar mediated type III

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0030-9923/2021/0001-0001 $ 9.00/0

secretion system (T3SS). This system contains six integral membrane proteins: FlhA, FlhB, FliO, FliP, FliQ, FliR (for *Salmonellae* and other species) at least. Among these flagellar proteins, FliI is the only established ATPase. It interacts with FliJ (no known function), and with a dimer of FliH (an inhibitor of FliI). ATP hydrolysis caused by FliI is important factor in gate-activation process. This suggested that FliI plays important role in energy provision to T3SS (Minamino *et al*., 2014).

Besides swimming motility, *S. typhimurium* is among earliest serovars to show morphological differentiation of swarmer cells (Harshey *et al*., 1994). Kim and Surette (2005) studied swarming motility in *S. typhimurium* and linked it to evolutionarily conserved behaviour in *Salmonella*. Therefore, in this study, we have looked into both swimming and swarming motility of *fliI* complemented *S. typhimurium* to check if surface swarming exist in *S. typhimurium*. Furthermore, we investigated the importance of flagellar mediated biofilm formation in perspective to FliI complementation in SJW2702 (Δ*fliI) strain.* We constructed *fliI* complemented strains by overexpressing FliI using pTrc99A vector and showed that *FliI* deleted SJW2702 (Δ *fliI)*) strains are inefficient in energy coupling mechanism of flagellar type III protein export system making them aflagellated. To our knowledge this study is first of its type to demonstrate the role of *fliI* gene in flagellation and biofilm formation

MATERIALS AND METHODS

Salmonella *strains and culture conditions*

Bacterial strains *Salmonella enterica* serovar Typhimurium SJW1103 and SJW2702 (Δ*FliI)* were used in this study. These were already available in lab obtained from Yamaguchi *et al*. (1986) and Kubori *et al*. (1992). The bacteria were routinely cultured in Luria–Bertani (LB) broth and agar at 37°C. When necessary, chloramphenicol (50 µgml^{-1}) was supplemented to the growth medium.

FliI cloning and complementation

To construct *fliI* complemented *Salmonella* strains, *fliI* coding regions were amplified from an existing clone of *Salmonela typhimurium* by performing *Pfu* PCR using *fliI* 5'*Nde*I, AAAAAAC-ATATGGAGTGCTCTGAATGACCAC and *fliI* 3'*EcoR*1, AAAAAAGAATTGCTTTGAGTGTTTCCAGAGC (designed in this study). The resulting 1.4kb product was digested with *NdeI* and *EcoRI* and ligated into pET-28a (+) plasmid, encoding a His-tagged. The *Nde*I-*EcoR*I were cloned into pTrc99A having *trc* promoter and transformed into *E. coli* BL21. Insertions of the *fliI* gene was confirmed by colony PCR, restriction digestion and DNA sequencing (BigDye v3.1, 3130 Genetic Analyzer; Applied Biosystems). *S. typhimurium* was transformed by electroporation (*E. coli* Pulser, Bio-Rad). *fliI* overproducing plasmid was used to transform *FliI deletion mutant Salmonella* strain SJW2702 (Δ *fliI)* for complementation.

SJW1103 (Wild type), SJW2702 (Δ *fliI)* and *fliI* complemented strains were grown and induced with 2mM isopropyl β-d-thiogalactoside (IPTG). Cell density was normalized and proteins were precipitated by 10% trichloroacetic acid (TCA), suspended in a tris-SDS loading buffer and coomassie brilliant blue (CBB) staining was performed.

Growth analysis

Culture samples were withdrawn at regular intervals to measure the optical density (OD_{600}). Replicate growth curve data from the SJW1103 (wild type), SJW2702 (Δ*fliI)* and *fliI* complemented strains were analyzed by drawing a logarithmic scale through the exponential-growth data points for each experiment (Riaz *et al*., 2018). Slope was used to calculate the specific growth rate constant.

Motility assay

Sixteen h old wild type and *fliI Salmonella* cultures were grown in motility medium supplemented with 0.1% glycerol and chloramphenicol (50 µgml^{-1}). Swimming motility was observed by incubating plates for 5 h without being inverted. For swarming motility, a region extending ~1 cm into the colony was observed by phase contrast microscopy following Turner *et al*. (2010).

Fluorescent staining of flagella

Fluorescent staining of flagella was performed by following protocol by Turner *et al*. (2010). Briefly, swarm cells were collected, washed and centrifuged. Pellet was gently suspended in motility medium and thiol-reactive dye (Alexa Fluor 488; Invitrogen-Molecular Probes) was added. Cells were washed with motility medium and Image J was used to measure the lengths of the cell bodies and numbers of flagella (http://rsb.info.nih.gov/ij/). This information was used to measure the polymorphic transition in flagella following Calladine (1975).

Biofilm formation analysis

Biofilm assay was performed as described previously by Liaqat *et al*. (2016). This was done in two stages. In first stage, time kinetics for biofilm formation by all three strains was performed following Liaqat and Sakellaris (2012). Second stage of biofilm formation was performed using two assays. In test tube essay, LB medium supplemented with antibiotic was prepared and inoculated and incubated for 96 h. In air-liquid interface method,

nutrient broth solution was inoculated and poured in petri plates. Coverslips were very cautiously placed aseptically followed by measurement of optical density (OD_{595}). Both test tubes and air liquid interface coverslip assays were performed two times for all *Salmonella* strains, and the averages and standard deviations were calculated for all repetitions of the experiment.

Statistical analysis

Statistical analyses were performed using student "t-test" for independent samples. All the experiments were performed three times. Data was analysed using Microsoft Excel and SPSS 18. The level of significance was $P<0.05$.

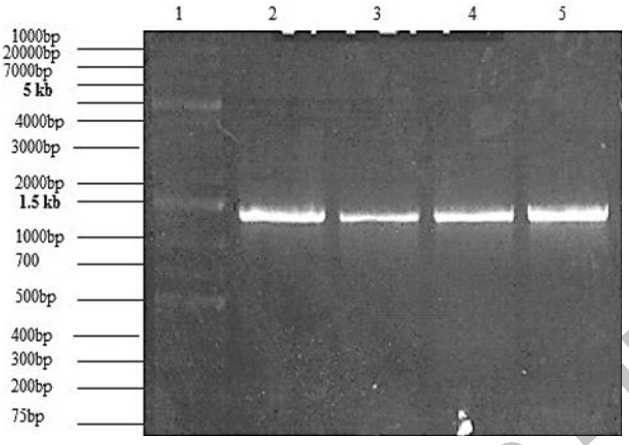

Fig. 1. Agarose gel electropherograms of the *fliI* from wild type and *fliI* complemented *Salmonella* using phusion PCR. 1st Lane, 1kb DNA ladder plus PageRuler™ Prestained Protein Ladder; 2nd Lane, *fliI* (wild type); 3rd – 5th Lane, *fliI* from *fliI* complemented *Salmonella* at different annealing temperatures of 60, 62 and 65°C.

RESULTS

FliI cloning and SDS-PAGE analysis of Salmonella *typhimurium*

To verify the role of FliI, we constructed *fliI* complemented strains. Figure 1 shows 1.4kb band of interest on the agarose gel electrophorses using phusion PCR amplification. This fragment encoding *fliI* was subcloned into pET-28a (+) vector. Subsequent restriction digestion and sequencing verified the recombinant plasmid. The double enzyme digestion of the recombinant plasmids demonstrated successful ligation into the vectors. Afterwards, sequencing confirmed that the sequence of cloned fragment to be accurate (data not shown).

We introduced *fliI* overproduction plasmid into Salmonella SJW2702 (Δ *fliI)*. SDS-PAGE anlaysis of each salmonella strain revealed that the amounts of overexpressed *fliI* was almost similar in both SWJ2102 (wild type) and *fliI* complemented strains, as seen in sodium dodecyl sulfate gels (Fig. 2, 2nd and 3rd lane). There was no apparent *FliI* bands in deletion mutants of Salmonella SJW2702 (Δ *fliI)* (Fig. 2, 1st lane), *fliI* bands were seen in wild type even in the absence of IPTG. In the presence of 2mM IPTG, *fliI* bands was the major ones in the whole-cell extracts (Fig. 2, 4th lane).

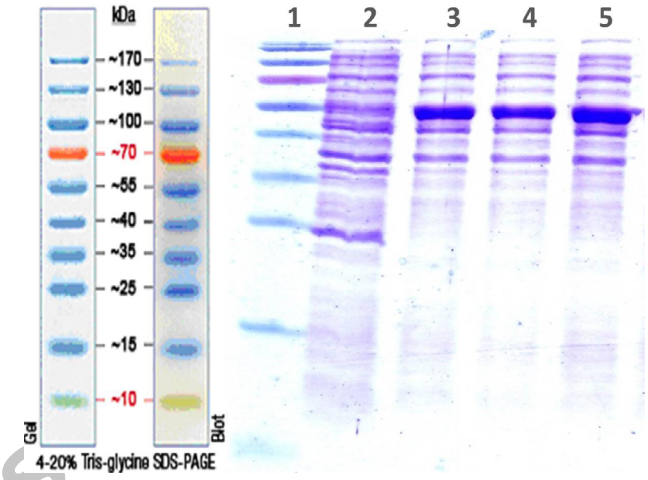

Fig. 2. Sodium dodecyl sulfate-polyacrylamide electrophoresis pattern of *FliI* in whole-cell extracts from SJW2702, SWJ1102 (wild type *Salmonella*), and *fliI* complemented *Salmonella* strains induced with IPTG. 1st Lane, PageRuler™ Prestained protein ladder; 2nd Lane, without plasmid; 3rd Lane, with plasmid and in the absence of IPTG; 4th and 5th Lanes, with plasmid and in the presence of 2mM IPTG.

Growth and motility assays

There was no calculated difference in growth rate of bacteria as was observed by their growth curve and specific growth rate calculations (Fig. 3A, B). Comparison of swimming and swarming motility assays among three strains showed that SJW1102 (wild-type) and *fliI* complemented *Salmonella* strains produced a large swim ring while the *fliI deletion* mutant *Salmonella* SJW2702 stayed at point of inoculation showing no motility (Data not shown). Phase contrast microscopy of *fliI* complemented cells showed that all cells had flagella (Fig. 4A). Following Calladine (1975), polymorphic transitions were observed in flagellated strains. Most of the flagella in our study were semicoiled in nature (Fig. 4B). To understand the role of *fliI* in swarming motility, we grew bacteria on swarm agar plates containing LB and 0.35% agar. SJW1102 and *fliI* complemented strains displayed motility on media. However, SJW2702 (Δ*fliI)* failed to exhibit any swarming motility on 0.35% agar.

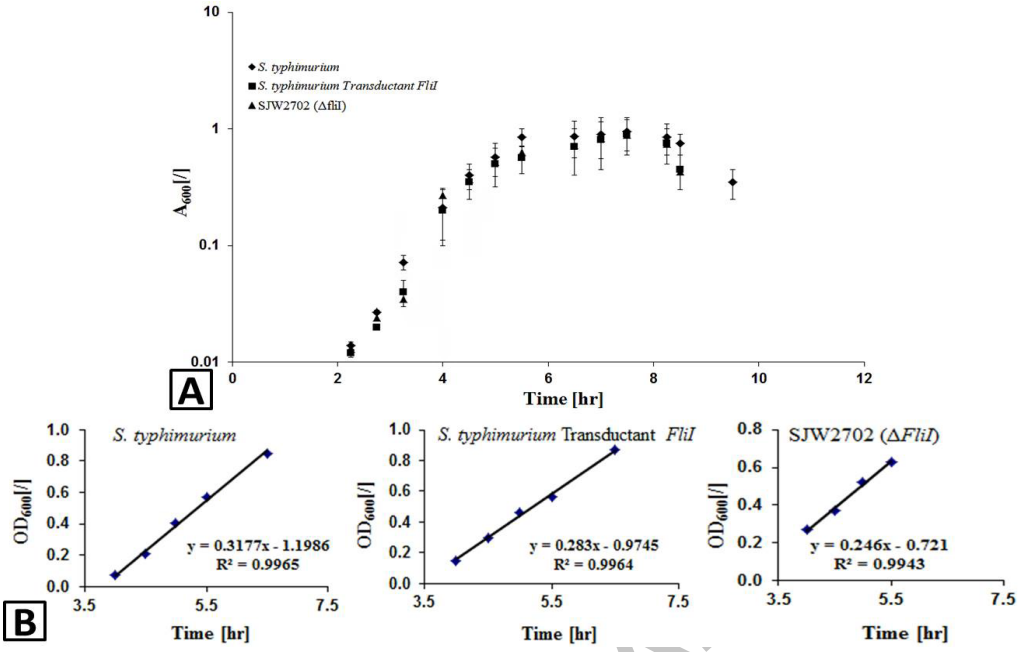

Fig. 3. A, *S. typhimurium* growth curves. Three strains of *Salmonella* including wild type, *S. typhimurium* complemented *fliI* and SJW2702 (*ΔfliI*) were grown in Luria-Bertani broth at 37°C with aeration. Bacterial growth was determined by OD_{600}. Data were obtained from the average of three independent experiments; B, specific growth rate of three strains.

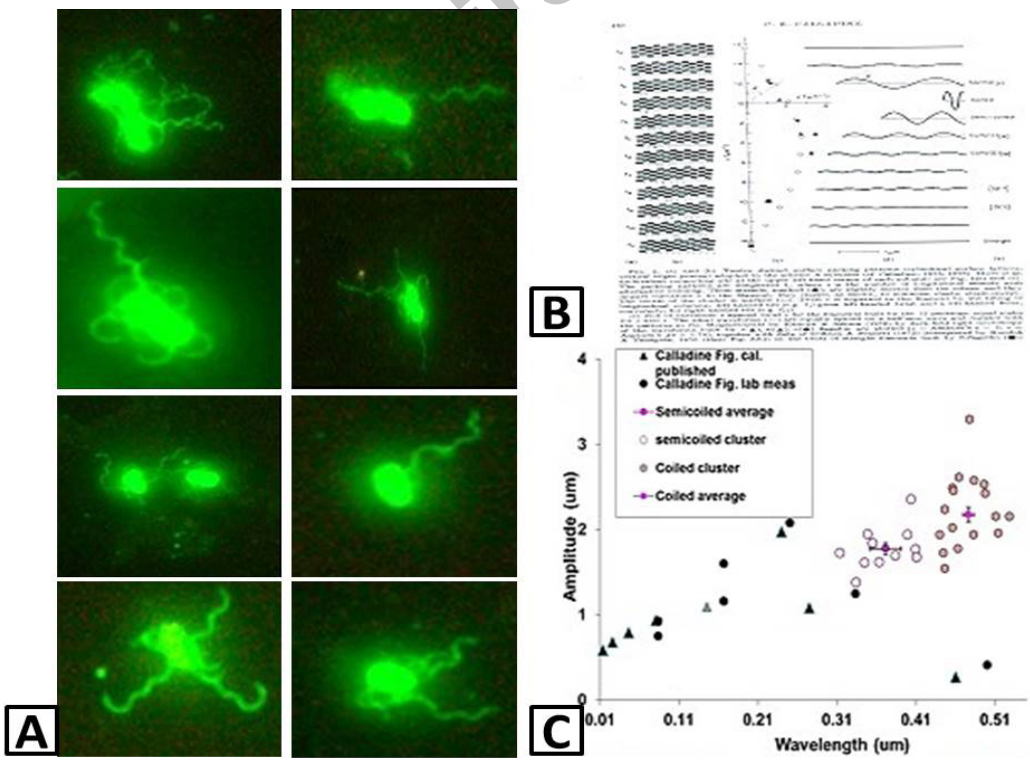

Fig. 4. A, phased contrast microscopy of Alexa flour-488 labeled flagellar strains of *fliI* complemented *Salmonella*; B, Calladine (1975) model; C, polymorphic transitions were calculated in flagella following Calladine (1975) model and placed in semi-coiled category.

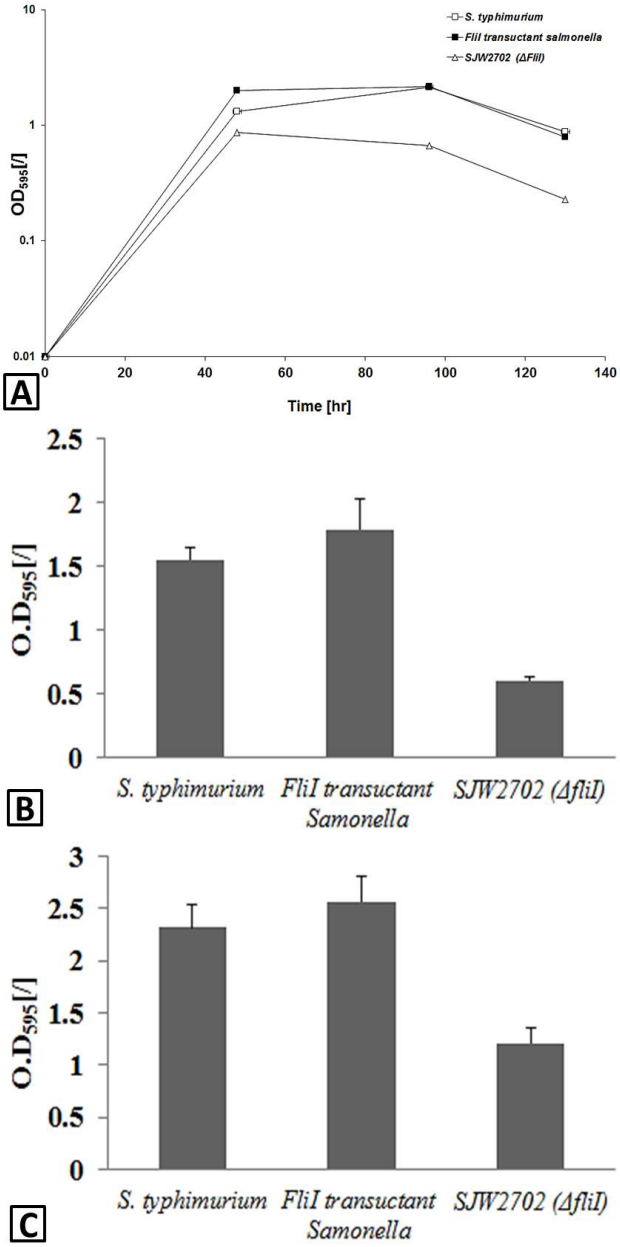

Fig. 5. A, Time kinetics for Biofilm formation. Bacteria were grown for 16 hours and diluted in fresh Luria Bertani medium supplemented with antibiotic. Data was obtained from the average of three independent experiments. B, biofilm formation assays by wild type and *fliI* complemented *Salmonella* strains by liquid-interface coverslip assay. C, biofilm formation assays by wild type and *fliI* complemented *Salmonella* strains by test tube method. All three strains were grown in LB medium for 96 h. O.D was measured at 595 nm. *fliI* complemented *Salmonella* showed best biofilm formation compared to both wild type and *fliI* deleted SWJ2702. Experiment was performed in duplicate.

Biofilm formation study

The biofilm-formation by all three strains of Salmonella was quantified using crystal violet staining method. Results of biofilm time kinetic indicated that all tested *Salmonella* isolates produced strong biofilm after 96 h. Afterwards, a decline in biofilm formation was observed (Fig. 5A). Once biofilm-forming capacity of different *Salmonella* isolates was assessed, we investigated further the difference in biofilm forming capacity of all three isolates using test tubes and liquid interface coverslip assays. We were interested to see whether *fliI* complemented strain has good biofilm forming capacity similar to swimming and swarming motility. As expected, strong biofilm formation by *fliI* complemented strain was observed compared to both wild type and *fliI* deleted strains strain (Fig. 5B, C) using both assays.

DISCUSSION

Flagellar apparatus has been observed to play crucial role in pathogenesis of a great diversity of intestinal pathogens. In this regard, the flagellar assembly of *S. typhimurium* is an interesting aspect to understand bacterial adherence mechanism and biofilm study. There is not much data about translocation of flagellar proteins from the cytosol to the distal end. Among several proteins studied so far, FliI is especially interesting because of its identical nature to catalytic β subunit of the F_1-ATPase and homology to various proteins in T3SS. Majority of bacteria including both Gram positive and Gram negative have FliI playing ATPase role in type three secretory system (T3SS). In this study, we have analyzed FliI role via complementation in flagellum-mediated rotation for swimming, swarming motility and biofilm formation.

We observed that *FliI* complemented strains have no difference in growth compared to motility and biofilm formation. Optimum swimming and swarming motility independent of planktonic growth observed in wild type and *FliI* complemented strains compared to SJW2702 (Δ *fliI*) is related to fact that fliI deletion lead to lack of energy for flagellar export. In a previous study, novel motility regulators were screened by genomic analysis and 130 mutations were found to be important to influence motility in *S. typhimurium* genome (Bogomolnaya *et al*., 2014). In fact, two energy sources used by flagellar export system include ATP and proton motive force (PMF). FliI forms a homo-hexamer and is the only ATPase of the export system. Although FliI makes export gate highly highly efficient, however, its role is still unclear because of limited information about ATPase mechanistic nature (Minamino *et al*., 2014).

It has been documented in several previous studies that either complete flagella or parts of it could promote bacterial adhesion and binding to the host's surfaces thus enhancing virulence. Biofilm formation is an adaptation by different bacterial species to enhance survival and pathogenesis. We observed biofilm formation by all Salmonella isolates in this study. This might be due to greater glycocalyx production at that stage as reported by MacFarlane *et al*. (2007). However, *Salmonella* strain lacking fliI gene exhibited almost one fold decrease in biofilm formation compared to wild type and fliI complemented strain. Wood *et al*. (2006) demonstrated that flagella are important both in biofilm initiation and development. Likewise, Olsen *et al*. (2013) reported that serovar-specific differences are important in determining in the flagellar involvement as well as chemotaxis genes in attachment and invasion of *Salmonella* to the host

Following time kinetics of biofilm formation, its quantification was performed using test tubes and liquid interface coverslips assays. Significantly decreased biofilm in *fliI* deficient strains observed in this study might be due to lack of flagella. Non-motile flagellar mutants might have decreased initial surface attachment hence showed poor biofilm formation (Liaqat *et al*., 2016). Additionally other factors including fimbriae, pili, curli may also contribute to decreased biofilm formation in non flagellar strains (Reisner *et al*., 2006; Lemon *et al*., 2007; MacFarlane and Dillon, 2007; Kim *et al*., 2008; Liaqat and Sakellaris, 2012). Importantly, higher biofilm formation observed by *fliI* complemented strain even compared to wild type. This might be due to the fact that *FliI* overexpression essentially means more energy production for flagellar export leading to enhanced flagellation hence initiating formation of biofilm. Previous studies by Lemon *et al*. (2007) and Gorski *et al*. (2009) are consistent with our finding that without flagella or flagella motility, biofilm formation was significant reduced.

The present study suggested that motility is stringently controlled by an organized flagellar assembly. We concluded that FliI is essential for flagellation, motility and biofilm formation in *Salmonella* strains. We are the first one to report that without *FliI*, motility is essentially lost leading to significantly reduced biofilm formation in *S. typhimurium*. More detailed studies on this aspect will lead to better understanding of various mechanisms involved in *Salmonella motility at molecular level. A*mong several questions about the role of flagella, one question which we are interested to address was the role of flagellation in attenuation of strain pathogenesis as was observed by Yang *et al*. (2012). Efforts will be made in our lab to study *fliI* complemented role through i*n vivo* trails.

ACKNOWLEDGEMENT

We are grateful to Shahid Khan, Molecular Biology Consortium, Lawrence Berkeley National Laboratory, Berkeley, CA-94720, USA for his criticism and support. Initial phase of this study was financially supported due to his collaboration with LUMS, Lahore, Pakistan.

Statement of conflict of interests

Authors declare no conflict of interests.

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