FliI Role in Flagellar Assembly of Salmonella ΔfliI Mutant Strain Determines Motility and Biofilm Formation

Iram Liaqat1,*, Safdar Ali Mirza2, Sumera Sajjad3, Shaukat Ali1,*, Muhammad Faiz Qamar4 and Ikram Ul Haq5

1Department of Zoology, Govt. College University, Lahore-54000
2Department of Botany, Govt. College University, Lahore-54000
3Department of Zoology, Lahore College for Women University, Lahore
4Department of Pathobiology, College of Veterinary and Animal Sciences, Sub-campus Jhang
5Institute of Industrial Biotechnology, Govt. College University, Lahore-54000

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. ΔfliI mutant Salmonella strain was used to transform fliI overproducing plasmid pTrc99A by electroporation. Using vital dyes (Alexafluor 488), visualization of motility was observed in wild type, ΔfliI mutant and fliI complemented strain which was further assessed by biofilm formation ability. Swimming, swarming motility along with significantly reduced biofilm formation was observed in ΔfliI 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, swimming, 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
secrption system (T3SS). This system contains six integral membrane proteins: FlhA, FlhB, FlhO, FliP, FliQ, FliR (for Salmonellae and other species) at least. Among these flagellar proteins, Fli 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 Fli is important factor in gate-activation process. This suggested that Fli 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 is among earliest serovars to show morphological typhimurium and linked it to evolutionarily conserved have looked into both swimming and swarming motility to T3SS (Minamino et al., 2014). Kim and Surette (2005) studied swimming motility in S. typhimurium and it linked to evolutionarily conserved behaviour in Salmonella. Therefore, in this study, we have looked into both swimming and swarming motility of flhI 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 flhI) strain. We constructed flhI complemented strains by overexpressing FliI using pTrc99A vector and showed that FlhI deleted SJW2702 (Δ flhI) 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 flhI gene in flagellation and biofilm formation.

**MATERIALS AND METHODS**

**Salmonella strains and culture conditions**

Bacterial strains Salmonella enterica serovar Typhimurium SJW1103 and SJW2702 (ΔfliI) 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 µg/ml) was supplemented to the growth medium. Salmonella Typhimurium SJW1103 and SJW2702 (Δ fliI) strain.

**FlhI cloning and complementation**

To construct flhI complemented Salmonella strains, flhI coding regions were amplified from an existing clone of Salmonella typhimurium by performing Pfa PCR using flhI 5’Ndel, AAAAAAC-ATATGGAGTGCTCTGAATGACCAC and flhI 3’EcoRI, AAAAAAGAATTTGCTTTGAGTGGTTTTCGAGC (designed in this study). The resulting 1.4kb product was digested with Ndel and EcoRI and ligated into pET-28a (+) plasmid, encoding a His-tagged. The Ndel-EcoRI were cloned into pTrc99A having trc promoter and transformed into E. coli BL21. Insertions of the flhI 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). flhI overproducing plasmid was used to transform Flh deletion mutant Salmonella strain SJW2702 (Δ flhI) for complementation.

SJW1103 (Wild type), SJW2702 (Δ flhI) and flhI 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 (OD600). Replicate growth curve data from the SJW1103 (wild type), SJW2702 (ΔflhI) and flhI 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 flhI Salmonella cultures were grown in motility medium supplemented with 0.1% glycerol and chloramphenicol (50 µg/ml) was supplemented to the growth medium.

To construct flhI complemented Salmonella strains, flhI coding regions were amplified from an existing clone of Salmonella typhimurium by performing Pfa PCR using flhI 5’Ndel, AAAAAAC-ATATGGAGTGCTCTGAATGACCAC and flhI 3’EcoRI, AAAAAAGAATTTGCTTTGAGTGGTTTTCGAGC (designed in this study). The resulting 1.4kb product was digested with Ndel and EcoRI and ligated into pET-28a (+) plasmid, encoding a His-tagged. The Ndel-EcoRI were cloned into pTrc99A having trc promoter and transformed into E. coli BL21. Insertions of the flhI 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). flhI overproducing plasmid was used to transform Flh deletion mutant Salmonella strain SJW2702 (Δ flhI) for complementation.

SJW1103 (Wild type), SJW2702 (Δ flhI) and flhI 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.

**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 Fluer 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.

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 electrophoresis 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.

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 (ΔfliI) 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.
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 fli 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 Fli 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 Liaqat et al. (2016). Additionally other factors including fimbrae, pili, curli may also contribute to decreased biofilm formation in non flagellar strains (Reisner et al., 2006; Lemon et al., 2007; MacFarlane and Dillon, 2008; Liaqat and Sakellaris, 2012). Importantly, higher biofilm formation observed by 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). 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 fimbrae, 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.

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 UUMS, Lahore, Pakistan.

Statement of conflict of interests

Authors declare no conflict of interests.

REFERENCES

Bigot, A., Pagniez, H., Botton, E., Fréhel, C., Dubail, I., Jacquet, C., Charbit, A. and Raynaud, C., 2005. Role of FliF and FliI of Listeria monocytogenes in flagellar assembly and pathogenicity. Infect. Immun., 73: 5530-5539. https://doi.org/10.1128/IAI.73.9.5530-5539.2005

Bogomolnaya, L.M., Aldrich, L., Ragoza, Y., Talamantes, M., Andrews, K.D., McClelland, M. and Andrews-Polymenis, H.L., 2014. Identification of novel factors involved in modulating motility of Salmonella enterica serotype Typhimurium. PLoS One, 9: e111513. https://doi.org/10.1371/journal.pone.0111513

Branda, S.S., Vik, S., Friedman, L. and Kolter, R., 2005. Biofilms: The matrix revisited. Microbiology, 13: 20-26. https://doi.org/10.1016/j.micr.2004.11.006

Calladine, C.R., 1975. Construction of bacterial flagella. Nature, 255: 121-124. https://doi.org/10.1038/255121a0

Chakroun, I., Mahdhi, A., Morcillo, P., Cordero, H., Cuesta, A., Bakhrouf, A., Mahdouani, K. and Esteban, M.Á., 2018. Motility, biofilm formation, apoptotic effect and virulence gene expression of atypical Salmonella Typhimurium outside and inside Caco-2 cells. Microb. Pathog., 114: 153-162. https://doi.org/10.1016/j.mppath.2017.11.010

Chevance, F.F. and Hughes, K.T., 2008. Coordinating assembly of a bacterial macromolecular machine. Nat. Rev. Microbiol., 6: 455-465. https://doi.org/10.1038/nrmicro1887

Fraser, G.M. and Hughes, C., 1999. Swarming motility. Curr. Opin. Microbiol., 2: 630-635. https://doi.org/10.1016/S1369-5274(99)00033-8

Gorski, L., Duhe, J.M. and Flaherty, D., 2009. The use of flagella and motility for plant colonization and fitness by different strains of the foodborne pathogen Listeria monocytogenes. PLoS One, 4: e5142. https://doi.org/10.1371/journal.pone.0005142
Hall-Stoodley, L. and Stoodley, P., 2009. Evolving concepts in biofilm infections. Cell. Microbiol., 11: 1034-1043. https://doi.org/10.1111/j.1462-5822.2009.01323.x

Harshey, R.M. and Matsuyama, T., 1994. Dimorphic transition in Escherichia coli and Salmonella Typhimurium: surface-induced differentiation into hyperflagellate swarmer cells. Proc. natl. Acad. Sci. USA, 91: 8631-8635. https://doi.org/10.1073/pnas.91.18.8631

Kearns, D.B., 2010. A field guide to bacterial swarming motility. Nat. Rev. Microbiol., 8: 634-644. https://doi.org/10.1038/nrmicro2405

Kim, T.J., Young, B.M. and Yong, G.M., 2008. Effect of flagellar mutations on Yersinia enterocolitica biofilm formation. Appl. environ. Microbiol., 74: 5466-5474. https://doi.org/10.1128/AEM.00222-08

Kim, W. and Surette, M.G., 2005. Prevalence of surface swarming behavior in Salmonella. J. Bact., 187: 6580-6583. https://doi.org/10.1128/JB.187.18.6580-6583.2005

Kubori, T., Shimamoto, N., Yamaguchi, S., Namba, K. and Aizawa, S.I., 1992. Morphological pathway of flagellar assembly in Salmonella typhimurium. J. mol. Biol., 226: 433-446. https://doi.org/10.1016/0022-2836(92)90958-M

Lemon, K.P., Higgins, D.E. and Kolter, R., 2007. Flagellar motility is critical for Listeria monocytogenes biofilm formation. J. Bact., 189: 4418-4424. https://doi.org/10.1128/JB.01967-06

Liaqat, I. and Sakellaris, H., 2012. Biofilm formation and binding specificities of CFA/I, CFA/II and CS2 adhesions of enterotoxigenic Escherichia coli and Cfae-R181A mutant. Braz. J. Microbiol., 43: 969-980. https://doi.org/10.1590/S1517-8382201200300018

Liaqat, I., Sumbal, F. and Sabri, A.N., 2009. Tetracycline and chloramphenicol efficiency against selected biofilm forming bacteria. Curr. Microbiol., 59: 212-220. https://doi.org/10.1007/s00284-009-9424-9

Liaqat, I., Pervaiz, Q., Bukhsh, S.J. and Jahan, N., 2016. Investigation of bactericidal effects of medicinal plant extracts on clinical isolates and monitoring their biofilm forming potential. Pak. Vet. J., 36: 159-164.

MacFarlane, S. and Dillon, J.F., 2007. Microbial biofilms in the human gastrointestinal tract. J. appl. Microbiol., 102: 1187-1196. https://doi.org/10.1111/j.1365-2672.2007.03287.x

Minamino, T., Morimoto, Y.V., Kinoshita, M., Aldridge, P.D. and Namba, K., 2014. The bacterial flagellar protein export apparatus processively transports flagellar proteins even with extremely infrequent ATP hydrolysis. Proc. natl. Acad. Sci. USA, 91: 8631-8635. https://doi.org/10.1073/pnas.91.18.8631

Olsen, J.E., Hoegh-Anderssen, K.H., Casadesus, J., Rosenkranzt, J., Chadfield, M.S. and Thomsen, L.E., 2013. The role of flagella and chemotaxis genes in host pathogen interaction of the host adapted Salmonella enterica serovar Dublin compared to the broad host range serovar S. Typhimurium. BMC Microbiol., 13:67. https://doi.org/10.1186/1471-2180-13-67

Riaz, A., Noureen, S., Qamar, M.F., Liaqat, I., Arshad, M. and Arshad, N., 2018. Characterization of bacteriocin like inhibitory substances from Enterococcus ratti MF183967. Pak. Vet. J., 39:1-6.

Reisner, A., Krogefelt, K.A., Klein, B.M., Zechner, E.L. and Molin, S., 2006. In vitro biofilm formation of commensal and pathogenic Escherichia coli strains: Impact of environmental and genetic factors. J. Bact., 188: 3572-3581. https://doi.org/10.1128/JB.188.10.3572-3581.2006

Riaz, A., Noureen, S., Qamar, M.F., Liaqat, I., Arshad, M. and Arshad, N., 2018. Characterization of bacteriocin like inhibitory substances from Enterococcus ratti MF183967. Pak. Vet. J., 39:1-6.

Turner, L., Zhang, R., Darnton, N.C. and Berg, H.C., 2010. Visualization of Flagella during bacterial Swarming. J. Bact., 192: 3259-3267. https://doi.org/10.1128/JB.00083-10

Wood, T.K., González Barrios, A.F., Herzberg, M. and Lee, J., 2006. Motility influences biofilm architecture in Escherichia coli. App. Microbial. Cell Physiol., 72: 361–367. https://doi.org/10.1007/s00253-005-0263-8

Yamaguchi, S., Aizawa, S.I., Kihara, M., Isomura, M., Jones, C.J. and Macnab, R.M., 1986. Genetic evidence for a switching and energy-transducing complex in the flagellar motor of Salmonella typhimurium. J. Bact., 168: 1172-1179. https://doi.org/10.1128/JB.168.3.1172-1179.1986

Yang, X., Thornburg, T., Suo, Z., Jun, S., Robison, A., Li, J., Lim, T., Cao, L., Hoyt, T., Avci, R. and Pascual, D.W., 2012. Flagella overexpression attenuates Salmonella pathogenesis PLoS One, 7: e46828. https://doi.org/10.1371/journal.pone.0046828