YfiB and the tripartite signaling system YfiBNR is involved in virulence of the gut invasive pathogen *Shigella flexneri.*

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ABSTRACT

Shigella flexneri is one of the principal cause of bacillary dysentery and contributes significantly to the worldwide implication of diarrheal infections. The presence and upsurge of multidrug resistance amongst *Shigella* strains, demands additional genetic analyses, advancement of new/improved drugs, and finding vaccine candidates against the pathogen. Whilst many features about the invasion of colonic cells by *Shigella* have been identified, fundamental gaps in information concerning in what way the bacteria transit, survive, and control gene expression, remain. Present study aims to illustrate the role of *yfiB* gene in *Shigella* virulence, which is a part of the periplasmic YfiBNR tripartite signaling system. This system is responsible for regulating cyclic-di-GMP levels inside the bacterial cells, which is a vital messenger molecule impacting varied cellular processes involving biofilm formation, cytotoxicity, motility, synthesis of exopolysaccharide, and other virulence mechanisms like adhesion and invasion of the bacteria. Through a combination of genetic, biochemical, and virulence assays, we show how knocking out the *yfiB* gene can disrupt the entire YfiBNR system and affect biofilm formation, bacterial invasion, surface attachment, and the virulence of *Shigella*. We then show how targeted mutagenesis of the significant amino acids of the YfiB protein can affect the proper functioning of the protein. This study eventually improves our understanding of the *in-vivo* persistence and survival of *Shigella* and provides a prospective

new target to design anti-infective drugs and vaccines against *S. flexneri* and other bacterial pathogens.

Keywords: *Shigella flexneri*; Biofilm; YfiBNR signaling; Virulence; Bacterial Invasion; Biofilm formation; Site-directed mutagenesis.

Introduction

Shigella cause an extreme enteric infection known as shigellosis, by attacking the colonic epithelium, it being gram negative in nature and a facultative intracellular pathogen **[1]**. Every year shigellosis leads to substantial morbidity and fatality worldwide, majorly in children below 5 years in under-developed and developing countries **[1,2].** Clinical features of shigellosis varies from minor diarrhea to bloody mucoid type of diarrhea alongside excruciating abdominal spasms with high fever [**3,4]**. Clinical manifestations are linked to the host's immune system plus the contributing *Shigella* species, varying in occurrence of vital virulence features, such as the Shiga toxin **[4,5]**. In developing countries, shigellosis infections are mostly due to the *S. flexneri* species and is accountable for roughly 10% of overall diarrhoetic incidents amongst children below 5 years of age **[6]**. An appropriate vaccine against *S. flexneri* has not been formulated yet, partially due to its considerable number of serotypes that have to be battled with for its vaccine to be effective globally **[6]**. The efficiency of the current antimicrobial treatments keeps on being compromised because of the persistence of poor hygiene and sanitation problems and an increase in the cost of treatment, followed by an upsurge of multidrug-resistant strains [**5,6]**.

Shigella's invasion process; the immune response it generates in the host and its intracellular survival has been comprehensively researched, but there still is a considerable gap in our understanding about how *Shigella* effectively extends to the colonic epithelium and establishes the infection [**7].** The bacteria encounters various stimuli, predominately bile in the small

intestine during its gastrointestinal passage [7], where the concentration of bile ranges between 0.2% to 2% (wt/vol) based on the individual, on its diet, and time of the day [**7,8]**. Bile is known to be bactericidal and gram-negative bacteria especially enteric pathogens have different mechanisms to resist bile and use it as a cell signal to control gene expression, control virulence, and boost the infection process [**7,8]**. Another indispensable step in the colonization of the pathogen is adherence to the host surface which prevents mechanical clearance and also aids in the invasion and intercellular spread of the bacteria [**9]**. *Shigella*'s pathogenic capability is associated with its capacity to adhere, invade, multiply, and eventually killing the host colonic epithelial cells [**9,10]**. This process involves the expression of various genes of the large virulence plasmid and also some chromosomal genes play a role [**10]**. Latest studies have revealed involvement of *ipA* genes, the *mxi-spa* locus of the large virulence plasmid, and genes like *icsA* a chromosomal gene, in effective biofilm production, adherence, and invasion process [**9,10].**

An abundant molecule that acts as a secondary messenger in *Shigella* and other bacteria facilitating cellular and molecular adaptations to their always-changing environment is Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) **[11].** Escalation in c-di-GMP levels inside the cell promotes flagella/pili biosynthesis, motility, biofilm formation, transcription, exopolysaccharide (EPS) production, surface adherence, invasion of host cells, and virulence of the bacteria [**11,12]**. A fall in c-di-GMP levels instead negatively affects all these above-mentioned virulence factors. The association between increased c-di-GMP levels and biofilm production and/or motility mechanisms have been extensively researched in *Escherichia coli*, *Salmonella enterica serovar* Typhimurium and *Pseudomonas aeruginosa* [**13]**. All the biofilm-linked targets are controlled at various pre and post transcriptional and translational stages by c-di-GMP, which include determinants like exopolysaccharide production, increase in motility, expression of surface adhesin, secondary metabolite production, biofilm dispersal,

antimicrobial resistance and additional stress responses]**13,14,15]**. c-di-GMP affects cellulose biosynthesis, poly-β-1,6-N-acetylglucosamine production, alginate biosynthesis, biosynthesis of important polysaccharides like Pel and Psl, all of these are crucial for biofilm formation [**15]**. Elevated levels of c-di-GMP also prompt an upsurge in motility which further leads to an initial increase in biofilm production, this is achieved by inducing the formation of pilli/flagella/fimbriae, which aids in biofilm formation, dispersal, and maturation which leads to a stable three-dimensional biofilm architecture [**15,16].**

Additionally, c-di-GMP is involved in adherence/attachment to host surfaces which prevents mechanical clearance and further helps in the effective invasion of the host. Non-fimbrial adhesins are objected to c-di-GMP regulation and are involved in stabilizing the extracellular matrix and cell surface adherence [**15]**. c-di-GMP signaling controls host cell adherence and invasion, intracellular spread, cytotoxicity, regulation of immune responses, and secretion of other virulence factors [**13,15,16,17]**. Moreover, c-di-GMP is also involved in regulating the fate of invading bacteria, following phagocytosis or an induced uptake with intracellular multiplication [**16,17]**. Therefore understanding how the levels of c-di-GMP are modulated and how this multistep signaling works is essential for developing novel and efficacious treatments against pathogenic bacteria.

The key protein in question for this study- YfiB, consists of an OmpA-like domain, present in the outer membrane and is a sensor protein, [**18,19]**. Complete functional understanding of YfiB remains incomplete, although it is predicted to transduce stress signals into a prompt escalation of intracellular c-di-GMP levels, exopolysaccharide production and affects downstream virulence factors such as inducing biofilm production [**19,20,21].** YfiB is part of the tripartite signaling system known as YfiBNR, first identified in *Pseudomonas aerouginosa* **[22]**; this modulates c-di-GMP levels as per the stress indicators detected at the periplasm and forms a crucial factor for the phenotype of small colony variant (SCV) in *Pseudomonas*

[22,23]. This signaling system has also been reported in other gram-negatives like *Klebsiella, E. coli, and Yersinia pestis* **[23].** YfiBNR system helps in outside-inside signaling and comprises of three different protein members- YfiN, YfiB, and YfiR [**22,23].** YfiN is situated at the inner membrane and is responsible for diguanylate cyclase (DGC) activity [**23,24]**. YfiR is placed in the periplasm and forms an association between the inner membrane YfiN and the outer membrane YfiB [**18]**; by binding to YfiN's periplasmic domain it allosterically inhibits or negatively regulates the YfiN's DGC activity [**18,23].** YfiB on the other hand sequesters YfiR to the outer membrane, preventing its binding to YfiN, can release the inhibition of YfiN [**19];** YfiB achieves this by membrane anchoring and peptidoglycan binding [**25]**. This leads to the stimulation of YfiN's DGC activity, c-di-GMP production, resulting in enhanced biofilm and various other virulence factors [**18,19,22,23]**. The working model for YfiBNR functioning adapted from *P. aeruginosa* YfiBNR system, developed by Malone, J. G. et al (2010) [**22]**, has been illustrated in **Figure 1**.

In this study, we examine the functional role of YfiB in the YfiBNR system and its effect on c-di-GMP production, which eventually determines the virulence of *Shigella flexneri*, hence determining the link between the DGC activity of YfiN and its positive regulator YfiB. To do this, we generated a *yfiB* knockout, using double homologous recombination and studied its effect on *Shigella*'s survival and pathogenesis, using various *in-vivo* and *in-vitro* virulence assays. We also studied the protein structure in comparison to the YfiB protein of *Pseudomonas*, which previously has been crystallized [**19,20]**. This helped in designing targeted mutagenesis experiments to establish which essential amino acids are involved in the proper functioning of YfiB in *Shigella.* This is the first known report to validate that YfiB regulation of YfiN and regulating c-di-GMP levels appropriately is indispensable for *S. flexneri* to cause an effective infection in the host.

Materials and Methods

Strains and growth conditions

S. flexneri serotype 1c (Y394) is a clinical strain from Bangladesh and was generously provided by Nils I. A. Carlin [**26]**. Using a sterile loop, Y394 and other *Shigella* strains were streaked from glycerol stocks and cultured on Luria Bertani (LB) agar plates and/or tryptic soy broth (TSB) agar plates with 0.01% (wt/vol) Congo red (CR) and were grown aerobically (180 rpm) at 30°C in LB broth to retain the virulence plasmid. Overnight cultures were then sub-cultured at 1:100 dilution and grown at 37°C with 180 rpm shaking, till an optical density of 0.5 to 0.8 (log-phage culture) was obtained at 600 nm (OD_{600}). The antibiotics were added as supplements where indicated at the concentrations: 50 µg/mL erythromycin, 25 µg/mL chloramphenicol, 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 20 µg/mL gentamicin.0.2% arabinose was added for all inducible plasmids. All plasmids, strains, and primers used in this study are listed in **Supplementary Table S1 and S2.**

Knocking out the *yfiB* gene to create the deletion mutant and complementation.

yfiB deletion mutant (SFL2641/ΔYfiB) was created by means of the lambda red double recombination approach established by Datsenko and Wanner with a few modifications [**27,28]** using the helper plasmid pKD46, and pKD3 plasmid containing chloramphenicol resistance (CM) for construction of the knockout template. A PCR-based methodology was used for the construction of the knockout template. The CM gene was amplified using the miniprep DNA of pKD3 using primer pairs containing 80bp overhangs, which were homologous to the downstream and upstream of the *yfiB* gene. *S. flexneri* 1c strain (Y394) containing the pKD46 plasmid, encoding the lambda red genes (*gam, beta, and exo*) when induced with 100mM arabinose, was transformed with the knockout template. The positive mutants were first

screened on LBA plates containing chloramphenicol and then via colony PCR followed by sequencing.

Complementation of *yfiB* deletion mutant was obtained by cloning the PCR-amplified *yfiB* gene from the genome of *S. flexneri* 1c SFL1613/Y394 strain, this PCR product was then purified and digested with *Nco*I and *Hind*III. Digested PCR product encoding *yfiB*, was cloned into the pBAD_Myc_HisA vector, which contains a C-terminal 6xHis tag fused to the protein for checking expression (SFL2642/YfiBComp).

Biofilm Assay

The protocol used for the biofilm assay was tailored from the method established by Christensen et al. with a few modifications [**29]**. Single colony of each bacterial strain was inoculated into a tube containing 10 ml of tryptone Soy broth (TSB). The cultures were incubated overnight at 30^0C with 180 rpm shaking. On the following day, each of the cultures was diluted in 1:100 in 5 ml into two tubes one with glucose (1%) and Bile salts (0.4%) and the other without the glucose and Bile salts. The OD_{600} of diluted cultures were checked to make sure the cultures were uniform. 100 µl control media and diluted cultures were inoculated per well in the sterile 96-well microplates (Thermo Fisher). The plates were incubated for 6, 12, and 24 hours at 37^0C statically. After incubation, the culture media were removed from the plate gently by slanting the plate and gradually removing the medium using a multichannel pipette and 1x PBS was used to rinse the wells. The wells were let dried for 30 minutes, after which methanol was used to fixed the dried surface biofilm and then 0.5% crystal violet was used to stain it. After staining, the wells were washed 4 times with MilliQ water, followed by drying the plate for over 3 hours. To each well, 200 µl of 95% ethanol was added after drying and incubated for an hour at 4^0C to avoid the evaporation of ethanol. The absorbance of all the wells was recorded at 595 nm wavelength (OD_{595}) using the Tecan plate reader. Biofilm Assay

with each strain was performed at least 4 times independently with three biological repeats, shown in the graph is the mean of all experiments. To calculate the significance between the biofilm produced by the WT and mutant strains, an unpaired student t-test was used.

in-vitro bacterial adhesion assay

A bacterial adhesion assay was carried out using Baby Hamster Kidney fibroblasts (BHK) cells [**30**]. *Shigella* strains were grown in TSB-Sodium deoxycholate medium until an OD_{600} = 0.6-0.7 (late log phase) was obtained and appropriately diluted in normal saline to a required $2x10^9$ CFU/ml concentration for infection. This prepared culture was used to infect the BHK cells (100 ul/each well), then incubated at 37^0C for 2-3 hours for the infection process. After incubation, the cells were rinsed 4 times using 1x PBS, to eliminate all non-adhered bacteria and were lysed using 1% Triton-X by incubating 10 minutes at room temperature on a shaker. LB medium was added to the lysed cells to recover the adhered and invading bacteria. Serial dilutions were prepared of the suspension, plated on LBA, and plates incubated at 37^0C, overnight. After incubation, counting of colonies was done and to calculate the total colony-forming unit (CFU). The percentage of adhered bacteria was estimated by dividing the total CFU of adhered bacteria by the total CFU of the inoculum. To calculate the significance between the percentage adhesion of the WT and mutant strains an unpaired student t-test was used.

in-vitro invasion assay and microscopy

The bacterial invasion assays [**31**] were performed using an epithelial cell line, Baby Hamster Kidney fibroblasts (BHK) cells. Cells were grown in tissue culture flasks (25 cm^2) to 80% confluency. The incubation of the cells was performed using a CO_2 incubator with 5% CO_2 at 37°C using the tissue culture media Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2mM Glutamine, and 1x non-

essential Amino Acids. Preparing the bacterial culture for invasion assay was done by taking a single isolated colony from each of the *S. flexneri* strains that need to be tested, these were cultured in 5 ml of LB having suitable antibiotics. Cultures were grown overnight at 30°C to preserve the virulence plasmid. Following day, the cultures were diluted 1:100 in LB with 0.1% deoxycholate, containing appropriate antibiotics and allowed to grow until OD_{600}=0.6-0.8 (mid-log phase) was obtained at 37°C. The appropriate amount of bacteria was harvested via centrifugation and resuspended in 1 ml of 1 x PBS to obtain the culture of 2 x 10^9 CFU/ml. From this culture, 0.1 ml was used per well of a 6-well tissue culture plate to infect the BHK cells. Centrifugation of the plates for 10 min at 1000X g was done and then incubated for 30 minutes at 37 °C in 5% CO_2 level. The media was then removed and wells were washed 4X with 1 ml of 1X PBS. The tissue culture media containing gentamycin (10 µg/ml) was added and incubation of the plate was done for further 60 – 90 minutes (2 hours maximum including 30 minutes above) at 37°C and 5% CO_2.

Staining and Data collection- After incubation, the media was aspirated and wells were rinsed with 1 ml of 1 x PBS two times. 0.5 ml of filtered Wright-Giemsa stain was added to each well for staining the cells. After 2 minutes, the stain was washed two times with 2 ml MilliQ water and the plates inverted for drying. The invasion of bacteria per BHK cells was determined by enumeration of at least 300 BHK cells and the total bacteria present using a 100X oil immersion microscope. The experiment was performed in duplicates with 3 repeates and to calculate the significance between the invasion of the WT and mutant strains, an unpaired student t-test was performed.

in-vitro Plaque Assay

Plaque assay was carried out using the HeLa cell line [**31-33]**, *Shigella* strains were grown in TSB medium until OD_{600} = 0.4-0.5 (mid-log phase) was obtained, and appropriately diluted in

normal saline to a required $1x10^7$ CFU/ml concentration. This prepared culture was further diluted to 10^6 to 10^4 CFU/ml, to infect the HeLa cells (100 ul/each well), and subsequently, the plates were incubated at 37^0C, 45-90 minutes for the infection process. Medium was removed after incubation, cells were washed twice with 1X PBS, and 2X gentamycin medium was added to each well. Gentamycin medium was used to remove all external bacteria, which results from futile spreading into the extracellular medium, rather than the neighboring cells. This ensures that each plaque formed, parallels to a sole invasion occurrence at the start of the experiment [**34-37]**. The plates were then re-incubated at 37^0C for 48-72 hours, plaques were mostly visible after 48 hours. When the plaques are visible, the plate was taken out, washed with 1x PBS, to wash away dead cells as they detach from the plate surface. Wright-Giemsa stain is used to counterstain the intact monolayer to make the plaques evident (clear spaces) against the stained background [**35]**. The over-all number of plaques were counted and the mean number of plaques per bacteria was plotted on the graph**.** The experiment was carried out for 50 technical repeats with two biological repeats each time and to calculate the significance between the number of plaques formed by the WT and mutant strains, an unpaired student t-test was performed. Few of the stained plaques were also observed under the microscope to see how the plaques looked under 40X magnification and if there were any observed morphological changes of the cells near and around the plaques.

in-vivo **bacterial Accumulation assay using** ***Caenorhabditis elegans***

The bacterial accumulation assay was executed using the *C. elegans* N2 strain (WT), these nematodes were cultured on modified nematode growth medium (mNGM), plates already containing *E. coli* OP50 grown on it [**37-39]**. L4 stage *C. elegans* of synchronized population was collected off these *E. coli* OP50 plates using S-basal buffer and were subjected for 3 hours to 200 µg/ml of gentamycin, to remove any *E. coli* OP50 cells present on the surface of the worms. Then, the worms were meticulously rinsed with S-basal buffer to eliminate any

remaining antibiotic. Bacterial strains were grown at 37^0C, overnight on mNGM plate to prompt the expression of genes encoded by the virulence plasmid. The washed worms were put onto these plates with the seeded *Shigella* strains and incubated for 24 hours, at 22°C. Next day, 15 worms were picked from each plate and to anesthetize these worms, they were rinsed meticulously using S-basal containing 1mM sodium azide. The worms were subjected for 3 hours, to 200 µg/ml of gentamycin to remove any bacterial cells present on the surface of the worms. After 3 hours, the worms were rinsed 3X times with S-basal buffer containing 1mM sodium azide. The washed worms were resuspended in S-basal buffer containing 0.1% Triton-X and were lysed mechanically using sterile glass beads. The lysate obtained was serially diluted using 1X PBS, and appropriate dilutions were plated on LB agar plates containing the suitable antibiotics, to obtain the intraluminal bacterial counts, these plates were incubated at 37°C for 16 hours. The number of bacteria per 15 nematodes was determined and the analysis was based on three independent repeats. To calculate the significance among the accumulation of WT and mutant strains in the gut of *C. elegans*, an unpaired student t-test was performed.

In-silico **analysis of YfiB protein.**

YfiB protein was present as a hypothetical protein in the *Shigella* genome, hence the 5 step identification system for a hypothetical protein was applied **[40]**. Functional analysis by identifying conserved domain was done using NCBI-protein BLAST **[41]** and conserved domain database **[42]**; followed by analysing its physicochemical parameter using Expasy's ProtParam tool **[43]**; determining the subcellular localization using CELLO [**44]**, PSORTb [**45]**, and PSLpred [**46]**; the presence of transmembrane helices using TMHMM [**47]** and HMMTOP [**48]** tools, and lastly to determine if they are concerned in virulence of *S. flexneri* using VICMpred [**49]** and VirulentPred [**50]**.

The protein sequence of *Shigella* Y394's YfiB was analysed and compared to *Pseudomonas aeruginosa*'s YfiB using NCBI-protein BLAST and Clustal-omega sequence alignment tool [**51**]. The similarity between the protein sequences and the conserved amino acid residues was evaluated. I-TASSER (Iterative Threading ASSEmbly Refinement) tool [**52,53]** was utilised to predict the three dimensional structure of YfiB protein of Y394, the already solved crystal structure of *Pseudomonas* YfiB protein [**19,20]** was used as a template to work out the 3D structure. Significant amino acids in YfiB protein involved in *Pseudomonas* virulence, known from previous studies [**19,20]** were identified and based on this analysis, targets for mutagenesis in Y394 YfiB protein were selected.

YfiB homologs were identified in various other gram-negative species, protein homologs (E-value<10^{-4}) were aligned using Clustal Omega, and conserved amino acid residues were determined. Sequence conservation at each position was evaluated using the WebLogo tool [**54]**, it creates a stack of symbols, the altitude of the pile at each position signifies the sequence conservation and the height of the symbols denotes the comparative incidence of individual amino acid at that locus [**54]**. Phylogenetic analysis was also carried out of the YfiB homologs and an illustrative tree based on the multiple sequence alignment of YfiB homolog protein sequences was generated using the MEGA software [**55]**, Maximum Composite Likelihood technique was used to determine the evolutionary distances [**56,57]**. UniProt accession number of all YfiB protein sequences used for the in-silico analysis have been listed in **Supplementary Table S3.**

Site-directed mutagenesis and protein expression

For the targeted mutagenesis experiment, four amino acid targets were selected, these being Cys19Gln20 mutated to Ala19Glu20; Pro22Gln23 mutated to Ala22Glu23; Glu29Gln30 mutated to Ala29Glu30, which are part of the YfiB peptidoglycan linker sequence, and Ser36

mutated to Ala36. Site directed mutants were synthesized by GenScript USA, cloned into the expression pBAD_Myc_HisA vector, using the *BamH*I and *Apa*I sites. For inspecting the expression of the YfiB protein, *Shigella* strains, containing the pBAD_Myc_HisA_YfiB vector were grown in LB broth medium having 100 µg/mL ampicillin and 50 µg/mL erythromycin at 37°C. Once the OD_{600} of the cultures reached 0.6, 1mL of the cell culture was pelleted down and suspended in an appropriate volume of protein loading dye containing β-mercaptoethanol as the reducing agent. This protein suspension was boiled at 100^0C for 5 minutes before loading into the wells of the SDS gel. Transfer and western blot were carried out with the whole-cell lysate using the anti-HisA [**58]** antibody to check for protein expression.

Results and Discussion

Biofilm formation is significantly slower in the *yfiB*-deleted *Shigella* strain

Structured groups of bacteria entrenched in a self-constructed matrix comprising of proteins, extracellular DNA, and majorly exopolysaccharides are known as biofilm [**7]**. Virulence genes associated with cellular invasion are interrelated to biofilm production in *S. flexneri* **[59]**, which eventually helps the pathogen to survive intracellularly and also evades the immune response of the host [**59]**. Enteric pathogens like *Shigella* produce biofilms to enhance survival and virulence due to the bile salts in the human GIT and bile being bactericidal [**7,59]**. We assessed whether the *Shigella* YfiB protein is involved in its production of biofilm, by knocking out the *yfiB* gene from *S. flexneri* 1c strain (SFL1613/Y394) and measured the extent of biofilm produced by the wild-type and mutant strain (SFL2641/ΔYfiB) at 6, 12, and 24 hours in presence and lack of 0.4% bile salts and 1% glucose. The *yfiB* gene was also complemented in the knockout strain (SFL2642/YfiBComp) by cloning the gene in the pBAD_Myc_HisA vector and introducing the plasmid in SFL 2641 strain, to see if the wildtype function is reinstated. As seen in **Figure 2A**, biofilm formation remained minimal in the lack of bile salts and glucose,

in comparison to a substantial increase of biofilm formation when bile salts and glucose is present. It was also observed that the capacity to produce biofilm was considerably slower in the *yfiB* deletion mutant (SFL2641/ΔYfiB) when compared to the WT (SFL1613/Y394) strain (**Figure 2B).** The biofilm produced by SFL2641 was considerably affected at 6 hours of incubation but showed no difference to the WT biofilm production at 12 and 24 hours. The complemented strain (SFL2642) showed similar amounts of biofilm production as WT and hence it could be concluded that complementing the *yfiB* gene in the mutant strain can reinstate the wildtype function (**Figure 2C**).

Even though biofilm production in bacteria is dependent on a multitude of cellular factors and coordinated pathways, this slow biofilm production in the *yfiB* deletion mutant can be explained by the functioning of *yfiBNR* operon model, previously studied in *P. aeruginosa* [**22]**. YfiB being a positive regulator of YfiN, which is a membrane-bound diguanylate cyclase (DGC), is accountable for the production of c-di-GMP [**21]**. Higher intracellular levels of c-di-GMP is responsible with an escalation of biofilm production and motility of bacteria [**17]**. In this case, when YfiB is absent, it cannot sequester YfiR to the outer membrane, which is the negative regulator of YfiN. Hence YfiN is always bound to YfiR, resulting in an inactivated state, and not being able to produce c-di-GMP needed for effective biofilm production in the presence of bile salts. Though in *E. coli*, it was observed that deleting genes encoding diguanylate cyclases such as *yfiN,* decreases c-di-GMP production, thereby showing an upsurge in motility and early biofilm production [**21]**. Motility affects biofilm formation as it has a functional role in preliminary surface attachment and to neighbouring bacterial cells [**21]**. Bacterial motility also affects the architecture of biofilms, as it is known that strains with reduced motility produce flat biofilms while strains with prominent motility form vertical biofilm structures [**14,21]**. The same kind of effect is not seen in *Shigella*, as they are non-motile bacteria, a decline in c-di-GMP directly decreases the biofilm formation. Experiments

in *E. coli* have proven that non-flagellated or bacterial cells with paralysed flagella show decreased biofilm production in lower concentration of c-di-GMP [**21]**, which explains the observed slow biofilm production in *Shigella-yfiB* deletion mutant.

***yfiB* deletion leads to a reduction in adhesion and invasion of BHK cells and displays an attenuated ability to form plaques in HeLa cells.**

The ability to infiltrate the colonic and rectal epithelium, proliferate intracellularly, and then spread cell to cell are the three critical features of virulent *S. flexneri* [**60]**, which leads to severe tissue damage in the host [**2]**. *Shigella* species primarily adhere to host, which is essential for consequent invasion and beginning an infection, how this is achieved, remains poorly understood [**39].** Virulence factor IcsA, a surface-displayed protein was recently discovered to play a crucial role in *Shigella*'s host cell adhesion, but other factors aiding in host cell adherence still need to be studied [**10]**. Various attachment and internalization mechanisms are required by *Shigella* for the invasion of mammalian cells, with the bacteria eventually residing in the host cell cytoplasm [**60].** After the invasion, the intercellular spread can happen in two ways- 1) lysis of infected cells and discharge of intracellular bacteria; 2) individual bacteria evading the initially infected cell before lysis and attacking the neighbouring cells without being exposed to the outside environment [**32].** Even in the second case, the initially infected cell is killed or lysed by the intracellular bacteria [**32].** This kind of interaction proposes that an individual *Shigella*-infected host cell would lead to an advanced infection of the surrounding cells and leave behind a region of dying or dead host cells, which is also known as a plaque [**32,33].** To determine the set of virulence genes responsible for the adhesion, invasion, and intercellular spread in host cells, cultured epithelial cell monolayers such as HeLa and BHK cell lines have been extensively used, which mimic the *in-vivo* infection model [**34]**.

To investigate the involvement of the *yfiB* gene and *yfiBNR* operon in the initial attachment or adherence of bacteria to the host cell, an *in-vitro* adhesion assay was carried out in which we infected BHK monolayers with the *Shigella* strains-WT (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB), and *yfiB* complement strain (SFL2642/YfiBComp). The percentage of adhered bacteria was computed by dividing the total colony forming unit (CFU) of adhered bacteria by the total CFU of the inoculum. The percentage of adhered bacteria of the WT strain (SFL1613/Y394) was seen to be four folds higher than the *yfiB* deletion mutant (SFL2641/ΔYfiB). Statistical analysis via an unpaired student t-test confirmed that the *yfiB* deletion mutant had a considerably decreased capacity to adhere to the epithelial cells of host, when paralleled with the wild-type (**Figure 3**). When the *yfiB* gene was complemented (SFL2642/YfiBComp), it was seen that the wild-type adherence ability was restored. This clearly shows the role of YfiB and YfiBNR system in *Shigella* virulence, as the very first step of its infection process is adherence to host cells.

Examining the consequence of deleting the *yfiB* gene and disrupting the *yfiBNR* operon on the invasive potential of *S. flexneri* serotype 1c, an *in-vitro* invasion assay was carried out in which BHK cells were infected with the strains- WT (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB), and *yfiB* complement strain (SFL2642/YfiBComp). The bacteria invading the BHK monolayers were stained and counted by microscopy, the average amount of bacteria invading per BHK cell was found to be 4-folds higher in wildtype when rivalled with the mutant strain (**Figure 4A**). It was additionally observed that the spread of bacteria to neighbouring cells was far less in the *yfiB* deletion mutant when paralleled to the wild type, demonstrating that the deletion has an effect on the intercellular spread of the infection. Statistical analysis confirmed that the *yfiB* deletion mutant had a considerably reduced capability to invade host epithelial cells when paralleled with the wild-type strain (**Figure 4 B-E**). When the *yfiB* gene was complemented, it was seen that the wild-type invasion ability was

restored, which clearly indicates the role of YfiB and YfiBNR system in the invasive potential *of S. flexneri* serotype 1c.

A plaque assay was also carried out using HeLa cells to understand the two vital steps involved in *Shigella* infection process, which are invasion and spreading of the bacteria [**33]**. The number of plaques evaluates the invasive ability of the bacteria and the size dimensions of the plaques reveals the bacteria's capability to spread within an epithelium [**33,34]**. We infected HeLa cells with *Shigella* strains- WT (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB), and *yfiB* complement strain (SFL2642/YfiBComp) for 48-72 hours. As seen from **Figure 5A**, which depicts the mean number of plaques produced by each strain, the number of plaques produced by the *yfiB* deletion mutant (SFL2641/ΔYfiB) was 2.5 times lesser than the wild-type strain (SFL1613/Y394). When the *yfiB* gene was complemented (SFL2642/YfiBComp), it was seen that the wild-type plaque-forming ability was restored. There is no substantial difference observed in the diameters of plaques formed between the strains or any other plaque characteristics (**Figure 5B**). Microscopy was also performed to observe the stained HeLa cells near and around the plaques under 40X (**Figure 5 C & D**). A low number of plaques seen in the *yfiB* deletion mutant indicates us about its inability to adhere to the host cells and disseminate from the initial site of infection, thereby showing significantly less intercellular spread, when rivalled with the wild type.

The consequence of *yfiB* deletion on *Shigella*'s ability of host cell adherence, invasion, and intercellular spread can be explained by the decreased intracellular levels of c-di-GMP and the disruption of the *yfiBNR* operon caused by the deletion of *yfiB* gene. The YfiBNR system regulates the c-di-GMP levels in the bacteria and as expected, absence of YfiB, leads to inactivating YfiN by it being permanently bound to the YfiR, causing decreased production of c-di-GMP which will eventually have an effect on the downstream virulence factors of *Shigella*. c-di-GMP signaling has been correlated with various virulence phenotypes like host

cell invasion, secretion of virulence factors, cytotoxicity, intracellular infection, host-cell adherence, cell motility, alteration of immune responses, and resistance to oxidative stress [**15,17]**. It also regulates intracellular mobility helping in cell to cell spread and regulates the type 3 secretion system in *Shigella*, which is needed to inject bacterial effectors into the host cells [**12,36]**. High concentration of c-di-GMP are known to have a progressive effect on the adhesion and invasion of the bacteria which eventually aids the pathogen in colonizing the host epithelial cells [**12]**. The relationship between biotic/abiotic surface attachment and c-di-GMP concentration has been inspected in the opportunistic human pathogen *P. aeruginosa*; it is known from these studies that on surface contact, there is an upsurge in levels of c-di-GMP, which induces flagella/pilli biosynthesis, surface adherence and boosts virulence in *Pseudomonas* [**13]**. This elucidates how deleting the *yfiB* gene and obstructing the proper functioning of the *yfiBNR* operon can severely affect the pathogenicity of *Shigella* by impeding its adhesion, invasion, and intercellular spreading capabilities.

yfiB deletion influences _C. elegans_ lifespan and survival during infection.

S. flexneri has a very limited host array in vertebrates, infecting just humans and primates. The highest frequently used mammalian *in-vivo* animal models for *Shigella* are the- murine pulmonary and guinea pig keratoconjunctivitis models [**39]**. However, these models lack clinical relevance of infection site and symptoms produced as compared with *S. flexneri* infection. Recently, *C. elegans*, a soil-dwelling nematode, is beginning to be extensively used to examine host-pathogen interaction for several enteric pathogens [**37,39]**. Its known that there are morphological resemblances between the intestinal cells of humans and *C. elegans* worms [**37-39]** and additionally, there are characteristic similarities in the innate immune system of human with that of *C.elegans* [**37]**. A variety of bacterial virulence determinants are required for pathogenesis in both nematode and mammals [**37]**, Burton et al. have previously revealed that infectious strains of *S. flexneri* get accumulated in the intestine of *C. elegans*, whereas the

avirulent strains get digested [**37]** . Thus, bacterial accumulation assay using *C. elegans* enabled us to inspect the functional role of *yfiB* gene and the *yfiBNR* operon system in the evasion of the nematode's defence system. Bacterial accumulation of pathogenic bacteria in the worm's intestine can drastically shorten the lifespan of worms and on the other hand, accumulation of non-pathogenic *E. coli* such as OP50, which is ingested as feed increases the lifespan of worms **[37-39]**.

A synchronized population of L4 larva stage hermaphrodite N2 nematodes was prepared and fed with wild-type (SFL1613/Y394), *yfiB* deletion mutant (SFL 2641/ΔYfiB), and the *yfiB* complemented strain (SFL2642/YfiBComp). Accumulation of the wild-type strain was around two-fold higher (average CFU/worm) than that of the mutant (average CFU/worm for SFL 2641/ΔYfiB). The average CFU/worm for the complemented strain (SFL2642/YfiBComp) was similar to that of the wildtype, indicating that the invading ability of the wild-type strain was restored. Statistical analysis confirmed that the accumulation in the *C. elegans* intestinal lumen by the wild-type strain was considerably higher when paralleled to the *yfiB* deletion mutant (**Figure 6**).

This experimental analysis validated that the positive regulator of c-di-GMP- *yfiB* gene, puts a significant impact on the intestinal accumulation of *S. flexneri*. Low accumulation of *yfiB* deletion mutant in the *C. elegans* intestinal lumen, indicates that it becomes less virulent when paralleled to the wild-type strain and eventually increases the lifespan and survival of worms when they are fed with the SFL2641/ΔYfiB strain. It is known that several bacterial species and non-virulent bacteria are unable to persistently colonize the nematode gut; are rapidly digested and expelled from the intestine. Earlier studies have discovered that c-di-GMP catabolizing enzymes have been known to promote intestinal gut colonization and intracellular survival of the pathogen. This result of a low bacterial accumulation of the *yfiB* deletion mutant in *C. elegans* gut sits consistent with the other observations like slow biofilm production;

reduced adherence and invasion; and decreased plaque numbers; when there is decreased c-di-GMP concentration inside the cells owing to the disruption of *yfiBNR* operon caused by the deletion of *yfiB* gene.

In-silico investigation of the YfiB protein elucidates its structure, function, and conserved nature across bacterial species.

YfiB was present as a hypothetical protein in the *Shigella* genome, *in-silico* functional analysis was carried out using NCBI-protein BLAST and the conserved domain database to find out homologs across other bacteria and conserved domains. Homologs across other gram-negative bacterial species were found, YfiB is a 17.2 KDa putative lipoprotein or an integral membrane protein, with an OmpA domain and Omp_C-like superfamily domain (**Figure 7A**). Functional prediction of YfiB as per the conserved domains showed that it might be involved in transmembrane transporter activity; cell growth and adherence; in response to stress and/or external encapsulating structure. Physiochemical properties, subcellular location, presence of transmembrane domains, and involvement in virulence were also studied, using various bioinformatics tools listed in the methods section. YfiB was predicted to be located in the outer membrane, comprising of a PAL-like peptidoglycan (PG) domain and no transmembrane helices. Consensus prediction of gene ontology (GO) showed that YfiB was involved in molecular and biological processes of the bacteria and the protein had a 0.271 of grand average of hydropathicity (GRAVY).

YfiB and YfiBNR system has been previously studied in *Pseudomonas* [**22]**, and protein crystal structures solved of all the three proteins involved in the operon [**18-20]**. When the YfiB protein sequence of *Shigella* was compared to that of *Pseudomonas* via NCBI nucleotide and protein BLAST, no similarity was found between the nucleotide or protein sequences as per the default BLAST settings. The protein sequence of YfiB from *Shigella* and *Pseudomonas* was aligned

using the ClustaW tool [**51**] to determine the conserved amino acid residues between the two proteins. It was observed that the crucial amino acids residues were conserved between the two proteins as seen in **Figure 7B**. The amino acids involved in PAL (peptidoglycan associate lipoprotein) domain which aid in proper binding to the peptidoglycan is conserved in both, these being Asparagine (N68), Aspartic acid (D102), and Glycine (G105) [**19,20]**. There is also the presence of an N-terminal, 13 amino acid long, linker sequence containing conserved critical residues. This linker sequence was predicted to be involved in binding to PAL domain, YfiB signaling, outer membrane sequesteration of YfiR, and affects biofilm formation and adhesion of bacteria [**19,20]**. An important amino acid cluster from position 35 to 55 is present in the YfiB protein which is involved in the activation of YfiB and plays a role in appropriate YfiR sequestration by YfiB in the periplasm [**19,20]**. Identifying these important amino acids helped in selecting the targets for the following site-directed mutagenesis experiments.

To determine the 3D structure of *Shigella* YfiB protein, the I-TASSER tool was used, which predicts protein structure and function based on structure, it identifies structural templates from the database by the multiple threading method known as LOMETS [**52]**. Functional understanding of the target sequence is then attained by re-threading the 3D models using BioLiP, which is a protein functional database [**52,53]**. To establish the structure of *Shigella* YfiB protein, I-TASSER utilised the solved crystal structure of YfiB protein from *Pseudomonas* [**19,20]**. It was found that the YfiB protein is composed of a core OmpA-like domain (27-160 amino acids), has an N-terminal signal peptide (1-26 amino acids), and consisted of α1-3 (three alpha helices) and a β1-β4-β2-β3 network (anti-parallel β-sheet topology) (**Figure 7C**). Its known from the solved YfiB crystal structure that residues 35–55 of the N-terminal are associated with membrane attachment and boosted YfiB activation. The YfiB sequestration of YfiR needs a comprehensive uncoiling of the N-terminal and the OmpA domain's β1 strand [**20]**.

Homologs of YfiB (E-value<10^{-4}) in other gram-negative bacteria were identified and selected for determining the sequence conservation at each position and phylogenetic analysis. Conservation of amino acids at each position of the protein was analysed using the WebLogo tool, all the YfiB homolog sequences were first aligned using ClustalW and then a sequence logo was created. **Figure 8A** shows the graphical representation of all amino acids, the altitude of the pile at each position indicates sequence conservation and the height of the characters characterises the comparative frequency of individual amino acid at that locus [**39]**. Using the ClustalW alignment of the homologs (**Supplementary Figure S1**), an illustrative phylogenetic tree was created using the MEGA software [**55]**, showing the evolutionary distance between the YfiB protein homologs from different gram-negative bacteria, calculated by the Maximum Composite Likelihood method (**Figure 8B**).

Mutational analysis of YfiB protein- Biofilm formation and Bacterial Adhesion Assays.

To determine whether some of the conserved amino acid residues of YfiB are involved in the proper functioning of the protein, targeted mutagenesis was carried out. The targets were selected based on previous studies in *Pseudomonas* YfiBNR proteins [**18-20]**. It was found that the 13 amino acids long linker sequence was the most critical for YfiB functioning, as it helps in binding to the PAL domain, YfiB signaling, and sequestering the YfiR protein. In *Shigella*, the linker sequence is only 11 amino acids long, with the majority of amino acids being conserved. Three pairs of conserved amino acid residues from the linker sequence were selected as targets, these being Cys19Gln20 mutated to Ala19Glu20; Pro22Gln23 mutated to Ala22Glu23; Glu29Gln30 mutated to Ala29Glu30 (**Figure 9A**). Another conserved amino acid serine at position 36, which is part of an important amino acid cluster (35-55) involved in the activation and functioning of YfiB, was selected as a target and was mutated to alanine (**Figure 9B**). Mutants were cloned into pBAD_Myc_HisA plasmid and the protein expression of these mutants was also confirmed using western blot via an anti-HisA antibody to check if the amino

acid mutations prevented the YfiB protein to be expressed properly (**Figure 9A & 9B**). To examine the function of these mutants, biofilm formation assay and bacterial adhesion assay were chosen as the functional tests to see if the amino acid mutation caused any changes in YfiB activity and to the overall surface attachment and biofilm formation of the bacteria. These assays were performed, as explained in the methods section.

Biofilm formation assay was carried with the wild-type strain (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and the *yfiB* complemented strain (SFL2642/YfiBComp); along with the YfiB site-directed mutant strains- SFL2645 (Cys19Gln20->Ala19Glu20), SFL2646 (Pro22Gln23->Ala22Glu23), SFL2647 (Glu29Gln30->Ala29Glu30), and SFL2648 (Ser36->Ala36). The empty pBAD_Myc_HisA vector strain SFL2650 (SFL2641/ΔYfiB strain containing the empty pBAD_Myc_HisA plasmid) used as the negative control. The assay was performed at 6, 12, and 24 hours and it was observed that there was a noteworthy difference in between the biofilm formation of the wild-type YfiB protein and the functional mutants of YfiB (**Figure 9C**). SFL2645 (Cys19Gln20->Ala19Glu20) mutant showed a decrease in biofilm at all three time points, whereas the SFL2646 (Pro22Gln23->Ala22Glu23) and SFL2647 (Glu29Gln30->Ala29Glu30) mutants only showed a decrease in observed biofilm at 6 and 12 hours. The mutant SFL2648 (Ser36->Ala36) showed only a slight decrease when compared to the wildtype YfiB strain, but the difference wasn't statistically significant (**Figure 9C**).

Bacterial adhesion assay was carried out using the BHK cell line, as described in the methods. We infected the BHK monolayers with the strains SFL1613/Y394 (WT), SFL2641/ΔYfiB, and SFL2642/YfiBComp; along with the YfiB site-directed mutant strains- SFL2645 (Cys19Gln20->Ala19Glu20), SFL2646 (Pro22Gln23->Ala22Glu23), SFL2647 (Glu29Gln30->Ala29Glu30), and SFL2648 (Ser36->Ala36), and the empty pBAD_Myc_HisA vector strain (SFL2650). It was observed that the YfiB site-directed mutants showed a significantly reduced capability to adhere to the epithelial cells of the host when compared with the wild-type YfiB

protein. Mutants SFL2645 (Cys19Gln20->Ala19Glu20), SFL2647 (Glu29Gln30->Ala29Glu30) and SFL2648 (Ser36->Ala36) showed a significant reduction in the percentage adhesion to BHK cells. Whereas mutant SFL2646 (Pro22Gln23->Ala22Glu23), showed a slight decrease when compared to the wildtype but the difference wasn't statistically significant (**Figure 9D**).

It can be concluded from these functional assays that the conserved amino acid pairs in the linker sequence (Cys19Gln20, Pro22Gln23, and Glu29Gln30) are crucial for the activation and proper functioning of the YfiB protein. The linker sequence acts as a lipid anchor and helps in peptidoglycan binding, which is critical for the full activity of the YfiB [**19]**. Anchoring of YfiB in the cell wall and outer membrane aids in proper signaling and accurate YfiR sequestration in the inner membrane, which eventually activates the YfiN and a higher concentration of c-di-GMP levels [**19]**. This c-di-GMP becomes a significant downstream signaling particle for all virulence-related progressions in bacteria such as formation of biofilm, attachment, and invasion of the host cells to name a few [**12]**. The serine at position 36 was also found to be conserved in most YfiB homologs and had the highest frequency of being present at this position when compared to other similar amino acids (**Figure 8A**). This was mutated to alanine to test its function (SFL 2648/Ser36->Ala36), it was observed that it doesn't exhibit any difference in the biofilm production when equated to the wild-type YfiB, but demonstrates a significant decrease in the percentage adhesion to BHK cells. Serine at position 36 is part of an important amino acid cluster of YfiB (35-55) which is involved in the activation of YfiB protein and proper YfiR sequestration [**20]**. We assume that mutating the serine 36 residue, causes improper activation of YfiB or results in an inappropriate sequestering of YfiR, leading to low intracellular c-di-GMP levels, hence causing the decline in adhesion capabilities of the bacteria [**20]**. This functional analysis proves that YfiN is under positive and negative control of YfiB and YfiR respectively and together this YfiBNR system regulates the

intracellular quantities of c-di-GMP, which aids in various virulence mechanisms of the bacteria.

Conclusions.

In this work, we remarkably show that altering intracellular c-di-GMP levels can severely affect the pathogenesis of *Shigella flexneri*. This study suggests that loss of outer-membrane YfiB, hinders the function of the inner membrane-bound YfiN (DGC activity), as the periplasmic YfiR is always bound to it, which causes a decreased concentration of c-di-GMP. Loss of YfiB and the apparent decrease in intracellular c-di-GMP levels, subsequently affects other downstream virulence factors of the bacteria, as seen as slower biofilm production; decreased adhesion, and invasion of host cells; weakened ability to form plaques; and lower accumulation in guts of *C. elegans*, which leads to a surge in the life span of the worms. From this work, it can be established that the YfiBNR regulatory system is involved *in S. flexneri*'s *in-vivo* persistence, biofilm formation, and its infection cycle. *Shigella* causes about 91 million infections worldwide each year, predominantly in developing and under-developed countries. It's also becoming a major public health concern as treatment alternatives are decreasing due to growing resistance to crucial antimicrobials and an absence of any licensed vaccine. Hence it becomes extremely important to understand every aspect of the *Shigella* infection model, to develop newer drug targets and possible vaccine candidates.

c-di-GMP is a significant player in bacterial virulence as it promotes prolonged survival of the pathogen and therefore c-di-GMP regulation in the cells is important to control and cure the infection. Homologs of YfiBNR exist in a majority of other gram negatives and this regulatory system can be used as an effective drug target in treating these bacterial infections. Drugs that can affect the functioning of YfiB or YfiN directly can be used effectively to control the spread and decrease the span of bacterial infections. This also decreases our dependency on

antimicrobials to treat infections and the risk of generating multidrug-resistant strains. This work provides a start point for additional mechanistic and functional research of the YfiBNR signaling system in *Shigella*, furthermore inspecting the prospect of targeting this signaling network, for discovering new therapeutics for bacterial infections like shigellosis or the ones caused by other bacterial pathogens.

Disclosure Statement

The authors declare no competing interest.

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Contributions-

T.S. - designed and conducted the experiments; analysed and interpretation of data; prepared figures and wrote the manuscript. N.K.V.-conceived and directed the study and critically revised the manuscript. Both the authors read and approved the final manuscript.

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Figure legends

Figure 1- The organization and interaction model of the YfiBNR system. YfiN, an inner-membrane-located DGC is repressed when bound to periplasmic YfiR. While dissociation of the complex by YfiB, which is an Pal-like protein located in the outer-membrane, sequestering YfiR and consequently stimulates YfiN and leads to the production of c-di-GMP.

Figure 2- Quantification analysis of biofilm formation by *S. flexneri* strains. (A) Examination of biofilm formation in plate wells when grown with and without bile salts in TSB media at 24 hours. Biofilm formation was performed in a 96 well plate, the wild-type *S. flexneri* serotype 1c (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) were cultured in tryptone soy broth (TSB) medium with and without bile salts and glucose for 6, 12 and 24 hours static. Observed biofilm was stained using crystal violet and assessed by determining the optical density at 595 nm (OD_{595}). **(B)** Biofilm formation of WT (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) at 6, 12, and 24 hours. **(C)** Biofilm formation of WT (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) at 6 hours, at which considerable difference was seen between the WT strain and *yfiB* deletion mutant. The Y-axis represents the biofilm amount relative to the blank media. A Student's t-test was applied for pairwise comparison of the biofilm formation and the analysis was based on three independent biological repeats with each assay having at least four technical repeats. The asterisks represent the variation observed is statistically significant ($p<0.05$) and error bars depict the SD of data based on the three independent experiments.

Figure 3- Graphical representation of percentage adhesion to BHK cells by *S. flexneri* strains. The wild-type *S. flexneri* serotype 1c (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) were used to infect the BHK cells. After infection, BHK cells were washed and lysed; lysates were serially diluted before plating on an LB agar plate for enumeration and CFU calculation. The percentage of adhered bacteria was calculated by dividing the total colony forming unit (CFU) of adhered bacteria by the total CFU of the inoculum. The Y-axis represents the percentage of adhered bacteria to BHK cells after 2 hours of post-infection at 37°C/ 5%

CO2. The results were obtained from six autonomous experiments and to analyse the statistical differences in the adhesion, a student's t-test was performed. The asterisks represent that the variance observed were statistically significant ($p<0.05$). SD of data from the six autonomous experiments is depicted by the error bars.

Figure 4- Invasion of BHK cell monolayers by *S. flexneri* strains. **(A)** The wild-type *S. flexneri* serotype 1c (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) were used to infect the BHK cells. The Y-axis represents the number of internalized bacteria per BHK cell after 2 hours of post-infection at 37°C/ 5% CO2. The results were obtained from three independent experiments and were derived from scoring at least 300 BHK cells. To analyse the statistical differences in the invasion, a student's t-test was performed. Asterisks represent that the variance observed were statistically significant ($p<0.05$) and the SD of data from three autonomous experiments is depicted by the error bars. Microscopic image of infected BHK cells, after 2 hours of post-infection at 37°C and at 5% CO_2, Giemsa stain was used to stain the monolayers, and the plates were observed under the microscope. **(B)** Microscopic image of non-infected BHK cells under 100X oil immersion. **(C)** Microscopic image of BHK cells infected with the wild-type strain (SFL1613/Y394) under 100X oil immersion**. (D)** Microscopic image of BHK cells infected with *yfiB* deletion mutant (SFL2641/ΔYfiB) under 100X oil immersion. **(E)** Microscopic image of BHK cells infected with *yfiB* complemented strain (SFL2642/YfiBComp) under 100X oil immersion.

Figure 5- Plaque formation in HeLa cells by *S. flexneri* strains. (A) Boxplot showing the mean number of plaques formed by each strain- wild-type (SFL1613/Y394), its isogenic *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp). Plaque assay was performed in 6-well plates by infecting confluent HeLa cell monolayers with *Shigella* strains. Infection was carried out for 48-72 hours, post-infection, the monolayer was washed and stained with Giemsa, resulting in clear plaques that are visible against the coloured background. The number of plaques formed were counted for each strain and then plotted. The results were obtained from 50 independent technical repeats with two biological repeats each time. To analyse the statistical variances in plaque formation between the strains, a student's t-test was performed and statistically significant ($p<0.05$) variations are

denoted by the asterisks. **(B)** Visual assessment of the plaques formed on the cellular monolayer at 72 hours post-infection, clear plaques representing dead cells in a well of 6-well plate with HeLa monolayer stained with Giemsa. **(C)** Microscopic view of an uninfected monolayer of HeLa cells stained with Giemsa, under 40X magnification. **(D)** Microscopic view of *Shigella* infected monolayer of HeLa cells stained with Giemsa, under 20X magnification, arrows point to an entire individual plaque.

Figure 6- Quantification of bacterial accumulation in *C. elegans.* The synchronized L4 stage N2 nematodes were fed with wild-type (SFL1613/Y394), *yfiB* deletion mutant (SFL2641/ΔYfiB) and *yfiB* complemented strain (SFL2642/YfiBComp) for 24 hours at 22°C. From each plate, 15 worms were picked; lysed utilising glass beads, after which suitable dilutions of individual lysate were plated on LB agar plates to attain bacterial accumulation numbers. To analyse the variance in accumulation seen between the strains, a students t-test was performed. Wild-type strain (SFL1613/Y394) and *yfiB* complemented strain (SFL2642/YfiB Comp) showed higher accumulation in *C. elegans*, when compared to *yfiB* deleted mutant (SFL2641/ΔYfiB) strain. Statistically significant (p. value < 0.05) variance is indicated by the asterisks and the analysis was based on three independent repeats.

Figure 7- Results for conserved domain analysis; predicted protein structure by I-TASSER and ClustalW alignment of YfiB from *Pseudomona*s and *Shigella*. (A) Conserved domains found in the YfiB protein, analysed using NCBI-protein BLAST and conserved domain database(CDD). **(B)** ClustalW protein sequence alignment of YfiB protein from *Pseudomonas and Shigella*, showing highly/weakly similar and dissimilar amino acids; it also highlights the conserved essential domains. *Blue- conserved linker sequence; Purple- 35-55 amino acid cluster important for activating YfiB; Yellow - PAL Domain associated amino acids. **(C)** The 3D protein structure predicted for YfiB, computed by the I-TASSER tool, using *Pseudomonas* YfiB solved crystal structure as a template; the 3D structure consists of Core OmpA-like domain, four-stranded anti-parallel β-sheet with topology β1-β4-β2-β3 and helices α1-3

Figure 8 - *In-silico* investigation of the YfiB protein. (A) Weblogo representations of the YfiB protein amino acid conservation. The height of amino acid code at each position reflects the comparative

incidence of the amino acid at that position and the total height of the pile denotes the degree of conservation at each individual locus (measured in bits). The consensus sequence was derived from multiple sequence alignment of YfiB protein sequences from various gram-negative bacteria.. **(B)** Phylogenetic tree illustrating the evolutionary distance between the YfiB protein homologs from various gram-negative bacteria. ClustalW alignment of the homologs was used to generate this phylogenetic tree and was created using the MEGA software, calculated by the Maximum Composite Likelihood method.

Figure 9- Analysing the effect of YfiB linker mutants and Serine36 mutant on YfiB activation and function. (A) The sequence of the wildtype N-terminal linker and the three site-directed linker mutants, the amino acid pairs mutated are highlighted in red. It also shows the immunoblot of the wild-type and mutant YfiB proteins in whole-cell lysate carried out with anti-HisA antibody. **(B)** The sequence of the wildtype YfiB activating domain with serine at position 36 and the mutant sequence with alanine at position 36, highlighted in red. It also shows the immunoblot of the wild-type and mutant YfiB proteins in whole-cell lysate carried out with anti-HisA antibody. **(C)** The consequence of various YfiB linker mutants and Ser36 mutant cloned in pBAD_Myc_HisA vector on biofilm formation, shown relative to the strain SFL2641/ΔYfiB containing the empty pBAD_Myc_HisA vector (SFL2650), used as control. The variance seen in the biofilm production between the strains was statistically evaluated using a student's t-test. SFL2645 (Cys19Gln20->Ala19Glu20) mutant showed a decrease in biofilm at all three time points, whereas the SFL2646 (Pro22Gln23->Ala22Glu23) and SFL2647 (Glu29Gln30->Ala29Glu30) mutants only showed a decrease in observed biofilm at 6 and 12 hours. SFL2648 (Ser36->Ala36) showed only a slight decrease in biofilm formation when compared to the wildtype YfiB, but the difference wasn't statistically significant. **(D)** The consequence of various YfiB linker mutants and Ser36 mutant cloned in pBAD_Myc_HisA vector on adherence to BHK cells, shown relative to the empty pBAD_Myc_HisA vector strain (SFL2650), used as control. To compute the variance seen in the percentage adhesion between the strains, a student's t-test was performed. Mutants SFL2645 (Cys19Gln20->Ala19Glu20), SFL2647 (Glu29Gln30-> Ala29Glu30) and SFL2648 (Ser36->Ala36) showed a significant reduction in the percentage adhesion to BHK cells. Whereas mutant SFL2646

(Pro22Gln23->Ala22Glu23), showed a slight decrease when compared to the wildtype but the difference wasn't substantial. Statistically significant (p. value < 0.05) variance is denoted by asterisks and the analysis was based on three independent repeats.

Figure 1

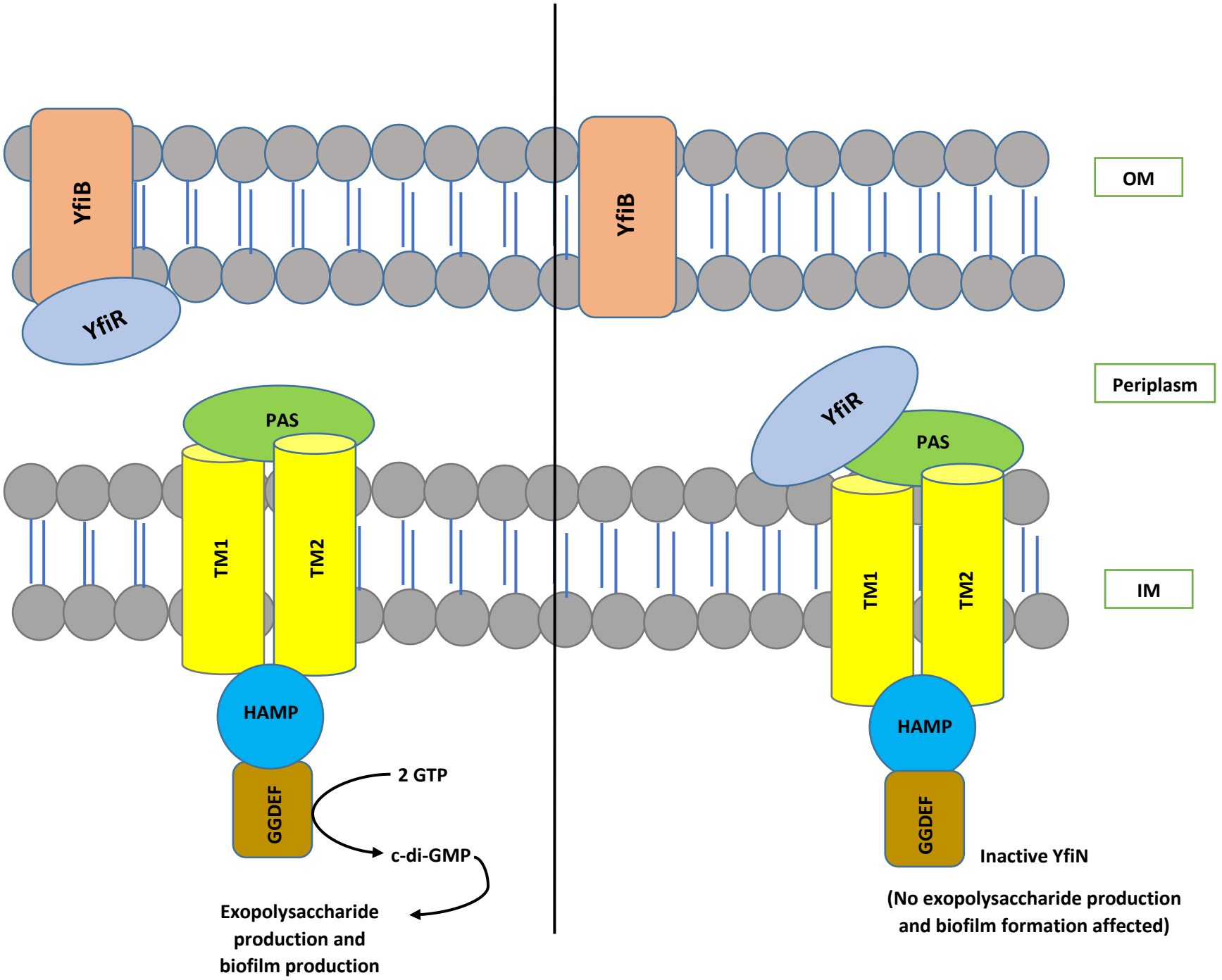

YfiB
YfiR
YfiB
OM
Periplasm
PAS
TM1
TM2
HAMP
GGDEF
2 GTP
c-di-GMP
Exopolysaccharide production and biofilm production
YfiR
PAS
TM1
TM2
IM
HAMP
GGDEF
Inactive YfiN
(No exopolysaccharide production and biofilm formation affected)

Figure 2

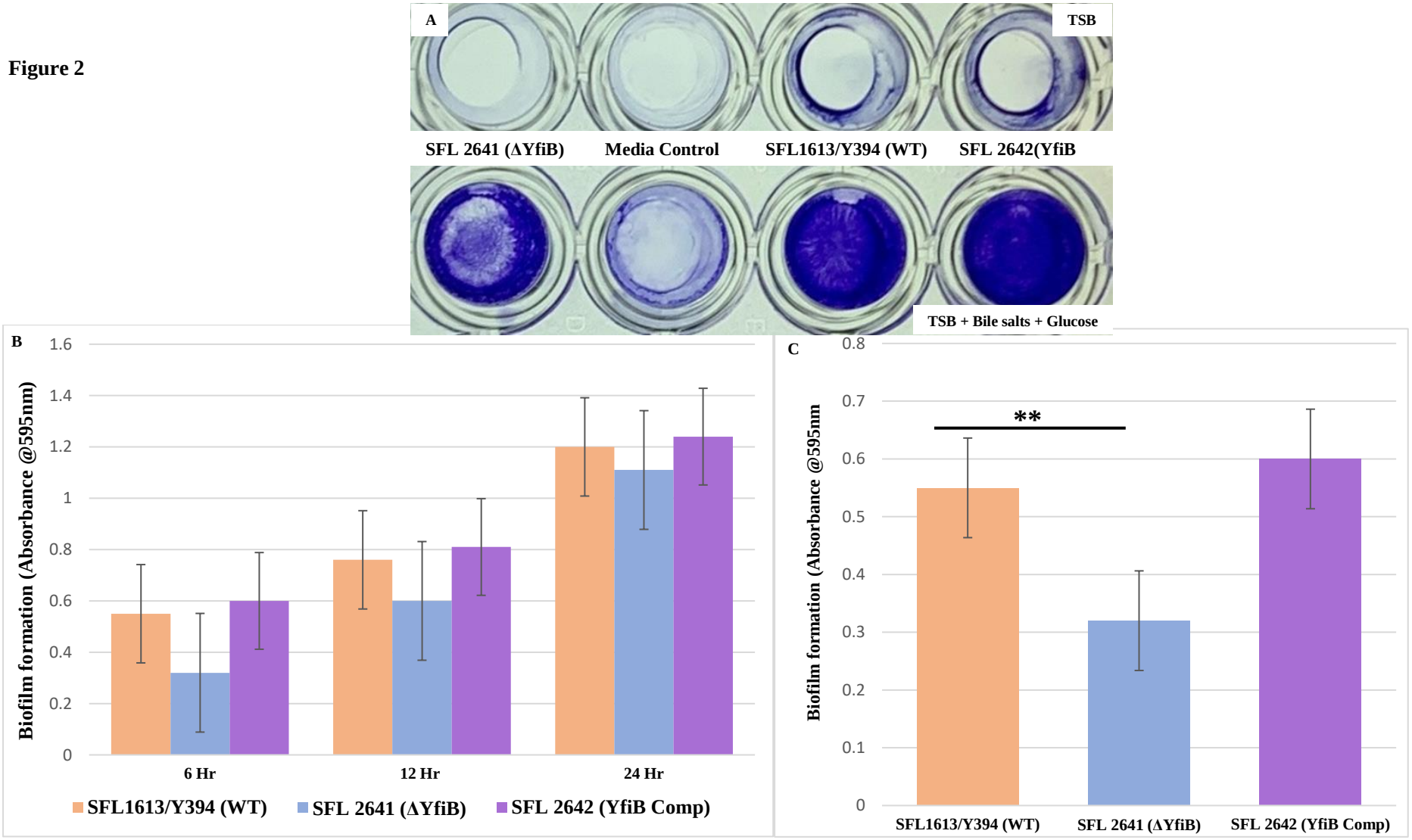

A
TSB
SFL 2641 (ΔYfiB)
Media Control
SFL1613/Y394 (WT)
SFL 2642(YfiB
TSB + Bile salts + Glucose
B
Biofilm formation (Absorbance @595nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
6 Hr
12 Hr
24 Hr
SFL1613/Y394 (WT)
SFL 2641 (ΔYfiB)
SFL 2642 (YfiB Comp)
C
Biofilm formation (Absorbance @595nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
SFL1613/Y394 (WT)
SFL 2641 (ΔYfiB)
SFL 2642 (YfiB Comp)

Figure 3

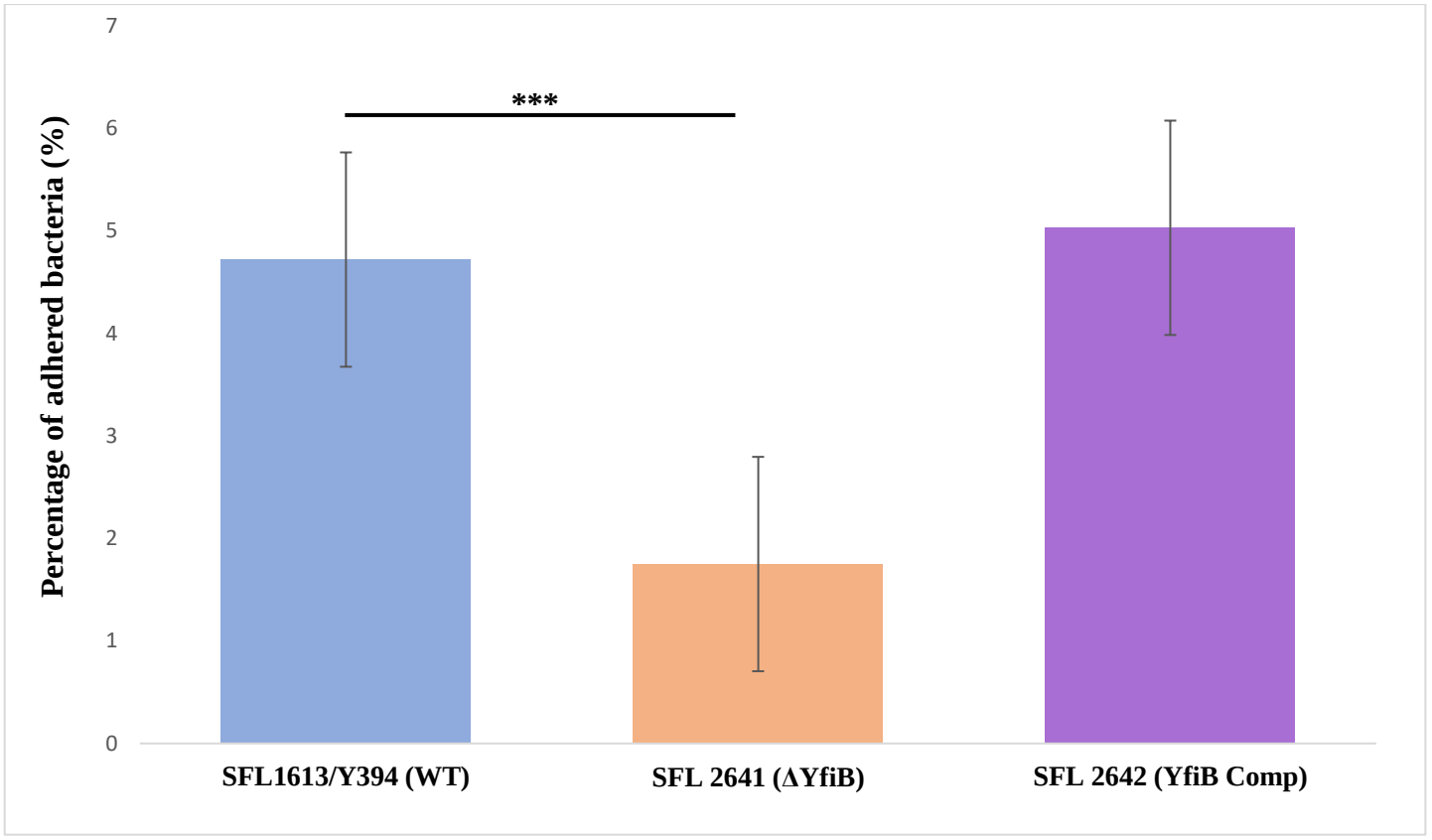

Percentage of adhered bacteria (%)
7
6
5
4
3
2
1
0
***
SFL1613/Y394 (WT)
SFL 2641 (ΔYfiB)
SFL 2642 (YfiB Comp)

Figure 4

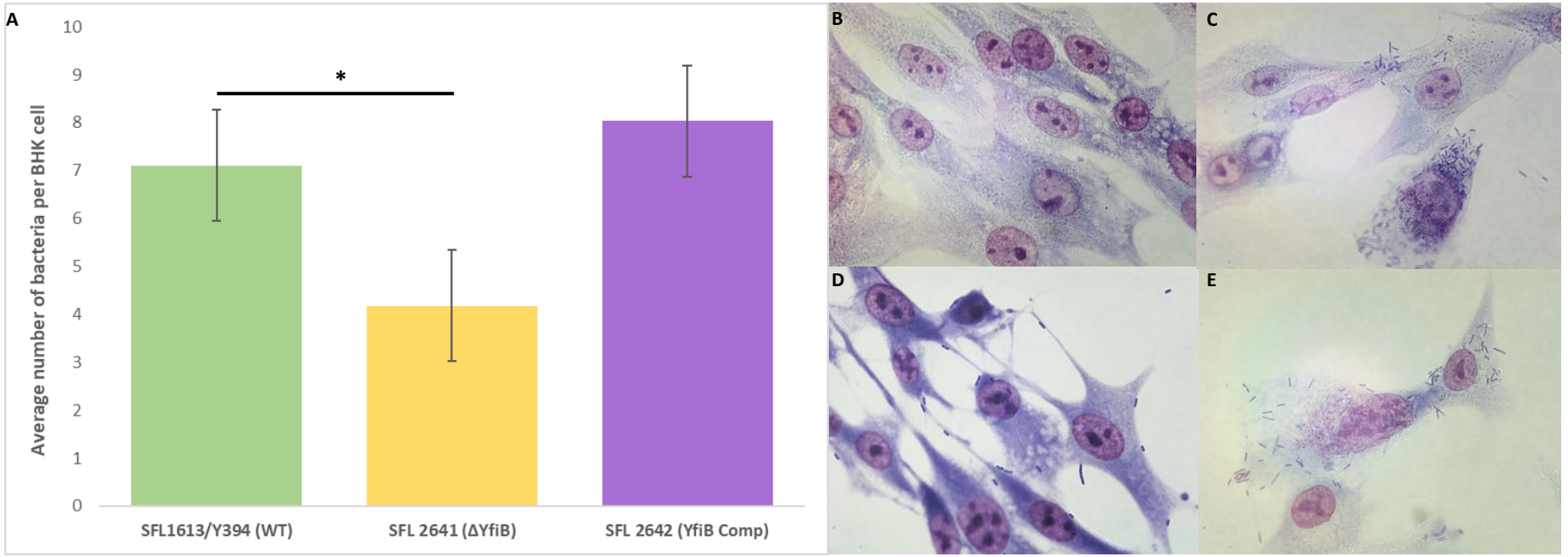

A
10
9
8
7
6
5
4
3
2
1
0
Average number of bacteria per BHK cell
*
SFL1613/Y394 (WT)
SFL 2641 (ΔYfiB)
SFL 2642 (YfiB Comp)
B
C
D
E

Figure 5

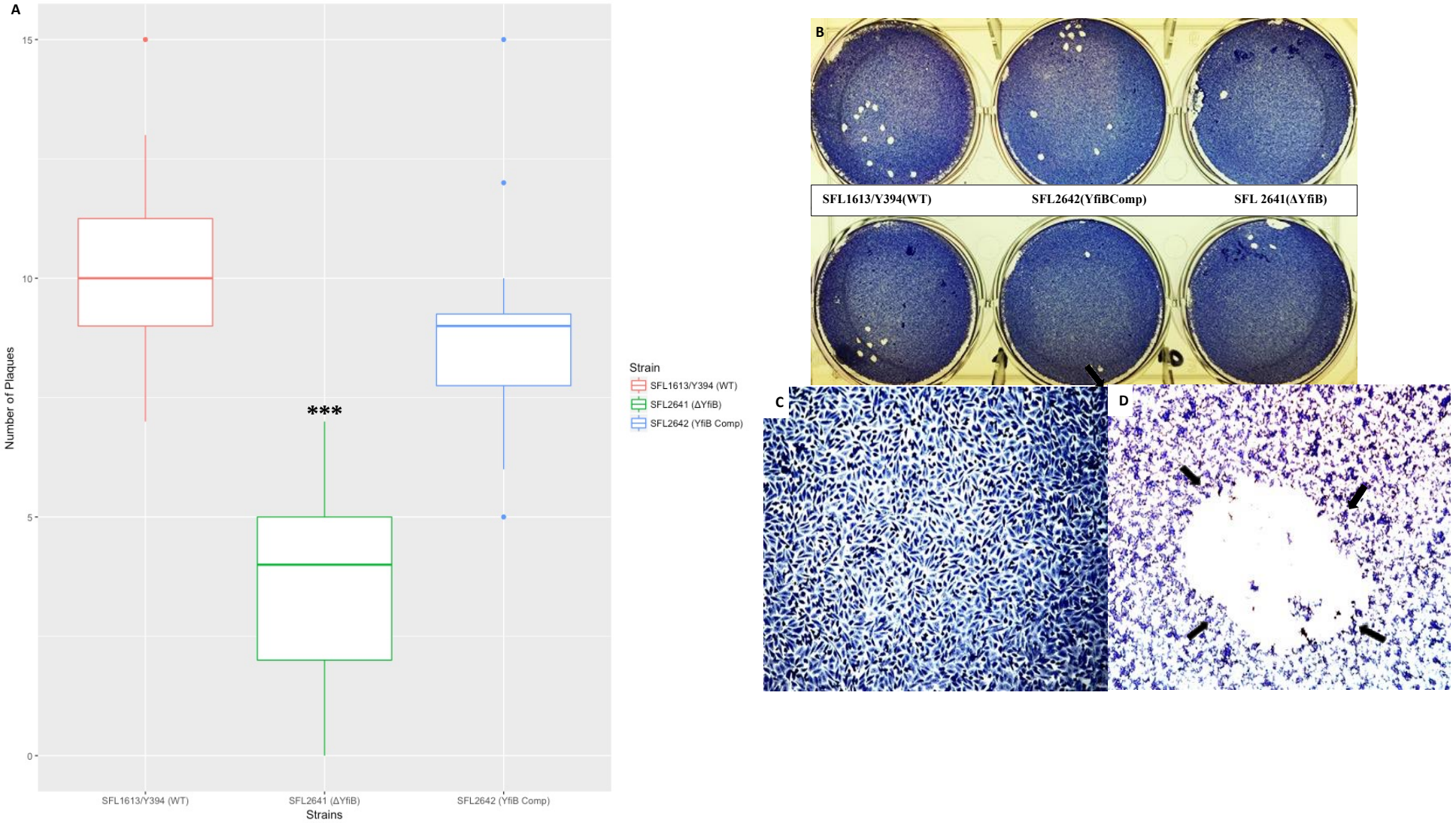

A
15
10
5
0
Number of Plaques
***
SFL1613/Y394 (WT)
SFL2641 (ΔYfiB)
SFL2642 (YfiB Comp)
Strains
Strain
SFL1613/Y394 (WT)
SFL2641 (ΔYfiB)
SFL2642 (YfiB Comp)
B
SFL1613/Y394(WT)
SFL2642(YfiBComp)
SFL 2641(ΔYfiB)
C
D

Figure 6

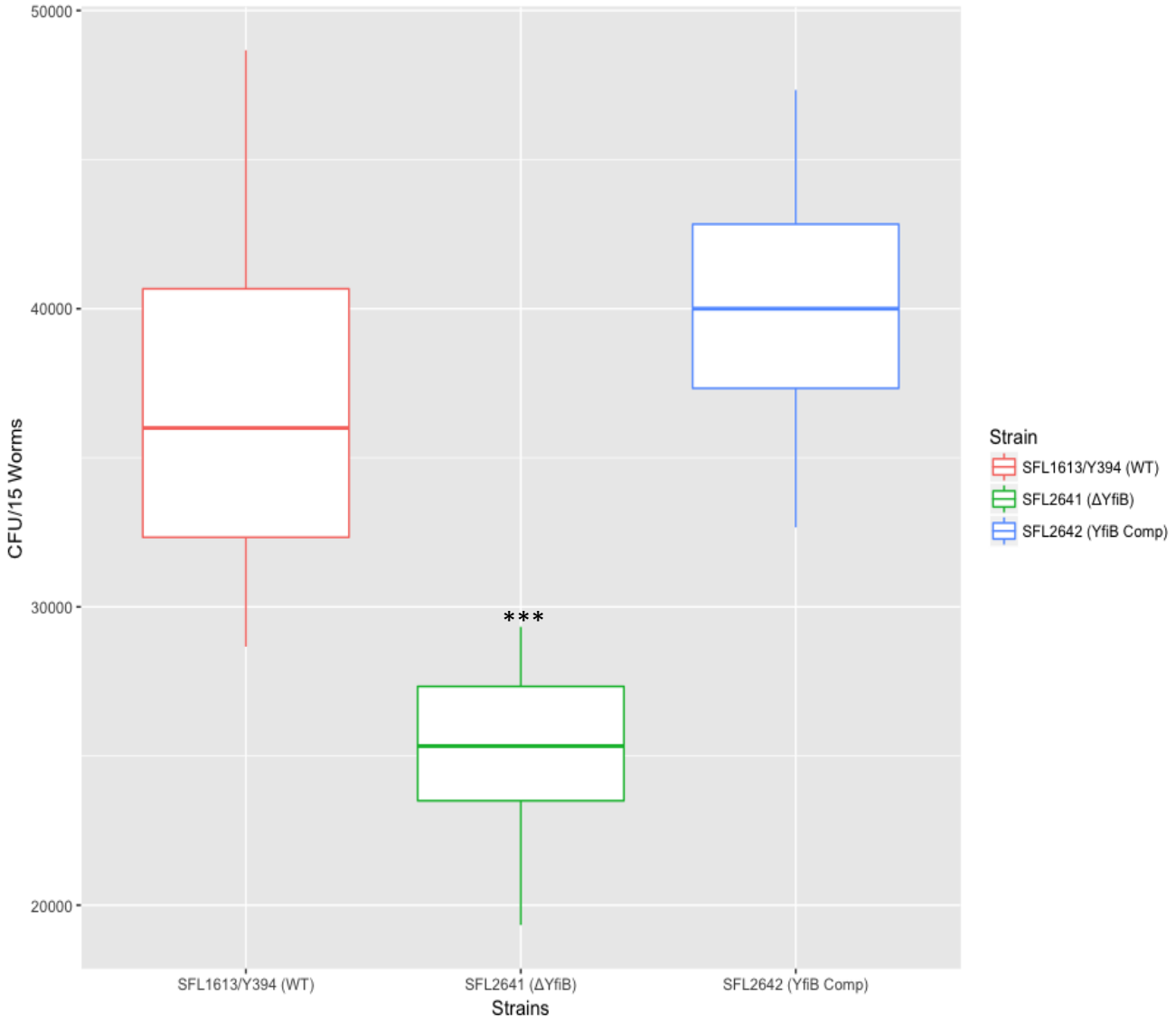
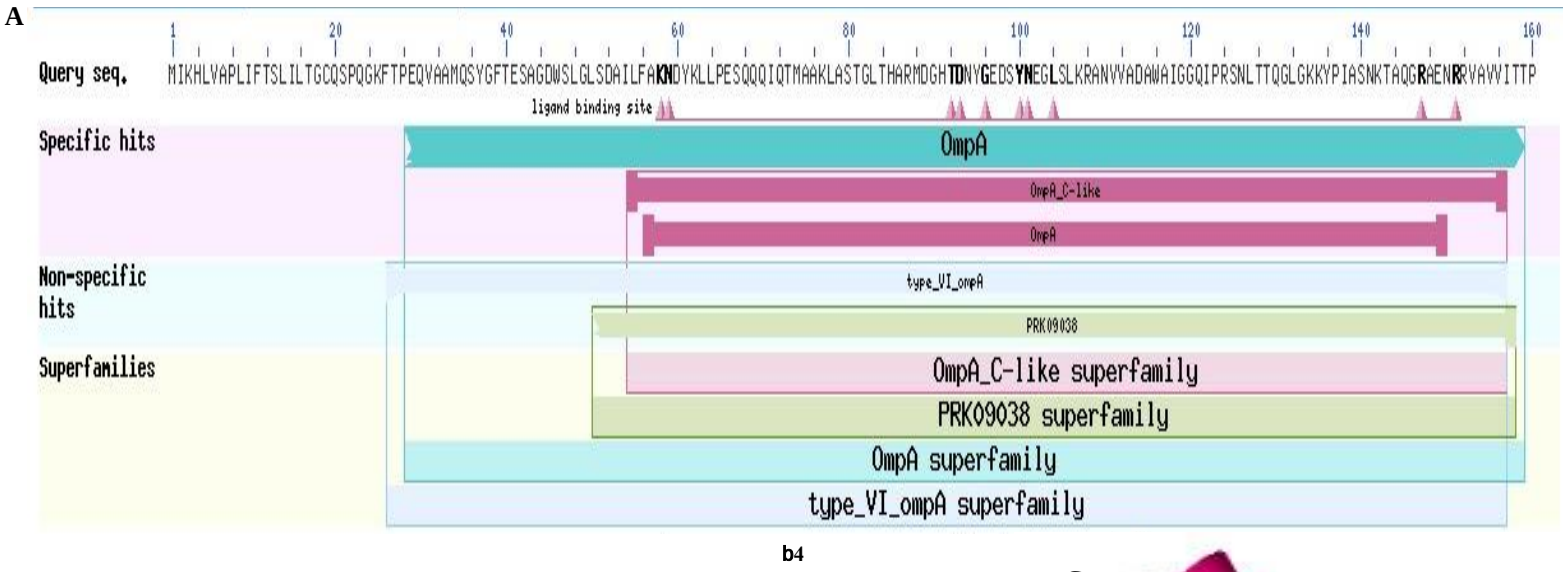

50000
40000
30000
20000
CFU/15 Worms
***
SFL1613/Y394 (WT)
SFL2641 (ΔYfiB)
SFL2642 (YfiB Comp)
Strains
Strain
SFL1613/Y394 (WT)
SFL2641 (ΔYfiB)
SFL2642 (YfiB Comp)

Figure 7



B

```
YfiB_Pseudomonas        MLPQRLHPSRLLALALFSLVLGLAGCQTKPPQTGLSAEQIAVLQEQGFELRDEGWEFGMS 60
YfiB_Shigella           MIKH-------LVAPLIFTSLILTGCQ--SPQGKFTPEQVAAMQSYGFTESAGDWSLGLS 51
                        *: :       *.  *:   * *:***   **  :: **:*.:*. **     .*.:*:*

YfiB_Pseudomonas        SKVLFGNNLDRLNPDSRNTLTKIARALLAVDIDKVRLEGHTDNYGDEGYNQKLSERRAES 120
YfiB_Shigella           DAILFAKNDYKLLPESQQQIQTMAAKLASTGLTHARMDGHTDNYGEDSYNEGLSLKRANV 111
                        . :**.:*  :* *:*:: : .:*  * :..: :.*::*******::.**: ** :**:

YfiB Pseudomonas        VAAVFREA-GMPAANIEVRGLGMSKPVADNKTRAGRSENRRVAIIVPAE   168
YfiB_Shigella           VADAWAIGGQIPRSNLTTQGLGKKYPIASNKTAQGRAENRRVAVVITTP   160
                        ** .:  .  :* :*: .:*** . *:*.***  **:******::: :
```

C

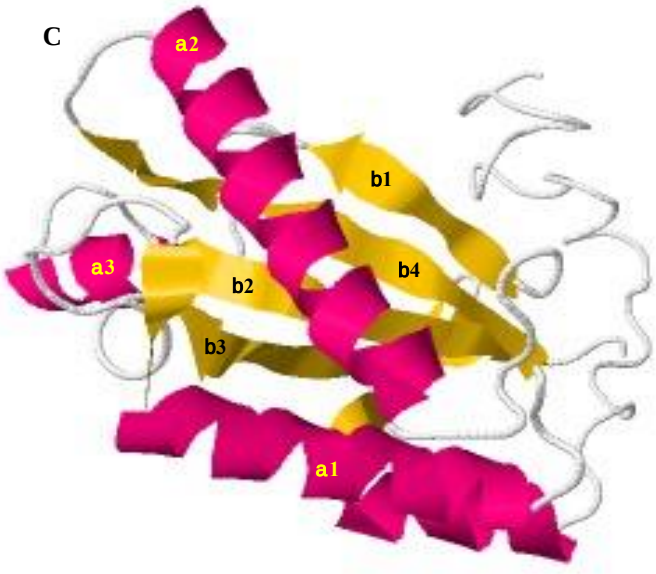


Figure 8A

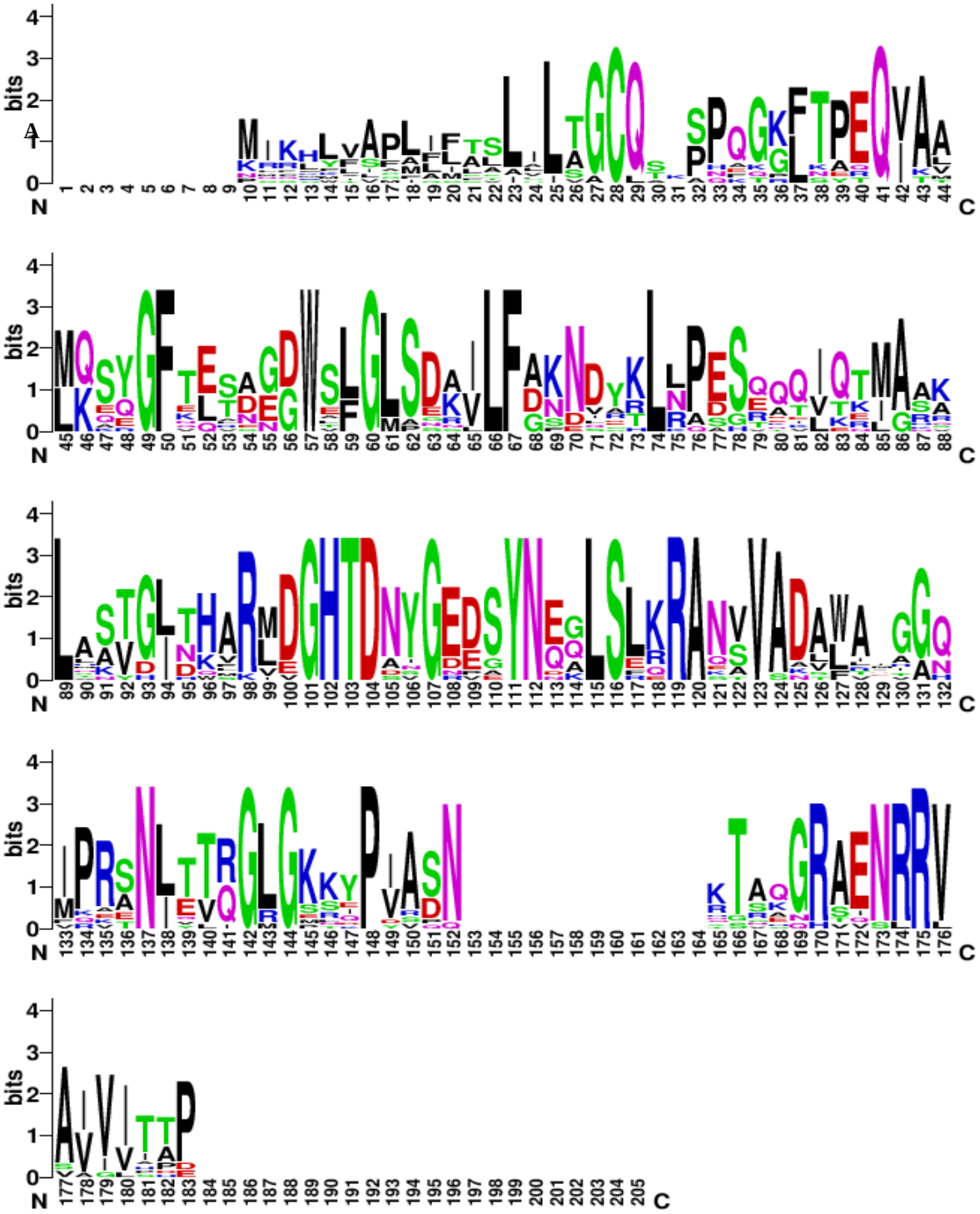
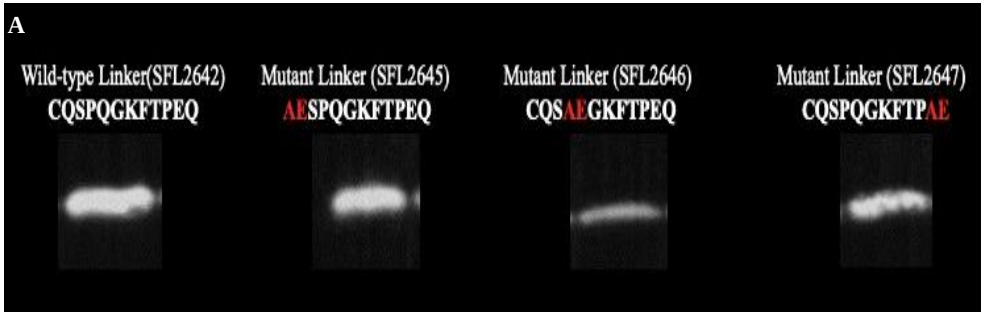


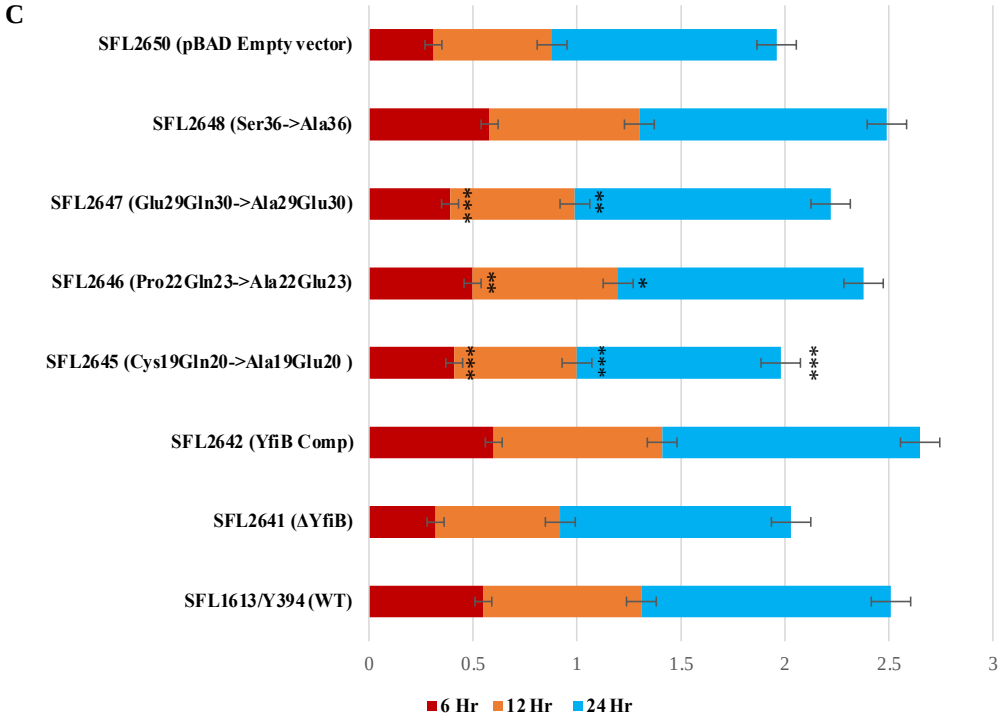
Figure 8B

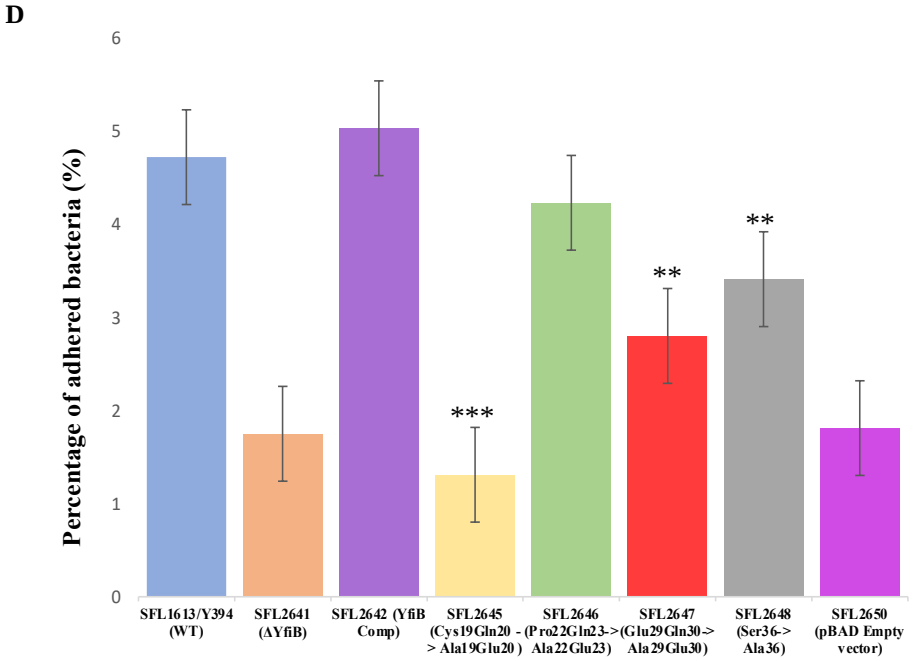

YfiB Yersinia enterocolitica
YfiB Acinetobacter baumannii
YfiB Pseudomonas aeruginosa
YfiB Pseudomonas chlororaphis
YfiB Serratia
YfiB Aeromonas
YfiB Xenorhabdus
YfiB Enterobacter
YfiB Klebsiella pneumoniae
YfiB Citrobacter
YfiB E.coli
YfiB S.dysenteriae
YfiB S.boydii
YfiB S.flexneri X
YfiB SFL1613/Y394
YfiB S.sonnei

Figure 9


A
Wild-type Linker(SFL2642)
CQSPQGKFTPEQ
Mutant Linker (SFL2645)
AESPQGKFTPEQ
Mutant Linker (SFL2646)
CQSAEGKFTPEQ
Mutant Linker (SFL2647)
CQSPQGKFTPAE


B
Wild-type conserved YfiB activating domain(SFL2642)
MQSYGFTESAGDW
Mutant YfiB activating domain (SFL26480)
MQAYGFTESAGDW


C
SFL2650 (pBAD Empty vector)
SFL2648 (Ser36->Ala36)
SFL2647 (Glu29Gln30->Ala29Glu30)
SFL2646 (Pro22Gln23->Ala22Glu23)
SFL2645 (Cys19Gln20->Ala19Glu20)
SFL2642 (YfiB Comp)
SFL2641 (ΔYfiB)
SFL1613/Y394 (WT)
0
0.5
1
1.5
2
2.5
3
6 Hr
12 Hr
24 Hr


D
Percentage of adhered bacteria (%)
0
1
2
3
4
5
6
SFL1613/Y394 (WT)
SFL2641 (ΔYfiB)
SFL2642 (YfiB Comp)
SFL2645 (Cys19Gln20 -> Ala19Glu20)
SFL2646 (Pro22Gln23-> Ala22Glu23)
SFL2647 (Glu29Gln30-> Ala29Glu30)
SFL2648 (Ser36-> Ala36)
SFL2650 (pBAD Empty vector)