Supplementary Materials

Salmonella STM1697 Coordinates Flagella Biogenesis and Virulence by Restricting Flagellar Master Protein $FlhD_4C_2$ from Recruiting RNA Polymerase

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Supplementary Methods

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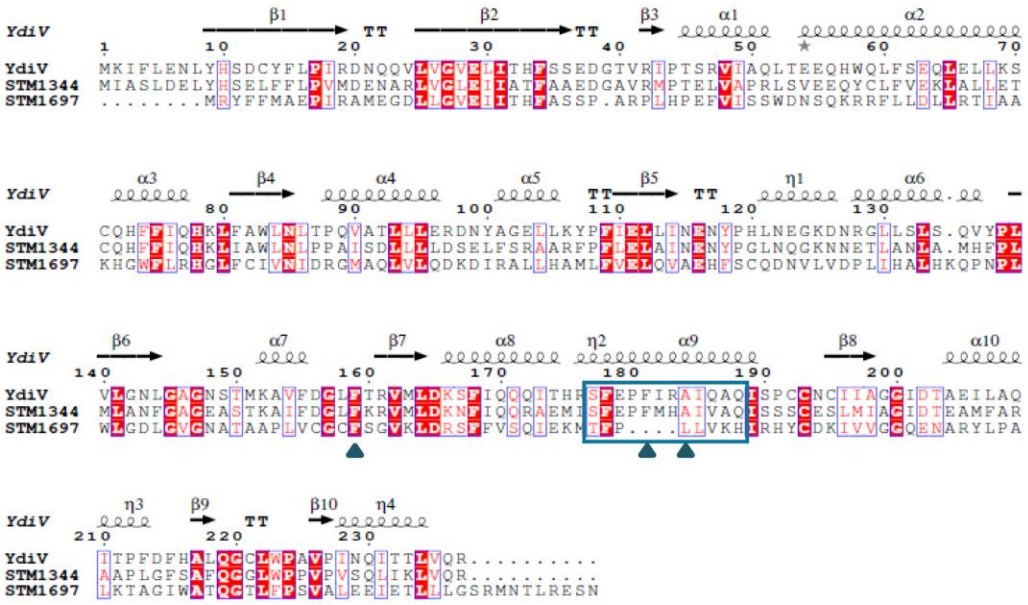

Figure S1. CLUSTALW alignment of YdiV, STM1344 and STM1697 proteins.
The residues of YdiV involved in interaction with FlhD are highlighted in blue triangle, while the most important region in the α9 helix of YdiV are surrounded by blue box.

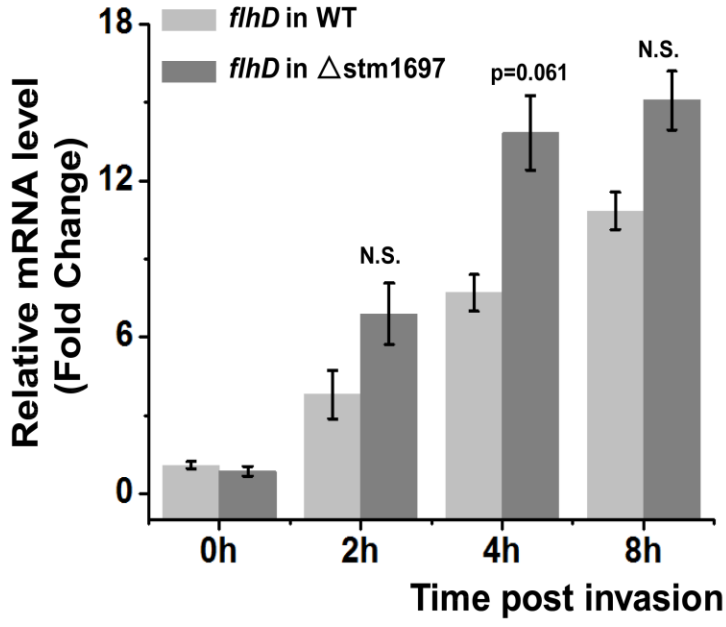

Figure S2. STM1697 presents no significant effect to the expression of FlhD gene.
The expression of flhD gene in wild type Salmonella and ∆ stm1697 strain before and after they enter into host cells was monitored by qRT- PCR. Statistical significance is compared with wild-type strain at the same time using a t-test.

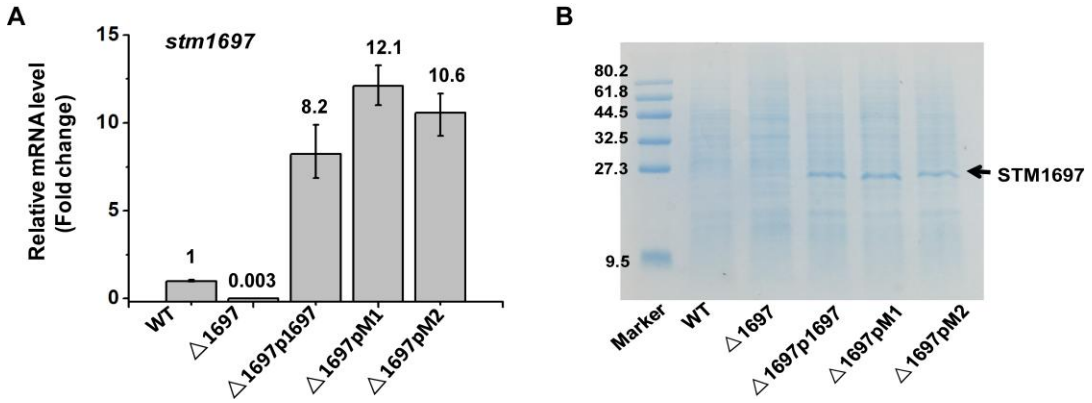

Figure S3. The expression of STM1697 in strains used in this study.
(A) The expression levels of STM1697 in wild type strain, STM1697 knockout strain and STM1697 or mutant over-expressed strains were tested by qRT-PCR. GADPH is used as internal reference. WT strain were set to 1. Bars represent derived from 3 independent experiments.
(B) The protein expression of STM1697 were detected by SDS-PAGE. The over-expressed bands of STM1697 were highlighted by a arrow.

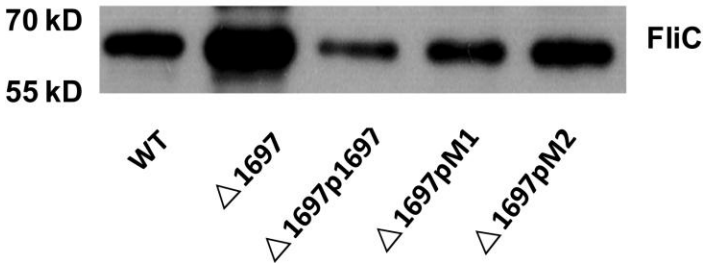

Figure S4. Western-blot analysis of FliC. The FliC protein was detected in wild type strain, STM1697 knockout strain and STM1697 or mutant over-expressed strains using Western-blot.

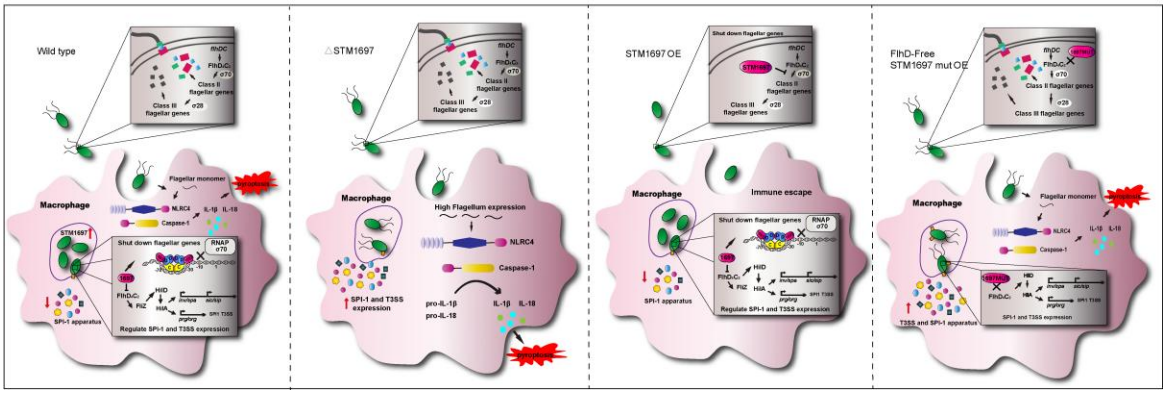

Figure S5. Model of STM1697-mediated flagellar control.

Table S1. Data collection and refinement statistics.

	STM1697-FlhD
Resolution range (Ã…)	32.74 - 1.998 (2.07 - 1.998)
Space group	P 31
Unit cell	61.01 61.01 166.89 90 90 120
Unique reflections	47026 (4713)
Completeness (%)	97.00 (100.00)
Mean I/sigma(I)	23.11 (4.04)
Wilson B-factor	35.19
R-factor	0.1780 (0.2138)
R-free	0.2061 (0.3109)
Number of non-hydrogen atoms	4682
Macromolecules	4388
Protein residues	552
RMS(bonds)	0.008
RMS(angles)	0.86
Ramachandran favored (%)	97
Ramachandran outliers (%)	0.74
Clashscore	4.62
Average B-factor	44.42
Macromolecules	43.94
Solvent	51.52

Statistics for the highest-resolution shell are shown in parentheses.

Table S2. Strains used in this study

Strain	Relevant characteristic(s)	Relevant results	Source or reference
E. coli BL21(DE3)	T7 expression host	Protein expression	Stock
WT *Salmonella*	ATCC14028	Fig.1	Stock
Δ1697	*stm1697* knockout strain	Fig.1	This study
WT *Salmonella* VC	Vector pBad24 control, Amp^+	Fig.7	This study
Δ1697 VC	Vector pBad24 control, Amp^+	Fig.7	This study
Δ1697p1697	p1697=STM1697 cloned into pBAD24, Amp^+	Fig.7	This study
Δ1697pM1	pM1= STM1697 F160A, L173A cloned into pBAD24, Amp^+	Fig.7	This study
Δ1697pM2	pM2= STM1697 L172A, L173A, H176A cloned into pBAD24, Amp^+	Fig.7	This study

Supplementary Methods

Construction of STM1697 knock-out strain

The gene disruption mutant of STM1697 was constructed using the lambda Red recombinase system described previously(1). The chloramphenicol resistance FRT cassette was PCR-amplified from pKD3 with primers homologous to the 5'and 3' flanking regions of the stm1697 gene. The amplified product was transformed into S. typhimurium ATCC14028 strain containing pKD46 by electroporation, and recombined colonies were selected on LB agar plates containing 25 μg/ml chloromycetin. Another two primers were used to confirm the deletion of the stm1697 gene. The pCP20 plasmid was used to express Flp recombinase to remove the chloromycetin resistance gene.

Cell Culture, Bacterial Infection Model and RNA Extracted

For Fig1, experiments were performed as described before(2). Briefly, the human colon adenocarcinoma cell line HT-29 was maintained at 37˚C in a humidified

atmosphere with 5% CO_2 in complete RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco). Cells were seeded at 1×10^7 in 100-mm-wide tissue culture dishes. S. typhimurium ATCC14028 and STM1697 knock-out strain were grown at 37 ℃ overnight in LB medium, and then subcultured in 5 mL LB medium with 3 mM NaCl to an OD600 of 0.5 (1×10^8 colony-forming units ml^{-1}), all strains were added to the HT-29 cell monolayer at a multiplicity of infection of 10 per cell. After incubation for 1 h at 37 ℃, the supernatant was removed and RPMI-1640 medium initially containing 100 μg ml^{-1} gentamicin was added to the cells to kill the extracellular bacteria. At each time point, 2, 4, 6, and 8 h after infection, HT-29 monolayers were washed, lysed in TRIzol reagent (TIANGEN) and stored at -70 ℃. RNA from each condition was extracted according to the TRIzol reagent manufacturer's instructions.

Plasmid construction

For *in vivo* study, STM1697 was amplified using primers stm1697-EcoRI-F and stm1697- HindIII-R from S. typhimurium ATCC14028. PCR products were digested with restriction endonucleases EcoRI and HindIII, then ligated into the pBAD24 vector cut similarly.

For expression of STM1344, STM1697, stFlhD, and stFlhD$_4$C$_2$ proteins using *E.coli*, the STM1344, STM1697, and stFlhD genes were cloned into the expression vector pGL01, a modified vector of pET15b with a Prescission Protease(PPase) cleavage site to remove the his-tag(3). The whole flhDC operon was cloned into pET21b such that FlhC contained a C-terminal his tag. The stFlhD gene was cloned into pET29b without a his-tag for STM1697-stFlhD production.

Mutational plasmids of STM1697 were amplified from above corresponding plasmids using a Site-Directed Mutagenesis system (TransGen biotech, Beijing, China).

Protein expression and purification

STM1697 and mutants used in the pull-down assay were expressed in *E. coli* BL21(DE3) using NZM medium (10 g/l Casein Tryptone digested, 5 g/l NaCl, Ph7.6-7.8) with 100 μg/ml Ampicillin. When the OD600 reached 0.5, cultures were cooled to 16^{o}C and induced overnight by addition of 0.1 mM IPTG. Harvested cells

were resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 200 mM NaCl) and lysed by sonication. After centrifugation at 28,500 ×g for 50min, STM1697 and mutants were purified by Ni^{2+}-NTA affinity column and Superdex 200 successively.
The STM1697-stFlhD complex was obtained by co-expression in *E. coli* BL21 (DE3) in LB medium as described before(3). After the Ni^{2+}-NTA affinity purification, the STM1697-stFlhD complex was lysed by 0.2 mg/ml trypsin for 30 min and immediately purified using a Source Q ion exchange column and Superdex 200 chromatography. The expression and purification of stFlhD$_4$C$_2$ was executed as described. The his-tag of FlhD protein was removed by PPase after purification by Ni^{2+}-NTA affinity column. FlhD without the his-tag was concentrated and purified by Superdex 200 chromatography.

Crystallization and Structure Determination

The STM1697-stFlhD complex were concentrated to 6 mg/ml. Crystals were grown by hanging drop vapour diffusion at 20^{o}C. The crystallization buffer contains 0.2 M KCl, 0.01 M $MgCl_2$, 0.05 M MES pH5.6, and 5% PEG 8000. Diffraction data were collected at the Shanghai Synchrotron Radiation facility (SSRF) beamline BL17u1. To prevent radiation damage, crystals were equilibrated in a cryoproctetant buffer containing 15% glycerol (v/v) plus reservoir buffer and then flash frozen in a 100K nitrogen stream. The data sets were processed using the HKL2000 software suite (4). The atomic model was built using Coot and refined using PHENIX (5,6). Data collection and structure refinement statistics are summarized in Table S1. The structural model was solved at 2.0 Å resolution by molecular replacement using the *E.coli* FlhD structure and YdiV structure as search models. Due to limited proteolysis, some residues are missing in this structure. Finally, two hundred and eleven residues (2-39 aa and 40-224aa) were built for STM1697 and 65 residues (5-69aa) were built for FlhD. Structural figures were generated using PyMol (http://www.pymol.org).

Protein Pull-down Assay

STM1697 and mutants with the his-tag were used as bait protein. FlhD without the his-tag was used as the prey protein. Approximately 0.05 mg of bait protein was immobilized onto Ni^{2+}-NTA beads and incubated with 0.3 mg of prey protein at 4^{o}C for 30 min. The mixture was washed three times using wash buffer (25 mM Tris-HCl pH 8.0, 100 mM NaCl). Proteins were eluted with elution buffer (25 mM Tris-HCl

pH8.0, 100 mM NaCl, 250 mM imidazole). The elution samples were analyzed by SDS-PAGE with coomassie blue staining. 0.3 mg of FlhD protein was incubated with Ni^{2+}-NTA beads alone as a negative control.

EMSA Experiment

For the EMSA experiment presented in Fig.4, a 49 bp DNA fragment containing the $FlhD_4C_2$ box of the flhB promoter in *Salmonella* was synthesized as target DNA. 10 pmol DNA was pre-incubated with different ratios of proteins in a reaction buffer containing 20 mM Tris-HCl pH8.0, 100 mM NaCl,1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 4%(v/v) glycerol for 10 minutes. Then samples were analyzed using a native 5% polyacrylamide gel at 4 ^oC in 0.5X TBE buffer (46 mM Tris base, 46 mM boric acid, 1 mM EDTA pH8.0) for 1 h and stained with Ethidium Bromide for 10 min. All gels were also stained with Coomassie brilliant blue.

Size-exclusion chromatography

For the SEC experiment presented in Fig.4, purified proteins were mixed at the corresponding ratio with concentrations varying between 50μM and 200μM for 10 min at at 4^oC and then injected for size exclusion chromatography using a superdex 200 column equilibrated in buffer containing 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl.

For the SEC experiment presented in Fig.6, a 145 bp DNA fragment containing the fliA promoter was amplified from the *Salmonella* genome. PCR products were purified using the E.Z.N.A.Cycle Pure Kit (OMEGA). Proteins and DNA were mixed at the corresponding ratio with concentrations varying between 10μM and 100μM for 10 min at 4^oC and then injected for size exclusion chromatography using a Superdex 200 increase column.

All data were processed by Origin 8.0.

Native Gel Experiment

For the native gel experiment shown in Fig.5, a 269 bp DNA fragment containing the fliA promoter was amplified from the *Salmonella* genome. PCR products were purified using the E.Z.N.A.Cycle Pure Kit (OMEGA). About 10 pmol DNA was pre-incubated with different ratios of protein in a reaction buffer containing 20 mM Tris-HCl pH8.0, 100 mM NaCl,1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 4%(v/v) glycerol.

Then samples were separated by native 4% polyacrylamide gel at 4 ^{o}C in 0.5X TBE buffer (46 mM Tris base, 46 mM boric acid, 1 mM EDTA pH8.0) for 3 h, stained with Gel Red (Biotium) for 10 min, and then stained by Coomassie brilliant blue G250.

Preparation of strains used in functional study

S. typhimurium ATCC14028 and mutant strains were grown overnight in 10 mL LB medium with 100 μg/ml Ampicillin, and then subcultured into 5 mL LB medium with 100 μg/ml Ampicillin and 0.1% L-arabinose to induce protein expression. To induce invasion phenotype, LB+0.3M NaCl were used to culture bacteria. When the OD600 reached 0.4-0.6, all strains were adjusted to the same OD600 by addition of 0.9% NaCl to get an equivalent quantity of bacterial cells. Bacterial cells were then harvested for functional study.

RNA isolation and real-time quantitative PCR

Total RNA was isolated using the RNAprep Pure Cell/Bacteria Kit (TIANGEN). The reverse transcription reactions were performed using the RevertAid cDNA Synthesis Kit (Thermo) according to manufacturer's instructions and incubated at 42℃ for 60min, followed by 10min at 70℃. The quantitative RT-PCR reactions were performed on an Applied Biosystems 7500 Sequence Detection system (Applied Biosystems, Foster, CA, USA) using iTaq Univerial SYBR Green Supermix (BIO-RAD). GADPH was used as an internal reference.

Western blot

Bacterial cells were lysed using 2×SDS-PAGE loading dye followed by heating at 95^{o}C for 10 min before SDS-PAGE. Total proteins on gels were transferred to nitrocellulose membranes at 250 mA for 2 h in transfer buffer (96 mM glycine, 12.5 mM Tris, and 10% methanol). The membranes were blocked in 5% milk in PBS-0.1% and Tween 20 (PBST) at 37^{o}C for 1h, followed by incubation with polyclonal antibody to FliC (abcam) diluted 1:10000, in PBST overnight at 4^{o}C. The membranes were incubated with HRP-Conjugated Goat anti Rabbit IgG (h+l) (abcam) diluted 1:10000 in PBST at 37^{o}C for 1h after three washes in PBST. The membranes were then washed three times for 10 min, and then detected by chemiluminescence.

Swimming motility assay

Bacterial cells were diluted to OD600=10. Then 0.5μl of each liquid was applied to a 0.3% LB agar plates with 100 μg/ml Ampicillin and 0.1% L-arabinose. Swimming motility was observed after 5 h at 30 ℃. At least six independent colonies were tested for each strain, and the diameter of each colony were measured.

Invasion assay

For the experiments in Fig.7, the human epithelial cell line HT-29 was maintained at 37˚C in a humidified atmosphere with 5% CO_2 in complete RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). Bacteria were diluted using RPMI-1640 medium and then seeded on confluent HT-29 cells grown in 24-well plates at a multiplicity of infection of 20, which approximately contain 10^7 cfu bacteria. One hour post- infection, the supernatant was removed from plates and RPMI-1640 medium containing gentamicin (100 μg ml^{-1}) was added to the cells for 1 h to kill the remaining extracellular bacteria. Cells were washed gently with PBS and then disrupted with 1% Triton X-100 (Sigma Chemical). The number of intracellular bacteria was detected by colony-forming units (CFU) counts of viable colonies. The CFU of the diluted bacteria were also determined to obtain the invasion rate. The presented results are based on at least three biological replicates.

Macrophage cytotoxicity assay

Bone marrow-derived macrophage (BMDM) were isolated as described(7). Macrophages were infected at the indicated MOI and then incubated at 37˚C with 5% CO_2 for 4 hours. Cytotoxicity was determined by LDH assay (CytoTox 96, Promega). IL-1β and IL-18 secretion was determined by ELISA kit (R&D Systems and MBL, respectively). Macrophages that were not infected with bacteria were used as negative controls.

In vivo infection of BALB/c mice

Six- to 8-week-old female BALB/c mice of weight between 16-18 g were purchased from Jinan Pengyue Laboratories (Jinan, China). The animals were raised and cared for

according to the guidelines of the National Science Council of the People's Republic of China. Experimental procedures were approved by the Institutional Animal Ethics Committee. For bacterial infections, mice were infected intraperitoneally (IP) with 1×10^6 of each *S.typhimurium* strain. The mice were sacrificed 6 h post-infection. The spleen and liver were homogenized in PBS and were plated on LB agar plates at appropriate dilutions. The number of bacteria located in the spleen or liver was detected by CFU counts of viable colonies. For survival, each cohort of mice (n = 5) was infected intraperitoneally with 1×10^6 bacteria in 100 μl, and the death of mice were observed every 2-3 hours.

Supplementary References

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