

Interactions of Flagellar Structural Subunits with the Membrane Export Machinery

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Abstract

During assembly of the bacterial flagellum, structural subunits synthesized inside the cell must be exported across the cytoplasmic membrane before they can crystallize into the nascent flagellar structure. This export process is facilitated by a specialized Flagellar Type III Secretion System (fT3SS) located at the base of each flagellum. Here, we describe three methods—isothermal titration calorimetry, photo-crosslinking using unnatural amino acids, and a subunit capture assay—used to investigate the interactions of flagellar structural subunits with the membrane export machinery component FlhB.

Key words *Salmonella*, Flagella, Flagellar type III secretion system (fT3SS), Protein export, Protein-protein interactions, Isothermal titration calorimetry (ITC), *p*-benzoyl-L-phenylalanine (*pBpa*) photo-crosslinking, Capture assay

1 Introduction

1.1 Background

A striking feature of the bacterial flagellum is that it self-assembles, aided by a dedicated Type III export machinery located at each flagellum base. The export machinery unfolds nascent structural subunits and delivers them across the cell membrane into a central 2 nm diameter channel that runs the entire length of the growing flagellum [1]. The unfolded subunits must then transit through this channel in the external flagellum to its tip where they crystallize beneath cap foldases [2]. In this way, the flagellar rod that spans the cell envelope is built first, followed by assembly of the hook and then the filament, both of which extend from the cell surface.

Structural subunits are thought to dock initially at the FliI export ATPase [3, 4]. Subunits of the rod and hook go on to bind a surface exposed hydrophobic pocket on the cytosolic domain of the FlhB export gate (FlhB_C, residues 212–383) via a conserved gate recognition motif (GRM sequence FxxxΦ, where Φ is any hydrophobic residue) near the subunit N-terminus [5]. Subunits

then move across the cytoplasmic membrane into the central channel of the external flagellum, where there is no conventional biological energy source, and transit to the assembly site at the distal tip.

There are a number of alternative theoretical models for the mechanism of subunit transit through the flagellar central channel [6–8], however, there is experimental evidence to suggest that the energy for transit is intrinsic to the unfolded subunits themselves [5]. It is proposed that transit could be achieved by linking of the subunit docked at the FlhB export gate to the free C-terminus of the preceding subunit that has already partly crossed the membrane into the channel [5]. The juxtaposed N- and C-terminal helices of successive subunits link as parallel coiled-coils and the newly linked subunit is then pulled from the export gate into the channel by the thermal motion of the unfolded subunit chain, which is anchored at its other end to the tip of the growing flagellum. Repeated crystallization of subunits at the tip causes the chain to shorten, stretch, and exert an increasing force on the next subunit docked at the export gate, eventually pulling this subunit into the channel. In this way, linking of consecutive subunits at the membrane export machinery is coupled to subunit folding at the flagellum tip to produce directional subunit transit [5].

1.2 Overview of Methods

Here, we describe the experimental techniques we used to investigate the mechanisms underlying the transfer of nascent flagellar subunits from the cytosol into the growing flagellum [5]. Specifically, interactions between the export gate component FlhB and subunits were analyzed using (1) Isothermal Titration Calorimetry (ITC) [9] to determine the binding affinities of subunits for FlhB_C (Subheading 3.7), (2) a photo-crosslinking assay based on the incorporation of unnatural amino acids [10] to identify subunit residues that directly bind FlhB_C (Subheading 3.8), and (3) a capture assay to examine linking and capture of FlhB_C-docked-subunit by free subunit (Subheading 3.9). These techniques rely on a common set of molecular microbiology procedures to maintain bacterial strains and plasmids (Subheading 3.1), construct recombinant plasmids harboring flagellar genes (Subheading 3.2), express recombinant flagellar proteins (Subheading 3.3), generate clarified bacterial cell lysates (Subheading 3.4), and purify proteins and/or protein complexes (Subheadings 3.5 and 3.6).

The biophysical technique ITC was used to measure the thermodynamics of (GST)FlhB_C binding to the hook cap subunit FlgD in solution (Subheading 3.7), generating quantitative data on the binding constant ($K_D = 39 \mu\text{M}$), stoichiometry (1:1), and enthalpy of binding ($\Delta H_b -1 \times 10^3$). Recombinant FlgD subunits in which the GRM (FlgD residues 36–40) was intact (wild-type) or deleted

(gate-blind subunit, FlgD Δ 36–40) were engineered to introduce a C-terminal hexa-histidine tag to enable purification using Ni²⁺-affinity chromatography. An N-terminal translational fusion of Glutathione S-transferase (GST) to FlhB_C and a GST control were purified using Glutathione affinity chromatography with Glutathione Sepharose resin (GSH-resin). Exhaustive dialysis of purified flagellar proteins into analysis buffer was carried out prior to performing ITC to prevent heats of dilution (ΔH_d ; background heats) masking observations.

Direct interactions between the subunit FlgD GRM (residues 36–40) and FlhB_C were identified using photo-cross-linkable unnatural amino acids (Uaa; Subheading 3.8) incorporated at specific sites in FlgD, either in the GRM (L₃₉Uaa or L₄₀Uaa) or elsewhere (L₅Uaa). This was achieved by mutating the corresponding codons in *flgD* to the amber codon (UAG). In the presence of an orthogonal tRNA and recombinant mutated aminoacyl-tRNA synthetase, the photo-cross-linkable Uaa *p*-benzoyl-L-phenylalanine (pBpa) was site-specifically incorporated into the polypeptide chain at the position encoded by the amber codon. Binding of the subunit GRM to FlhB_C was demonstrated by covalent linkage of FlgD to (GST)FlhB_C via the engineered ultraviolet (UV)-activated site-specific crosslinking residues. Soluble extracts of *E. coli* individually expressing FlgD wild-type or photo-cross-linkable derivatives were incubated with purified (GST)FlhB_C, exposed to UV light, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting using anti-FlgD sera to identify cross-linked protein complexes. In addition to assessing subunit binding to (GST)FlhB_C, our study also used a GST fusion to the autocleavage-defective FlhB_CN₂₆₉A [11] to enhance the shift in the migration of the cross-linked protein adducts when analyzed by SDS-PAGE. Binding of FlgD to (GST)FlhB_C and GST-FlhB_CN₂₆₉A was shown to be comparable [5].

The N-terminus of a subunit docked at the FlhB_C export gate can be captured by the free C-terminus of the preceding subunit already in the channel. It is thought that the docked subunit can be pulled into the central channel by the entropic force generated by the thermal motion of the chain of unfolded subunits in the channel. To characterize the interactions between FlhB_C-docked subunits and free subunits, and to demonstrate subunit release from FlhB_C, we developed an in vitro capture assay (Subheading 3.9). In this assay, subunits docked at the FlhB_C export gate are captured and released from FlhB_C by free gate-blind subunits, which are deleted for the GRM and cannot interact with FlhB_C [5]. Below, we describe the protocol used to show that the flagellar hook subunit FlgE can, in a concentration-dependent manner, capture and release FlgD subunit from a preformed (GST)FlhB_C-FlgD complex.

2 Materials

Prepare all solutions using sterile Milli-Q water.

2.1 Materials for the Maintenance of Bacterial Strains and Plasmids

1. Bacterial strains for molecular biology and protein expression (Table 1).
2. 37 °C static incubator for culture plates.
3. 37 °C shaking incubator for liquid culture tubes and flasks.
4. Luria-Bertani (LB) broth.
5. Plastic tubes with lids (e.g., Falcon®), 5 mL, 14 mL, and 50 mL for bacterial cell culture.
6. Conical flasks for bacterial cell culture.
7. Luria-Bertani (LB) 1.5% agar plates.
8. Spectrophotometer to measure absorbance from ultraviolet to visible light or densitometer to measure bacterial culture density.
9. Floor-standing centrifuge with rotors (e.g., Avanti, Beckman Coulter) and thick-wall polypropylene tubes (e.g., 25 × 89 mm Beckman Coulter) to collect bacterial cell pellets.
10. Plastic microcentrifuge tubes, 0.5 mL or 1.5 mL for storage of electrocompetent cells.
11. Ice-cold sterile Milli-Q water.
12. 10% (v/v) glycerol.
13. Liquid nitrogen in a Dewar flask.
14. Ultra-low temperature (−80 °C) freezer.
15. Ice.
16. Electroporation apparatus (e.g., Bio Rad Gene Pulser™ set at 12.5 kVcm^{−1} field strength, 25 μFD and 200 Ω at 2.5 kV).
17. Electroporation cuvette, 0.1 cm gap.

Table 1
Bacterial strains

Bacterial strain	Description	Source
<i>Salmonella enterica</i> serovar Typhimurium SJW1103	Wild-type	Yamaguchi et al. [12]
<i>Escherichia coli</i> DH5α	F [−] <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20</i> φ80d <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169, <i>hsdR17(rK[−]mK⁺)</i> , λ [−]	Hanahan [13]
<i>Escherichia coli</i> C41 (DE3)	F [−] <i>ompT gal dcm hsdS_B(r_B[−] m_B[−])</i> DE3	Miroux and Walker [14]

18. Super Optimal broth with Catabolite repression (SOC) medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM Glucose (add filter-sterilized MgCl₂ and glucose after sterilization of broth at 121 °C).
19. Antibiotics for plasmid selection (Table 2): ampicillin (100 µg/mL), chloramphenicol (25 µg/mL), tetracycline (12.5 µg/mL).

2.2 Materials for the Construction of Recombinant Plasmids Harboured Flagellar Genes

1. Oligonucleotide primers (Table 3) for amplification of flagellar genes using the polymerase chain reaction (PCR).
2. High-fidelity DNA polymerase, template genomic DNA, deoxynucleotide triphosphates (dNTPs), and buffers for PCR.
3. Thermal cycler.
4. Equipment for agarose gel electrophoresis (separation of nucleic acids).
5. Plasmid vectors (Table 2).
6. Enzymes (restriction endonucleases, DNA ligase) for the construction of recombinant plasmids.

2.3 Materials for the Expression of Recombinant Flagellar Proteins

1. 2× Tryptone-Yeast Extract (2× TY) broth medium.
2. Two-liter conical flasks for bacterial cell culture.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 M stock solution.
4. 20% (w/v) L-arabinose.
5. *p*-benzoyl-L-phenylalanine (*p*Bpa).
6. Sodium hydroxide (1 M).
7. Equipment for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
8. SDS-polyacrylamide gels for SDS-PAGE (10%, 12.5%, or 15% acrylamide).

Table 2
Plasmids

Plasmid (antibiotic resistance)	Origin of replication	Restriction sites	Transcription promoter	Source
pGEX-4T-3 (Amp ^R)	pBR322	BamHI, XhoI	T7	Kaelin et al. [16]
pDULE (Tet ^R)	p15A	n/a	lpp	Farrell et al. [10]
pBAD18 (Amp ^R)	pBR322	XbaI, HindIII/SalI	P _{BAD}	Guzman et al.[17]
pET20b (Amp ^R)	pBR322	NdeI, HindIII	T7	Studier and Moffatt [18]
pACT7 (Cm ^R)	p15A	NdeI, BamHI	T7	Evans et al. [19]

Table 3**Primers for amplification of recombinant *Salmonella* flagellar genes by PCR**

Gene	Description	Primer sequence (5' to 3')
<i>flhBC</i>	BamHI site; (GST) fusion; residue 219; forward; for the construction of pGEX-4 T-3- <i>flhBC</i>	CCGCGTGATCCGTGGCAGAAGAGAGCGACGACGA
<i>flhBC</i>	Residue 383 Stop; XhoI site; reverse; for the construction of pGEX-4 T-3- <i>flhBC</i>	GTCAGCCTCGAGTTAGCCATCAGTATTCTT
<i>flhB</i>	N ₂₆₉ A; forward; for the construction of pGEX-4 T-3- <i>flhBC</i> N ₂₆₉ A	CGGACGTCATTGTCACTGCCCCGACGCACT
<i>flhB</i>	N ₂₆₉ A; reverse; for the construction of pGEX-4 T-3- <i>flhBC</i> N ₂₆₉ A	GAATAGTGCGTCGGGGCAGTGACAATGACGT
<i>flgD</i>	XbaI site; RBS, residue 1; forward for the construction of pBAD18 flgD derivatives	GCTCCTTCTAGAAGGAGAGCCCAAATGTCTATTGCCGT AAATATGAATG
<i>flgD</i>	Residue 232 stop; HindIII; reverse for the construction of pBAD18 <i>flgD</i> derivatives	GCATGCAAGCTTGATTATTTGCCGAAC TCGTCGAGTGT
<i>flgD</i>	Deletion of GRM residues 36–40; forward for the construction of “gate-blind” flgD derivatives	GATCTGCAAAGCAGTGTGCGCAATTGAA
<i>flgD</i>	Deletion of GRM residues 36–40; reverse for the construction of “gate-blind” flgD derivatives	CTTCAATTGCGGACACTGCTTTGCAGATC
<i>flgD</i>	V ₅ Uaa; forward for the construction of amber codon containing <i>flgD</i> derivative	GCTCCTCTCTAGGAGATATACCATGTCTATTGCCTAGA ATATGAATGACCCGA
<i>flgD</i>	L ₃₉ Uaa; forward for the construction of amber codon containing <i>flgD</i> derivative	GCAGTTTCCTGACCTAGCTGGTCGCGCAATTGA
<i>flgD</i>	L ₃₉ Uaa; reverse for the construction of amber codon containing <i>flgD</i> derivative	CAATTGCGGACCAGCTAGGTCAGGAAACTGCT

(continued)

Table 3
(continued)

Gene	Description	Primer sequence (5' to 3')
<i>flgD</i>	L ₄₀ Uaa; forward for the construction of amber codon containing <i>flgD</i> derivative	CAGTTTCCTGACCTTATAGGTCGCGCAATTGAA
<i>flgD</i>	L ₄₀ Uaa; forward for the construction of amber codon containing <i>flgD</i> derivative	CAATTGCGCGACCAGCTAGGTCAGGAAACTGCT
<i>flgD</i>	NdeI site; residue 11; forward for the construction of N-terminal truncate of FlgD	CGCCTGCATATGACCAACACGGGCGTCAAAACGACG ACCGGCA
<i>flgD</i>	FlgD residue 232; FLAG3; stop; SalI site; reverse for the construction of full-length <i>flgD</i> with C-terminal FLAG × 3 tag	CGTAGTGTCGACTTACTTGTTCATCGTCATCCTTTATAAT CAATATCATGATCTTTATAGTCGCCGTCATGATCTTT ATAATCGATTATTTGCCGAACCTTCGTCGAGTGTGG
<i>Flag tag</i>	FLAG × 3; stop; BamHI site; reverse for the construction of C-terminally tagged flagellar gene derivatives	CGTAGTGGATCCTTACTAGTCATCGTCATCCTTTAT
<i>flgE</i>	NdeI site; Residue 1; forward	CGTAGTCATATGTCTTTTCTCAAGCGGTTAGC
<i>flgE</i>	HindIII site; 403, no stop; reverse for the construction of full-length <i>flgE</i> C-terminally FLAG × 3 tagged	GCTATCCCGTCAAGCTTGCGCAGGTTA
<i>flgE</i>	HindIII site; 359, no stop; reverse for the construction of C-terminally truncated and FLAG × 3 tagged <i>flgE</i>	CGTAGTAAGCTTGCCGTTTCGTCAGCTTACCGAAGTT
<i>flgE</i>	Deletion of GRM residues 39–43; forward for the construction of “gate-blind” <i>flgE</i> derivative	GTCCGGTACGGCATCAGCCGGTTCCAAAGTGGGGCT
<i>flgE</i>	Deletion of GRM residues 39–43; reverse for the construction of “gate-blind” <i>flgE</i> derivative	CACTTTGGAACCGGCTGATGCCGTACCGGACTTAAA

Table 4
Buffers for purification of recombinant proteins from *E. coli*

Protein	Resin	Lysis buffer	Wash buffer	Elution buffer
His-tagged proteins	Nickel	50 mM Tris-HCl, pH 7.4 400 mM NaCl, 10 mM imidazole Protease inhibitor (one tablet in 20 mL of lysis buffer) DNase I (10 µg/mL)	50 mM Tris-HCl, pH 7.4 400 mM NaCl 10 mM imidazole	50 mM Tris-HCl, pH 7.4 200 mM NaCl 600 mM imidazole
GST-fusion proteins	GSH	50 mM Tris-HCl, pH 7.4 200 mM NaCl Protease inhibitor (one tablet in 20 mL of lysis buffer) DNase I (10 µg/mL)	50 mM Tris-HCl, pH 7.4 200 mM NaCl	50 mM Tris-HCl, pH 7.4 200 mM NaCl 10 mM glutathione
Untagged proteins	Q HP	50 mM sodium phosphate buffer, pH 7.0–7.5 5–50 mM NaCl Protease inhibitor (one tablet in 20 mL of lysis buffer) DNase I (10 µg/mL)	50 mM sodium phosphate buffer, pH 7.0–7.5 5–50 mM NaCl	50 mM sodium phosphate buffer, pH 7.0–7.5 1 M NaCl

9. SDS-PAGE running buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS.
10. SDS-PAGE sample buffer: 100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol (DTT).
11. Prestained protein molecular weight marker for SDS-PAGE.
12. Coomassie Brilliant Blue G-250 stain: 0.1% (w/v) Coomassie Brilliant Blue G-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid.

2.4 Materials for the Production of Clarified Bacterial Cell Lysates

1. Cells lysis buffers (Table 4) for purification of recombinant proteins from *E. coli*.
2. Plastic tubes with lids (e.g., Falcon®), 5 mL, 14 mL, and 50 mL.
3. Cell disruptor or French® Pressure Cell.
4. cOmplete® protease inhibitor cocktail, EDTA-free (Roche).
5. DNase I (Thermo Scientific).

2.5 Materials for the Purification of Recombinant Proteins and Recombinant Protein Complexes

1. Buffers for purification of recombinant proteins (Table 4) and recombinant subunit/export gate complexes (Table 5) from *E. coli*.
2. Plastic tubes with lids (e.g., Falcon®), 5 mL, 14 mL, and 50 mL.
3. Ni²⁺-Sepharose HisTrap® Excel column (GE Healthcare).
4. Ni²⁺-nitrilotriacetic acid (NTA) resin.

Table 5
Buffers for purification of recombinant subunit/export gate complexes

Buffer	Components
Lysis buffer	50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM β -mercaptoethanol, protease inhibitor, DNase 1
Wash buffer	50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM β -mercaptoethanol
Elution buffer	50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM β -mercaptoethanol, 10 mM reduced glutathione

5. Glutathione Sepharose 4B resin (GSH-resin; GE Healthcare).
6. Anion exchange resin (Q HP; GE Healthcare).
7. Bench-top tube rotator.
8. Fast protein liquid chromatography (FPLC) system.
9. Peristaltic pump.
10. Plastic beaker, 5 L.
11. Dialysis tubing, 6–8 kDa, 10 kDa, and 25 kDa molecular weight cutoff (MWCO).
12. Pasteur pipettes.

2.6 Additional Materials for ITC

1. Vacuum pump for degassing solutions.
2. ITC assay buffer: 50 mM Tris-HCl pH 7.4, 100 mM NaCl.
3. Spin concentrators, 3.5–5 kDa membrane cutoff.
4. DCTM Protein Assay (Bio-Rad).
5. Heating block.
6. Degassed sterile Milli-Q water for cleaning calorimeter.
7. VP-Isothermal Titration Calorimeter (MicroCal) with loading needle.
8. Plastic syringes, 2 mL, 5 mL, and 10 mL.
9. Computer with Origin[®] analysis software.

2.7 Additional Materials for Photo-Crosslinking

1. Phosphate buffered saline (PBS).
2. Polystyrene 24-well plate.
3. Shallow box.
4. Lamp that emits 350 nm light and cooling fan.

5. Trichloroacetic acid (TCA), 100%.
6. Acetone, 4 °C.
7. Bench top microcentrifuge.
8. Wet blotting transfer equipment (e.g., Hoefer TE22).
9. Nitrocellulose membrane for immunoblotting.
10. Blotting transfer buffer: 10 mM CAPS pH 11.0, 10% (v/v) methanol.
11. Phosphate buffered saline (PBS), pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄.
12. PBS-Triton: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.05% (v/v) Triton X100.
13. Immunoblotting blocking buffer: PBS-Triton, 5% (w/v) skimmed milk powder.
14. Specific antisera (rabbit) raised against purified *Salmonella* FlhB or FlgD, and commercial antibodies raised against glutathione S-transferase (GST).
15. IRDye-conjugated goat-anti-rabbit secondary antibodies (Licor).
16. Licor Odyssey® CLx imaging system.

2.8 Additional Materials for Capture Assay

1. Ni²⁺-nitrilotriacetic acid (NTA) Resin (Qiagen).
2. Bovine serum albumin (BSA).
3. Specific antisera (rabbit) raised against purified *Salmonella* FlhB or FlgE, and commercial antibodies raised against the FLAG epitope.

3 Methods

3.1 Maintenance of Bacterial Strains and Plasmids

1. Grow bacterial strains *Salmonella enterica* serovar Typhimurium SJW1103 [12], *Escherichia coli* DH5α [13], and *Escherichia coli* C41 [14] (Table 1) at 37 °C in Luria-Bertani (LB) broth with vigorous shaking (200 rpm) or on LB agar (1.5%) plates.
2. To prepare electrocompetent bacteria cells [15] for plasmid propagation and maintenance (*E. coli* DH5α) and for protein expression (*E. coli* C41), inoculate LB broth (500 mL) using an overnight bacterial broth culture to a starting density of approximately A₆₀₀ 0.005 and grow at 37 °C, with shaking (200 rpm), to mid-exponential phase (A₆₀₀ 0.6–0.8). Carry out all subsequent steps at 4 °C or on ice. Harvest cells by centrifugation (10 min, 5000 × g). Resuspend cells in 200 mL ice-cold sterile Milli-Q water, and repeat harvest and wash steps twice. After final harvest, resuspend cells in 100 mL ice-cold 10% (v/v) glycerol and pellet cells by centrifugation (10 min, 5000 × g). Finally, resuspend cells in 0.5 mL 10% (v/v) glycerol

and snap freeze 50 μL aliquots of the cell suspension in plastic microcentrifuge tubes (e.g., 1.5 mL tubes) by immersing in liquid nitrogen in a Dewar flask. Store electrocompetent cells at -80°C .

3. Isolate and purify plasmids from *E. coli* DH5 α (or comparable strain, deleted for *endA* and *recA1*) using standard laboratory techniques [15].
4. Transform *E. coli* with plasmids harboring recombinant flagellar genes by mixing 0.5 μg of plasmid DNA with 50 μL electrocompetent cells that have been thawed on ice. Transfer mixture to a chilled electroporation cuvette (0.1 cm gap) and electroporate the bacterial cells using a Gene PulserTM (Bio Rad) set at 12.5 kVcm^{-1} field strength, 25 μFD and 200 Ω at 2.5 kV. Immediately add 1 mL SOC medium to the electroporation cuvette, resuspend cells, and transfer to a 5 mL plastic tube with lid. Incubate transformed cells at 37°C for 1 h with shaking (200 rpm). Select cells carrying appropriate plasmid(s) by plating onto LB agar (1.5%) plates containing, where appropriate (Table 2), ampicillin (100 $\mu\text{g/mL}$), tetracycline (12.5 $\mu\text{g/mL}$), and/or chloramphenicol (25 $\mu\text{g/mL}$).

3.2 Generation of Recombinant Plasmids Harboring Flagellar Genes

1. Isolate genomic DNA from *Salmonella enterica* serovar Typhimurium SJW1103 (*Salmonella*) using standard laboratory techniques [15].
2. To construct recombinant plasmids, amplify *Salmonella* flagellar genes (e.g., *flhB*, *flgD*, or *flgE*) using the polymerase chain reaction (PCR; [15]) with specific oligonucleotide primers (Table 3), high fidelity DNA polymerase, dNTPs, *Salmonella* genomic DNA as template and recommended buffer conditions in a thermal cycler. Use overlap-extension PCR [15] to introduce site-specific point mutations (e.g., in the gene-encoding FlhBN₂₆₉A), the amber codon UAG for photo-crosslinking (see **Note 1** on mutagenic primer design) or deletions (e.g., in the gene-encoding FlgD Δ 36–40). Perform restriction digestions and ligations using standard laboratory techniques [15].
3. Validate recombinant plasmids by DNA sequencing.

3.3 Protein Production in *E. coli* Cells Carrying Expression Plasmids Harboring Flagellar Genes

1. Following transformation of *E. coli* C41 with appropriate expression plasmid(s) (Subheading 3.1, **step 4**), select fresh colonies to inoculate 50 mL 2TY medium containing appropriate antibiotics (Table 2) and grow at 37°C with shaking (200 rpm) to late stationary phase ($A_{600} > 2.0$).
2. For plasmids harboring *flgD* derivatives with amber codons for incorporation of photo-cross-linkable pBpa, transform into competent *E. coli* cells that already carry pDULE.

3. Pre-warm (30 °C) 500 mL 2TY medium containing appropriate antibiotics (Table 2) in a 2 L conical flask.
4. Inoculate the pre-warmed 2TY with the late stationary phase ($A_{600} > 2.0$) *E. coli* C41 culture (**step 1**) to a starting density of A_{600} 0.005.
5. Grow cells to mid-exponential phase (A_{600} 0.6–0.8) and induce protein expression using 0.8 mM IPTG for pGEX-4 T3 and pDULE or 0.2% (w/v) L-arabinose for pBAD18 (Table 2).
6. For expression of photo-cross-linkable FlgD derivatives, dissolve 108 mg of *p*-benzoyl-L-phenylalanine (*p*Bpa) in 440 μ L of 1 M sodium hydroxide, then add to 400 mL of pre-warmed 2TY medium to give a final concentration of 1 mM *p*Bpa (*see* **Note 2**).
7. Incubate expression cultures at 30–37 °C for 4–6 h, with shaking (200 rpm). Harvest cells by centrifugation at $8000 \times g$ for 10 min. Store bacterial cell pellets at –80 °C.
8. Assess protein expression by analyzing preinduced and postinduced whole cell samples by SDS-(15%)PAGE followed by staining with Coomassie Brilliant Blue G-250 stain.

3.4 Preparation of Cleared Cell Lysates

Prepare cleared lysates for purification of proteins and/or protein complexes (Subheadings 3.5 and 3.6) or to use directly in the capture assay (Subheadings 3.9). Preparation of cleared lysates is typically performed in parallel and, to minimize degradation, all steps are carried out on ice.

1. Resuspend *E. coli* C41 cells producing flagellar proteins in the appropriate Lysis Buffer (Table 4) containing protease inhibitors (1 tablet in 20 mL of lysis buffer) and 10 μ g/mL DNase I.
2. For small-scale *E. coli* C41 cultures (<5 g wet weight cells, culture volume < 500 mL) lyse cells using a precooled French® pressure cell at 30 kpsi.
3. For larger-scale *E. coli* C41 cultures (> 5 g wet weight, culture volume > 500 mL) lyse using a cell disruptor (Constant Systems) at 30 kpsi.
4. Centrifuge ($40,000 \times g$, 1 h) cell lysates to remove insoluble cell debris and then decant the soluble fraction, which is the cleared cell lysate, into a plastic tube with lid.
5. Store the cleared cell lysate on ice for later use.

3.5 Purification of Flagellar Proteins

1. Equilibrate Ni^{2+} -sepharose resin, Ni^{2+} -nitrilotriacetic acid (NTA) resin, GSH-resin, or anion exchange resin (e.g., Q HP, GE Healthcare) in 50 volumes of the appropriate Lysis Buffer (Table 4), where one volume equals the volume of resin used.
2. If performing batch purification, equilibrate the resin [3 mL resin slurry, 50% (v/v)] in a 50 mL plastic tube with lid, allow

the resin to settle to the bottom of the tube, and then carefully remove the Lysis Buffer with a Pasteur pipette to leave only the resin in the tube. Carefully add the cleared cell lysate (*see* Subheading 3.4) to the resin and incubate for 1 h at room temperature on a bench-top tube rotator.

3. If using resin in a column, pass the cleared cell lysate over the equilibrated resin using a peristaltic pump or FPLC at 1 mL/min.
4. Wash the resin with 10 volumes of the appropriate Wash Buffer (*see* Table 4 and **Note 3**).
5. Elute bound protein from the resin using the appropriate Elution Buffer (Table 4). It is optimal to elute in multiple fractions over 3–5 volumes (*see* **Note 4**).
6. In a 5 L plastic beaker, dialyze (using, e.g., dialysis tubing, 8–10 kDa MWCO) the eluted protein at 4 °C in 4 L of the buffer appropriate for the downstream experiment (*see* **Note 5**).

3.6 Purification of Recombinant Subunit/Export Gate Protein Complexes

1. Place 1 mL of GSH-resin slurry [50% (v/v)] into a 50 mL plastic tube with lid and wash with 5 mL of sterile Milli-Q water followed by 5 mL of the appropriate Lysis Buffer (Table 5). Allow the washed resin to settle to the bottom of the plastic tube and then carefully remove the Lysis Buffer with a Pasteur pipette to leave only the resin.
2. Decant the cleared cell lysate containing (GST)FlhB_C into the 50 mL plastic tube containing the washed GSH-resin.
3. Incubate tube-containing lysate and resin on a bench-top tube rotator at room temperature for 1 h.
4. Place the 50 mL plastic tube containing lysate and resin on ice for 10 min to allow the resin to settle and then carefully remove the supernatant with a Pasteur pipette to leave only the resin.
5. Wash the resin with 10 mL of Wash Buffer (Table 5).
6. Apply the cleared cell lysate containing recombinant FlgD (*see* **Notes 6** and **7**) to the washed resin and incubate on a bench-top tube rotator at 4 °C for 2 h.
7. Repeat **step 4**.
8. Wash the resin with 2 mL of wash buffer (*see* **Note 8**).
9. Repeat **step 4**.
10. For the capture assay protocol (Subheading 3.9), store the resin on ice and carry out the capture assay on the same day. For the photo-crosslinking protocol proceed to **step 11**.
11. Elute the protein complex by adding 1 mL Elution Buffer (Table 5) per 1 mL of resin and incubate in a 50 mL plastic tube with lid on a bench-top tube rotator at room temperature for 10 min.

12. Place the 50 mL plastic tube with lid on ice to allow the resin to settle to the bottom of the tube, collect the supernatant containing the eluted protein, and store in a plastic tube with lid on ice.
13. Repeat **steps 11** and **12** two more times.
14. Store the elution fractions on ice.
15. Confirm protein purity by analyzing the elution fractions by SDS-(15%)PAGE and Coomassie Brilliant Blue G-250 staining.

3.7 ITC

All buffers and samples used in ITC are degassed under a vacuum prior to use.

1. Proteins to be analyzed by ITC should be dialyzed (dialysis tubing, 6–8 kDa MWCO) into ITC assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl) at 4 °C (*see Note 5*).
2. Concentrate solutions of purified and dialyzed GST or (GST)FlhB_C to a concentration of 50 µM using a spin concentrator (3.5–5 kDa MWCO). Ligand (in this case untagged FlgD or untagged FlgDΔGRM) should be concentrated to 1.6 mM (*see Note 9*).
3. For each ITC experiment, use a heating block to pre-warm (to 20 °C) 2.0 mL of ITC assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), 2 mL of 50 µM GST or (GST)FlhB_C in ITC assay buffer, and 200 µL 1.6 mM protein ligand (untagged FlgD or untagged FlgDΔGRM) in ITC assay buffer.
4. Pass 100 mL degassed, sterile Milli-Q water through the sample cell of the calorimeter using a vacuum pump.
5. Fill the reference cell with ITC assay buffer.
6. Equilibrate the sample cell of the calorimeter by passing 10 mL ITC assay buffer through the cell using a 5 mL syringe and the loading needle provided with the calorimeter. The loading needle should be as straight as possible to avoid touching the sample cell walls (*see Note 10*).
7. Load 1.8 mL degassed and pre-warmed GST, (GST)FlhB_C or ITC assay buffer control into the sample cell (*see Note 11*).
8. Load the calorimeter syringe with 200 µL protein ligand in ITC assay buffer (in this case untagged FlgD or untagged FlgDΔGRM). Use the system software to slowly push the plunger until the air is expelled from the syringe (*see Note 11*).
9. Set experimental parameters on the calorimeter's control software. Input the protein and ligand concentrations. Sequentially inject the protein sample until it is fully titrated against the ligand. The parameters provided below are specific for (GST)FlhB_C and FlgD measurements, but may need to be modified for other proteins and ligands.

- (a) Initial injection of ligand: 0.5 μL for 200 s.
- (b) Remaining injections (29): 4 μL , 200 s between injections. The final titration volume is 116.5 μL (*see Note 12*).
10. Using Origin[®] software, find the integral of the calorimetric signal after each injection and apply a best-fit line. The baseline, where Origin[®] calculates the top of each data point, can be altered by the investigator but this is not advised (*see Note 13*).
11. Using Origin[®], produce a final figure from the experimental data. This will show both the $\mu\text{cal/s}$ of each injection (top plot) and kcal/mol of injectant against the molar ratio of protein and ligand (bottom plot) with a best (single) fit line. Origin[®] will provide an association constant (K_a) that can be converted into the dissociation constant using $K_d = 1/K_a$.
12. Other data provided by Origin[®] include the Chi-squared value, the R score, the stoichiometry (n), and the enthalpy of binding (ΔH_b). These are automatically calculated by Origin[®] and provided with the K_a .

3.8 Photo-Crosslinking of Purified Subunit-Export Gate Complexes

1. Prepare co-purified protein complexes (as described under Subheading 3.6) of (GST)FlhB_CN₂₆₉A with FlgD subunit derivatives containing the Uaa p*Bpa* in either the GRM (L₃₉Uaa or L₄₀Uaa) or elsewhere (L₅Uaa). A 1 mL elution fraction from 1 mL GSH-resin yields $\sim 100 \mu\text{M}$ of FlgD-(GST)FlhB_CN₂₆₉A complex.
2. Dialyze the FlgD-(GST)FlhB_CN₂₆₉A complexes for 2 h against 2 L of PBS in a 5 L plastic beaker at 4 °C.
3. Transfer 200 μL aliquots of the co-purified complexes (100 μM) of FlgD-(GST)FlhB_CN₂₆₉A into the wells of a polystyrene 24-well plate mounted in a shallow box containing ice.
4. Set aside equivalent amounts of protein complexes as non-irradiated control samples.
5. Place the samples in the polystyrene 24-well plate mounted in a shallow box containing ice 5 cm beneath a lamp that emits 350 nm light.
6. With the cooling fan on, UV irradiate (350 nm) the samples for 5 min, and then remove the samples in the polystyrene 24-well plate mounted in a shallow box containing ice and allow to cool for 5 min. Repeat this process eight times, replacing the ice surrounding the plate as necessary.
7. Precipitate 200 μL of each sample with 10% (v/v) TCA at 4 °C for 1 h, then centrifuge at $16,000 \times g$ for 15 min to pellet the precipitated proteins. Decant supernatant and wash precipitated protein pellet with two volumes of ice-cold 100% acetone, centrifuge at $16,000 \times g$ for 15 min, decant the supernatant, and air-dry the resulting protein pellet. Resuspend

the dried protein pellet in 100 μ L of SDS-PAGE sample buffer and heat at 100 °C for 10 min. Cool the samples on ice and load 15 μ L directly onto SDS-PAGE gels (15% and 12.5% polyacrylamide). Run the electrophoresis at 200 V until the loading dye migrates out of the gel.

8. The formation of cross-linked protein adducts is assessed by immunoblotting with specific antisera against FlhB, FlgD, or GST. Briefly, transfer separated protein complexes from SDS-PAGE gels to nitrocellulose membranes using wet blotting transfer equipment and blotting transfer buffer at 400 mA for 1 h. Transfer nitrocellulose membranes into 20 mL immunoblotting blocking buffer in a square plastic dish and incubate overnight at 4 °C. Wash blots with PBS and then incubate blots in 20 mL PBS containing the appropriate antisera. Wash blots with 20 mL PBS-Triton for 15 min. Repeat wash step five times. Incubate blots in 20 mL PBS containing the appropriate IRDye-conjugated goat-anti-rabbit secondary antibodies (Licor) for 1 h in the dark. Wash blots with 20 mL PBS-Triton for 15 min in the dark. Repeat wash step five times. Image blots using a Licor Odyssey® CLx imaging system.
9. Compare irradiated samples to non-irradiated samples. Determine the percentage of cross-linking by integrating the band density corresponding to the cross-linked and non-cross-linked material. Typically, efficiency of cross-linking is relatively low (>30%).

3.9 Capture Assay

1. Purify FlgE Δ 39–43 (Δ GRM) and FlgE Δ 39–43, Δ 360–403 (Δ GRM, Δ Ct) (*see* Subheading 3.5 and **Note 14**) and store on ice.
2. Prepare a co-purified protein complex of (GST)FlhB_C-FlgD_{FLAG} (*see* Subheading 3.6).
3. Split the GSH-resin-bound complex of (GST)FlhB_C-FlgD_{FLAG} into 50 μ L aliquots in 2 mL microcentrifuge tubes.
4. Prepare 1 mL solutions of increasing concentrations (0, 0.1, 1, and 10 μ M) of purified FlgE Δ 39–43 (Δ GRM) and FlgE Δ 39–43, Δ 360–403 (Δ GRM, Δ Ct).
5. In 2 mL microcentrifuge tubes, mix each 1 mL aliquot of the FlgE variants described in **step 4** with a 50 μ L aliquot of resin-bound complex of (GST)FlhB_C-FlgD_{FLAG} and incubate on a bench-top tube rotator at room temperature for 20 min.
6. Place the samples in the 2 mL microcentrifuge tubes on ice for 10 min to allow the resin to settle.
7. Collect the supernatant fractions containing unbound/captured proteins using a pipette and take a sample (900 μ L) for analysis. Precipitate the proteins in the analysis samples, as described under Subheading 3.8, **step 11**.

8. Wash the resin with five volumes of wash buffer (for GST-fusion proteins, Table 4).
9. Repeat **step 6** and discard the supernatant.
10. Elute remaining resin-bound proteins ([GST]FlhB_C-FlgD_{FLAG}) by adding one volume of SDS-PAGE loading buffer to the resin and heating at 80 °C for 10 min.
11. Adjust the 10 µM subunit-challenged unbound/captured fractions (from **step 7**) to contain 5 mM imidazole and 0.5% BSA (w/v).
12. Add 80 µL of 50% suspension of Ni-NTA resin prewashed in buffer (50 mM sodium phosphate pH 7.4, 200 mM NaCl, 1 mM β-mercaptoethanol) to the 10 µM subunit-challenged unbound/captured fractions and incubate on a bench-top tube rotator at room temperature for 1 h.
13. Repeat **step 6** and discard the supernatant.
14. Elute resin-bound (subunit-captured) proteins by adding one volume of SDS-PAGE loading buffer to the resin and heating at 80 °C for 10 min.
15. For the unbound/captured samples (from **step 7**) and the subunit-captured samples eluted from the Ni-NTA (from **step 14**), load 5 µL onto a SDS-(15%)PAGE gel. Run the electrophoresis at 200 V until the loading dye migrates out of the gel and analyze by immunoblotting with antisera against FlhB_C, FlgE, and the FLAG epitope (as described under Subheading 3.8, **step 12**).

4 Notes

1. When selecting codons for substitution to amber codons, avoid codons for highly conserved residues as substitution may alter protein function or expression.
2. Always freshly prepare the *p*Bpa sodium hydroxide solution. We occasionally observe *p*Bpa precipitating out of solution on addition, but this has no effect on protein expression.
3. It is best to perform the wash in two or three steps. When purifying a single protein, you can use more than ten volumes of wash buffer; however, you may lose some protein with additional washes.
4. For untagged proteins, use an increasing gradient (usually over five to ten volumes) of NaCl to elute protein. Collect 0.5 mL elution fractions.
5. For some proteins, it is possible to dialyze overnight with 100–300 times more buffer than sample with little loss of protein. Other proteins might precipitate over this time, in which case

use shorter dialysis times, e.g., 2–3 h, with a buffer change every hour. It is important to be sure that all proteins are in the same buffer before beginning an experiment (such as ITC).

6. To prepare the cleared cell lysate containing FlgD_{FLAG}, lyse the resuspended cells expressing recombinant FlgD_{FLAG} using a French© pressure cell. Aim to use a cell wet weight-to-buffer ratio of 1:1 to obtain a high FlgD_{FLAG} concentration.
7. Ensure that you saturate the (GST)FlhB_C-bound resin with FlgD_{FLAG}. The binding capacity of glutathione sepharose 4B resin is >25 mg horse liver GST/mL resin. Aim to saturate the GST-FlhB_C-bound resin with at least twice the molar ratio of FlgD_{FLAG} to (GST)FlhB_C.
8. The dissociation constant of FlgD for FlhB_C is 39 μ M [5]. As this is a low affinity interaction, it is important to wash the subunit-gate complex (GST)-FlhB_C-FlgD_{FLAG} with a low volume of wash buffer to avoid dissociating the complex.
9. You can use a spectrophotometer, such as a Nanodrop (Thermo), or a commercial Bradford assay (e.g., DCTM Protein Assay, Bio-Rad) to measure protein concentration. It is critical to use a technique that gives a precise and reliable measure of protein concentration for use in ITC.
10. Be sure not to scratch the sides of the sample cell as this can damage the experiment and lead to poor data.
11. The MicroCal control software allows control of the plunger with 1 μ L increments. This is handy to lower the plunger until only protein is left in the syringe. Be sure to purge and refill once the air has been expelled from the syringe. The plunger should sit at the liquid interface and there should not be any empty space between it and your protein. If there is, the volume injected into the sample cell may be incorrect.
12. You may need to adjust the length of each injection to allow for a return to a temperature baseline, depending on the protein injected into the sample cell.
13. The heats of reactions of the buffer with (GST)FlhB_C and the buffer with ligand should be subtracted from the heat of reaction of (GST)FlhB_C with ligand. This is accomplished by collecting that experimental data and adding it to the spreadsheets containing the FlhB_C-ligand reaction data. A final column with the recalculated data should be used to analyze the association of FlhB_C and ligand (e.g., FlgD).
14. FlgE has a short motif comprising a conserved phenylalanine followed by three residues and a hydrophobic residue (Fxxx Φ) termed the gate recognition motif (GRM). The two FlgE derivatives used in the capture assay lack the GRM and can no longer interact with FlhB_C and hence are unable to compete with FlgD_{FLAG} docked on GST-FlhB_C.

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