Abstract

For assembly of the bacterial flagellum, most of flagellar proteins are transported to the distal end of the flagellum by the flagellar type III protein export apparatus powered by proton motive force (PMF) across the cytoplasmic membrane. FlhA is an integral membrane protein of the export apparatus and is involved in an early stage of the export process along with three soluble proteins, FliH, FliI, and FliJ, but the energy coupling mechanism remains unknown. Here, we carried out site-directed mutagenesis of eight, highly conserved charged residues in putative juxta- and trans-membrane helices of FlhA. Only Asp-208 was an essential acidic residue. Most of the FlhA substitutions were tolerated, but resulted in loss-of-function in the ΔfliH-fliI mutant background, even with the second-site flhB(P28T) mutation that increases the probability of flagellar protein export in the absence of FliH and FliI. The addition of FliH and FliI allowed the D45A, R85A, R94K and R270A mutant proteins to work even in the presence of the flhB(P28T) mutation. Suppressor analysis of a flhA(K203W) mutation showed an interaction between FlhA and FliR. Taken together, we suggest that Asp-208 is directly involved in PMF-driven protein export and that the cooperative interactions of FlhA with FliB, FliH, FliI, and FliR drive the translocation of export substrate.

Introduction

The flagellum of Salmonella enterica is a supermolecular motor powered by an electrochemical potential difference of protons (PMF) across the cytoplasmic membrane. The flagellum consists of at least three parts: the basal body, the hook, and the filament. Flagellar assembly begins with the basal body, followed by the hook and finally the filament. Almost all the substructures of the flagellum lie beyond the cytoplasmic membrane. Most of flagellar proteins are transported to the distal end of the growing flagellum by the flagellar type III protein export apparatus [1–4]. The components of the export apparatus are highly homologous not only to those of the type III secretion system of pathogenic bacteria, which directly injects virulence effectors into eukaryotic host cell [5] but also to those of FOFl-ATP synthase, which consists of a water soluble F1 part, which is a ring complex having three catalytic sites for ATP synthesis/hydrolysis, and a membrane-integrated FO part, which mediates proton translocation [6–8].

The flagellar type III protein export apparatus consists of three soluble proteins (FliH, FliI, FliJ) and six integral membrane proteins (FlhA, FlhB, FlhO, FlhP, FlhQ, FlhR) (Figure S1) [9,10]. The export apparatus is believed to be located in the putative central pore of the basal body MS ring [11–13]. FliI is an ATPase [14] and forms a complex with FliH and FljJ [7,10,15,16]. FliI and FliJ bind to the FlgN-FlgK and FliT-FliD chaperone-substrate complexes [17–19]. The FlhI-FlhJ delivers export substrates to the export gate complex made up of the six integral membrane proteins [20,21]. A specific interaction of the FlhX-FlhL-FljJ ring complex with the docking platform formed by the cytoplasmic domains of FlhA and FlhB induces the initial entry of the substrates into the gate [7,22,23]. The export gate complex utilizes PMF across the cytoplasmic membrane as the energy source for the translocation of the substrates [22,24]. It has been shown that a fliO null mutant displays a weakly motile phenotype, suggesting that FljO is not directly involved in flagellar protein export [25]. Interestingly, a homologue of FljO is apparently absent in some other type III secretion systems [25].

FlhA is composed of an N-terminal integral membrane domain with eight predicted transmembrane (TM) helices (FlhATM, residues 1–327, 34.5 kDa) and a C-terminal cytoplasmic domain (FlhAC, residues 328–692, 40.5 kDa) (Figure 1) [26]. A well-conserved hydrophilic cytoplasmic loop between TM-4 and TM-5 is indispensable for FlhA function, but little is known about its role in flagellar protein export [27]. FlhAC interacts with FliH, FliI, FliJ, the C-terminal cytoplasmic domain of FlhB (FlbBC), and the Fls-SflC and FliT-FliD chaperone-substrate complexes and initiates the translocation of the substrates [10,23,28,29]. FlhAC consists of four subdomains (D1, D2, D3, and D4) and a linker connecting FlhAC to FlhATM (Figure 1) [29–31]. The linker is involved in an interaction with FljJ [29]. The D2 subdomain is responsible for an interaction with the FljT-FliD and Fls-SflC complexes [29]. The D4 subdomain is dispensable
between FlhA and FliR.

We also show an interaction protein export and that most of substitutions are tolerated by the charged residue at position 208 of FlhA is critical for PMF-driven membrane helices of FlhA. We show that only a negatively conserved charged residues in the putative juxta- and trans-membrane helices of FlhB. Closed circles in red and blue indicate invariant acidic and basic residues, respectively, which were identified by multiple sequence alignment of FlhA homologs (Figure S1).

doi:10.1371/journal.pone.0022417.g001

for its function but is involved in the substrate specificity switching of the export apparatus [32]. Although FlhATM is required for the association of FlhA with the MS ring [12], it remains unknown whether it is directly involved in the PMF-driven protein export process.

The flagellar type III protein export apparatus shows many similarities with FOF1-ATP synthase [3,6–8]. An inward-directed proton translocation through the FO part drives the rotation of the FO-c-ring as a rotor, and the γ and ε subunits act as a drive shaft to cause conformational changes in the F1 part that result in ATP synthesis. ATP hydrolysis by F1 drives the reverse rotation of the rotor, resulting in an outward-directed proton pumping. Two highly conserved charged residues, Arg-210 in the a subunit and Asp-61 in the c subunit of the E. coli FOF1-ATP synthase are critical for proton translocation [33]. Since FlhATM is the main component of the PMF-driven flagellar protein export gate, there is the possibility that the highly conserved charged residues of FlhATM may be involved in the energy transduction mechanism.

In order to clarify the role of FlhATM in PMF-driven flagellar protein export, we performed genetic analyses of eight highly conserved charged residues in the putative juxta- and trans-membrane helices of FlhA. We show that only a negatively charged residue at position 208 of FlhA is critical for PMF-driven protein export and that most of substitutions are tolerated by the presence of FlhB, FliH and FliI. We also show an interaction between FlhA and FliR.

**Results**

**Alanine mutagenesis of conserved charged residues of FlhATM**

To test the hypothesis that highly conserved charged residues of FlhATM is involved in the energy transduction mechanism, we identified eight highly conserved charged residues, Asp-45, Arg-85, Arg-94, Lys-203, Arg-206, Asp-208, Asp-249 and Arg-270, in putative juxta- and trans-membrane helices of FlhA by multiple sequence alignment (Figures 1 and S2) and then replaced each with alanine. Immunoblotting with polyclonal anti-FlhAC antibody detected all the point mutant variants at the wild-type level (Figure 2A), indicating that these substitutions do not affect protein stability.

We next analyzed the motility of the flhA null mutant expressing each of the FlhA point mutant variants in soft agar plates (Figure 2B). FlhA(R206A) and FlhA(R270A) fully complemented, FlhA(D208A) and FlhA(R206A) restored motility to a significant degree, and FlhA(K203A) to some degree. However, mutants with alanine substitution for Arg-94, Asp-208 and Asp-249 did not complement at all. To test whether their poor motility is due to their reduced export activity, we prepared the culture supernatants from the overnight culture of these flhA point mutants and analyzed the secretion levels of the hook-capping protein FlgD by immunoblotting with polyclonal anti-FlgD antibody (Figure 2C). FlgD was detected at the wild-type level in the culture supernatants from the D45A, R85A, K203A, R206A, and R270A mutants but not from the R94A, D208A, and D249A mutants. FlhA(R94A), FlhA(D208A) and FlhA(D249A) also inhibited motility when expressed in the wild-type strain (Figure 2D), indicating that these mutant proteins exert a negative dominance. This suggests that they can be incorporated into the export apparatus. Therefore, we conclude that Arg-94, Asp-208, and Asp-249 are critical for protein export.

**Importance of the charge of critical residues**

To probe the role of Arg-94, Asp-208 and Asp-249 in protein export, we mutated these three residues to the following two types: the same charge with a different length of side chain (Arg-to-Lys and Asp-to-Glu); and the oppositely charged residue (Arg-to-Asp and Asp-to-Lys). Immunoblotting with the polyclonal anti-FlhAC antibody revealed that all the mutant variants were as stable as the wild-type (Figure 3A). At positions 94 and 249, neither types of mutations affected the secretion level of FlgD (Figure 3B, lanes 3, 4, 8 and 9), indicating that these residues maintain the function of FlhA regardless of the charge type. While the D208E replacement still permitted the export of FlgD at the wild-type level (Figure 3B, lane 5) the D208K and D208N mutations totally diminished the export (lanes 6 and 7). In agreement with these results, the D208K and D208N mutants as well as the flhA null mutant harboring the vector control accumulated much higher amounts of FlgD in the cytoplasm than the wild-type while the others did so more or less at the wild-type levels. FlhA(D208K) and FlhA(D208N) also exerted a dominant negative effect on wild-type motility (data not shown), indicating that they retain the ability to be incorporated into the export apparatus. These results suggest that a negatively charged residue at position 208 of FlhA is essential for the export function.

**Protein secretion rate of slow motile mutants**

We found that the motility of the flhA(D208E) (data not shown) and flhA(K203A) (Figure 2B) mutants was worse than that of wild-type cells in soft agar plates whereas the levels of FlgD secretion by these mutants were at the wild-type level when grown overnight in
LB (Figure 3B, lane 5, and Figure 2C, lane 6, respectively). These results raise the possibility that these mutants are slow secretors. Secretion rate measurement of the flagellar proteins requires the external onset control of flagellar gene expression. To do this, we inserted a Tn10d (T-POP) transposon upstream of the flagellar master flhDC operon, which is required for the expression of the entire flagellar regulon. As flhDC is transcribed from a tetracycline-inducible promoter PtetA only in the presence of tetracycline, flagellar gene expression can be externally controlled [34]. A flhA null mutant containing T-POP was transformed with pUC19-based plasmids encoding wild-type FlhA or each of the FlhA mutants. The transformants were grown at 30°C in LB until OD600 reached ca. 0.5–0.6. After induction with tetracycline, the culture supernatants were collected at regular time intervals and analyzed by immunoblotting with polyclonal anti-FlgD antibody (Figure 4).

For wild-type FlhA, FlgD was detected at 60 min after induction and the amount of FlgD gradually increased as the incubation was continued. The D208E mutation caused a 15 min delay in the detection of FlgD. In agreement with this, when comparing the length of flagellar filaments labeled with a fluorescent dye, the filaments of these mutants were significantly shorter than those of the wild-type at 120 minutes after tetracycline induction (data not shown). When the incubation was further continued for 12 hours, the filaments grew nearly to the wild-type level. These results suggest that the D208E mutation reduces the rate of protein export. For the flhA(K203A) mutant, FlgD was not detected until 120 min after induction, indicating that this is a slow secretor than the flhA(D208E) mutant.

Effect of flhA mutations on flagellar protein export in the ΔflhH-flhI double null mutant background

It has been previously reported that a ΔflhH-flhI double null mutant is weakly motile [22]. We tested if the flhA mutations that did not affect protein export are also tolerated in the absence of FlhH and FlhI (Figure 5). The weakly motile phenotype of the ΔflhH-flhI mutant was totally abolished by the flhA mutations (Figure 5A), indicating that these mutant FlhA proteins have a more synergistic phenotype when combined with the flhH-flhI double null mutation.

Minamino & Namba [22] isolated a ΔflhH-flhI bypass mutant whose second-site P28T mutation in FlhB significantly increases the probability of substrate entry into the gate in the absence of FlhH and FlhI. Therefore, we tested if the mutant FlhA proteins can work in this ΔflhH-flhI bypass mutant (Figure 5B). Wild-type FlhA restored the motility of the ΔflhH-flhI flhB(P28T) ΔflhA mutant, indicating that these mutant FlhA proteins have a more synergistic phenotype when combined with the flhH-flhI double null mutation.

Tryptophan mutation can probe the structure and environment of transmembrane domains of membrane proteins [35–37]. We

Figure 2. Effect of alanine substitutions in FlhATM on motility and flagellar protein export. (A) Expression levels of alanine-substituted variants of FlhA in cells. Immunoblotting, using polyclonal anti-FlhAC antibody, of whole cells protein (Cell) prepared from a flhA null strain, NH0001 (ΔflhA), transformed with pUC19-based plasmids encoding various alanine-substituted forms of FlhA. V, pUC19; WT, wild-type FlhA; D45A, FlhA(D45A); R85A, FlhA(R85A); R94A, FlhA(R94A); K203A, FlhA(K203A); R206A, FlhA(R206A); D208A, FlhA(D208A); D249A, FlhA(D249A); R270A, FlhA(R270A). (B) Motility of the flhA null mutant transformed with the above plasmids in soft agar. The plate was incubated at 30°C for 6 hours. (C) Secretion of FlgD. Immunoblotting, using polyclonal anti-FlgD antibody, of culture supernatants (Sup) prepared from the above strains. (D) Dominant negative effect on motility of the wild-type strain SJW1103 in soft agar. The plate was incubated at 30°C for 6 hours.

doi:10.1371/journal.pone.0022417.g002
performed tryptophan mutagenesis to test if the ΔflhB(P28T) mutation and the FliH-FliI complex affect the structural architecture of FlhATM. The flhA(K203W) and flhA(R270W) mutants in the otherwise wild-type background remained functional mostly and to some degree, respectively, while the others were nonfunctional (Figure S3). Since tryptophan substitution for residues facing the lipid preserves the function but is likely to disrupt the structure and function for residues within or adjacent to transmembrane segments, the results indicate that Lys-203 and Arg-270 of FlhA are likely to face the lipid or water-exposed channel of the export gate complex. We then analyzed the effect of the K203W and R270W mutations on motility of the ΔflhB(P28T) mutant and the ΔflhB(P28T) mutant. The motility of these mutants was significantly reduced compared with the wild-type (Figure 6C). Since Lys-203 and Arg-270 are predicted to be located in the conserved cytoplasmic loop between TM-4 and TM-5 and within TM-6, respectively (Figure 1), these residues may face the interior of the gate complex in the presence of the flhB(P28T) mutation.

Isolation of pseudorevertants from the ΔfliH-fliI flhB(P28T) flhA(K203W) mutant

To investigate how the K203W mutation significantly reduces the export activity in the ΔfliH-fliI bypass mutant, gain-of-function mutants were isolated from the ΔfliH-fliI flhB(P28T) flhA(K203W) mutant. In total three pseudorevertants were obtained with significantly improved motility although not as good as that of the wild-type (Figure 7A). DNA sequencing revealed that the second-site mutations are located in FliR, and they were G103A, G103C and G117D (Figure 7B). These mutated residues are not conserved among FliR homologs (Figure S4). They were all located in a cytoplasmic loop between TM-2 and TM-3, suggesting that the conserved loop between TM-4 and TM-5 in FlhA may interact with the cytoplasmic loop of FliR.

To test whether these extragenic fliR suppressors are allele specific for the parental flhA(K203W) mutation, we carried out P22-mediated transductional crosses. The ΔfliH-fliI ΔflhA flhB(P28T) fliR::Tn10 strain harboring the pUC19-based plasmid encoding each mutant FlhA protein was used as a recipient and the three pseudorevertants isolated were used as donors. A 50 μl solution of the overnight culture of the recipient and a 5 μl aliquot of phage lysates prepared from the donors were mixed together and then streaked on soft agar plates. After 40 hours incubation at 30°C, we looked for motility halos resulting from homologous recombination between the fliR::Tn10 and the suppressor fliR alleles. Motility halos appeared from the recipient expressing...
FlhA(K203W) but not from those expressing FlhA(K203A) or FlhA(R270W) as shown by the data of the fliR(G103A) allele (Figure S5A). These results indicate that these fliR suppressors display allele specificity. This suggests an interaction between the conserved cytoplasmic loop of FlhA and the cytoplasmic loop of FliR.

To test the effect of each second-site fliR mutation by itself on motility, we isolated the second-site fliR mutants and analyzed their motility on soft agar plates. In all cases motility was nearly normal, just slightly less than the wild-type (Figure S5B), indicating that they do not affect the function of FliR significantly.

**Discussion**

FlhAC not only acts as part of the docking platform of the export gate complex for the soluble export components, FliH, FliI, and FliJ, but also plays a critical role in PMF-driven flagellar protein export along with FliH, FliI, and FliB [23]. However, little is known about the role of FlhATM in PMF-driven flagellar protein export. Here, we carried out genetic analysis of eight highly conserved charged residues of FlhA TM and provide evidence suggesting that Asp-208 is critical for the translocation of export substrate and that the cooperative interactions of FlhA with FlhB, FliH, FliI, and FliR facilitates PMF-driven flagellar protein export.

**Asp-208 of FlhA is essential for flagellar protein export**

Two conserved charged residues of *E. coli* F0F1-ATP synthase, Arg-210 of the a subunit and Asp-61 of the c subunit, are critical for proton flow though F0, which is coupled with ATP synthesis/hydrolysis by F1 [38-40]. Asp-61 is directly involved in proton translocation [39]. Arg-210 is essential for preventing the short-circuiting of proton flow without c-ring rotation in the F0 proton channel [40]. Since there are many similarities between the flagellar type III protein export apparatus and F0F1-ATP synthase [3], the highly conserved acidic and basic residues of the transmembrane components of the flagellar export apparatus may be directly involved in the inwardly-directed proton flow. If so, the function of putative proton-binding sites in the export gate is presumably critical. In this study, we probed the function of highly conserved charged residues in trans- or juxta-transmembrane helices of FlhA by mutating these charged residues and found that only Asp-208 of FlhA is critical for the functioning of the export apparatus (Figure 2 and 3). The D208A, D208W, D208K, and D208N mutant alleles were dominant to the wild-type *flhA* gene and hence totally inhibited motility of wild-type cells. This suggests that these mutants retain the ability to be incorporated into the export apparatus. Because only the conservative D208E replacement permitted any function, the important feature of this residue appears to be either a negative side-chain charge or the ability to bind a proton. Asp-208 of FlhA is predicted to be located in the cytoplasmic juxtamembrane region (Figure 1), whereas both Arg-210 of a subunit and Asp-61 of e subunit are located at the center of a transmembrane helix.

This suggests that Asp-208 in FlhA may not directly be involved in the proton translocation mechanism. But since Asp-208 is involved in PMF-driven flagellar protein export, we assume that a negative side-chain charge of Asp-208 may be required for an electrostatic interaction with other export component(s) and/or export substrates.

A degree of defectiveness for the individual fliA alleles is dependent on the presence of FliB, FliH and FliI

In this study, we found that most of substitutions of evolutionally conserved charged residues except for Asp-208 were tolerated when FliH and FliI were present. In contrast, these substitutions totally abolished a weakly motile phenotype of a ΔfliH-fliI double null mutant. The P28T mutation in FliB increases the probability...
of export substrate entry into the narrow pore of the export gate in the absence of FliH and FliI [22]. Here, we also found that those FlhA mutants did not function in the ΔfliH-fliI flhB(P28T) bypass mutant (Figure 5B). FliH and FliI overcame the D45A, R85A, R94K and R270A defects even in the presence of the flhB(P28T) allele (Figure 6A), indicating that the flhB(P28T) significantly affects the function of these mutant FlhA proteins and that FliH and FliI restore their function. Taken all together we suggest that these mutant FlhA proteins require the FliH-FliI complex and FlhB to exert their export function.

Tryptophan substitution at positions 203 and 270 in FlhATM significantly reduced the export activity when the FlhB(P28T) mutation was present (Figure 6C). Tryptophan substitution is assumed to preserve the protein function when the mutated residues face lipid or water but to disrupt structure and function when the residues face adjacent protein segments [35–37]. Mutant FlhA(K203W) and FlhA(R270W) proteins retained the FlhA function when FlhB, FliH, and FliI were present (Figure 6C), indicating that the orientation of the side-chain of these tryptophan residues is significantly affected by FlhB, FliH and FliI. Therefore, we propose that cooperative interactions among FlhA, FlhB, FlhI, Flh and export substrate may induce conformational changes in FlhATM to facilitate PMF-driven protein export.

Interaction of the well-conserved cytoplasmic loop between TM-4 and TM-5 of FlhA with a cytoplasmic loop of Flir

A highly conserved hydrophilic cytoplasmic loop is located between TM-4 and TM-5 of FlhA, but little is known about its role in flagellar protein export [27]. In this study, we found that the reduced motility of the ΔfliH-fliH flhB(P28T) flhA(K203W) mutant was significantly improved by extragenic mutations in the cytoplasmic loop between TM-2 and TM-3 of Flir (Figure 7). Interestingly, these suppressors were allele specific for the flhA(K203W) mutation, suggesting an interaction between the conserved cytoplasmic loop of FlhA and the cytoplasmic loop of Flir. The Flir-FlhB fusion protein is partially functional, suggesting that Flir interacts with FlhB [13]. Therefore, it is likely that FlhA, Flhb and FliI are very close to each other in the export gate complex. This is supported by our finding that the P28T bypass mutation, which is close to the interface of TM-1 of FlhB, significantly affects the function of mutant FlhA.

The K203A mutation considerably reduced the secretion rate of FlgD in a way similar to the D208E mutation (Figure 4). Since Lys-203 is likely to be close to Asp-208, we propose that the precise conformation of a negatively charged residue at position 208 of FlgD's cytoplasmic loop is critical for PMF-driven export.
FlhA may be important for protein export and that the interaction of the conserved cytoplasmic loop of FlhA with the cytoplasmic loop of FliR is required for maintaining this conformation.

Materials and Methods

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. L-broth and soft tryptone agar plates were used as described previously [9,10]. Tetracycline-sensitive (TcS) plates, in which the tetracycline-resistant cells cannot grow, were prepared as described by Maloy and Nunn [41]. Ampicillin and tetracycline were added to LB at a final concentration of 100 \( \mu \text{g/ml} \) and 15 \( \mu \text{g/ml} \), respectively.

Transductional crosses and DNA manipulations

P22-mediated transductional crosses were carried out using p22HTint as described previously [42]. DNA manipulations were carried out as described before [10]. Site-directed mutagenesis was carried out using QuickChange site-directed mutagenesis method as described in the manufacturer’s instructions (Stratagene) with a plasmid, pMMHA004 (pUC19/His-FlhA), as a template DNA. The pairs of oligonucleotides shown in Table 2 were used to construct each FlhA mutant. All of the flhA mutations were confirmed by DNA sequencing. DNA sequencing reactions were carried out using BigDye v3.1 as described in the manufacturer’s instructions (Applied Biosystems), and then the reaction mixtures were analyzed by a 3130 Genetic Analyzer (Applied Biosystems).

Construction of the flhA null strains

To delete the flhA gene from the chromosome, we used the \( \lambda \) Red homologous recombination system developed by Datsenko and Wanner [43]. First, the entire flhA gene was replaced with the tetRA genes to construct a \( \Delta \text{flhA}::\text{tetRA} \) strain as described [44]. Then, to remove the tetRA genes from the chromosome, the 112-bp DNA was first synthesized and then was purified using a QIAquick PCR purification kit (QIAGEN). The \( \Delta \text{flhA}::\text{tetRA} \) strain carrying pKD46, which has a temperature sensitive replicon [43], was grown in 5-ml L-broth containing ampicillin and 0.2% L-arabinose at 37°C until OD\(_{600}\) had reached 0.6. The cells were washed three times with ice-cold H\(_2\)O and suspended in 50 \( \mu \text{l} \) of ice-cold H\(_2\)O. 50 \( \mu \text{l} \) of cells were electroporated with 100 to 200 ng of purified 112-bp DNA using 0.1-cm cuvettes at 1.8 kV. Shocked cells were added to 1 ml SOC, and incubated for 1 h at 37°C. Then, one-half were spread onto TcS plates and incubated at 42°C overnight. The constructs were confirmed by DNA sequencing.

Motility assay

Fresh transformants were inoculated onto soft tryptone agar plates containing ampicillin and incubated at 30°C.

Preparation of whole cell and culture supernatant fractions and immunoblotting

Salmonella cells were grown at 30°C with shaking until the cell density had reached an OD\(_{600}\) of ca. 1.4–1.6. Aliquots of culture proteins containing a constant number of cells were clarified by centrifugation. Cell pellets were resuspended in SDS-loading buffer normalized by cell density to give a constant amount of cells. The proteins in the culture supernatants were precipitated by 10% trichloroacetic acid (TCA), suspended in a Tris-SDS loading buffer and heated at 95°C for 5 min. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting with polyclonal anti-FlgD or anti-FlhAC antibody was carried out as described previously [9]. Detection was done with an ECL immunoblotting detection kit (GE Healthcare).

Tetracycline-induced protein export rate assay

Salmonella cells with a T-POP insertion between the flhDC promoter and the transcription start site [34], were grown overnight in LB culture at 30°C and diluted 50-fold into 30 ml of fresh LB. When the cell density reached an OD\(_{600}\) of 0.5, tetracycline was added to a final concentration of 15 \( \mu \text{g/ml} \). Samples were taken at 0, 15, 30, 45, 60, 75, 90, 120 and 180 min after tetracycline addition. Proteins in the culture supernatant fraction were prepared by TCA precipitation as described above.
Table 1. Strains and Plasmids used in this study.

| Strains and Plasmids | Relevant characteristics | Source or reference |
|----------------------|--------------------------|---------------------|
| **Salmonella**        |                          |                     |
| SJW1103              | Wild type for motility and chemotaxis [42] |                     |
| NH0001               | ΔflhA flhB(P28T)         | This study          |
| NH0002               | ΔflhA flhB(P28T)         | This study          |
| NH0003               | ΔflhA flhB                 | This study          |
| NH0004               | ΔflhA flhB flhA flhB(P28T) | This study          |
| NH0005               | ΔflhA P_{pOC2}:T-POP(DEL-25) | This study          |
| NH0006               | ΔflhA flhB flhA flhB(P28T) fliR-Tn10 | This study          |
| NH0007               | ΔflhA flhB flhB(P28T) fliR(G103C)/pNH001(K203W) | This study          |
| NH0008               | ΔflhA flhB flhB(P28T) fliR(G103D)/pNH001(K203W) | This study          |
| NH0009               | ΔflhA flhB flhB(P28T) fliR(G117D)/pNH001(K203W) | This study          |
| NH0010               | fliR(G103C)               | This study          |
| NH0011               | fliR(G103D)               | This study          |
| NH0012               | fliR(G117D)               | This study          |
| **Plasmids**         |                          |                     |
| pUC19                | Cloning vector            | Invitrogen          |
| pMMHA004             | pUC19/His-FlhA            | [45]                |
| pNH001(D45A)         | pUC19/His-FlhA(D45A)      | This study          |
| pNH001(R85A)         | pUC19/His-FlhA(R85A)      | This study          |
| pNH001(R94A)         | pUC19/His-FlhA(R94A)      | This study          |
| pNH001(K203A)        | pUC19/His-FlhA(K203A)     | This study          |
| pNH001(R206A)        | pUC19/His-FlhA(R206A)     | This study          |
| pNH001(D208A)        | pUC19/His-FlhA(D208A)     | This study          |
| pNH001(D249A)        | pUC19/His-FlhA(D249A)     | This study          |
| pNH001(R270A)        | pUC19/His-FlhA(R270A)     | This study          |
| pNH001(D45W)         | pUC19/His-FlhA(D45W)      | This study          |
| pNH001(R85W)         | pUC19/His-FlhA(R85W)      | This study          |
| pNH001(R94W)         | pUC19/His-FlhA(R94W)      | This study          |
| pNH001(K203W)        | pUC19/His-FlhA(K203W)     | This study          |
| pNH001(R206W)        | pUC19/His-FlhA(R206W)     | This study          |
| pNH001(D208W)        | pUC19/His-FlhA(D208W)     | This study          |
| pNH001(D249W)        | pUC19/His-FlhA(D249W)     | This study          |
| pNH001(R270W)        | pUC19/His-FlhA(R270W)     | This study          |
| pNH001(R94K)         | pUC19/His-FlhA(R94K)      | This study          |
| pNH001(R94E)         | pUC19/His-FlhA(R94E)      | This study          |
| pNH001(D208E)        | pUC19/His-FlhA(D208E)     | This study          |
| pNH001(D208K)        | pUC19/His-FlhA(D208K)     | This study          |
| pNH001(D208N)        | pUC19/His-FlhA(D208N)     | This study          |
| pNH001(D249E)        | pUC19/His-FlhA(D249E)     | This study          |
| pNH001(D249K)        | pUC19/His-FlhA(D249K)     | This study          |

doi:10.1371/journal.pone.0022417.t001
Table 2. Primers used in this study.

| Mutated Residue | Primer | Sequence (5'-3') |
|-----------------|--------|-----------------|
| D45A            | F      | CCGCTACCTGCTTTATCCTCGCCTATTGTTTACCTTTAATATT |
|                 | R      | AATATTTAAAGTAAACATATTAAAGGCGAATTAAGCAAGTACGG |
| R85A            | F      | GATTACCACCTGCTGCTGGCGTCTAGCGTGAAGTGTG |
|                 | R      | CAACGTTAAGAGGCGAGACAGGTACGCTAGGAACATG |
| R94A            | F      | CTTAAGGCGCCTCAACGCGCCTATTGTTTACGTTTAAATT |
|                 | R      | CCGCAGTTAAGGGCGAGACAGGTACGCTAGGACGTG |
| K203A           | F      | GGACGGGGCAAGTGCATTTGTACGCGGCGAGG |
|                 | R      | GTCGCCGCGTACAAATGCACTTTGCAAGTGGAG |
| R206A           | F      | GCAATCCTGCTGCTGGCGTCTAGCGTGAAGTGTG |
|                 | R      | CAACGTTAAGAGGCGAGACAGGTACGCTAGGAACATG |
| R208A           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| R249A           | F      | CGTACCGTCTGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| K203W           | F      | ATGACGGGGCAAGTGCATTTGTACGCGGCGAGG |
|                 | R      | GTCGCCGCGTACAAATGCACTTTGCAAGTGGAG |
| R206W           | F      | GCAATCCTGCTGCTGGCGTCTAGCGTGAAGTGTG |
|                 | R      | CAACGTTAAGAGGCGAGACAGGTACGCTAGGAACATG |
| R208W           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D249W           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D45W            | F      | CCGCTACCTGCTTTATCCTCGCCTATTGTTTACCTTTAATATT |
|                 | R      | AATATTTAAAGTAAACATATTAAAGGCGAATTAAGCAAGTACGG |
| R85W            | F      | CTTAAGGCGCCTCAACGCGCCTATTGTTTACGTTTAAATT |
|                 | R      | CCGCAGTTAAGGGCGAGACAGGTACGCTAGGACGTG |
| R94W            | F      | GCGCTTAACGCGGCGCCTGCACTCGCCGCTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| R206W           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D208W           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D249K           | F      | CGTACCGTCTGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| R94K            | F      | GCGCTTAACGCGGCGCCTGCACTCGCCGCTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| R270W           | F      | GCGCTTAACGCGGCGCCTGCACTCGCCGCTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| R270K           | F      | GCGCTTAACGCGGCGCCTGCACTCGCCGCTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D208E           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D208K           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D208N           | F      | AAATTTGACGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D249E           | F      | CGTACCGTCTGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |
| D249K           | F      | CGTACCGTCTGCGGCGGCGGCGCTACGCGTATTC |
|                 | R      | GACCTACGCGCGGCGGCGCTACGCGTATTC |

For site-directed mutagenesis of FlhA.
F, forward; R, reverse.
doi:10.1371/journal.pone.0022417.t002
Multiple sequence alignment

PSI-BLAST searches were initiated on the NCBI's BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) under default conditions to obtain FlhA homologs. Multiple sequence alignment was done by CLUSTAL-W (http://clustalw.ddbj.nig.ac.jp/top-j.html).

Supporting Information

Figure S1  Schematic diagram of the bacterial flagellar type III protein export apparatus. The flagellar type III protein export apparatus consists of three soluble proteins, FlhI, FliJ, and Flj and six integral membrane proteins, FlhA, FlhB, FlhO, FliP, FliQ, and FliR. The integral membrane proteins are postulated to be located within the central pore of the MS ring and form the PMF-driven export gate complex, FlhA and FlhB have large cytoplasmic domains which project into the cavity within the C ring and form the docking platform for FlhI, Flil, and Flj as well as the substrates. Fili forms a heterotrimer with the FlhI dimer in the cytoplasm. The FliH2Flil complex, along with Flj and export substrates, is localized to the basal body C-ring through a specific interaction between FlhI and Fln. Fln hexamersizes upon docking of the FliH-Flil-Flj-substrate complex to the FlhA-FlhB platform and facilitates the entry of the N-terminal segment of a substrate into the gate. ATP hydrolysis by the Flil hexamer induces the dissociation of the FliHX-Flil-Flj complex from the gate. The exit gate utilizes PMF across the cytoplasmic membrane as the energy source for the translocation of the export substrates into the central channel of the growing flagellar structure.

Figure S2  Multiple sequence alignment of FlhA homologs. Multiple sequence alignment was carried out by CLUSTAL-W (http://clustalw.ddbj.nig.ac.jp/top-j.html). Green boxes encircle putative transmembrane domains. UniProt Accession numbers: Salmonella (P40729); Escherichia (P76298); Yersinia (O56887); Pseudomonas (Q44674); Aerifex (O62765); Caulibacter (Q30345); Vibrio (Q59764); Bacillus (Q30345); Helicobacter (O66758); InvA_Salmonella (P0A131); LcrD_Yersinia (P66555); SsaV_Salmonella (P74856). Red and blue shades stars indicate conserved acidic and basic residues, respectively, which are labeled with various colors. Putative transmembrane helices were encircled by green boxes. UniProt Accession numbers: Salmonella (P547029); Escherichia (P76298); Yersinia (O56887); Pseudomonas (Q44674); Aerifex (O62765); Caulibacter (Q30345); Vibrio (Q59764); Bacillus (Q30345); Helicobacter (O66758); InvA_Salmonella (P0A131); LcrD_Yersinia (P66555); SsaV_Salmonella (P74856). Red and blue shades stars indicate conserved acidic and basic residues, respectively, which are selected for site-directed mutagenesis.

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Acknowledgments

We acknowledge M. Kihara for critical reading of the manuscript and helpful comments, D. F. Blair and K. Paul for discussion and suggestions and Y. V. Morimoto for technical assistance. We also think M. Kihara, S. Nakamura and K. Ikeda for continuous support and encouragement.

Author Contributions

Conceived and designed the experiments: NH KN TM. Performed the experiments: NH TM. Analyzed the data: NH TM. Wrote the paper: NH KN TM.
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