The flexible linker of the secreted FliK ruler is required for export switching of the flagellar protein export apparatus

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The hook length of the flagellum is controlled to about 55 nm in Salmonella. The flagellar type III protein export apparatus secretes FliK to determine hook length during hook assembly and changes its substrate specificity from the hook protein to the filament protein when the hook length has reached about 55 nm. Salmonella FliK consists of an N-terminal domain (FliKn, residues 1–207), a C-terminal domain (FliKc, residues 268–405) and a flexible linker (FliKL, residues 208–267) connecting these two domains. FliKn is a ruler to measure hook length. FliKc binds to a transmembrane export gate protein FlhB to undergo the export switching. FliKn not only acts as part of the ruler but also contributes to this switching event, but it remains unknown how. Here we report that FliKL is required for efficient interaction of FliKc with FlhB. Deletions in FliKL not only shortened hook length according to the size of deletions but also caused a loose length control. Deletion of residues 206–265 significantly reduced the binding affinity of FliKc for FlhB, thereby producing much longer hooks. We propose that an appropriate length of FliKL is required for efficient interaction of FliKc with FlhB.

The bacterial flagellar hook is a tubular structure composed of the hook protein FlgE and acts as a universal joint to smoothly transmit torque produced by the flagellar motor to the long helical filament that functions as a propeller. The hook length of the Salmonella flagellum is controlled to about 55 nm, and the length control is important for the universal joint mechanism.

The flagellar type III protein export apparatus is located at the flagellar base and transports FlgE subunits from the cytoplasm to the distal end of the growing hook structure for hook assembly. Newly exported FlgE monomers polymerize onto the nascent hook structure with the help of the hook cap (FlgD), which is located at the hook tip. Interactions between FlgD and FlgE suppress the leakage of FlgE subunits into the culture media so that each FlgE subunit can be efficiently incorporated into the hook structure.

When hook length has reached about 55 nm, the flagellar type III protein export apparatus changes its substrate specificity, thereby terminating hook assembly and initiating filament assembly. FliK, FlhA, FlhB and Flk are directly involved in the export switching process. If certain mutations in FliK, FlhB or FlhA inhibits the export switching of the flagellar type III protein export apparatus, unusually elongated hooks called polyhooks are generated. FlhA and FlhB are transmembrane proteins of the flagellar protein export apparatus, and their C-terminal cytoplasmic domains (FlhAc, FlhBc) project into the cavity within the basal body C ring and provides binding-sites for cytoplasmic export components (FliH, FliI, FliJ) and flagellar export chaperones (FlgN, FliS, FliT) in with their cognate substrates. Interactions of FlhAc with FliH, FliI and FliJ seems to support the strict order of flagellar assembly. The interaction of FlhBc with FliK induces the export switching of the flagellar type III protein export apparatus. FlhBc binds to FliI, FliJ and export substrates such as FlgD and FlgE. Conformational changes of FlhAc and FlhBc...
are required for the substrate specificity switching of the flagellar protein export apparatus upon hook completion. FliK interferes with premature switching of the protein export apparatus during hook-basal body assembly. The flagellar type III protein export apparatus transports several FliK molecules during hook assembly to determine hook length in a way that the protein export apparatus switches its substrate specificity when the hook length has reached about 55 nm. Salmonella FliK is a 405 amino-acid protein consisting of the N-terminal ruler domain (FliK_N, residues 1–207), the C-terminal export switch domain (FliK_C, residues 268–405) and a flexible linker (FliK_L, residues 208–267) connecting these two domains. FliK_N contains a hook-type export signal recognized by the flagellar type III protein export apparatus. Insertions and deletions in FliK_N make the hook longer and shorter, respectively, suggesting that FliK_N is a molecular ruler. FliK_N binds to FlgD and FlgE and FliC. Immunoblotting using polyclonal anti-FliK (1st row), anti-FlgE (2nd row) or anti-FliC (3rd row) antibody, of whole cell proteins (Cell) and culture supernatants (Sup) from the above strains. The regions of interest were cropped from original immunoblots shown in Fig. S6a in the Supplemental information using a software, Photoshop CS6, and then the contrast and brightness were adjusted. The positions of molecular mass markers (kDa) are indicated on the left.  

Figure 1. Effect of deletions of five residues within the N-terminal region of FliK_N on FliK function. (a) Domain organization of FliK ruler. FliK consists of the N-terminal ruler domain (FliK_N), the C-terminal export switch domain (FliK_C) and a flexible linker (FliK_L) connecting these two domains. FliK_L has an intrinsically disordered C-terminal tail (FliK_CT). Amino acid sequence of FliK_L is shown. Proline residues are highlighted in red. (b) Motility of TH8426 harboring pTrc99AFF4 (Δflk), pMK002 (WT), pMMK1001 (Δ206–210), pMMK1002 (Δ211–215), pMMK1003 (Δ216–220), pMMK1004 (Δ221–225), pMMK1005 (Δ226–230) or pMMK1006 (Δ231–235) in 0.35% soft agar. Plates were incubated at 30°C for 7 hours. (c) Secretion assays of FliK, FlgE and FliC. Immunoblotting using polyclonal anti-FliK (1st row), anti-FlgE (2nd row) or anti-FliC (3rd row) antibody, of whole cell proteins (Cell) and culture supernatants (Sup) from the above strains. The regions of interest were cropped from original immunoblots shown in Fig. S6b using Photoshop CS6, and then the contrast and brightness were adjusted.
FliK<sub>c</sub> contains ten proline residues (Fig. 1a)<sup>39</sup> and hence is intrinsically disordered<sup>41</sup>. Hook lengths of the *Salmonella* fliK(Δ238–269) (32 residues deletion) and fliK(Δ248–269) (22 residues deletion) mutants are 40.2 ± 6.1 nm [mean ± standard deviation (SD)] and 51.0 ± 8.8 nm, where their average lengths are shorter than that of the wild-type strain (52.7 ± 4.5 nm)<sup>32</sup>. The length of the hook produced by the fliK(Δ161–216) mutant (56 residues deletion) is 48.7 ± 22.3 nm, where the average is also shorter than that of the wild-type strain by 4 nm. However, the SD value is larger than the wild-type one, indicating a much looser length control of the hook structure<sup>42</sup>. Furthermore, deletions of residues 161–223 and 161–244 cause polyhooks without the filament attached whereas a deletion of residues 208–269 results in the polyhooks with the filament attached (polyhook–filmament phenotype)<sup>42</sup>. These observations raise the possibility that FliK<sub>c</sub> not only acts as part of the ruler but also contributes to substrate specificity switching of the flagellar protein export apparatus. To clarify this hypothesis, we constructed a series of mutant variants of FliK<sub>c</sub> with in-frame deletions within FliK<sub>c</sub>. We show that a proper length of FliK<sub>c</sub> between FliK<sub>n</sub> and FliK<sub>c</sub> is required for efficient interaction of FliK<sub>n</sub> with FlhB<sub>c</sub>.

Results

**Effect of deletions of five amino-acid residues within residues 206–235 on hook length control.**

It has been shown that the N-terminal portion of FliK<sub>c</sub> is responsible for proper measurement of hook length<sup>42</sup>. To clarify the role of residues 206–235 of FliK<sub>c</sub> in the hook length control, we constructed a series of mutant variants of FliK<sub>c</sub> with sequential 5-amino-acid deletions within a region of residues 206–235, namely FliK(Δ206–210), FliK(Δ211–215), FliK(Δ216–220), (Δ221–225), (Δ226–230) and FliK(Δ231–235) (Table 1). These six fliK deletion variants fully restored motility of the ΔfliK mutant in 0.35% soft agar plates when they were expressed from the pTrc99A-based plasmid (Fig. 1b). Consistently, the levels of FlgE and FliC secreted by these deletion mutants were detected at the wild-type levels (Fig. 1c, 2nd and 3rd rows). These fliK deletions did not affect either protein stability or protein secretion into the culture media (Fig. 1c, 1st row). Therefore, we conclude that these in-frame deletions do not affect FliK function at all.

The length of the most extended polypeptide chain is 0.37 nm per residue. If FliK<sub>c</sub> adopts a fully extended conformation to act as part of the ruler, we predicted that these 5-amino-acid deletions within residues 206–235 of FliK<sub>c</sub> would reduce the hook length by 1.9 nm. Therefore, we measured the hook length of these fliK deletion mutants. The average hook length of the fliK(Δ206–210), fliK(Δ211–215), fliK(Δ216–220), fliK(Δ221–225), fliK(Δ226–230) and fliK(Δ231–235) mutants were 48.5 ± 4.5 nm (n = 113), 49.3 ± 5.2 nm (n = 168), 49.0 ± 5.6 nm (n = 130), 48.6 ± 4.2 nm (n = 118), 49.2 ± 4.2 nm (n = 232) and 49.6 ± 5.4 nm (n = 126), respectively, which were shorter than the length of the wild-type hook (53.3 ± 6.5 nm, n = 154) (Fig. S1). Over-expression of FliK slightly shortens the hook length. In contrast, when the expression level of FliK is reduced, the cell produces polyhooks, sometimes with the filament attached<sup>47,48</sup>. Polyhooks are frequently observed when FlgE is overproduced in wild-type cells<sup>47,48</sup>. Thus, the balance between the secretion levels of FlgE and FliK seems to be critical for the proper termination of the hook assembly. Since 5-amino-acid deletions within residues 206–235 shorten the hook length by 4 nm, which is shorter than the predicted value, we assume that these deletions may not affect only hook length measurements but also the secretion process of FliK by the type III protein export apparatus and/or the export switching process of the protein export apparatus induced by the interaction of FliK<sub>c</sub> with FlhB<sub>c</sub>.

To directly test if these 5 amino-acid deletions affect the interaction of FliK<sub>c</sub> with FlhB<sub>c</sub>, it is necessary to analyze the protein transport process of FliK and the export switching process of the flagellar type III protein export apparatus separately. To do so, we used a fliK(Δ2–99) allele, which is an export deficient variant of FliK, because it retains the ability to catalyze export switching of the flagellar type III protein export apparatus when over-expressed<sup>49</sup>. We introduced deletions of residues 206–210, 211–215, 216–220, 221–225, 226–230 or 231–235 into the fliK(Δ2–99) allele by inverse PCR method and analyzed motility of the ΔfliK mutant cells over-expressing FliK(Δ2–99) with these deletions in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). These additional deletions did not affect motility of the cells over-expressing FliK(Δ2–99) (Fig. 1d). Consistently, the inner and extra-cellular amounts of FliC seen in the fliK(Δ2–99 + Δ206–210), fliK(Δ2–99 + Δ211–215), fliK(Δ2–99 + Δ216–220), fliK(Δ2–99 + Δ221–225), fliK(Δ2–99 + Δ226–230) and fliK(Δ2–99 + Δ231–235) cells were the same as those in the fliK(Δ2–99) cells (Fig. 1e, 1st row). Since these deletions did not affect the expression level of FliK(Δ2–99) (Fig. 1e, 2nd row), we suggest that they do not affect the interaction of FliK(Δ2–99) with FlhB<sub>c</sub>. Therefore, we propose that these deletions may affect the protein transport process of FliK as well as hook length measurement.

To further understand the ruler function of residues 206–235 in FliK<sub>c</sub>, we constructed larger deletion variants, FliK(Δ206–215), FliK(Δ216–225), FliK(Δ226–235), FliK(Δ206–220), FliK(Δ221–235) and FliK(Δ206–235) and analyzed their motility in 0.35% soft agar plates (Fig. S2a). Motility of the fliK(Δ206–215), fliK(Δ216–225), fliK(Δ206–220), fliK(Δ221–235) and fliK(Δ206–235) cells was almost the same as wild-type motility whereas that of the fliK(Δ216–225) mutant was slightly less than the wild-type level (Fig. S2a). The cellular and secretion levels of these deletion variants were essentially the same as the wild-type levels (Fig. S2b). Because these deletion mutants were expressed from the pTrc99A vector, it is also possible that their over-expression results in motility comparable to the wild-type level. To verify this possibility, the wild-type fliK allele on the chromosomesal DNA was replaced by the fliK(Δ206–235) allele. Motility of the fliK(Δ206–235) mutant was slightly less than that of wild-type cells (Fig. S2c). To test whether the deletion of residues 206–235 affect hook length, we isolated hook-based bodies from the fliK(Δ206–235) mutant and measured the hook length (Figs. 2 and S3). A major peak of the hook length distribution was shifted to a shorter value than that of the wild-type (Fig. 2). While the majority of wild-type hook length was distributed within a range from 50 nm to 60 nm, the hook length distribution of the fliK(Δ206–235) mutant showed a major peak population between 41 nm and 50 nm. However, longer hooks were also observed albeit shorter than those of polyhooks produced by the ΔfliK mutant [362.8 ± 200.9 nm (N = 146)]. As a result, the average hook length of the fliK(Δ206–235) mutant was 54.5 ± 15.5 nm (n = 112), compared to 53.8 ± 5.6 nm (n = 130) for the wild-type. The SD value of the fliK(Δ206–235) mutant was larger than the wild-type one, indicating that the deletion of residues 206–235 in FliK<sub>c</sub> cause a looser hook length control.
than the wild-type. Therefore, we suggest that a deletion of residues 206–235 not only shortens the hook length according to the size of deletion but also affects the interaction of FliKC with FlhBC during hook assembly.

Effect of much larger deletions within FliK on hook length control and substrate specificity switching. To clarify the role of FliKL in the export switching process of the flagellar type III protein export apparatus, the wild-type fliK allele on the chromosome was replaced by the fliK(Δ206–245), fliK(Δ206–255) or

| Strains and Plasmons | Relevant characteristics | Source or reference |
|----------------------|--------------------------|---------------------|
| E. coli BL21 Star (DE3) | Overexpression of proteins | Novagen |
| Salmonella SJW1103 | Wild type for motility and chemotaxis | |
| THB426 |ΔfliK | |
| MMK1012 | fliK(Δ206–235) | This study |
| MMK1013 | fliK(Δ206–245) | This study |
| MMK1014 | fliK(Δ206–255) | This study |
| MMK1015 | fliK(Δ206–265) | This study |

Table 1. Strains and plasmids used in this study.
α∆fliK levels of FliC seen in these pseudorevertants were much higher than those in the ∆FliKC.

motility considerably (Fig. 3e). This suggests that these three deletions reduce the export switching function of FliK. FliK(∆2–99) mutants were 54.4 ± 1.7 nm (n = 226), 75.0 ± 8.9 nm (n = 160) and 107.1 ± 65.0 nm (n = 300), respectively, indicating that the hook length control becomes worse in the fliK(Δ206–255) and fliK(Δ206–265) mutants.

To investigate whether these larger deletions directly affect the export switching function of FliK, we introduces deletions residues 206–245, 206–255 or 206–265 into the fliK(Δ2–99) allele. Motility of the cells over-expressing FliK(Δ2–99) with these three deletions was reduced significantly (Fig. 3d), and especially the deletion of residues 206–265 reduced the cellular and extracellular levels of FliC by about 3-fold, thereby reducing motility considerably (Fig. 3e). This suggests that these three deletions reduce the export switching function of FliK.

To clarify why the deletion of residues 206–265 considerably reduces the export switching activity of FliK, we isolated fourteen pseudorevertants from the cells over-expressing FliK(Δ2–99) with an in-frame deletion of residues 206–265. Motility of these pseudorevertants was much better than that of its parent cells and was slightly better than that of the cells over-expressing FliK(Δ2–99) (Fig. 4a). Consistently, the cellular and extracellular levels of FliC seen in these pseudorevertants were much higher than those in the fliK(Δ2–99) cells and slightly larger than those in the fliK(Δ2–99) cells (Fig. 4b). These results indicate that the suppressor mutations enhance the probability of export switching to occur. DNA sequence analysis revealed that all suppressor mutations are insertion mutation of a DNA fragment encoding 100 amino-acid residues corresponding to residues 111–270 without residues 206–265 due to gene duplication (Fig. 4c). As a result, this intragenic FliK(∆2–99) mutant harbored pMMK1015SP [FliK(∆2–99) + pBPA] reproducibly formed a 51 kDa photo-crosslinked product along with FliBC (Fig. 4e, lane 2), in agreement with a previous report. However, FliK(∆2–99 + pBPA) did not form any photo-cross-linked products with FliBC (Fig. 4e, lane 4), indicating that the binding affinity of FliK(Δ2–99) for FliBC is lower than that of wild-type FliK. FliK(Δ2–99 + pBPA) reproducibly formed a 51 kDa photo-cross-linked product along with FliBC (Fig. 4e, lane 8) whereas FliK(Δ2–99 + pBPA) did not (lane 6). This suggests that the inserted sequence of the pseudorevertant increases the binding affinity of FliK(Δ2–99) for FliBC so that the export switching of the type III protein export apparatus occurs more efficiently.

Interaction between FliK(Δ2–99) and FlhBc

Targeted photo-crosslinking experiments have shown that Val-302 and Ile-304 of FliKc are in relatively close proximity of FliBc, allowing FliK to form a photo-crosslinked product with FlhBc. To investigate whether the deletion of residues 206–265 of FliKc and its suppressor insertion mutation reduces and increases the binding affinity of FliKc for FlhBc, respectively, we introduced an amber mutation at position 304 of FliK(Δ2–99), FliK(Δ2–99 + Δ206–265) and FliK(Δ2–99 + Δ206–265SP) to incorporate p-benzoyl-phenylalanine (pBPA), which is a photo-reactive phenylalanine and carried out photo-crosslinking experiments. We used FliK(I304amber) as a positive control. FliK(I304pBPA) produced a ca. 53 kDa photo-crosslinked product with FlhBc after UV irradiation (Fig. 4e, lane 2), in agreement with a previous report. However, FliK(Δ2–99 + I304pBPA) did not form any photo-cross-linked products with FlhBc (Fig. 4e, lane 4), indicating that the binding affinity of FliK(Δ2–99) for FlhBc is lower than that of wild-type FliK. FliK(Δ2–99 + Δ206–265SP + I304pBPA) reproducibly formed a 51 kDa photo-cross-linked product along with FlhBc (Fig. 4e, lane 8) whereas FliK(Δ2–99 + Δ206–265 + I304pBPA) did not (lane 6). This suggests that the inserted sequence of the pseudorevertant increases the binding affinity of FliK(Δ2–99) for FlhBc so that the export switching of the type III protein export apparatus occurs more efficiently.

Effect of the 100 residues suppressor insertion on the length of hook produced by the fliK(Δ206–265) mutant

To investigate whether the insertion mutation of the fliK(Δ2–99 + Δ206–265) suppressor mutant also improves the export switching function of the fliK(Δ206–265) mutant, we introduced the extra 100 amino-acid insertion sequence of the fliK(Δ2–99 + Δ206–265SP) allele by the overlap PCR method to generate the fliK(Δ206–265SP) allele. Motility of the ΔfliK mutant harboring pMMK1015SP [FliK(Δ206–265SP)] was better than that of the ΔfliK mutant harboring pMMK1015 [FliK(Δ206–265)] although it was worse than that of the ΔfliK mutant transformed with pKM002 (wild-type FliK) (Fig. 6a). Neither cellular nor extracellular FliK level was affected by the inserted sequence (Fig. 6b, 1st row). The amounts of FliC secreted by the fliK(Δ206–265SP) mutant were slightly less than that by the fliK(Δ206–265) mutant and almost the same as the wild-type level (Fig. 6b, 2nd and 3rd rows). There was no difference in the cellular and extracellular FliC levels between the fliK(Δ206–265) and fliK(Δ206–265SP) mutants. These results indicates that the inserted sequence of the fliK(Δ2–99 + Δ206–265) suppressor mutant is also capable of improving the export switching function of FliK(Δ206–265).

The amino acid sequence of FliK(Δ206–265SP) is longer by 40 amino-acids than that of wild-type FliK, from which the average hook length is predicted to be about 70 nm, thereby reducing motility. To verify this hypothesis, we isolated hook-basal bodies from the ΔfliK mutant carrying pMMK1015 [FliK(Δ206–265SP)] or pMMK1015SP...
[FliK(Δ206–265SP)] and measured their hook length (Figs. 6c and S5). The average hook length of the ΔfliK mutant carrying pMMK1015 was 225.4 ± 167.7 nm (n = 127), which were longer than that of the MMK1015 strain (107.1 ± 65.0 nm). Since FliK(Δ206–265) was expressed from the pTrc99AFF4 vector, we assume that such a length difference may be a consequence of the multicopy effect of FliK(Δ206–265SP). The hook length distribution of the ΔfliK mutant carrying pMMK1015SP showed a major peak population between 61 nm and 100 nm, but much longer hooks and polyhooks were observed as well. As a result, the average hook length of the ΔfliK mutant carrying pMMK1015SP was 204.0 ± 183.9 nm (n = 518), which was shorter than that of the ΔfliK mutant carrying pMMK1015. This suggests that the suppressor insertion mutation increases the probability of the interaction between FliKc with a deletion of residue 206–265 and FlhBc, thereby increasing the export switching probability of the flagellar type III protein export apparatus.

Discussion

The bacterial injectisome directly transports virulence effector proteins into the cytosol of host cells for bacterial infection. The injectisome consists of basal body rings and a tubular structure called the needle and looks similar to the flagellar hook-basal body. The injectisome uses a secreted molecular ruler, SctP (originally referred to as YscP and Inv) in the Yersinia and Salmonella injectisomes, respectively) to determine the needle length in a way similar to FliK. The core domain of FliKc is conserved among FliK/SctP family and possesses a fold similar to the C-terminal domain of SctP of the injectisome of Pseudomonas aeruginosa. It has been shown that residues 301–350 of FliKc are directly involved in substrate specificity switching of the flagellar type III protein export apparatus. Recent photo-crosslinking experiments have demonstrated that the conserved core domain of FliKc directly binds to FlhBc. Similar protein-protein interactions have been observed in the injectisome. These suggest that length control and substrate specificity switching mechanisms are conserved in both flagellar and injectisome systems. However, it remains unknown how the length measurement process by the secreted ruler is linked to the substrate specificity switching process of the type III protein export apparatus.

It has been reported that FliKc forms part of the ruler to determine hook length, but a deletion of residues 208–269 results in polyhooks with the filament attached, having led to a hypothesis that residues 208–235 may contribute to efficient substrate specificity switching of the flagellar type III protein export apparatus. To verify this hypothesis, we introduced systematic deletions into FliKL and found that a deletion of residues 206–235 not only shortened hook length according to the size of deletion but also caused a loose hook length control (Fig. 2). The hook length control became much worse in the fliK(Δ206–255) and fliK(Δ206–265) mutants (Fig. 3c). The deletion of residues 206–265 considerably reduced the export switching function of FliKc (Fig. 3d,e). An insertion of 100 amino-acids between Glu-270 and Trp-271 residues in the core domain of FliKc considerably improved the switching function of FliK(Δ2–99 + Δ206–265), thereby shortening the hook length considerably and increasing the probability of filament formation significantly (Figs. 4 and 5). Consistently, this inserted sequence allowed FliK(Δ2–99 + Δ206–265 + I304pBPA) to form a photo-crosslinked product with FlhBc in a way similar to FliK(I304pBPA) (Fig. 4e). Therefore, we suggest that the inserted sequence of the suppressor mutant significantly increases the binding affinity of the core domain of FliKc for FlhBc. Although FliK(Δ2–99) is not secreted via the flagellar type III protein export apparatus during hook assembly, it retains the ability to catalyze the substrate specificity switching of the flagellar type III protein export apparatus to a considerable degree. Therefore, we suggest that FliKc is also required for efficient interaction between the core domain of FliKc and FlhBc. When the length of FliKc was shortened by deletions, the export switching activity of FliKc was reduced depending on the size of deletions (Fig. 3). Furthermore, when the linker length became longer by 40 amino-acids compared to the wild-type length, the switching function of FliKc became worse (Fig. 6). Therefore, we propose that a proper length of FliKc between FliKc and FliKc may be important for FliKc to bind to FlhBc to switch the substrate specificity of the flagellar type III protein export apparatus. Assuming that FliKc suppresses the switching activity of FliKc when FliKc gets close to FliKc via deletion of residues in FliKc, it is also possible that FliKc may push FliKc away from FliKc to allow these two domains to fully exert their own functions.
The core domain of FliKC consists of four β-strands, β1, β2, β3 and β4 and two α-helices, α1 and α2. Three parallel β1, β3 and β4 strands and one anti-parallel β2 strand form a hydrophobic core with the α1 and α2 helices (Fig. 4d)⁴⁶. Highly conserved Val-302 and Ile-304 residues in the β2 strand, which are critical for the switching function of FliK ⁴⁷, form hydrophobic interaction networks in FliKC⁴⁶. Photo-crosslinking experiments have shown that Val-302 and Ile-304 are in very close proximity to FlhBC, suggesting that these two residues are exposed on the molecular surface of FliK when it binds to FlhB.C⁴⁵. Since FliK(Δ2-99 + Δ206-265SP + 1304pBPA) formed a photo-crosslinked product with FlhB.C whereas neither FliK(Δ2-99 + I304pBPA) nor FliK(Δ2-99 + Δ206-265 + 1304pBPA) did (Fig. 4e), this suggests that the inserted sequence of the suppressor mutant induces a conformational change of the N-terminal portion of the core domain of FliKC to allow Ile-304 to bind to FlhB.C. Therefore, we propose that FliK may be required for efficient conformational rearrangements of FliKC to interact with FlhB.C. However, it is also possible that deletion of residues 206–265 makes FliKC very close to FliK, to suppress the interaction of FliKC with FlhB.C and that the inserted sequence of the suppressor mutant may push FliK away from FliK, allowing FliK to bind to FlhB.C.
FliK$_C$ associates with and dissociates from FliK$_C$ in solution. When FliK$_C$ adopts a fully extended conformation, the N-terminal portion of the core domain of FliK$_C$ becomes disordered. Because the length of the most extended polypeptide is 0.37 nm per residue, the stretch of FliK$_C$ sequence inside the channel of the hook-basal body must be longer than 250 residues to measure the hook length of about 55 nm together with the rod length of 35 nm. Therefore, we propose that FliK$_C$ may adopt a fully extended conformation when the hook length reaches about 55 nm, allowing Val-302 and Ile-304 in the hydrophobic core domain of FliK$_C$ to be in very close proximity to FlhBC to catalyze substrate specificity switching of the flagellar type III protein export apparatus.

**Figure 4.** Isolation of pseudorevertants from the fliK(Δ99 + Δ206–265) mutant. (a) Motility of TH8426 transformed with pTrc99A (ΔfliK), pNM201 (Δ99), pMMK1030 (Δ99 + Δ206–265) or pMMK1030SP (Δ99 + Δ206–265SP) in 0.35% soft agar containing 1 mM IPTG. Plates were incubated at 30°C for 9 hours. (b) Secretion assay of FliC. Immunoblotting using polyclonal anti-FliC (1st row) or anti-FliK (2nd row) antibody, of whole cell proteins (Cell) and culture supernatants (Sup) prepared from the above strains. The regions of interest were cropped from original immunoblots shown in Fig. S8a in the Supplemental information using Photoshop CS6, and then the contrast and brightness were adjusted. The positions of molecular mass markers (kDa) are indicated on the left. (c) Location of the intragenic suppressor insertion mutation on FliK(Δ99 + Δ206–265) and schematic bar diagram representations of FliKΔ99 products caused by a deletion of residues 206–265 and the intragenic suppressor insertion of residues 111–270 lacking residues 206–265 (L$_{111}$ - S$_{205}$ + L$_{266}$ - E$_{270}$) between Glu-270 and Trp-271 residues. Red bar indicates a core domain of FliK$_C$. (d) NMR structure of a core domain of FliK$_C$ (PDB ID: 2RRL). The Co backbone is color-coded from green to orange, going through the rainbow colors from the N- to the C-terminus. A highly conserved Ile-304 residue is involved in the interaction with FlhB. The inserted suppressor sequence is located between Glu-270 and Trp-271 residues. (e) Photo-crosslinking between FliK(Δ2–99) and FlhB$_C$. E. coli BL21(DE3) cells co-expressing FliK(I304pBPA), FliK(Δ99 + I304pBPA), FliK(Δ99 + Δ206–265 + I304pBPA) or FliK(Δ99 + Δ206–265SP + I304pBPA) with FlhB$_C$ were UV-irradiated for 5 min (+) or not irradiated (−), and then analyzed by immunoblotting with polyclonal anti-FliK antibody. The regions of interest were cropped from original immunoblots shown in Fig. S8b in the Supplemental information using Photoshop CS6, and then the contrast and brightness were adjusted. The positions of molecular mass markers (kDa) are indicated on the left. The positions of free FliK and FliK-FlhB$_C$ photo-crosslinked products are shown by blue and red balls, respectively. C-terminal truncated variants of FliK are shown by cyan asterisk.
Methods

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used in this study are listed in Table 1. To construct the *Salmonella* fliK(Δ2–99), fliK(Δ206–235), fliK(Δ206–255) and fliK(Δ206–265) mutant strains, the fliK gene on the chromosome was replaced by the fliK(Δ2–99), fliK(Δ206–235), fliK(Δ206–255), fliK(Δ206–265), fliK(Δ206–265SP) gene on the chromosome was replaced by the fliK(Δ2–99), fliK(Δ206–235), fliK(Δ206–255), fliK(Δ206–265SP) or pMMK1030 (Δ99 + Δ206–265) or pMMK1030SP (Δ99 + Δ206–265SP).

**Figure 5.** Effects of deletion of residues 206–265 and its intragenic suppressor insertion mutation on length distribution of the hooks produced by the fliK(Δ2–99) mutant. Histograms of hook length distribution of TH8426 harboring pNM201 (Δ99), pMMK1030 (Δ99 + Δ206–265) or pMMK1030SP (Δ99 + Δ206–265SP).

**Figure 6.** Effects of the inserted sequence of the intragenic fliK(Δ2–99 + Δ206–265) suppressor mutant on length distribution of the hooks produced by the fliK(Δ206–265) mutant. (a) Motility of TH8426 harboring pTrc99A (ΔfliK), pKM002 (WT), pMMK1015 (Δ206–265) or pMMK1015SP (Δ206–265SP) in 0.35% soft agar. Plates were incubated at 30°C for 6.5 hours. (b) Secretion assays of FlgE and FliC. Immunoblotting using polyclonal anti-FliK (1st row), anti-FlgE (2nd row) or anti-FliC (3rd row) antibody, of whole cell proteins (Cell) and culture supernatants (Sup) prepared from the above strains. The regions of interest were cropped from original immunoblots shown in Fig. S9 using Photoshop CS6, and then the contrast and brightness were adjusted. The positions of molecular mass markers (kDa) are indicated on the left. (c) Histograms of hook length distribution of TH8426 harboring pMMK1015 or pMMK1015SP.
and fliK(Δ206–265) alleles, using the λ Red homologous recombination system. L-broth contained 10 g of Bacto-
Tryp-tone, 5 g of yeast extract and 5 g of NaCl per liter. Soft agar plates contained 10 g of Bacto Tryptone, 5 g of NaCl and 0.35% Bacto-Agar per liter. Ampicillin was added at a final concentration of 100 μg/ml if necessary.

DNA manipulations. DNA manipulations were carried out as described. A series of mutant variants of FliK with deletions within FliK were generated by inverse PCR using pKM0024 or pNM20144 as a template. The fliK(Δ206–265SP) allele were generated by overlap PCR method. All of the fliK deletions were confirmed by DNA sequencing. DNA sequencing reactions were carried out using BigDye v3.1 (Applied Biosystems) and then the reaction mixtures were analyzed by a 3130 Genetic Analyzer (Applied Biosystems).

Motility assays in soft agar. Fresh colonies were inoculated onto 0.35% soft tryptone agar plates and incubated at 30 °C. At least seven independent measurements were carried out.

Secretion assays. Secretion assays were performed as described previously. Salmonella cells were grown in 5 ml L-broth containing 100 μg/ml ampicillin at 30 °C with shaking until the cell density had reached an OD600 of ca. 1.2–1.6. 1.5 ml of each culture was transferred into a 1.5 ml Eppendorf tube. After centrifugation (15,000 g, 5 min, 4 °C), cell pellets and culture supernatants were collected, separately. The cells were resuspended in OD600 x 250 μl of SDS-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) containing 1 μl of 2-mercaptoethanol and heated at 95 °C for 3 min. Trichloroacetic acid was added to each culture supernatant at a final concentration of 10%. After leaving on ice for 1 h, proteins in the culture supernatants were precipitated by centrifugation at 20,000 g for 20 min. Pellets were suspended in OD600 x 25 μl of a Tris-SDS loading buffer (one volume of 1 M Tris, nine volumes of 1 × SDS loading buffer) containing 1 μl of 2-mercaptoethanol and heated at 95 °C for 3 min. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting with polyclonal anti-FlgE, anti-FliC or anti-FliK antibody was carried out as described previously. Detection was done with an ECL prime western blotting detection reagent (GE Healthcare). Chemiluminescence signals were captured by a Luminoimage analyzer LAS-3000 (GE Healthcare). The regions of interest were cropped from original immunoblots shown in the Supplemental information using a software, Photoshop CS6, and then the contrast and brightness were adjusted. At least three independent experiments were performed.

Electron microscopy. Osmotically shocked Salmonella cells were prepared described previously. After centrifugation (18,500 g, 30 min), the cell pellets were resuspended in 200 μl of H2O. Samples were applied to carbon-coated copper grids and were negatively stained with 1.0% (W/V) phosphotungstic acid, pH 7.0. Micrographs were recorded at a magnification of × 5,000 with a JEM-1200EXII transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

Hook-based bodies and polyhook-based bodies were isolated as described before. Salmonella cells were grown in 5 l L-broth containing ampicillin at 30 °C with shaking until the cell density had reached an OD600 of ca. 1.0. The cells were harvested by centrifugation (10,000 g, 10 min, 4 °C) and suspended in 20 ml of ice-cold 0.1 M Tris-HCl pH 8.0, 0.5 M sucrose, followed by addition of EDTA and lysozyme at the final concentrations of 10 mM and 0.1 mg/ml, respectively. The cell suspensions were stirred for 30 min at 4 °C and then were solubilized on ice for 1 hour by adding Triton X-100 and MgSO4 at final concentrations of 1% and 10 mM, respectively. The cell lysates were adjusted to pH 10.5 with 5 M NaOH and then centrifuged (10,000 g, 20 min, 4 °C) to remove cell debris. After ultracentrifugation (45,000 g, 60 min, 4 °C), pellets were resuspended in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, and the solution was loaded a 20–50% (w/w) sucrose density gradient in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100. After ultracentrifugation (49,100 g, 13 h, 4 °C), intact flagella were collected and ultracentrifuged (60,000 g, 60 min, 4 °C). Pellets were suspended in 50 mM glycine, pH 2.5, 0.1% Triton X100, and were incubated at room temperature for 30 min to depolymerize flagellar filaments. After ultracentrifugation (60,000 g, 60 min, 4 °C), pellets were resuspended in 50 μl of 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% Triton X100. Samples were negatively stained with 2% (w/v) uranyl acetate. Samples were applied to carbon-coated copper grids and were negatively stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) operated at 100 kV and equipped with a F415 CCD camera (TVIPS, Gauting, Germany). Hook length was measured by ImageJ version 1.48 (National Institutes of Health).

Photo-crosslinking. E. coli BL21(DE3) cells harboring pEVOl and a pETDuet-based plasmid encoding both FliK with an amber mutation and FlhB were exponentially grown at 30 °C in L-broth containing 1 mM pBPA. Then, 100 μM IPTG and 0.02% arabinose were added and the incubation was continued until the culture density had reached an OD600 of ca. 1.4–1.5. Photo-crosslinking was carried out as described previously. The cell pellets were harvested by centrifugation, suspended in SDS-loading buffer, and heated at 95 °C for 3 min. After SDS-PAGE, immunoblotting with polyclonal anti-FliK antibody was carried out. Detection was done with an ECL prime western blotting detection reagent. Chemiluminescence signals were captured by a Luminoimage analyzer LAS-3000. The regions of interest were cropped from original immunoblots shown in the Supplemental information using a software, Photoshop CS6, and then the contrast and brightness were adjusted. At least three independent experiments were performed.

Data availability
All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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Author contributions
M.K., N.K., S.I.A. and T.M. conceived and designed research; M.K., S.T., Y.I., S.I.A. and T.M. performed research; M.K., N.K., S.I.A. and T.M. wrote the paper based on discussion with other authors.

Competing interests
The authors declare no competing interests.

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