



Role of the Dc domain of the bacterial hook protein FlgE in hook assembly and function

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The bacterial flagellar hook acts as a universal joint to smoothly transmit torque produced by the motor to the filament. The hook protein FlgE assembles into a 55 nm tubular structure with the help of the hook cap (FlgD). FlgE consists of four domains, D0, Dc, D1 and D2, arranged from the inner to the outer part of the tubular structure of the hook. The Dc domain contributes to the structural stability of the hook, but it is unclear how this Dc domain is responsible for the universal joint mechanism. Here, we carried out a deletion analysis of the FlgE Dc domain. FlgEΔ4/5 with deletion of residues 30 to 49 was not secreted into the culture media. FlgEΔ5 and FlgEΔ6 with deletions of residues 40 to 49 and 50 to 59, respectively, still formed hooks, allowing the export apparatus to export the hook-filament junction proteins FlgK and FlgL and flagellin FliC. However, these deletions inhibited the replacement of the FlgD hook cap by FlgK at the hook tip, thereby abolishing filament formation. Deletion of residues 50 to 59 significantly affected hook morphology. These results suggest that the Dc domain is responsible not only for hook assembly but also for FlgE export, the interaction with FlgK, and the polymorphic supercoiling mechanism of the hook.

Key words: Bacterial flagella, hook cap, hook-filament junction, self-assembly, supercoiled structure

The flagellum of *Salmonella enterica* functions as a rotary nanomachine consisting of at least three parts: the basal body, the hook and the filament. The basal body is embedded in the cell surface and functions as a reversible rotary motor powered by proton motive force across the cytoplasmic membrane. The hook (FlgE) and filament (FliC) extend out into the cell exterior. The filament works as a helical propeller to produce thrust. The hook connects the basal body with the filament and functions as a universal joint to smoothly transmit torque produced by the motor to the filament for high-speed rotation and frequent reversal^{1–3}. The bending flexibility of the hook is important for flagellar bundle formation in swimming cells⁴. Because the hook is relatively flexible while the filament is much more rigid for its propeller function, the hook-filament junction (FlgK and FlgL) acts as a buffering structure to connect these two filamentous structures with distinct mechanical characteristics^{5,6}.

The flagellar type III protein export apparatus recognizes the N-terminal amino-acid sequence of FlgE as an export signal and then transports FlgE monomers to the distal end of the rod and the growing hook². FlgE requires the support of the FlgD cap to assemble onto the distal end of the rod and the growing hook structure⁷. FlgD consists of a flexible N-terminal domain (FlgD_N) and a compactly folded C-terminal domain (FlgD_C)⁸. Genetic analyses of polymerization-deficient *flgE* point mutants have shown that FlgD_N attaches to the distal end of the hook to promote hook polymerization and that FlgD_C prevents newly exported FlgE monomers from leaking out, allowing FlgE to have more time to assemble into the hook⁹. Upon completion of hook assembly, FlgK displaces the FlgD cap prior to filament formation⁷. How-

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ever, it remains unknown how this occurs.

The length of the hook is relatively well controlled at ca. 55 nm¹⁰. Hook length control is required to ensure the universal joint function of the hook¹¹. FliK, which is secreted during hook assembly¹², is postulated to act as an infrequent molecular ruler to measure hook length^{13–16}. When the hook has reached its mature length of 55 nm, temporal association of the N-terminal ruler domain of FliK with the FlgD cap and the inner surface of the hook during FliK secretion allows the C-terminal domain of FliK to interact with an export apparatus membrane protein FlhB, thereby terminating the export of proteins required for assembly of the rod and hook and initiating the export of proteins responsible for filament formation^{17–19}.

The FlgE monomer is composed of four domains, D0, Dc, D1 and D2, arranged from the inner to the outer part of the hook structure (Fig. 1A)^{20–22}. The hook is a highly curved tubular structure with its subunits arranged in a helical manner. The basic helical line that passes through all the subunits is called 1-start helix, and there are approximately 11 subunits per two turns of the 1-start helix. The structure is also viewed as it is made of 11 protofilaments that are axially staggered by about a half subunit in their lateral interactions²¹. The axial packing of the subunits in the outer part of the tube made of the D1 and D2 domains is relatively loose, which is primarily responsible for the bending flexibility of the hook structure (Fig. 1B)^{20,22}. The N- and C-terminal α -helices form a coiled coil in the inner core domain D0, which structurally and mechanically stabilizes the hook structure²². FlgE(Δ 9–20) (thereafter referred to as FlgE Δ 2) missing residues 9 through 20 fails to polymerize and form hooks, suggesting that the D0-D0 interactions are required for hook formation¹³. The orientations and axial packing interactions of the terminal two α -helices of FlgE provide enough space for axial compression and extension for the bending flexibility of the hook without impairing its mechanical stability²².

The Dc domain consists of 46 residues from Asn26 to Arg71 and presumably ten residues from Asn358 to Asp366. The Dc domains are connected along the left-handed 5-start helical arrays with a large gap between them (Fig. 1C), contributing to the mechanical stability of the hook structure along with the right-handed 6-start helical arrays of the D2 domains²². However, it remains unclear how the Dc domains contribute to the universal joint mechanism of the hook because the resolution of the electron density map of the hook structure is limited.

In present study, we constructed and characterized sequential in-frame deletion variants of FlgE (Fig. 2). We show that residues 30–49 of FlgE are required for rapid and efficient FlgE export, residues 40–59 for the initiation process of flagellar filament assembly, and residues 50–59 for hook morphology.

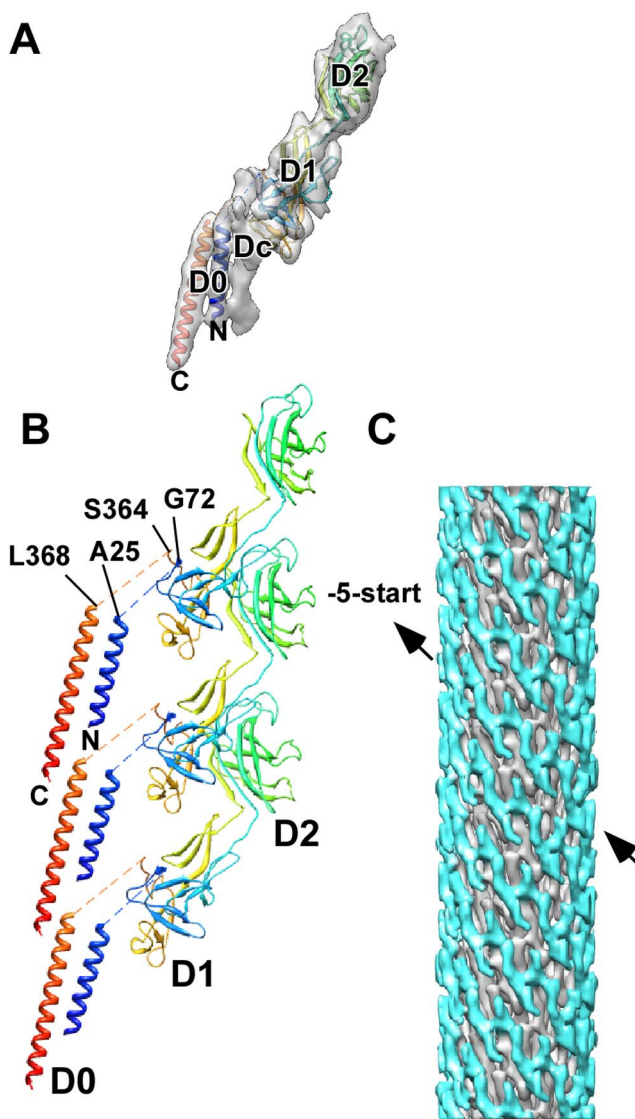


Figure 1 Partial atomic model of the hook. (A) Three-dimensional density map of the hook subunit with a fitted atomic model of the FlgE31 fragment (PDB ID: 1WLG) and terminal α -helices²². FlgE is composed of four domains, D0, Dc, D1, and D2, which are arranged from the inner to the outer part of the tubular structure of the hook. The N- and C-terminal α -helices, which are disordered in the monomeric form in solution, form a coiled coil in the D0 domain²². The C α backbone is color-coded from blue to red, going through the rainbow colors from the N- to the C-terminus. (B) Protofilament structure of the hook. The three subunits of the hook are shown. (C) Dc domain interactions in the hook structure. The array of Dc domains in light blue revealed by the removal of the D1 and D2 domains shows the connection along the left-handed 5-start with a gap between them.

Materials and methods

Bacterial strains, plasmids, P22-mediated transduction, DNA manipulations, and media

Bacterial strains and plasmids used in this study are listed in Table 1. Procedures for DNA manipulation were carried out as described previously^{23,24}. L-broth (LB) and soft agar

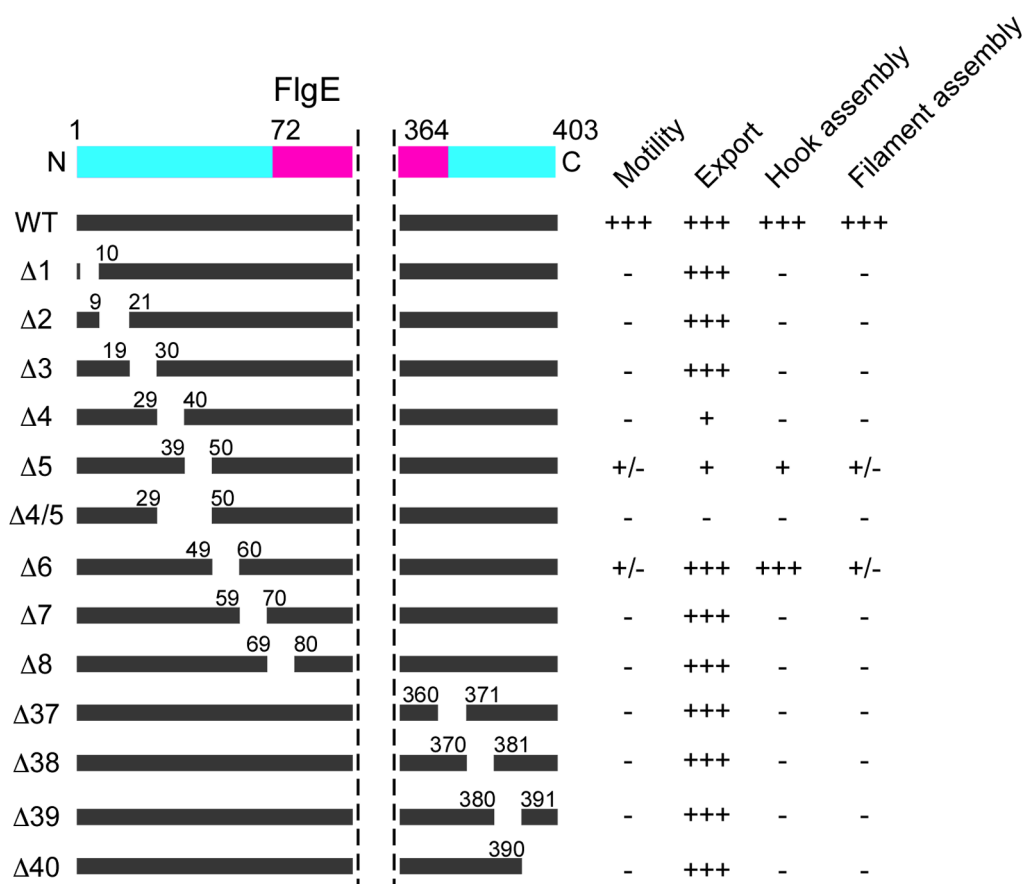


Figure 2 The primary structure of FlgE and the effect of scanning deletions on motility, FlgE export, hook assembly and filament assembly. *Salmonella flgE* gene encodes a 403-amino-acid sequence (UniProt ID: P0A1J1). In the present study, the amino acid residues are numbered including the initiator methionine (Met1), which is removed in the cell after translation. A FlgE fragment corresponding to residues 72–370, which are colored magenta, have been crystallized and its structure has been solved 1.8 Å by X-ray crystallography. The N and C-terminal regions that are disordered in monomeric form in solution but form the D0 and Dc domains in the hook structure are shown in light blue. In-frame deletion variants of FlgE labeled Δ1, Δ2, Δ3, etc. here are referred to in the text as FlgEΔ1, FlgEΔ2, FlgEΔ3 and so on. The deletion variants contain in-frame-deletion of residues 2 to 9, 9 to 20, 20 to 29 and so on. To the right is a summary of various properties associated with these variants, where ‘+++’ refers to a positive result for the relevant property, ‘+++’ an intermediate positive, ‘+’ a weakly positive, ‘+/-’ a very weakly positive and ‘-’ a negative result.

Table 1 Strains and Plasmids used in this study

Strains and Plasmids	Relevant characteristics	Source or reference
<i>Salmonella</i>		
MMEK001	Δ <i>flgE</i> Δ <i>fliK</i>	Minamino <i>et al.</i> (2009)
NME001	Δ <i>flgE</i>	Minamino <i>et al.</i> (2009)
<i>Plasmids</i>		
pNM001	pTrc99AFF4 / FlgE	Moriya <i>et al.</i> (2006)
pNM007	pTrc99AFF4 / FlgE(Δ9-20)	This study
pMKM25	pTrc99AFF4 / FlgE(Δ2-9)	This study
pHF26	pTrc99AFF4 / FlgE(Δ20-29)	This study
pMKM27	pTrc99AFF4 / FlgE(Δ30-39)	This study
pMKM28	pTrc99AFF4 / FlgE(Δ40-49)	This study
pHF29	pTrc99AFF4 / FlgE(Δ50-59)	This study
pMKM30	pTrc99AFF4 / FlgE(Δ60-69)	This study
pHF31	pTrc99AFF4 / FlgE(Δ70-79)	This study
pMKM31	pTrc99AFF4 / FlgE(Δ30-49)	This study
pMKM37	pTrc99AFF4 / FlgE(Δ361-370)	This study
pMKM38	pTrc99AFF4 / FlgE(Δ371-380)	This study
pMKM39	pTrc99AFF4 / FlgE(Δ381-390)	This study
pMKM40	pTrc99AFF4 / FlgE(Δ391-403)	This study

plates were prepared as described before^{25,26}. Ampicillin was added at a final concentration of 100 µg/ml.

Motility assay on soft agar plates

Fresh transformants were inoculated onto soft agar plates and incubated at 30°C. At least seven independent experiments were performed.

Secretion assay

A *flgE* null mutant strain of *Salmonella enterica*, NME001 ($\Delta flgE$), harboring pTrc99A-based plasmids encoding wild-type FlgE or FlgE deletion variants were grown at 30°C with shaking until the cell density had reached an OD₆₀₀ of ca. 1.6. After centrifugation, the whole cellular and culture supernatant fractions were collected separately. Cell pellets were resuspended in SDS-loading buffer normalized by the cell density to give a constant amount of cells. Proteins in the culture supernatants were precipitated by 10% trichloroacetic acid, suspended in a Tris/SDS loading buffer and heated at 95°C for 5 min. After SDS-PAGE, immunoblotting with polyclonal anti-FlgE, anti-FlgK, anti-FlgL, or anti-FliC antibody was carried out as described²⁵. At least three independent experiments were performed.

Preparation of hook-basal body structures

Polyhook-basal bodies were prepared from MMEK001 ($\Delta flgE \Delta fliK$) expressing FlgE, FlgE Δ 5 or FlgE Δ 6 as described before¹³. Samples were negatively stained at room temperature with 3% phosphotungstic acid (pH 4.5) on carbon-coated copper grids. Micrographs were recorded at a magnification of $\times 50,000$ with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

Visualization of flagellar filaments

Flagellar filaments were labeled with Alexa Fluor 594 as described previously²⁷. The $\Delta flgE$ mutant transformed with pTrc99A, pNM001 (wild-type FlgE), pMKM028 (FlgE Δ 5) or pHF029 (FlgE Δ 6) were attached to a cover slip (Matsunami glass, Japan), and unattached cells were washed away with motility medium (10 mM potassium phosphate, 0.1 mM EDTA, and 10 mM sodium lactate; pH 7.0). 1 µl aliquot of anti-FliC antibody suspended in 50 µl of motility medium was applied to the cells attached to the cover slip. After washing twice with motility medium, 1 µl of anti-rabbit IgG conjugated with Alexa Fluor[®] 594 (Invitrogen) suspended in 50 µl of motility medium was applied. After washing twice with motility medium, cells were observed by an inverted fluorescence microscope (IX-71, Olympus) with a 150 \times oil immersion objective lens (UApo150XOTIRFM, NA 1.45, Olympus) and an Electron-Multiplying Charge-Coupled Device (EMCCD) camera (C9100-02, Hamamatsu Photonics).

Fractionation of cell membrane

Fractionation of cell extracts of the $\Delta flgE$ mutant harboring pTrc99A, pNM001, pMKM028 or pHF029 was carried out as described previously²⁸. Cells were grown exponentially in 40 ml LB at 30°C with shaking. The cells were harvested, resuspended in 3 ml PBS (8 g of NaCl, 0.2 g of KCl, 3.63 g of Na₂HPO₄·12H₂O, 0.24 g of KH₂PO₄, pH 7.4 per liter), and sonicated. After the cell debris was removed by low-speed centrifugation, the cell lysates were centrifuged (100,000 g, 30 min, 4°C). After carefully removing the soluble fractions, membranes were resuspended in 300 µl of SDS-loading buffer. The protein concentration was normalized and heated at 95°C for 5 min. After the membrane proteins in each fraction were separated by SDS-PAGE, immunoblotting with polyclonal anti-FliG, anti-FlgD, or anti-FlgK antibody was carried out. At least three independent experiments were carried out.

Results

Complementation of FlgE deletion variants

The FlgE31 fragment consisting of residues 72–365 has been crystallized and the structure has been solved by X-ray crystallography at 1.8 Å resolution²⁰. FlgE31 has two domains, D1 and D2, connected with a short stretch of two-stranded anti-parallel β -sheet²⁰. To clarify the role of the terminal disordered regions of FlgE, forming the D0 and Dc domains in the hook structure, in the universal joint mechanism, we constructed a series of FlgE variants with sequential in-frame deletions within the N-terminal region from residues 2 to 79 and the C-terminal region from residues 361 to 403. These constructs were cloned into the expression vector pTrc99A^{FF4} to produce a set of 13 plasmids (Table 1 and Fig. 2). We transformed a *Salmonella flgE* null mutant ($\Delta flgE$) with these FlgE deletion constructs and analyzed motility of the resulting transformants in soft agar (Fig. 3). Wild-type FlgE fully complemented the $\Delta flgE$ mutant. FlgE Δ 1 through FlgE Δ 4, FlgE Δ 7, FlgE Δ 8 (Fig. 3A) and FlgE Δ 37 through FlgE Δ 40 (Fig. 3B) did not complement at all. FlgE Δ 5 and FlgE Δ 6 restored motility to some degree after a prolonged incubation (Fig. 3A, bottom panel). Immunoblotting with polyclonal anti-FlgE antibody revealed that the cellular level of all the FlgE deletion variants was detected more or less at the wild-type level (data not shown), indicating that these in-frame deletions did not affect the stability of FlgE itself. These results suggest that these deletions impair the function of FlgE considerably.

Effect of FlgE deletions on its export

It has been reported that residues 38 to 58 of *Caulobacter* FlgE are essential for its export²⁹. Therefore, we tested whether the in-frame deletions of FlgE reduce the secretion level of FlgE. When the hook has reached its mature length of ca. 55 nm, the flagellar type III export apparatus switches export specificity from rod- and hook-type to filament-type

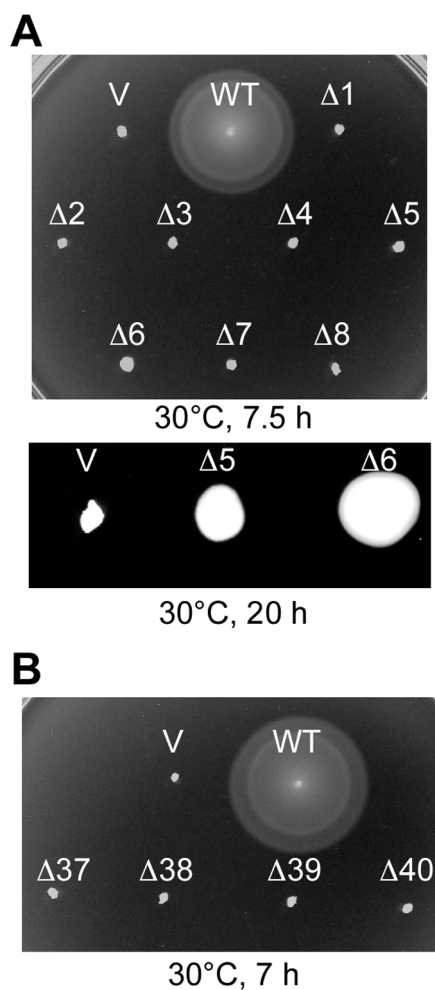


Figure 3 Effect of in-frame deletions of FlgE on motility. (A) Motility assay for the complementation of the motility of NME001 ($\Delta flgE$) transformed with pTrc99A-based plasmids encoding various N-terminal in-frame deletion variants of FlgE. V, pTrc99A-based plasmid encoding wild-type FlgE; $\Delta 1$, FlgE $\Delta 1$; $\Delta 2$, FlgE $\Delta 2$; $\Delta 3$, FlgE $\Delta 3$; $\Delta 4$, FlgE $\Delta 4$; $\Delta 5$, FlgE $\Delta 5$; $\Delta 6$, FlgE $\Delta 6$; $\Delta 7$, FlgE $\Delta 7$; $\Delta 8$, FlgE $\Delta 8$. Soft tryptone agar plates inoculated with the transformants were incubated at 30°C for 7.5 h (top panel) or 20 h (bottom panel). (B) Motility of the $\Delta flgE$ mutant expressing various C-terminal deletion variants of FlgE in soft agar. $\Delta 37$, FlgE $\Delta 37$; $\Delta 38$, FlgE $\Delta 38$; $\Delta 39$, FlgE $\Delta 39$; $\Delta 40$, FlgE $\Delta 40$.

substrates, thereby terminating hook polymerization and starting filament formation. At least two flagellar proteins, FliK and FlhB, are responsible for the switching process. Certain mutations in *fliK* or *flhB* prevent the export apparatus from switching export specificity, thereby causing the bacteria to produce abnormally long hooks called polyhooks¹¹. Because assembly-deficient *flgE* mutants do not produce the hook structure and hence cannot change substrate specificity of the export apparatus^{9,13}, they secrete a much higher amount of mutant FlgE subunits into the culture media than wild-type cells. Therefore, to carry out a quantitative comparison of the FlgE levels secreted by the *flgE* deletion variants with that by the wild-type, we used a

flgE fliK double null mutant ($\Delta flgE \Delta fliK$) as the host. To evaluate the export of in-frame deletion variants of FlgE, the whole cellular and culture supernatant fractions were prepared from the $\Delta flgE \Delta fliK$ mutant transformed with pTrc99A-based plasmids encoding FlgE deletion variants and analyzed by immunoblotting with polyclonal anti-FlgE antibody (Fig. 4). The cellular levels of FlgE were almost at the wild-type level in *flgE* $\Delta 5$, *flgE* $\Delta 6$, and *flgE* $\Delta 37$ through *flgE* $\Delta 40$ (upper panel, lanes 7, 8, and 15–18), slightly higher in *flgE* $\Delta 2$ through *flgE* $\Delta 4$ (lanes 4–6) and *flgE* $\Delta 4/5$ (lane 12), and slightly lower in *flgE* $\Delta 1$, *flgE* $\Delta 7$ and *flgE* $\Delta 8$ (lanes 3, 9 and 10). The secretion levels of FlgE were higher than the wild-type in *flgE* $\Delta 1$ through *flgE* $\Delta 3$ (lower panel, lanes 3–5), *flgE* $\Delta 6$ through *flgE* $\Delta 8$ (lanes 8–10) and *flgE* $\Delta 37$ through *flgE* $\Delta 40$ (lanes 15–18) and less than the wild-type in *flgE* $\Delta 4$ and *flgE* $\Delta 5$ (lanes 6–7). No FlgE $\Delta 4/5$ was detected in the culture supernatants (lane 12), indicating that deletion of residues 30–49 abolishes the export of FlgE. These results suggest that residues 30–49 in *Salmonella* FlgE act as the export signal recognized by the flagellar export apparatus.

Effects of in-frame deletions of FlgE on hook formation

To investigate whether these FlgE deletion variants retain the ability to assemble into the hook structure, the polyhook-basal bodies were isolated from the *flgE fliK* double null mutant transformed with pTrc99A-based plasmids encoding in-frame deletion variants of FlgE (Figs. 2 and 5). The *flgE* $\Delta 5 \Delta fliK$ and *flgE* $\Delta 6 \Delta fliK$ mutants formed polyhooks at the tip of the basal body (Fig. 5, 2nd and 3rd rows) while the other mutants did not (data not shown). These results indicate that FlgE $\Delta 5$ and FlgE $\Delta 6$ retain the polymerization ability to form hooks while the others do not (Fig. 2). The length of polyhooks produced by the *flgE* $\Delta 5 \Delta fliK$ mutant was much shorter than the $\Delta fliK$ mutant (Fig. 5, 2nd row) presumably due to its reduced secretion activity (Fig. 4, lane 7). In contrast, the length of polyhooks produced by the *flgE* $\Delta 6 \Delta fliK$ mutant was almost the same as those made by the $\Delta fliK$ mutant. Interestingly, straight polyhooks were often observed in the *flgE* $\Delta 6 \Delta fliK$ mutant. Because the hook shows polymorphic transformations of its supercoiled forms³⁰, this result suggests that the Dc domain is involved in the supercoiling mechanism.

Filament-type export substrates, such as FliC (flagellin), FlgK, and FlgL, are exported only after the completion of hook assembly³¹. Therefore, we analyzed proteins secreted by the *flgE* null mutant transformed with pTrc99A-based plasmids encoding in-frame deletion variants of FlgE by Coomassie brilliant blue (CBB) staining and immunoblotting with polyclonal anti-FliC, anti-FlgK, or anti-FlgL antibody (Fig. 6). FliC, FlgK, and FlgL were detected almost at wild-type levels in the culture supernatants of the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ mutants (lanes 6–8) but not in the other *flgE* mutants (data not shown). Therefore, we conclude that the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ mutants form hooks and subsequently

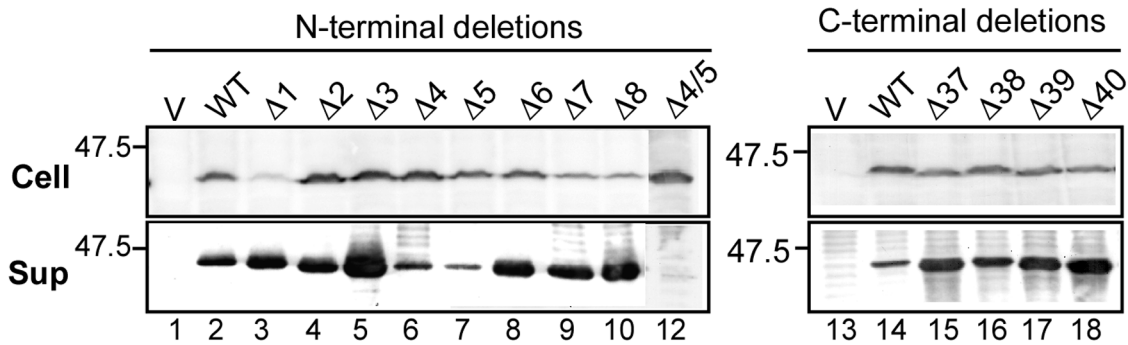


Figure 4 Secretion ability of in-frame deletion FlgE variants. Immunoblotting, with polyclonal anti-FlgE antibody, of the whole cellular (Cell) and culture supernatant (Sup) fractions prepared from MMEK001 ($\Delta flgE \Delta fliK$) transformed with pTrc99A-based plasmids encoding various deletion variants of FlgE. WT, wild-type FlgE; $\Delta 1$, FlgE $\Delta 1$; $\Delta 2$, FlgE $\Delta 2$; $\Delta 3$, FlgE $\Delta 3$; $\Delta 4$, FlgE $\Delta 4$; $\Delta 5$, FlgE $\Delta 5$; $\Delta 6$, FlgE $\Delta 6$; $\Delta 7$, FlgE $\Delta 7$; $\Delta 8$, FlgE $\Delta 8$; $\Delta 4/5$, FlgE $\Delta 4/5$; $\Delta 37$, FlgE $\Delta 37$; $\Delta 38$, FlgE $\Delta 38$; $\Delta 39$, FlgE $\Delta 39$; $\Delta 40$, FlgE $\Delta 40$.

switch the substrate specificity of the export apparatus upon completion of hook assembly while the others do not.

Effects of FlgE $\Delta 5$ and FlgE $\Delta 6$ deletions on filament formation

To examine whether the FlgE $\Delta 5$ and FlgE $\Delta 6$ deletions affect filament formation, the *flgE* null mutant transformed with pMKM028 (FlgE $\Delta 5$) or pHF029 (FlgE $\Delta 6$) were grown overnight in L-broth and then flagellar filaments were stained with a fluorescent dye. Much fewer flagellar filaments were produced by the *flgE $\Delta 5$* and *flgE $\Delta 6$* mutants than wild-type cells (Fig. 7A). The length of the filaments produced by these mutants was shorter than those of the wild-type as well. In agreement with this, the amount of FliC was much less in the whole cell fractions of the *flgE $\Delta 5$* and *flgE $\Delta 6$* mutants (Fig. 6, lanes 3 and 4) than the wild-type (lane 2).

We found that the *flgE $\Delta 5$* and *flgE $\Delta 6$* mutant cells secrete FliC into the culture media at wild-type levels (Fig. 6) but did not form the filaments efficiently (Fig. 7A). The hook-filament junction proteins FlgK and FlgL and the filament cap protein FliD are required for filament formation. The hook-filament junction formed by FlgK and FlgL in this order connects the hook and filament. The filament cap is located at the tip of the filament to promote filament formation^{5,32–34}. When FlgK, FlgL, or FliD is missing, FliC cannot assemble into the flagellar filament at the tip of the hook and is excreted into the culture media³⁵. Taking this into account, we postulated that the $\Delta 5$ and $\Delta 6$ deletions of FlgE affect FlgK assembly onto the distal end of the hook. To test this, we prepared the insoluble membrane fractions from the $\Delta flgE$ mutant harboring pTrc99A, pNM001 (wild-type FlgE), pMKM028 (FlgE $\Delta 5$) or pHF029 (FlgE $\Delta 6$) and analyzed FliG, FlgD, and FlgK in the membrane fractions by immunoblotting (Fig. 7B). Essentially the same level of the basal-body C ring protein FliG was detected in all the isolated membrane fractions (1st row), indicating that these membrane fractions contain almost the same number of the

basal bodies. FlgK was detected in the membrane fractions isolated from wild-type cells (3rd row, lane 4) but not from the vector control (lane 2). Much smaller amounts of FlgK were observed in the membrane fractions isolated from the *flgE $\Delta 5$* and *flgE $\Delta 6$* mutants compared to the wild-type level (lanes 6 and 8). These results suggest that these FlgE deletions significantly reduce the binding affinity of FlgK to the hook tip.

The FlgD cap is displaced by FlgK prior to filament formation⁷. Therefore, we tested whether FlgD is still associated with the tip of the hook produced by the *flgE $\Delta 5$* and *flgE $\Delta 6$* mutants (2nd row). As expected, FlgD was detected in the membrane fractions isolated from the vector control (lane 2) but not from the wild-type (lane 4). The amount of FlgD was slightly lower in the membrane fraction prepared from the $\Delta 5$ and $\Delta 6$ deletion mutants than the vector control but significantly higher than the wild-type (lanes 6 and 8). These results suggest that the $\Delta 5$ and $\Delta 6$ deletions inhibit the replacement of the FlgD cap by FlgK at the tip of the hook structure, thereby reducing the probability of filament assembly considerably.

Discussion

FlgE polymerizes into the 55 nm-long hook structure with the help of the FlgD cap. Upon completion of hook assembly, the FlgD cap is displaced by FlgK prior to flagellar filament formation. The hook is a flexible helical tubular structure consisting of 11 protofilaments. FlgE consists of four domains, D0, Dc, D1 and D2 (Fig. 1A). The D0 domains form the inner core of the hook and stabilize the structure. In contrast, the packing of the D1 domains is loose in all directions, conferring the bending flexibility on the hook structure. The D2 domains are connected along the right-handed 6-start helical lines while the Dc domains are connected along the left-handed 5-start helical lines. These subunit arrays form a mesh-like structure that contributes to the twisting rigidity as well as the structural stability of the

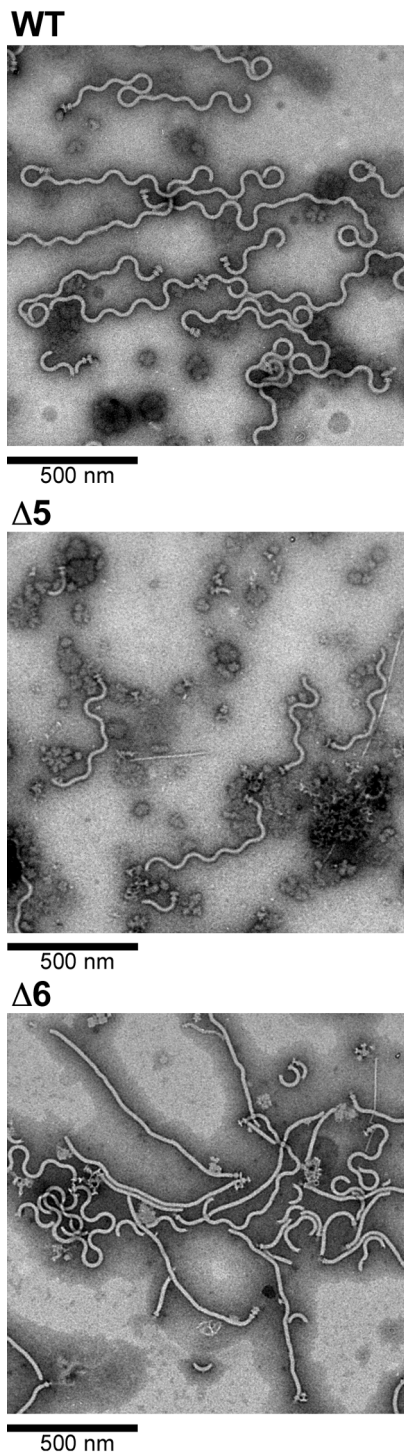


Figure 5 Effect of in-frame deletions of FlgE on hook assembly. Electron micrographs of the polyhook-basal bodies produced by the $\Delta flgE \Delta fliK$ mutant transformed with pNM001 (WT), pMKM028 (FlgE Δ 5, indicated as Δ 5) or pHF029 (FlgE Δ 6, indicated as Δ 6). Bar = 500 nm.

hook necessary to transmit motor torque to the filament²². Nevertheless, little is known about the role of the Dc domain in the universal joint mechanism. To clarify this, we carried

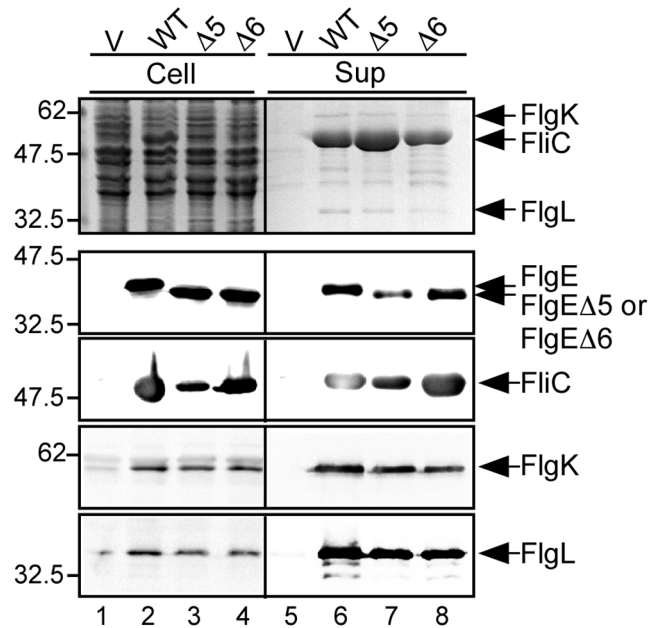


Figure 6 Effect of the FlgE Δ 5 and FlgE Δ 6 deletions on the secretion of filament-type substrates. Secretion of FliC, FlgK, and FlgL was analyzed by Coomassie brilliant blue (CBB) staining (1st row). Secretion of FlgE (2nd row), FliC (3rd row), FlgK (4th row) and FlgL (5th row) were measured by immunoblotting with polyclonal anti-FlgE, anti-FliC, anti-FlgK, and anti-FlgL antibodies, respectively. Cell and Sup indicate whole cell proteins and culture supernatant fractions, respectively. The positions of molecular mass markers (kDa) are shown on the left.

out deletion analyses of the Dc domain and provided evidence that the Dc domain is responsible not only for hook assembly but also for FlgE export, polymorphic supercoiling, and the assembly of FlgK at the tip of the hook.

Most flagellar proteins are transported to the distal end of the growing flagellar structure by a specific export apparatus¹⁻³. N-terminal polypeptides of flagellar proteins retain the ability to be secreted via the flagellar export pathway into the culture media^{19,29,36,37}. Flagellar protein export can occur post translationally³⁸. These observations indicate that N-terminal amino acid sequences of the export substrates function as the export signal recognized by the export apparatus³⁶. Here, we found that the secretion levels of FlgE Δ 4 missing residues 30–39 and FlgE Δ 5 lacking residues 40–49 were lower than that of the wild-type FlgE protein whereas not the case in the other deletions (Fig. 4, lanes 6 and 7). In-frame deletion of residues 30–49 totally abolished the secretion of FlgE into the culture media (Fig. 4, lane 12), which is consistent with a previous report that residues 38–58 of *Caulobacter* FlgE are essential for its export²⁹. It has been shown that residues 7 to 25 of FlgM and residues 26 to 47 of FliC form the export signal recognized by the flagellar export apparatus^{37,39}. Therefore, we conclude that the export signal is located within residues 30–49 in *Salmonella* FlgE. Residues 30–49 in FlgE do not show significant sequence similarity to the previously iden-

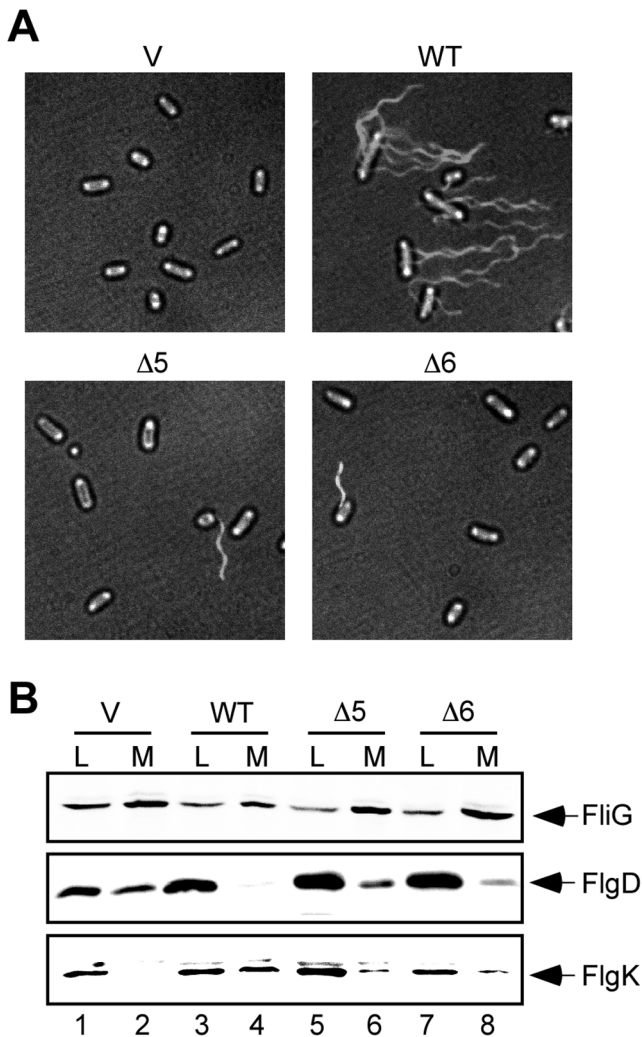


Figure 7 Effect of the $\Delta 5$ and $\Delta 6$ deletions on flagellar filament formation. (A) Visualization of flagellar filament produced by the $\Delta 5$ and $\Delta 6$ deletion mutants. The $\Delta flgE$ mutant was transformed with pTrc99A (V), pNM001 (WT), pMKM028 ($\Delta 5$) or pHF029 ($\Delta 6$) and then, the resulting transformants were treated with polyclonal anti-FliC antibody and Alexa Fluor 594-conjugated anti-rabbit secondary antibody. The bright field and fluorescence images of the filament were merged. (B) Membrane localization of the C ring protein FliG, the hook-capping protein FlgD, and the hook-filament junction protein FlgK. The membrane fractions of the above transformants were prepared after sonication and ultracentrifugation. Then, the cell lysates (L) and the membrane fractions (M) were subjected to SDS-PAGE, and analyzed by immunoblotting with polyclonal anti-FliG, anti-FlgD, or anti-FlgK antibody. Positions of FliG, FlgD and FlgK are indicated by arrows on the right.

tified export signal of flagellar proteins. However, these export signal is commonly located within their N-terminal region that is disordered in monomeric form in solution but form the amphipathic helix upon their polymerization. Therefore, it is possible that a common amphipathic helical conformation in the export substrate mediates recognition by the flagellar protein export apparatus as suggested before³⁹.

The extensive intermolecular axial packing interactions

between the D0 domains are responsible for structural and mechanical stability of the hook. The left-handed 5-start helical arrays of Dc also contribute to the structural stability of the hook²². As expected, the *flgE* $\Delta 1$ through *flgE* $\Delta 3$, *flgE* $\Delta 7$, *flgE* $\Delta 8$ and *flgE* $\Delta 37$ through *flgE* $\Delta 40$ mutants did not form the hook although higher amounts of FlgE were detected in the culture media of these deletion mutants than the wild-type (Fig. 2). This suggests that not only the intermolecular D0-D0 interactions but also the intermolecular Dc-Dc interactions are responsible for stable hook formation. Interestingly, FlgE $\Delta 5$ and FlgE $\Delta 6$ missing residues 40–49 and 50–59, respectively, retained the ability to form the hook structure (Fig. 5), indicating that residues 40–59 are not critical for hook assembly. In agreement with this, the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ deletion mutants were able to export filament-type substrates, such as FlgK, FlgL and FliC, at the wild-type levels (Fig. 6). Therefore, we conclude that residues 40–59 are not involved in the intermolecular Dc-Dc interactions responsible for hook formation.

FlgK, FlgL and FliD are required for filament formation^{32,33}. When these proteins are missing, FliC subunits do not polymerize at the distal end of the hook structure and hence are excreted into the culture media³⁵. In this study, we found that the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ mutants form motility halos on soft agar plates after a prolonged incubation (Fig. 3A). These deletion mutants secreted FlgK, FlgL, and FliC at the wild-type levels but did not form flagellar filaments efficiently (Fig. 7A), indicating that these deletions considerably affect the initial process of filament assembly. FlgK was detected in the membrane fractions prepared from the *flgE* null mutant harboring a plasmid encoding wild-type FlgE (Fig. 7B, 3rd row, lane 4) but not from the vector control (lane 2). A much smaller amount of FlgK was present in the membrane fractions prepared from the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ mutants (lanes 6 and 8). Because these membrane fractions contain almost the same number of hook-basal bodies (Fig. 7B, 1st row), we conclude that the very poor motility of these deletion mutants result from the inability of FlgK to assemble onto the tip of the hook. Therefore, we suggest that residues 40–59 in the Dc domain are required for the interaction with FlgK so that the FlgE $\Delta 5$ and FlgE $\Delta 6$ deletions significantly reduce the binding affinity of the hook tip for FlgK. Completion of hook assembly induces the replacement of the FlgD cap by FlgK at the hook tip⁷. Here, we showed that FlgD was detected in the membrane fractions from the *flgE* $\Delta 5$ and *flgE* $\Delta 6$ mutants (Fig. 7B, 2nd row, lanes 6 and 8) but not from the wild-type (lane 4), indicating that these deletions significantly affect the replacement of FlgD by FlgK. Therefore, we propose that an interaction of FlgK with the Dc domain of FlgE may induce the release of the FlgD cap from the tip of the hook.

It has been reported that *flgE* mutations affect the shape of the hook structure^{40,41}. Purified polyhooks show polymorphic transformations of its supercoiled form in response to changes in the salt concentration, pH and temperature of the

solution. The supercoiled forms of the polyhooks are classified into four groups: normal (right-handed supercoil), coiled without a helical pitch, left-handed and straight types³⁰. It has been proposed that the supercoiled forms of the hook are produced by close packing interactions between the right-handed 6-start helical connections of the D2 domains on the inner side of the curved hook structure²⁰. In this study, we showed that the *flgEΔ6 ΔfliK* mutant produced not only normal-type polyhooks but also straight-type ones (Fig. 5). Because the curvature and twist of each supercoil would be dependent on the direction of the intermolecular D2-D2 interactions²⁰, in-frame deletion of residues 50 to 59 in the Dc domain may have affected the direction of the D2-D2 interactions. Therefore, we propose that the Dc domain may act as a switch to induce polymorphic transformations of the supercoiled forms of the hook.

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