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Regular Article

Evidence for the hook supercoiling mechanism of the bacterial flagellum

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The bacterial flagellar hook is a short, highly curved tubular structure connecting the basal body as a rotary motor and the filament as a helical propeller to function as a universal joint to transmit motor torque to the filament regardless of its orientation. This highly curved form is known to be part of a supercoil as observed in the polyhook structure. The subunit packing interactions in the *Salmonella* hook structure solved in the straight form gave clear insights into the mechanisms of its bending flexibility and twisting rigidity. *Salmonella* FlgE consists of four domains, D0, Dc, D1 and D2, arranged from inside to outside of the tube, and an atomic model of the supercoiled hook built to simulate the hook shape observed in the native flagellum suggested that the supercoiled form is stabilized by near-axial interactions of the D2 domains on the inner surface of the supercoil. Here we show that the deletion of domain D2 from FlgE makes the hook straight, providing evidence to support the proposed hook supercoiling mechanism that it is the near-axial interactions between the D2 domains that stabilize the highly curved hook structure.

Key words: hook curvature, universal joint, deletion mutation, structure, electron microscopy

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Many bacteria swim in liquid environments by rotating the flagella. The bacterial flagellum can be roughly divided into the following three parts: the basal body acting as a rotary motor, the hook as a universal joint and the filament as a helical propeller. In *Escherichia coli* and *Salmonella enterica* (hereafter referred to as *Salmonella*), the filament is in a left-handed supercoiled form to act as a helical propeller, and the flagellar motor rotates in the counterclockwise direction to produce thrust for forward swimming. *E. coli* and *Salmonella* cells have several flagella, and they form a bundle behind the cell body to amplify the thrust and push the cell forward. But when a quick reversal of motor rotation occurs every few seconds to the clockwise direction, this produces a twisting force in the base region of the filament and transforms its left-handed supercoiled form to a right-handed one. Then, the flagellar bundle falls apart to make the cell tumble and change its swimming direction [1]. During this dynamic process, the hook plays an essential role as a universal joint with high rigidity against twisting and flexibility in bending.

The flagellar motor has a drive shaft called the rod, which extends on the rotor ring called the MS ring that spans the cytoplasmic membrane. The rod is composed of four rod proteins, FlgB, FlgC, FlgF and FlgG, and the LP ring surrounds the distal part of the rod made of FlgG as a bushing to support high-speed rotation of the motor. The proximal

◀ ***Significance*** ▶

The bacterial flagellar hook is a short, highly curved tubular structure connecting the flagellar motor with the helical filament acting as a propeller to function as a universal joint to transmit motor torque to the filament regardless of its orientation. The highly curved form is part of a supercoil as observed in the polyhook structure. The essential role of the outermost domain D2 of the hook protein in the supercoiling mechanism is revealed by its deletion that made the polyhook straight.

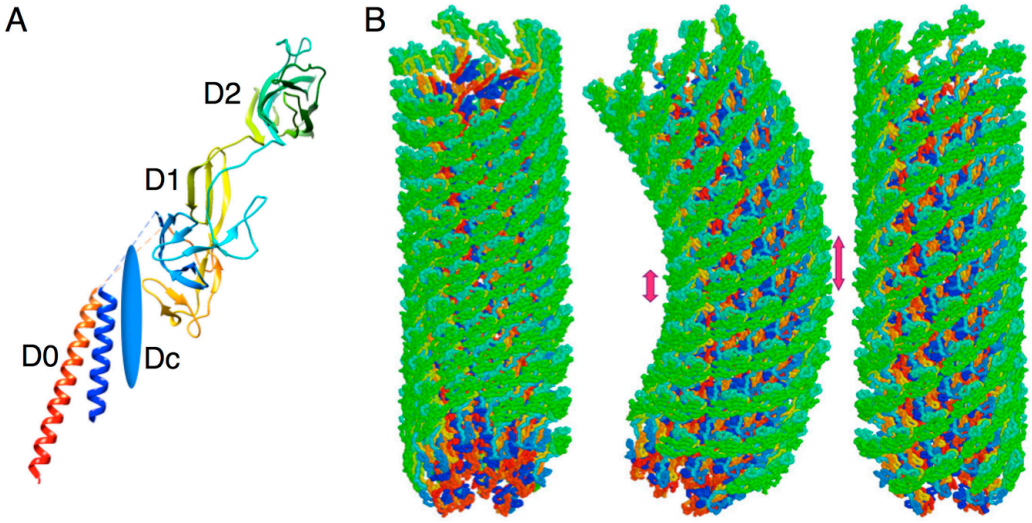

Figure 1 Cα backbone ribbon models of *Salmonella* FlgE and supercoiled hook. (A) A FlgE subunit, in which four domains are labeled as D0, Dc, D1 and D2 from inside to outside of the tubular structure of the hook. (B) The supercoiled hook in three different views: left, viewed from the inner side of the supercoil; middle, from the side; right, from the outer side. The models are color coded in rainbow from blue to red according to the sequence from the N-terminus to the C-terminus. Therefore, domains D0 and D1 located inside of the tubular structure are colored blue/red and yellow, respectively, and domain D2 is colored green.

end of the hook is directly attached to the distal rod, and its distal end is connected to the filament through the hook-filament junction to transmit the motor torque to the filament regardless of its orientation, on and off the axis of the motor rotation. So the twisting rigidity and bending flexibility of the hook are the important mechanical properties required for flagellar bundle formation for swimming and the dynamic process of the bundle falling apart for tumbling as described above [2,3].

The *Salmonella* hook is a helical, tubular structure made of about 120 subunits of the hook protein FlgE. The diameter is 18 nm while the length is about 55 nm, which is controlled within ±7 nm [4] by at least two flagellar proteins, FliK and FlhB [5]. The hook is highly curved in the absence of mechanical force, orienting the filament nearly 90° off-axis of the motor, and this makes it easy for the flagellar bundle to form behind the cell during swimming.

The structure of *Salmonella* hook has been solved by a complementary use of X-ray crystallography of the subunit protein FlgE and electron cryomicroscopy (cryoEM) and helical image analysis of the straight polyhook produced by deletion mutation of FliK and by incubating it at 0°C [6–8]. *Salmonella* FlgE (*FlgE*) is a 402 amino-acid residue protein composed of four domains, D0, Dc, D1 and D2, which are arranged from the inner to the outer part of the hook structure (Fig. 1a) [6,8]. Domain Dc is a small domain connecting Domains D0 and D1, and the atomic model of domain Dc is missing because the resolution of the cryoEM map [8] is not high enough to trace the chain in this region, but the structure should be similar to that of *Campylobacter* hook, for which the complete structure has been published recently [9], because their sequences and density maps are similar to each other. Domain D0 is composed of the N- and C-terminal α-helices, which form a coiled coil at the innermost part of the hook structure to form the central channel with a diameter of 1.5 nm. The hook protein subunits are arranged in a helical manner to form the tubular structure composed of 11 protofilaments [7,8]. Each of the four domains of FlgE forms extensive interactions with the same domains in the neighboring subunits along either or both of the major helical lines, namely the left-handed 5-stranded and right-handed 6-stranded helical lines, but not along the near-axial 11-stranded helical lines that are recognized as the protofilaments [8]. This specific structural feature makes the hook structurally and mechanically stable and rigid against twisting while highly flexible in bending because each of the 11 protofilaments can be compressed or elongated relatively freely to allow the flexible bending of the entire hook structure [6,8].

The structure of *Salmonella* distal rod has also been solved by cryoEM helical image analysis [10]. Although the diameter of the distal rod is 13 nm, which is markedly smaller than that of the hook, the rod is far more rigid against bending than the hook to function as a drive shaft to transmit motor torque to the hook and filament. Based on careful comparison of the hook and rod structures and the highly homologous amino acid sequences of FlgE and FlgG, a FlgG specific sequence consisting of 18 residues was identified as an insertion in the region corresponding to domain Dc, and the actual insertion of this FlgG specific sequence into FlgE made the hook straight and rigid [3].

The hook is not a simply curved tube but is part of a supercoil, as indicated by the supercoiled structure of the

Table 1 Strains and Plasmids used in this study

Strains	Relevant characteristics	Source or reference
Salmonella enterica serovar Typhimurium		
SJW1353	*flgE*	[14]
HK1010	*flgE* Δ*fliK::tetRA*	This Study
HK1012	*flgE* Δ*fliK::tetRA* / pHMK624	This Study
Plasmids	**Relevant characteristics**	**Source or reference**
pUC19	multi-copy vector for cloning, Ap^r	NEW ENGLAND BioLabs
pHMK11	modified pTrc99A vector, Ap^r	[13]
pHMK605	*flgE* in pUC19	This Study
pHMK615	*flgE* (Ser1-Ala144 and Pro285-Arg402) in pUC19	This Study
pHMK624	*flgE* (Ser1-Ala144 and Pro285-Arg402) in pHMK11	This Study
pNM001	pTrc99AFF4/ wild-type FlgE	[16]

polyhook, which undergoes polymorphic transformations in response to changes in the salt concentration, pH and temperature of the solution [11]. Since the curvatures of those supercoils are much larger than those of the filament acting as a helical propeller, the mechanism of hook supercoiling was thought to be distinct from that of the filament, which is based on segregated arrangements of the protofilaments in two different conformations called the L-type and R-type that have slightly longer and shorter subunit repeats, respectively, in the 11 protofilament tubular structure [1,12]. An atomic model of the supercoiled hook built to simulate the hook shape observed in the native flagellum suggested that the supercoiled form is stabilized by near-axial interactions of the D2 domains on the inner surface of the supercoil (Fig. 1b) [6]. Here we studied the effect of deletion of domain D2 on the shape of the polyhook by constructing *Salmonella* in-frame deletion mutants lacking domain D2. We show that the deletion of domain D2 makes the hook straight, providing evidence to support the proposed hook supercoiling mechanism that it is the near-axial interactions between the D2 domains that stabilize the highly curved hook structure.

Materials and Methods

Bacterial strains, plasmids, DNA manipulations and media

A DNA fragment except for the D2 region corresponding to residues from Ala145 through Lys284 of *Salmonella* FlgE (UniProt P0A1J1, ΔMet1) was amplified by the inversed PCR method with primers (5'-CCGGGCGACCTGGTGAGC-3' as forward site and 5'-CGCCATCAGCGTGTTCGGAATGG-3' as reverse site) using pHMK605 as a template. The DNA fragment was self-circularized in the presence of T4 polynucleotide kinase and T4 ligase to create pHMK615. The NdeI-BamHI fragment containing $flgE_{\Delta D2}$ of pHMK615 was inserted downstream of the *trc* promoter in pHMK11 [13] to create pHMK624. A *fliK*-knockout strain of SJW1353 [14], HK1010, was constructed by homologous recombination using TetRA cassette [15]. Finally, pHMK624 was introduced into HK1010 by electroporation, and this cell construct was used to isolate the polyhook-basal body for the initial preliminary observation of the polyhook morphology by electron microscopy (EM) for a relatively crude specimen preparation. For the final EM observation presented in this study, pNM001 (pTrc99AFF4/ wild-type FlgE) [16] was modified to prepare a pTrc99A-based plasmid encoding wild-type FlgE or $FlgE_{\Delta D2}$ (lacking residues P139-K284) and was introduced into *Salmonella ΔflgE ΔfliK* strain to prepare the polyhook-basal bodies in a highly purified form. L-broth and soft agar plates were prepared as described before [17,18]. Strains and plasmids used in this study are listed in Table 1.

Preparation of polyhook-basal body

The polyhook-basal bodies were isolated from *Salmonella* cells as described before [19,20].

Electron microscopy

The isolated polyhook-basal bodies were negatively stained at room temperature with 2% phosphotungstic acid on carbon-coated copper grids 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) at a magnification of x5,500, which corresponds to 2.75 nm per pixel.

Results and Discussion

FlgE is a 402 amino-acid residue protein composed of four domains, D0, Dc, D1 and D2, arranged from the inner to the outer part of the hook structure (Fig. 1A) [6,8]. In order to study and compare the morphologies of the flagellar hooks made of wild-type FlgE and a FlgE mutant lacking domain D2, we used polyhooks that are produced by loss-of-function mutations in FliK [4] instead of short hooks of wild-type length on the hook-basal body, because it is easier to evaluate the morphology and estimate the curvature and

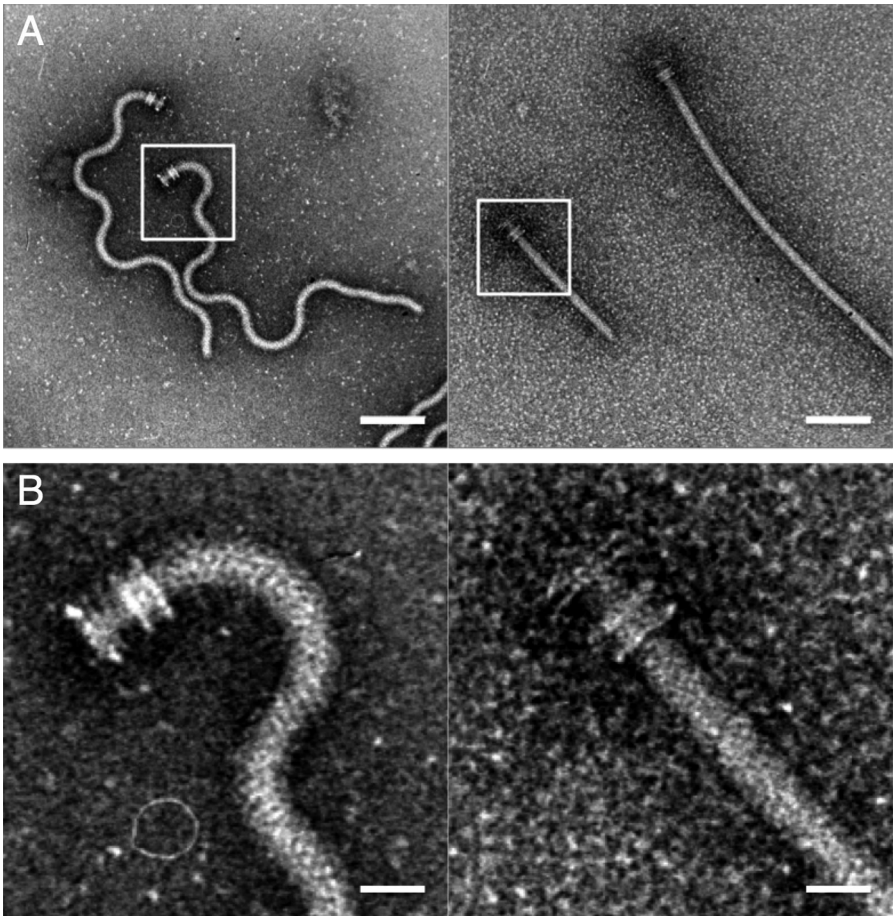

Figure 2 Electron micrographs of polyhooks produced by native FlgE and a mutant FlgE missing domain D2. (A) Wild-type FlgE forms supercoiled polyhooks (left) whereas the FlgE mutant made by deleting domain D2 forms straight polyhooks (right). (B) Magnified image of the boxed area in (A). The wild-type polyhook shows a helical feature of the D2 domains on the surface whereas the surface of the mutant polyhook is smooth due to the lack of domain D2. The polyhooks were negatively stained with 2% phosphotungstic acid (pH 6.5) at room temperature. Scale bar, 100 nm in (A) and 20 nm in (B).

supercoiling status. The FlgE mutant lacking domain D2 was made by deleting residues Ala 145 to Lys 284 for the initial observation and Pro 139 to Lys 284 for the final observation we carried out later. We isolated the flagellar polyhook-basal bodies from the Δ*fliK* and $flgE_{\Delta D2}$ Δ*fliK* double mutant cells and carried out EM observation of their morphologies by negative staining with 2% phosphotungstic acid (pH 6.5) at room temperature (Fig. 2). The polyhooks produced by the Δ*fliK* mutant cells adopted highly curved and supercoiled conformations (left). In contrast, the $flgE_{\Delta D2}$ Δ*fliK* double mutant cells produced straight polyhooks (right). In magnified images, the wild-type polyhook (Fig. 2B left) shows a feature of the helical array of the D2 domains on the surface whereas the surface of the mutant polyhook (right) is smooth due to the lack of D2 domains. This result clearly indicates that the deletion of domain D2 makes the otherwise supercoiled hook straight and therefore that it is the near-axial interactions between the D2 domains that stabilize the highly curved hook structure, as previously proposed based on the atomic model of the supercoiled hook built to simulate the shape of the short hooks observed in the wild-type flagellum and also that of supercoiled polyhooks [6].

It is known that the flagellar polyhooks can be converted into a straight form by lowering the solution pH to a range between 3.2 and 2.6, where the lower limit is to avoid disintegration of the polymer form [21]. It has also been known that hooks can be made straight by incubating them at nearly 0°C [6–8]. Our present result suggests that the near-axial interactions between the D2 domains are of electrostatic nature that is suppressed by lowering the pH or the temperature.

It would be interesting to see the functional consequence of the deletion of D2 domain in FlgE on *Salmonella* cell motility. Although the polyhook made of $FlgE_{\Delta D2}$ was observed as a straight form, the EM observation was made in the absence of external force that may deform the polyhook structure into curved forms. The bending flexibility of the hook should still be retained even after the deletion of

domain D2 because it is determined by the rather loose subunit packing interactions along the protofilament. Then, as far as the mutant hook retains the mechanical stability to function as a universal joint, the cell motility should not be impaired by the D2 deletion mutation. A functional study is now underway.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Author Contributions

T. F. and Y. I. isolated polyhook-basal bodies from the wild-type and mutant strains and observed them by electron microscopy. H. M. prepared the *flgE* mutant strains for the initial observation of polyhook morphology. T. F., H. M. and K. N. wrote the manuscript. K. N. supervised the study.

References

[1] Namba, K. & Vonderviszt, F. Molecular architecture of bacterial flagellum. *Q. Rev. Biophys.* **30**, 1–65 (1997).
[2] Brown, M. T., Steel, B. C., Silvestrin, C., Wilkinson, D. A., Delalez, N. J., Lumb, C. N., *et al.* Flagellar hook flexibility is essential for bundle formation in swimming *Escherichia coli* cells. *J. Bacteriol.* **194**, 3495–3501 (2012).
[3] Hiraoka, K. D., Morimoto, Y. V., Inoue, Y., Fujii, T., Miyata, T., Makino, F., *et al.* Straight and rigid flagellar hook made by insertion of the FlgG specific sequence into FlgE. *Sci. Rep.* **7**, 46723 (2017).
[4] Hirano, T., Yamaguchi, S., Oosawa, K. & Aizawa, S. Roles of FliK and FlhB in determination of flagellar hook length in *Salmonella typhimurium*. *J. Bacteriol.* **176**, 5439–5449 (1994).
[5] Minamino, T., Imada, K. & Namba, K. Mechanisms of type III protein export for bacterial flagellar assembly. *Mol. Biosyst.* **4**, 1105–1115 (2008).
[6] Samatey, F. A., Matsunami, H., Imada, K., Nagashima, S., Shaikh, T. R., Thomas, D. R., *et al.* Structure of the bacterial flagellar hook and implication for the molecular universal joint mechanism. *Nature* **431**, 1062–1068 (2004).
[7] Shaikh, T. R., Thomas, D. R., Chen, J. Z., Samatey, F. A., Matsunami, H., Imada, K., *et al.* A partial atomic structure of the flagellar hook of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **102**, 1023–1028 (2005).
[8] Fujii, T., Kato, T. & Namba, K. Specific arrangement of α-helical coiled coils in the core domain of the bacterial flagellar hook for the universal joint function. *Structure* **17**, 1485–1493 (2009).
[9] Matsunami, H., Barker, C. S., Yoon, Y. H., Wolf, M. & Samatey, F. A. Complete structure of the bacterial flagellar hook reveals extensive set of stabilizing interactions. *Nat. Commun.* **7**, 13425 (2016).
[10] Fujii, T., Kato, T., Hiraoka, K. D., Miyata, T., Minamino, T., Chevance, F. F. V., *et al.* Identical folds used for distinct mechanical functions of the bacterial flagellar rod and hook. *Nat. Commun.* **8**, 14276 (2017).
[11] Kato, S., Okamoto, M. & Asakura, S. Polymorphic transition of the flagellar polyhook from *Escherichia coli* and *Salmonella typhimurium*. *J. Mol. Biol.* **173**, 463–476 (1984).
[12] Yamashita, I., Hasegawa, K., Suzuki, H., Vonderviszt, F., Mimori-Kiyosue, Y. & Namba, K. Structure and switching of bacterial flagellar filaments studied by X-ray fiber diffraction. *Nat. Struct. Biol.* **5**, 125–132 (1998).
[13] Kojima, S., Furukawa, Y., Matsunami, H., Minamino, T. & Namba, K. Characterization of the periplasmic domain of MotB and implications for its role in the stator assembly of the bacterial flagellar motor. *J. Bacteriol.* **190**, 3314–3322 (2008).
[14] Ohnishi, K., Ohto, Y., Aizawa, S.-I., Macnab, R. M. & Iino, T. FlgD is a scaffolding protein needed for flagellar hook assembly in *Salmonella typhimurium*. *J. Bacteriol.* **176**, 2272–2281 (1994).
[15] Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645 (2000).
[16] Moriya, N., Minamino, T., Hughes, K. T., Macnab, R. M. & Namba, K. The type III flagellar export specificity switch is dependent on FliK ruler and a molecular clock. *J. Mol. Biol.* **359**, 466–477 (2006).
[17] Minamino, T. & Macnab, R. M. Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J. Bacteriol.* **181**, 1388–1394 (1999).
[18] Minamino, T. & Macnab, R. M. Interactions among components of the *Salmonella* flagellar export apparatus and its substrates. *Mol. Microbiol.* **35**, 1052–1064 (2000).
[19] Aizawa, S.-I., Dean, G. E., Jones, C. J., Macnab, R. M. & Yamaguchi, S. Purification and characterization of the flagellar hook-basal body complex of Salmonella typhimurium. *J. Bacteriol.* **161**, 836–849 (1985).
[20] Kubori, T., Okumura, M., Kobayashi, N., Nakamura, D., Iwakura, M. & Aizawa, S.-I. Purification and characterization of the flagellar hook-basal body complex of *Bacillus subtilis*. *Mol. Microbiol.* **24**, 399–410 (1997).
[21] Kagawa, H., Aizawa, S.-I. & Asakura, S. Transformations in Isolated Polyhooks. *J. Mol. Biol.* **124**, 333–336 (1979).