A structural model of flagellar filament switching across multiple bacterial species

Fengbin Wang¹, Andrew M. Burrage², Sandra Postel³, Reece E. Clark², Albina Orlova¹, Eric J. Sundberg⁴, Daniel B. Kearns² & Edward H. Egelman¹

The bacterial flagellar filament has long been studied to understand how a polymer composed of a single protein can switch between different supercoiled states with high cooperativity. Here we present near-atomic resolution cryo-EM structures for flagellar filaments from both Gram-positive Bacillus subtilis and Gram-negative Pseudomonas aeruginosa. Seven mutant flagellar filaments in B. subtilis and two in P. aeruginosa capture two different states of the filament. These reliable atomic models of both states reveal conserved molecular interactions in the interior of the filament among B. subtilis, P. aeruginosa and Salmonella enterica. Using the detailed information about the molecular interactions in two filament states, we successfully predict point mutations that shift the equilibrium between those two states. Further, we observe the dimerization of P. aeruginosa outer domains without any perturbation of the conserved interior of the filament. Our results give new insights into how the flagellin sequence has been “tuned” over evolution.
Most motile bacteria have complex structures known as flagella, with extracellular filaments that grow up to 15 µm long and span at hundreds of revolutions per second. There are three parts to the flagellar structure: the trans-membrane basal body that functions as the motor, the connecting rod and hook, and the flagellar filament that acts like a helical propeller. The bacterial flagellar filament has been intensively studied for many years. It has served as an enlightening system for understanding how a protein polymer composed of a single protein, flagelin (except for the cap protein at the end that acts as an assembling chaperone), switches among different states to supercoil. This supercoiling allows the filament to behave as an Archimedean screw and produce thrust. The filament can adopt different conformational states due to mechanical forces, such as when the motor switches the sense of rotation, allowing the bacteria to swim forward, backward, in a screw-like fashion and to tumble. With the motor linked to sensory receptors, the bacteria are capable of moving towards nutrients and away from dangerous environments, resulting in a significant survival advantage. On the other hand, mutations within the flagelin protein that fail to form supercoiled filaments generate no thrust when such straight filaments are rotated, leading to non-motile bacteria.

Our current understanding of supercoiling in bacterial flagellar filaments, referred to as polymorphic switching, is based upon the notion that protofilaments within the flagellar filament can exist in two discrete states, that differ slightly in length. The filaments curve due to shorter protofilaments forming the inside of supercoils, with longer protofilaments on the outside, generating periodic waveforms. Given 11 protofilaments in the intensively studied *Salmonella* flagellar filament, 12 different supercoiled states have been proposed, ranging from all protofilaments in the “short” state to all 11 protofilaments in the “long” state. The “short” state results from a protofilament having a right-handed inclination (the R-state), while the “long” state results from a protofilament having a left-handed inclination (the L-state). The structure of wild-type flagellar filaments with non-straight waveforms cannot be analyzed at high resolution easily, because the filaments do not have a simple helical symmetry in which every subunit is in an equivalent environment. To reconstruct filaments at high resolution, all protofilaments must be “locked” into the same state, either L- or R-type, producing straightened filaments that lead to non-motile bacteria. Based upon extensive work from the Namba laboratory using X-ray fiber diffraction, X-ray crystallography, and cryo-EM, atomic models have been proposed for straight *Salmonella enterica* filaments with all protofilaments in either the R-state or the L-state.

While these two atomic models represent a significant advance in understanding polymorphic switching of bacterial flagellar filaments, they do not provide sufficient mechanistic understanding of how switching occurs. Indeed, these models raise numerous questions that will need to be addressed so that we can develop drugs to inhibit the flagellar functions of pathogenic bacteria and engineer novel nano-machines for controlled movement of molecular cargoes. First, each model is based upon only a single amino acid variant, which came from the selection of flagellin mutants that cause loss of motility. Whether other motility mutants lead to the same or similar atomic models, or if a multiplicity of different states might arise from different mutants is unclear. Second, it has emerged that there is a divergence of quaternary structure in bacterial flagellar filaments, with those from *Campylobacter jejuni* having seven, rather than 11 protofilaments. As *C. jejuni* and *Salmonella* are both Gram-negative bacteria, the degree of divergence among not only Gram-negative organisms but also among the Gram-positive populations has not been resolved, as is the question of whether Gram-positive bacteria share a conserved flagellar filament structure with Gram-negative bacteria. Third, enormous advances have been made in resolution using cryo-EM over the past 4 years, largely driven by the availability of direct electron detectors. These detectors were not available with the *Salmonella* models were proposed. Here, we attempt to answer these questions and others using a direct electron detector by generating and studying locked, straight flagellar filaments from the Gram-positive bacterium *B. subtilis* and the Gram-negative *P. aeruginosa* with high resolution cryo-EM.

**Results**

**Cryo-EM structures of *B. subtilis* flagellar filaments.** Flagelin protein in *Bacillus subtilis* is expressed from the gag gene, which is homologous to flicC in *Salmonella enterica*. To obtain straight flagellar filaments suitable for cryo-EM analysis, we used low-fidelity PCR to randomly mutagenize an allele of the gag gene, gag209C, and screened for non-motile mutants in *B. subtilis*. The non-motile mutants could be fluorescently labeled with a mal- eimide dye due to the Hag209C allele and were assessed by fluorescence microscopy to identify straight structures (Blair et al. 2008). Of the mutants screened, 30 were non-motile and, of those, six were determined to have straight flagellar filaments by fluorescent microscopy. We also included a previously identified straight flagament mutant, Hag-A233V, in the study. Flagella were isolated from the mutants and cryo-EM images were collected using a Falcon II direct electron detector (Fig. 1a). Filaments were boxed and cut into overlapping segments, filament segments were sorted by the considerable structural polymorphism (both rise and rotation), and reconstructed to near-atomic resolution (the helical parameters and statistics of all seven reconstructions are listed in Table 1). Notably, all seven mutants (three L-type and four R-type mutants) have right-handed 1-start, left-handed 5-start and right-handed 6-start helices (Figs. 1b, c, Supplementary Fig. 1a, b). While the three L-type mutants (E115G, S285P, and S17P) have a left-handed 11-start helix, the four R-type mutants (N226Y, A233V, H84R and A39V/N133H) have a right-handed 11-start helix, and the designations L and R correspond to the hand of these 11-start helices. The L-type 11-start helices are tilted left by ~1.7° and the R-type 11-start helices are tilted right by ~3.9° (Figs. 1b–e).

We obtained near-atomic resolution reconstructions for both L- and R-type straight filament mutants. The first atomic filament model (R-type H84R) was built de novo using an established Rosetta protocol, and the other filament models were built by RosettaCM using the H84R model as the starting template. The best resolution reached for L- and R-type mutants reconstructions are 4.5 and 3.8 Å, respectively (Figs. 1d, e, Table 1) based on Fourier shell correlation (FSC) of the cryo-EM density map with the resulting filament model (Supplementary Fig. 2). We confirmed that this model:map FSC yields a similar estimate of resolution compared with the more traditional “gold standard” FSC between two independent map reconstructions (map:map). We calculated both FSC plots in the A39V/N133H dataset and found that the model:map FSC (4.3 Å) is similar but more conservative compared with the map:map FSC (4.1 Å, Supplementary Fig. 2f). A top view of these reconstructions clearly shows that both L- and R-type mutants have 11 protofilaments, forming annular tubes with inner diameters of ~25 Å and outer diameters of ~125 Å (Figs. 1d, e, and c), and these dimensions are similar to *Salmonella* if one excludes the D2/D3 domains. The Hag subunit is composed of two domains labeled D0 and D1, which are arranged on the inside and outside, respectively, of the flagellar filaments (Figs. 1d, e). For all seven mutants, the filament
core (D0) has a slightly better resolution than the outer region (D1), presumably due to packing stability (Supplementary Fig. 2). These observations are similar to previous reports of other helical assemblies where the outer residues are less ordered than the inner ones.

The subunits of L-type and R-type Hag share highly similar secondary architecture (Supplementary Fig. 3a, b). The D0 domain is composed of two α-helices (ND0 and CD0) that form a short coiled-coil. The D1 domain is composed of a β-hairpin and three α-helices (ND1a, ND1b and CD1), which form a longer coiled-coil than in D0. The D0 and D1 domains are connected by two loop regions: residues S31-D47 (NL) connect ND0 and ND1a and residues R264-D267 (CL) connect CD0 and CD1 (Supplementary Fig. 3a, b). The entire backbone, except for the first four residues at the N-terminus, can be traced unambiguously and built in the reconstruction for both L- and R-type subunits. Side chain densities can be seen for most of the residues (Supplementary Fig. 3a, b), which allows us to accurately determine the register of the amino acid sequence in the cryo-EM density. These are the highest resolution reconstructions of bacterial flagellar filaments reported to date.

Cryo-EM structures of *P. aeruginosa* flagellar filaments. Next, we analyzed flagellar filaments from the Gram-negative bacterium *Pseudomonas aeruginosa* to compare with a Gram-negative species. Since the D0 and D1 domains of *P. aeruginosa* and *S. enterica* share a high degree of sequence identity (55%), we tested the conserved L- and R-type mutations characterized in *S. enterica* (G426A and A449V, respectively) which correspond to G420A and A443V in *P. aeruginosa*, respectively. We found that these mutations also generate straightened filaments in this organism. Filaments from these two FliC mutants of *P. aeruginosa* were sheared off the bacteria, concentrated, plunge-frozen and cryo-EM imaged (Fig. 2a). Each mutation results in L- and R-type straight filaments as expected, which suggests that the D0/D1 architecture is indeed conserved between *P. aeruginosa* and *S. enterica*.

For both the L- and R-type *P. aeruginosa* filaments, we identified three pseudo layer-lines in the power spectra from the filaments, besides the 1-, 5-, 6- and 11-start layer-lines that are similar to *B. subtilis*, which correspond to non-helical perturbations (Supplementary Fig. 1c, d). Similar perturbations have been observed before in a particular *Salmonella* strain as well as in other bacteria, and correspond to a larger asymmetric unit containing two flagellin subunits. The non-helical nature of the pairing is due to the fact that a seam is introduced into the structure, which is a discontinuity in the helical surface lattice (Fig. 2c). To further investigate whether this non-helical
After one cycle, inter-subunit pairing can be seen clearly in the parameters were applied (Fig. 2c, d). The rotation and shift for the Dα atoms twist more when they are further from the Dβ subunits are aligned by their D0 domains, which comprise the inner part of the filament, the D1 domain of the L-type subunit was twisted clockwise from the D0 domain of the R-type subunit (Fig. 3a). The rotation and shift for the Dα atoms in the D1 domain range from 2 to 10° and 1.5–11 Å, respectively. The D1 domain Dα atoms twist more when they are further from the D0 domain, which places them at the outer region of the filament. A similar twisting motion was observed when L- and R-type subunits were aligned by the D1 domain. The small RMSD of 1 Å, suggests a highly conserved fold between Gram-positive and -negative bacteria, and between the L- and R-states (Fig. 3b, bottom left). However, if the RMSD is calculated from the connected D0-D1 helices, it can be seen from Fig. 2c that the structure can actually be viewed as an ideal helix, but one with an asymmetric unit containing 2 flagellin subunits. This very large asymmetric unit would be related to adjacent ones by an axial rise of 22*4.61 Å = 101.5 Å and a rotation of 6.41°, generating a 1-start helix with a pitch of 5700 Å having 56.16 units per turn. These symmetry deviations were applied (Fig. 2d) to an IHRSR reconstruction. After one cycle, inter-subunit pairing can be seen clearly in the reconstructed volume across the 5-start helices (Figs. 2c, d), where the D3 domain of the subunitSN (green dots in Fig. 2c) interacts with the D2 domain of subunit SN+1 (red dots in Fig. 2c). The pseudo layer-lines reflecting this pairing can be seen from the power spectrum of the projection of this volume (Supplementary Fig. 4), matching the layer lines seen in the actual images. Unfortunately, the reconstruction fell apart very quickly with additional cycles so a higher resolution reconstruction could not be obtained. This was not surprising and is due to the very large asymmetrical unit combined with the poor signal-to-noise ratio in the micrographs.

Overall comparison of the L- and R-type structures. Since these are the first near-atomic resolution structures of L- and R-type filaments from both Gram-positive and -negative strains, we set out to compare detailed differences in the molecular architecture between L- and R-type filaments. First, we compared a single subunit from each filament type by superimposing their Cα backbones (Fig. 3a), which shows that the L- and R-type subunits of B. subtilis share the same secondary structure. When two subunits are aligned by their D0 domains, which comprise the inner part of the filament, the D1 domain of the L-type subunit was twisted clockwise from the D0 domain of the R-type subunit (Fig. 3a). The rotation and shift for the Dα atoms in the D1 domain range from 2 to 10° and 1.5–11 Å, respectively. The D1 domain Dα atoms twist more when they are further from the D0 domain, which places them at the outer region of the filament. A similar twisting motion was observed when L- and R-type subunits were aligned by the D1 domain. The small RMSD of 1 Å, suggests a highly conserved fold between Gram-positive and -negative bacteria, and between the L- and R-states (Fig. 3b, bottom left). However, if the RMSD is calculated from the connected D0-D1 domains, we can see a clear clustering by handedness: domains of the same hand from two species share a very conserved backbone

| Table 1 Refinement statistics for the flagellar filament models |
|---------------------------------------------------------------|
| Mutation site (s) | B. subtilis | P. aeruginosa |
| S285P | E115G | S17P | N226Y | A39VN133H | H84R | A233V | G420A | A443V |
| Filament hand (1-start) | Left | Left | Left | Right | Right | Right | Right | Left | Left |
| **Helical symmetry** | | | | | | | | | |
| Rise (Å) | 4.72 | 4.72 | 4.68 | 4.64 | 4.65 | 4.64 | 4.64 | 4.73 | 4.61 |
| Rotation (°) | 65.30 | 65.30 | 65.29 | 65.83 | 65.81 | 65.81 | 65.81 | 65.27 | 65.75 |
| CTF selected images | 569 | 285 | 539 | 604 | 826 | 490 | 324 | 104 | 637 |
| **Total segments** | 138,327 | 41,587 | 67,195 | 97,487 | 315,847 | 165,589 | 74,183 | 17,450 | 209,965 |
| **Sorted segments** | 55,403 | 22,682 | 13,899 | 72,005 | 134,766 | 58,771 | 33,992 | 17,450 | 102,119 |
| **Resolution** (Å) | 4.5 | 5.7 | 6.7 | 3.8 | 4.3 | 4.4 | 5.5 | 4.2 |
| **Clash score, all atoms** | 6.3 | 3.7 | 3.1 | 4.3 | 3.3 | 1.9 | 4.0 | 9.1 | 3.6 |
| **Protein geometry** | | | | | | | | | |
| Ramachandran favored (%) | 88.9 | 90.5 | 87.3 | 91.0 | 90.7 | 91.7 | 90.6 | 93.5 | 90.2 |
| Ramachandran outliers (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rotamer outliers (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Cβ deviations > 0.25 Å** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **RMS deviations** | | | | | | | | | |
| Bond (Å) | 0.006 | 0.007 | 0.007 | 0.008 | 0.005 | 0.007 | 0.006 | 0.010 | 0.008 |
| Angels (°) | 0.98 | 1.16 | 1.03 | 1.11 | 0.88 | 0.96 | 0.96 | 1.36 | 1.11 |
| Molprobity score | 1.93 | 1.70 | 1.72 | 1.73 | 1.65 | 1.44 | 1.72 | 1.91 | 1.69 |
| Molprobity percentile | 99 | 99 | 99 | 99 | 99 | 99 | 99 | 99 | 99 |
| PDB ID | 5WJY | 5WJZ | 5WJX | 5WJT | 5WJU | 5WJV | 5WJW | 5WK6 | 5WK5 |
| EMD ID | 8852 | 8853 | 8851 | 8847 | 8848 | 8847 | 8848 | 8850 | 8856 | 8855 |

| Notes: |
|---|
| 1Resolution of all filaments was estimated by model-map FSC (0.38 cutoff). This approach was validated by using a mapmap FSC from non-overlapping data sets for the A39VN133H mutation, starting with volumes filtered to 10 Å resolution as starting references. This yielded an FSC at 0.138 of 4.1 Å. |
| 2Reconstruction of sorted P. aeruginosa G420A segments gave a worse resolution than unsorted segments, presumably due to limited number of total segments |

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trace (RMSD 0.6 ~ 0.9 Å), whereas the RMSD of different hands ranges from 1.5 to 3.3 Å (Fig. 3b, upper right). Altogether this structural investigation reveals strong conservation in the individual D0 and D1 fold between L-type and R-type filaments, and even between different bacterial species. Furthermore, it shows that the major difference between L- and R-type subunits resides in the rotation angle mediated by the two connecting loops (NL and CL).

Next, we compared the subunit-subunit interactions of the L- and R-type filaments. A subunit Sβ in the flagellar filament interacts with eight other subunits: Sα5, Sα6, Sα11, Sβ15, Sβ25, Sβ46, Sβ11, Sβ16. Since the interactions between Sα and Sβ are the same as the interactions between Sα and Sα only five subunits (Sβ0, Sβ5, Sβ10, Sβ15, Sβ16) were used to analyze all the unique interactions in the flagellar filaments as shown in Fig. 3c. PISA interface analysis shows that for both L- and R-type structures, Sβ is making major contacts with Sα5 and Sα11 with an interfacial area of ~1900 Å², minor contacts with Sα16 with an interfacial area of ~270 Å², and intermediate contacts with Sα6 with an interfacial area of ~600 Å² (Supplementary Table 1). In the 5-start interface, we detected contacts of Sβ-ND1a/b to Sα5-CD1 and Sβ-ND1b to Sα5-β-hairpin. In the Sβ and Sα11 interface, we detected that the Sβ-CD1 makes contacts with Sα11-ND0, ND1 and β-hairpin, and Sβ-ND1a interacts with Sα11-ND1 and β-hairpin. There are also contacts between Sβ-CD0 and Sα11-ND0 and CD0. Even in the relatively smaller interface of Sβ and Sα10, we were able to detect interactions between D1 domains: Sβ-ND1b and Sα16-ND1a. The interface of Sβ and Sα6 on the other hand, only involves the interactions of D0 domains: Sβ-CD0 and Sα6-ND0 and CD0 (Fig. 3c, d). These observations differ from previous conclusions derived from Salmonella filament structures, in which the 5-start interface has been considered as the only inter-subunit D1 domain interaction and the only important interface for the L/-R- switch mechanism. By analyzing homologous flagellar filaments reconstructions with much higher resolution from two other bacterial species, we suggest that the D1 domain interactions along the 5-start helices in Salmonella exist but are not the only interactions.

Finally, we compared the filament packing of all the L- and R-type structures by calculating the RMSD of the five subunits
containing all of the unique interactions (Fig. 3e). Similar to single subunit RMSD calculation (Fig. 3b), we observed striking clustering/conservation among filaments of the same hand regardless of the species. In fact, the average five-subunit RMSD of R-type filaments between two species is only 0.8 Å, significantly lower than the average five-subunit RMSD (greater than 2.0 Å) between any L- and R-type filaments (Fig. 3e). These calculations strongly suggest that filaments of the same hand are not only conserved in their single subunit architecture, but also highly conserved in terms of...
**Fig. 4** Comparison with flagellar filaments of other bacterial species. 

(a) A comparison of L-type flagellar structures in *B. subtilis* (S285P), *P. aeruginosa* (G420A) and *S. enterica* (PDB: 3A5X, EMD-1641). A comparison of the segmented maps corresponding to a single subunit is shown on top. A diameter comparison from the top view of the filaments is shown on the bottom. 

(b) Superimposition of the single L-type subunits: three from *B. subtilis* (yellow, S285P, S17P, E115G), one from *P. aeruginosa* (orange, G420A) and one from *S. enterica* (dark green, 3A5X). The dashed line indicates where the crystal structure of *S. enterica* ends.

(c) Alignments of the flagellin amino acid sequence from four gram-positive bacteria and four gram-negative bacteria. Single mutants in *B. subtilis* are marked with filled red stars, the double mutants are marked with empty red stars, and the mutants in *P. aeruginosa* are marked with filled blue squares.
helical parameters (rise and rotation), inter-subunit interactions and overall filament packing. This provides a comprehensive structural confirmation of the bi-state mechanism proposal, by using nine different structures from two different species.

Domains and interfaces responsible for polymorphic switching. Extensive work has been done in S. enterica to identify the residues responsible for the L- and R-type polymorphic switching, including mutagenesis, computational simulations and cryo-EM approaches. To date, only two lower resolution cryo-EM

Fig. 5 Mutation sites of B. subtilis and their molecular basis that lead to straight filaments. a Locating the mutation sites on the single flagellin subunit. b Locating the mutation sites on the 5-start interface (left) and the 11-start interface (right). c–f Subunits S₀, S₁₁, S₁₆ and S₅ are colored in dark yellow, purple, green and blue, respectively. Un-mutated residues are shown in gray, and adapted from the other mutants in the same hand. Mutation sites for: left-handed mutation E₁₁₅G c, right-handed mutation H₈₄R e, and right-handed mutation H₈₄R e, and right-handed mutation A₂₃₃V f. g Top view of two mutant structures N₂₂₆Y (gray) and S₂₈₅P (purple) aligned by upper part of D₀ domain (amino acids 18–32 and 268–284). h Top view of two mutant structures N₂₂₆Y (gray) and S₁₇P (blue) aligned by the same upper part of D₀ domain.
structures of S. enterica flagellar filaments have been reported24, 25. A comparison between S. enterica, B. subtilis and P. aeruginosa filament structures shows that their D0/D1 cores share a very conserved 11-protofilaments packing, with an inner diameter of ~25 Å and an outer diameter of ~125 Å (Fig. 4a). In fact, like P. aeruginosa we were able to generate the same-hand straight filaments using mutations corresponding to those reported in S. enterica (Fig. 2, Table 1). In contrast, in Campylobacter jejuni which has been reported to have seven and not 11 protofilaments, a mutation corresponding to the Salmonella A449V mutation does not lock the filaments into a straight form27. This strongly suggests that D0/D1 core is very conserved among species that have 11 protofilaments, and only the D0/D1 core is responsible for the L/R switching in these bacteria.

To determine whether S. enterica shares the same D0/D1 core, five L-type subunits were superimposed in Fig. 4b. As mentioned previously, the three L-type subunits of B. subtilis and one L-type subunit of P. aeruginosa aligned very well in both D0 and D1 domains. However, the L-type subunit of S. enterica only aligns well with other subunits in most of its D1 domain (above the dashed line) where a crystal structure was available28, while its D0 domain is shifted ~8 Å, presumably due to a poor map with very low resolution in this region (Supplementary Fig. 5) and the absence of a corresponding crystal structure. Thus, we present here the first accurate models of the D0-D1 connecting loop and the D0 domains.

In contrast to D0/D1, the D2/D3 domains are not conserved among species in terms of sequence, packing and oligomeric state within the filament (Figs. 4a, c). The flagellar filaments in B. subtilis and other Gram-positive species lack most of D2/D3; instead the β-hairpin and CD1 is connected by a small loop (Figs. 4a, c). On the other hand, S. enterica, P. aeruginosa and other gram-negative bacteria have D2/D3 domains, but with no detectable sequence identity (Supplementary Fig. 6). From our structures (Fig. 2d), in P. aeruginosa the D3 domain extends along the 11-start and forms a dimer with the D2 domain of another subunit; while in S. enterica, the D3 domain extends from the filament and does not interact with other subunits. Although the D2/D3 domains of Gram-negative bacteria contain ~200 amino acids, they may be considered unnecessary for the polymorphic switching for two reasons: (1) two spontaneous mutants of S. typhimurium previously reported with deletion of most or part of D2/D3 domains are still capable of swimming29, and (2) we show here in B. subtilis that switching occurs in the absence of D2/D3 domains (Figs. 4a, c).

During the random mutagenesis process to screen for straight mutants, we identified several new sites in B. subtilis not previously implicated in polymorphic switching (Fig. 4c). Strikingly, we found those sites are not necessarily conserved among different species or participate in unique inter-subunit interactions. These include three straight mutations outside of the D1 helices: S17P, S285P (both within D0) and the double mutant A39V/N113H (within the loop region) (Fig. 5a). We also identified several straight mutations that are only involved in the 11-start interactions: S17P, H84R and A39V in the double mutants (Fig. 5b). The existence of these mutations strongly suggests that the previous hypothesis, which proposes that only D1 domain interactions along the 5-start helices are involved in determining the L/R switching4, is incomplete and oversimplified.

Molecular basis for polymorphic switching. From the analysis of seven straight mutants in B. subtilis and two straight mutants in P. aeruginosa, we confirmed the bi-state mechanism of polymorphic switching: that only two types of subunit-subunit interactions (L-type and R-type) exist. This suggests that the wild type flagellar filaments with swimming motility must adopt an intricate “balance” in the flagellin sequence, so that the sequence does not generate a strong preference for either the L- or R-type conformation. Such a balance is required for the sharp transition of flagellar filaments switching between different waveforms during bacterial swimming and tumbling. The fact that straight phenotypes can be readily found due to single point mutations indicates that this balance is exquisitely sensitive to small changes, such as a single mutation, and can be easily tipped towards a dominant conformation, either all L or all R, which eliminates motility. To date, the molecular basis for the role of these mutations in abrogating switching has not been established.

To better understand the molecular basis of each mutation, we compared the local interactions of L- and R-type subunits in B. subtilis by examining ~20 amino acids around the mutation sites (Figs. 5c–f). In R-type subunits of B. subtilis, the side chain of E115 in S0 forms two conserved hydrogen bonds with the side chain of N240 in subunit S5. These two sites (E115G and N240) are mostly conserved among different bacterial species (Fig. 4c), and we also detected the same interaction in the R-type A443V mutant in P. aeruginosa. In the B. subtilis E115G mutant, the filaments lose this conserved R-type interaction and therefore formed a left-handed filament (Fig. 5c). The residue N226 in B. subtilis is not a conserved site and does not form any conserved interactions with other subunits. In the R-type N226Y mutant, the side chain of Y226 in subunit S5 forms new hydrogen bonds with the side chain of Q110 and the main chain of T107 in subunit S0. But in the L-type subunits, Y226 likely causes major steric clashes. Therefore, N226Y filaments adopt a right-handed form (Figs. 4c, 5d). Similarly, residue H84 in B. subtilis is not conserved in terms of sequence and subunit-subunit interactions. In the R-type mutation H84R, the R84 side chain in subunit S0 forms new hydrogen bonds with the side chain of S229 in subunit S5, and the E50 side chain in subunit S11, enhancing the R-type interaction. As a result, the H84R filaments adopt a right-handed form (Figs. 4c, 5e). Another example is the R-type mutant A233V in B. subtilis. A233 is conserved among Gram-positive species but not in Gram-negative species. In the A233V mutant, valine in subunit S0 makes stronger contacts than an alanine residue with the hydrophobic pockets formed by A43, L46 and A47 in subunit S11, and A233V filaments adopt a right-handed form (Figs. 4c, 5e). We also detected two mutations, S17P and S285P, located in the middle of the small coiled-coil D0 domain (Fig. 5a). These two residues are not conserved in other species, and proline is not found at this position in any wildtype species. This is likely because the proline cannot donate an amide hydrogen bond and its sidechain interferes sterically with the α-helical backbone. Therefore, mutation S17P or S285P in B. subtilis forces a local bend of ~30° in the helix axis, and this conformational change makes the D0 coiled-coil adopt an L-type form more easily (Figs. 5g, h).

Designing mutations that lead to R-type transformation. A stringent test of the model presented above is whether we can design new mutants that switch the wildtype filament into other waveforms. We hypothesized that constructing A237V or S71L mutants individually, or generating an A47CA233C double mutant (where an intermolecular disulfide would form between these residues) would shift filaments towards the R-type, while R915 or F132V individual mutants would shift filaments towards the L-type. These mutants were built and visualized by fluorescence microscopy (Figs. 6a, b).

Predictions were based on the comparison of the models of these mutations on the L- and R-type scaffolds (Supplementary
structures do not exist and the cryo-EM maps were limited in resolution. These incomplete structural models therefore led to inconsistent predictions at the single amino acid level\textsuperscript{21}. Here our high-resolution reconstructions expand our knowledge and we can now observe how mutations in D0 can cause a switching of the flagellar structure.

Our observations do not support some predictions from previous studies. A molecular dynamics simulation of switching in Salmonella flagellar filaments\textsuperscript{22} described three classes of inter-subunit residue pairs: “permanent” (those interactions that would be conserved between the L- and R-states), “sliding” (those variable hydrophobic or hydrophilic interactions with new partners that allow inter-subunit shear without a large change in energy), and “switching” (those key interactions that when made or broken cause a shift of the equilibrium from the L- to R-state or vice versa). We map the three classes proposed for Salmonella onto the corresponding observed residue pairs in B. subtilis (Supplementary Fig. 8) for both the L- (orange circles) and R-state (blue circles). For “permanent” interactions the distances should be conserved between the L- and R-states, while for both “sliding” and “switching” interactions the distances should differ between L and R. What we observe, however, is that some of the “sliding” and “switching” interactions are more conserved than the proposed “permanent” ones, and that most of the “permanent” ones are not permanent at all.

We have directly visualized how the outer D2/D3 domains within P. aeruginosa dimerize while not perturbing the conserved interactions that take place within the D0 and D1 core. However, the functional importance of D2/D3 domains still remains unknown, and they are dispensable in Salmonella for swimming motility\textsuperscript{23}. Instead, they have been considered to contribute as radial spokes for bacteria that swim in a high viscosity environment\textsuperscript{46} or provide antigenic variability used to escape immune surveillance\textsuperscript{47}.

The bacterial flagellar filament is an exquisitely tuned system that represents evolutionary development over hundreds of millions of years. In contrast to the simplicity of the bacterial flagellum, the eukaryotic flagellum, which has no homology to the bacterial one, is based upon microtubules and dynein rather than a homolog of flagellin and is currently estimated to contain more than 400 different proteins\textsuperscript{48}. The archaean flagellum, which has no homology to either the bacterial or eukaryotic ones, has only recently been solved at near-atomic resolution\textsuperscript{47, 48} showing how the core of these archaean filaments is formed by a domain that is homologous to the N-terminal domain of bacterial Type IV pilin.
Convergent evolution has thus yielded three very different flagellar filaments that all allow cells to swim, although by entirely different mechanisms. We are now entering a new era where the structure of such filaments can be solved at a near-atomic level of resolution using cryo-EM. We expect that the present study and future studies will yield new insights into how flagella-based swimming motility has independently arisen at least three different times in evolution using very different components, and how these convergent adaptations use very different mechanisms to achieve a similar function.

**Methods**

### Strain and growth conditions

*P. aeruginosa* strains were grown in lysogenic broth (LB) (10 g tryptone, 5 g yeast extract, 5 g NaCl per 1 L) or on LB plates fortified with 1.5% Bacto agar at 37 °C. When appropriate, antibiotics were included at the following concentrations: 100 µg ml⁻¹ spectinomycin, 5 µg ml⁻¹ chloramphenicol, 5 µg ml⁻¹ kanamycin, Isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) was added to the medium at the indicated concentration when appropriate.

For swimming motility assays, swim agar plates were 85% confluent with a single overnight colony, incubated in a humidity chamber at 37 °C, and scored for motility after incubation for 18 h.

### Hag mutagenesis

To produce the initial straight filament mutants, genomic DNA from DS1919 (*Δhag* amyE::P₅₁-hagT²⁰⁰C spec) was PCR amplified using primers 953/1009 and the Expand Long Template low-activity polymerase system (Roche) to introduce random base pair mutations into the amyE::P₅₁-hagT²⁰⁰C spec construct. The products were transformed into XLI-Blue Mopped E. coli strain deleted for the highly competent PT79 lab strain background. Transformants were streaked on swim agar plates and swim-deleted mutants were isolated. The mutated amyE::P₅₁-hagT²⁰⁰C spec locus was then transduced to the DS1677 Δhag 3610 ancestral strain background via SPPI-mediated generalized phage transduction. Strains were assessed for swarming motility (which like swimming motility requires functional flagella) as described below, and straight filament mutants were verified by fluorescent microscopy. Genomic DNA was isolated, and the amyE::P₅₁-hagT²⁰⁰C spec construct was amplified with primers 953/1009. The products were sequenced using primers 1008/3459. To increase flagellar expression, the P₅₅ₑ promoter was replaced with an IPTG-inducible Pₐₔ₈ₐₛ₉ promoter by transducing the Pₐₔ₈ₐ₄₉ promoter into each filament mutant background by SPPI-mediated generalized transduction using DK14 as a donor. All strains used in this study are listed in Supplementary Table 2. All primers are listed in Supplementary Table 3.

For site-directed mutageneses, *hag* was inserted into the *apre* ectopic locus by Gibson assembly. The *apre* upstream and downstream homology regions were amplified from 3610 chromosomal DNA as a template with primers 4440/4894 and 4349/4894, respectively. The *hag* gene and its native promoter were amplified with primers 4893/4549. To increase flagellar expression, the P₅₅ₑ promoter was replaced with an IPTG-inducible Pₐₔ₈ₐ₄₉ promoter by transducing the Pₐₔ₈ₐ₄₉ promoter in each filament mutant background by SPPI-mediated generalized transduction using DK14 as a donor. All strains used in this study are listed in Supplementary Table 2. All primers are listed in Supplementary Table 3.

### Purification of flagellar filaments of *Bacillus subtilis*

Straight filament strains were transduced with DK14 to produce flagella overexpression constructs. Flagellar filaments were isolated using a modified protocol from Aizawa et al. Strains cultured overnight in 250 ml flask were back-diluted to 0.1 OD₅₆₂₀ into 500 ml LB containing the proper antibiotic. Cultures were shaken at 37 °C and 220 rpm for approximately 1.5 h. A sample was withdrawn and stained using the conventionally accepted concentration point (demarcated by the India ink) were saved for further experimentation.

### Fluorescence microscopy and waveform calculation

For fluorescence microscopy of flagella, 1 ml of cell culture at mid-log phase was harvested in 1.5 ml microcentrifuge tubes at 7000 RPM for 1 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml of PBS. Alexa Fluor 488 C₅ maleimide (Molecular Probes) was added to the sample to achieve a final concentration of 2 µM and incubated at 37 °C for 40 min. The solution was ultracentrifuged in a SW40 Ti rotor (60,000 g, 18 h), and resuspended in PBS. Flagella were sheared off the cells by passing the suspension through a 20 gauge needle and syringe, dialyzed in 0.1 M Tris-HCl buffer, and resuspended in 11 ml sodium citrate, and 4.7 g of CaCl₂ was added. The protein solution was ultracentrifuged in a SW40 Ti rotor (60,000 g, 18 h), and filaments formed an opaque white band approximately halfway down the tube. Filaments were collected by a 18ga needle and syringe, dialyzed in 0.1 M Tris-HCl buffer, and stored at 4 °C prior to imaging.

### Sample preparation of flagellar filaments of *P. aeruginosa*

The *flc* sequence from *Pseudomonas aeruginosa* PAO1 was aligned with *flc* from *Salmonella typhimurium* to identify residues corresponding to the mutations G426A and A449V that were shown to result in straight filaments. The *P. aeruginosa* *flc* mutations G426A and A449V were introduced into pUCP12 and transformed into the transposon knockout line PW2971 (*Δflc*) obtained from the Manoil Lab at the University of Washington using electroporation as described. *P. aeruginosa* PAO1 strains were grown overnight in LB liquid culture, cells were spun down and resuspended in PBS. Flagella were sheared off the cells by passing the suspension through a 20 gauge needle and syringe 25 times. After centrifugation, the supernatant containing flagella was concentrated using a centrifuge.

### Cryo-electron microscopy and image processing

Flagellar filament samples (3.5–4.5 µl) were applied to lacy carbon grids and vitrified with the Vitrobot Mark IV (FEI). The grids were imaged at a Titan Krios operating at 300 kV using a Falcon II camera with a 1.05 Å pixel sampling, with the CTF parameters controlled by the EPU software. Images were collected using a defocus range of 0.5–3.0 µm, with a total exposure of 2 × dose-fractionated into seven chunks. All the images were first
motion corrected by the MotionCorr v2.1 \cite{8852} and then the CTFFIND3 program \cite{8853} was used for detecting the actual defocus of the images. Images with poor CTF estimation as well as defocus > 3 μm were discarded. Total number of images selected for each mutation are listed in Table 1. Filaments of varying lengths were boxed using the e2helixboxer program within EMAN2 \cite{8855}. The SPIDER software package \cite{8856} was used for most other operations. The CTF was corrected by multiplexing. The first two chunks (containing a dose of ~ 20 electrons per Å²) with the theoretical CTF, which is a Wiener filter in the limit of a very poor signal-to-noise ratio (SNR). Overlapping long boxes (512 pixels for bacillus subtillis mutant N2267F, 384 pixels for the other mutants) with a shift of 7 pixels (± 1.5 times of the box) were cut from the long filaments. A reference-based sorting procedure was used to bin the segments based on the axial rise and azi-muthal rotation. The number of total segments and the segments used after sorting for each mutation are listed in Table 1. After sorting, the segments were processed by the IHRRS method \cite{8857} to produce the final reconstructions.

**Model building.** The first model B. subtillis H84R was built using the de novo model-building protocol of Rosetta\cite{8858}. First, the map corresponding to a single flagellar subunit was segmented from the experimental filament density in Chimera\cite{8860}. Then a fragment library that contains pieces of experimentally determined structures was generated for the Rosetta server\cite{8861}. This allowed for approximately 70% of the backbone of one H84R subunit being successfully built in Rosetta. Then the full-length H84R subunit was rebuilt with the RosettaCM protocol\cite{8862}. A total of 1000 full-length models were generated based on the segmented map, and the top ~15 models were selected according to the Rosetta's energy function. Those selected models were then combined into one model by manual editing in Coot\cite{8863} using the criteria of the local fit to the density map and the geometry statistics of the model. This model was used as the starting point for a whole filament RosettaCM rebuilding restrained by the determined helical symmetry and the whole experimental filament map. A total of 500 filament models were generated, and the best 15 models were combined again into one model in Coot\cite{8864} using the e2helixboxer program within EMAN2\cite{8855}.

**Data availability.** The reconstruction maps were deposited in the Electron Microscopy Data Bank with accession numbers of 8847, 8848, 8849, 8850, 8851, 8852, 8853, 8855, and 8856. The corresponding filament models were deposited in the Protein Data Bank with accession numbers of 5WJT, 5WJU, 5WJV, 5WJW, 5WX, 5WJY, 5WJZ, 5WK5 and 5WK6. The additional data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
A.M.B. performed site directed mutagenesis, phenotyping, and prepared the B. subtilis filament samples; R.E.C. screened for randomly generated non-motile alleles of B. subtilis hag and identified straight filament mutants; S.P. prepared the P. aeruginosa filament samples; A.O. and F.W. collected cryo-EM data; F.W. and E.H.E. performed image processing; F.W. did the structural modeling; F.W., A.M.B. and E.H.E. prepared figures; F.W. and E.H.E. wrote the manuscript; E.H.E., E.J.S. and D.B.K. conceived the study.

Additional information
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Supplementary Figure 1. Power spectra of flagellar filaments. The power spectra of raw image stack of L-type (a) and R-type (b) straight filaments of B. subtilis. The power spectra of raw image stack of L-type (c) and R-type (d) straight filaments of P. aeruginosa. (e) An image of negatively stained wild-type flagellar filaments of P. aeruginosa, and the corresponding averaged power spectrum from these filaments showing that the non-helical perturbation observed in mutants also exists in the wild-type.
Supplementary Figure 2. Resolution of the flagellar filament structures of *B. subtilis* (a-g) and *P. aeruginosa* (h, i), derived from Fourier Shell Correlation (FSC) calculation. The red and blue lines are FSC of the full map and the D0 core, respectively, calculated between the refined atomic model and the map. The resolution in these plots is estimated using FSC=0.38, which is sqrt(0.143). A “gold standard” FSC between two half maps was also calculated for the A39VN133H dataset (f, green line) yielding a resolution of 4.1 Å at FSC=0.143. This suggests that the model:map FSC curves provide a similar estimate of resolution as the more conventional map:map FSC, and if anything are more conservative.
Supplementary Figure 3 The structure of a single flagellin subunit and its corresponding map density in *B. subtilis* and *P. aeruginosa*. (a) L-type subunit (S285P) of *B. subtilis* (b) R-type subunit (N226Y) of *B. subtilis* (c) L-type subunit (G420A) of *P. aeruginosa* (d) R-type subunit (A443V) of *P. aeruginosa*
Supplementary Figure 4 power spectra of *P. aeruginosa* filaments. (a) The power spectrum of raw filament images of *P. aeruginosa*. (b) The power spectrum of the reconstructed volume projection, considering one subunit as the asymmetrical unit (D0 and D1 domains) (c) The power spectrum of the reconstructed volume projection, considering 22 subunits as the asymmetrical unit (D2 and D3 domains)
Supplementary Figure 5. The density maps comparison between *Salmonella* (EMD-1641) and *Bacillus* (N226Y). Corresponding cryo-EM map of D0 and D1 domains with similar threshold are shown in (a) and (b). The atomic models were aligned to the maps.
Supplementary Figure 6. Alignments of the flagellin amino acid sequence from four Gram-positive bacteria and four Gram-negative bacteria. This alignment contains full sequences with D2 and D3 domains. Dark blue indicates higher sequence identities among species and light blue indicates intermediate sequence identities among species.
Supplementary Figure 7. Predicted mutation sites in *B. subtilis*. (a-c) Straight filaments S285P and N226Y structures were used as the modeling template, and the resulting interfaces are shown in (a) A237V (b) S71L and (c) A47CA233C. (d) Fluorescence images of three mutant filaments: F123V and R91S.
Supplementary Figure 8. Mapping three types of interactions in Salmonella onto seven Bacillus mutants.

Three types of interactions were described in Salmonella, called “permanent”, “sliding” and “switching” interactions (Kitao et al., 2006), and many Salmonella residues are involved in those interactions. The corresponding residues in Bacillus can be identified and mapped based on the sequence alignment. Here we are showing the Cα distances of the corresponding Bacillus residues that belong to those three types of interactions. The orange solid circles are the three L-type straight mutants (E115G, S17P and S285P), and the blue empty circles are the four R-type straight mutants (H84R, N226Y, A233V, and A39VN133H).
## Supplementary Table 1: Subunit-subunit interfacial area along different helices

| Mutation site(s) | B. subtilis | P. aeruginosa |
|------------------|-------------|---------------|
|                  | 5-start (Å²) | 11-start (Å²) | 6-start (Å²) | 16-start (Å²) | 5-start (Å²) | 11-start (Å²) | 6-start (Å²) | 16-start (Å²) |
| S285P            | 1850        | 1954          | 552          | 238          | 1800        | 1630          | 495          | 211          |
| E115G            | 1823        | 1810          | 485          | 237          | 1758        | 1922          | 542          | 286          |
| S17P             | 1893        | 1833          | 574          | 265          |             |               |              |              |
| N226Y            | 1819        | 1985          | 683          | 278          |             |               |              |              |
| A39VN133H        | 1673        | 1874          | 596          | 330          |             |               |              |              |
| H84R             | 1671        | 1924          | 516          | 275          |             |               |              |              |
| A233V            | 1779        | 1867          | 571          | 271          |             |               |              |              |
|                  |             |               |              |              |             |               |              |              |

**B. subtilis**

- S285P
- E115G
- S17P
- N226Y
- A39VN133H
- H84R
- A233V

**P. aeruginosa**

- G420A
- A443V
### Supplementary Table 2 Strains and mutations

| Strain | Genotype |
|--------|----------|
| 3610   | Wild type |
| DS1677 | Δ*hag* |
| DS1919 | Δ*hag* Δ*hag* |
| DK29   | [PY79] Δ*hag* *sfp* *swrA* |
| DK620  | Δ*hag* Δ*hag* |
| DK1343 | Δ*hag* Δ*hag* |
| DK1362 | Δ*hag* Δ*hag* |
| DK2750 | Δ*hag* Δ*hag* |
| DK2956 | Δ*hag* Δ*hag* |
| DK2977 | Δ*hag* Δ*hag* |
| DK3400 | Δ*hag* Δ*hag* |
| DK3965 | Δ*hag* Δ*hag* |
| DK4152 | Δ*hag* Δ*hag* |
| DK4153 | Δ*hag* Δ*hag* |
| DK4229 | Δ*hag* Δ*hag* |
| DK4230 | Δ*hag* Δ*hag* |
| DK4324 | Δ*hag* Δ*hag* |
| DK4399 | Δ*hag* Δ*hag* |
| DK4859 | Δ*hag* Δ*hag* |
| DK4860 | Δ*hag* Δ*hag* |
| DK4864 | Δ*hag* Δ*hag* |
| DK4879 | Δ*hag* Δ*hag* |
| DK4884 | Δ*hag* Δ*hag* |
| DK4894 | Δ*hag* Δ*hag* |
| PY79   | Δ*hag* Δ*hag* |
|        | Δ*hag* Δ*hag* |

- *Ahag* (Kearns and Losick, 2005)
- *Ahag* *P*hyspank fla/che operon *kan* (Guttenplan et al., 2013)
- *Ahag* *sfp* *swrA* (Blair et al., 2008)
| Primers | Genotype |
|---------|----------|
| 953     | GGAGTGTCAGAATGTTTGCAAAAAAC |
| 1008    | TGCAAGCCGCTGAAAGATATGCA    |
| 1009    | TGCAGGTATGAGAAGAGAGGA      |
| 1115    | AGGAGGAGATTIGATTGATGCTGTAATG |
| 1116    | CTCTCTCGAGGCCAGAACCAGCAGGAGCCAGGAAACCTGATCCCGAAGGGACTGCTTCG |
| 1117    | AGGAGGAGATTIGATTGATGCTGTAATG |
| 1118    | CTCTCTCGAGGCCAGAACCAGCAGGAGCCAGGAAACCTGATCCCGAAGGGACTGCTTCG |
| 3251    | CTCACCTAAGGGAACAAAAGCGTG |
| 3459    | GCTAAATCGAATCTACTATAGGTAATGTAGTACGCCAGGAGGA |
| 4439    | CGTTAGAAAGCACTGCAACA      |
| 4440    | CGCCTCGTAAAAGATTAACGT    |
| 4893    | TACCACGATGGCTGCACTA       |
| 4894    | GTACTGGGACACCACATCAAA     |
| 4895    | TGTTTCAGCTTCGCTTAAACGGCGCCGCTATCCCGAAGGTGATGATG |
| 4897    | ACGTTAACCTTTAGGATCGCGAGCGACTCTATAGGGCCGAAATTG |
| 4932    | CTITTTGTCCTCCTTTCATGTAGCGCTGCAACGCCAGGTTTTTTTTAAAAA |
| 5518    | GAAACTGCTTCTCAAGCTCCTCGAATCAAC |
| 5519    | GGAGGAGGTITTCATGATTTCTTTTG |
| 5520    | GAAATCCCTTCTAATACACGCTGAG |
| 5521    | GATAAGAGATTTCTCCGCTTTAGAGAG |
| 5522    | GGTAGGCTGCTTGCATTGTTCAAGCTCG |
| 5523    | CTIACCTACAGCTGAGGATGCAATCG |
| 5524    | GAAGTCAGTAAAAAGTACCTCG    |
| 5525    | CATTGACTTCTGACGATTGTAATAC |
| 5526    | GGTGTGTAACAAAATCCTGCTAAGC |
| 5527    | GTACCACAACAGCTGTCAGCGTTG |
| 5528    | CTITGCTACCTGAAAAATGAGAGG |
| 5529    | GATGCCAAAGAAGCTGCCTGCTGTCATTC |
| 5530    | CGTTGCAAGCCTTGGTGCTGTAACAAATC |
| 5531    | GCTTTGCAACGTTGAGAAGAATGTTTG |
Reference

Kitao, A., Yonekura, K., Maki-Yonekura, S., Samatey, F.A., Imada, K., Namba, K., and Go, N. (2006). Switch interactions control energy frustration and multiple flagellar filament structures. Proc Natl Acad Sci U S A 103, 4894-4899.