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Flagellin outer domain dimerization modulates motility in pathogenic and soil bacteria from viscous environments

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Flagellar filaments function as the propellers of the bacterial flagellum and their supercoiling is key to motility. The outer domains on the surface of the filament are non-critical for motility in many bacteria and their structures and functions are not conserved. Here, we show the atomic cryo-electron microscopy structures for flagellar filaments from enterohemorrhagic Escherichia coli O157:H7, enteropathogenic E. coli O127:H6, Achromobacter, and Sinorhizobium meliloti, where the outer domains dimerize or tetramerize to form either a sheath or a screw-like surface. These dimers are formed by 180° rotations of half of the outer domains. The outer domain sheath (ODS) plays a role in bacterial motility by stabilizing an intermediate waveform and prolonging the tumbling of E. coli cells. Bacteria with these ODS and screw-like flagellar filaments are commonly found in soil and human intestinal environments of relatively high viscosity suggesting a role for the dimerization in these environments.
Bacteria use their flagella to swim towards or away from various environmental signals. The flagellum is divided into several parts: the motor (basal body), the rod, the hook, and the filament. The rotating flagellar filament primarily functions as a propeller, acting as an Archimedian screw, and supercoiling of the filament is essential as rotations of a straight filament do not generate thrust.

Bacterial species from the *Escherichia* and *Salmonella* genera have peritrichous flagella. During running mode, the flagellar motors at the base of the flagellum are rotating counterclockwise, which orients the filaments in a bundle toward one end of the cell. When the direction of rotation of one or several flagella changes clockwise, the affected flagellar filaments break out of the bundle, and the filament switches from the normal left-handed waveform to right-handed semi-coiled and curly forms. This causes the cell to tumble, allowing the trajectory of the cell to change. During chemotaxis, the alternation between running and tumbling modes depends on the presence of both positive and negative stimuli and result in a "biased random-walk" swimming pattern.

The mechanism of flagellar filament supercoiling has been attributed to the existence of the 11 protofilaments in a mixture of two subunit conformations that differ very slightly in length. The short protofilaments are located on the inside of the supercoil, while the long protofilaments are on the outside. It is this path length difference between the short and long protofilaments that causes the supercoiling. Most previous structural studies analyzed straight mutant flagellar filaments so that helical symmetry could be imposed for the purposes of averaging. Recently, high-resolution cryo-EM structures of supercoiled flagellar filaments were achieved with helical reconstruction, assuming all the protofilaments are in a single state. These studies provided valuable information related to flagellar filament structure, but very few insights into the mechanism of polymorphic switching. However, they showed that a high-resolution cryo-EM structure can be generated without using straight mutant flagellar filaments.

The polymorphic switching of the flagellar filament has been attributed to the central flagellin region containing the "hyper-variable" outer domains, which have been shown to have non-critical roles in motility for some bacterial species. Large deletions of up to 100 amino acid residues in the outer domains D2 and D3 in *Salmonella typhimurium* flagellin FliC had no effect on bacterial motility. In addition, many bacterial species have flagellar filaments assembled from flagellins with just the core domains D0 and D1 and no outer domains. Rather than affecting motility, many outer domains are thought to provide their flagellar filaments with non-conserved species-specific functions such as adhesion, modulation of host immune responses, or protease activity.

The flagellar filaments of certain soil bacteria such as *Sinorhizobium meliloti* have been called “complex” due to the pairing of outer domain subunits to produce a criss-cross pattern on the surface of the filament. This pairing was regarded as a perturbation of the underlying symmetry modeled in part on the work done with the Dahlemense strain of tobacco mosaic virus. This model proposed that pairing of subunits across the helical groove, involving displacements of ~3 Å, served to break the helical symmetry. In TMV, subunits are rigidly locked into a helical lattice and major rearrangements of subunits would not be possible. We discovered the existence of non-canonical flagellar filaments in *Achromobacter* sp. MFA1 R4, a soil bacterium that was identified as a cell culture contaminant. These flagellar filaments have a sheath surrounding the core of the filament similar to those previously described for several H-serotypes of *E. coli*.

Here, we show the high-resolution cryo-EM structures of the *S. meliloti*, enterohemorrhagic *E. coli* O157:H7 (EHEC O157:H7), *Achromobacter*, and enteropathogenic *E. coli* O127:H6 (EPEC O127:H6) flagellar filaments. The three former structures would have been classified as "helically perturbed" while the latter as "non-helically perturbed". In the non-helical perturbation model, subunit pairing introduced a seam causing a discontinuity in the helical lattice, much as such a seam or discontinuity exists in microtubules. In the present study, we show that the outer domains in these flagellar filaments have considerable freedom. In physics and mathematics, perturbations are regarded as small deviations, such as the influence of the gravitational field of the sun on the orbit of the moon around the earth. Instead of the previously predicted small perturbations, half of the outer domain population rotated by 180° to form symmetrical dimers or tetramers with other outer domains. These outer domain dimers form either a screw-like surface surrounding the filament core domains (D0 and D1) or an outer domain sheath (ODS) around the core as in EHEC O157:H7, *Achromobacter* sp., and EPEC O127:H6.

The ODS surrounding the EHEC H7 and *Achromobacter* flagellar filaments produces an intermediate waveform that is not adopted by the "canonical" *S. typhimurium* and *E. coli* K-12 H48 flagellar filaments. In addition, we provide evidence that the ODS of the EHEC H7 flagellar filament prolongs the average time *E. coli* cells spent in the tumbling mode and suggest that this behavior is due to an additional step in flagellar polymorphism during tumbling created by the intermediate waveform. We hypothesize that extended tumbling mediated by ODS flagellar filaments offers an advantage to both intestinal pathogens and soil bacteria by allowing for better reorientation of cells in their respective environments.

**Results**

**Cryo-electron microscopy structures of screw-like and ODS flagellar filaments.** Cryo-electron microscopy (cryo-EM) was used to determine the structures of flagellar filaments from *Sinorhizobium meliloti*, enterohemorrhagic *E. coli* O157:H7 (EHEC O157:H7), *Achromobacter*, and enteropathogenic *E. coli* O127:H6 (EPEC O127:H6). Differences in filament surface structures were observable in the cryo-electron micrographs by the naked eye (Fig. 1a–d). The power spectrum of each flagellar filament (Supplementary Fig. 1b–e) was very different from that of any published canonical flagellar filament (Supplementary Fig. 1a) indicative of the uniqueness of each structure.

The *S. meliloti*, EHEC O157:H7, and *Achromobacter* flagellar filament structures were solved using helical reconstruction to 3.7, 3.6, and 3.7 Å resolution, respectively (Supplementary Table 1). Since a seam breaks the helical symmetry in the EPEC O127:H6 flagellar filament, reconstruction was performed asymmetrically to 4.0 Å. Clear side-chain densities and well-resolved β-sheets were observed for the flagellin subunits of each filament (Supplementary Fig. 2). For the *S. meliloti* flagellar filament, a prominent 3-start helix is created by the outer domains surrounding the flagellar core (Fig. 1c), which gives the *S. meliloti* filament surface the appearance of a 3-start screw. A simple screw can be described by a 1-start helix, where there is a single continuous helix forming the threads on the surface of the screw. For a 3-start screw, there are three separate strands that form the surface threads. The outer domains of the EHEC O157:H7, *Achromobacter*, and EPEC O127:H6 flagellar filaments form a sheath-like structure surrounding the core domains D0 and D1 (Fig. 1f–h), which we named an ODS. The *S. meliloti* filament possesses a single outer domain, D2 (Fig. 1i), while the ODS filaments have three outer domains, D2, D3, and D4.
Dimeric outer domain interactions of the *S. meliloti* screw-like filament. The asymmetric unit (ASU) of the *S. meliloti* flagellar filament consists of two flagellin conformations, which are identical in the core domains, but the outer domains are rotated 180° from each other resulting in an “up” and a “down” conformation (Fig. 2a). The D2 domains in the *S. meliloti* filament form dimers between an arbitrary “up” conformation subunit (Sn) and two “down” conformation subunits (Sn+5) and (Sn+11) as depicted in Fig. 2b. Since the outer surface is bipolar, a “down” D2 will also interact with two “up” D2s. These interactions form a 3-start helix surrounding the flagellar core, which resembles the grooves in a helical screw (Fig. 2c). They can be described as a reduction in helical symmetry along the 6-start helix, resulting in a 3-start helix (Supplementary Fig. 5a). Due to this subunit pairing, the axial rise and helical twist of the outer domains are twice those of the flagellar core (Table 1).

It has been hypothesized that flagellin dimers in *S. meliloti* are comprised of one each of FlaA and FlaX where FlaX can be any of the other three flagellins. A multiple sequence alignment of all four *S. meliloti* flagellins reveals key similarities and differences among them (Supplementary Fig. 6). FlaD is composed of 321 amino acid residues, and therefore is too short to fit the structure. In addition, it is transcribed at very low levels and expected to have a minor structural function. FlaA, B, and C have the correct size, but a few key residues in FlaC clearly do not fit the density map (Fig. 2d). FlaA and FlaB are very similar in sequence. In positions with residue variations between FlaA and FlaB, there is support for FlaA at position 205 (Fig. 2e) and FlaB at position 16 (Fig. 2f) in both flagellins of the ASU. This suggests that there is likely a mixture of FlaA and FlaB in the flagellar filament segments of our images. The fact that FlaA is the primary component of the flagellins is supported by the strongly reduced motility of a flaA mutant strain due to severely truncated flagellar filaments.

Outer domain dimerization of the EHEC H7 flagellar filament. Similar to the *S. meliloti* flagellar filament, the EHEC O157:H7 flagellar filament has a dimer symmetry (Table 1) with two flagellins in the ASU, one with an up and one with a down outer domain conformation (Fig. 3a). The much larger EHEC H7 outer domains (D2, D3, and D4) interact with outer domains from three other subunits forming a mesh-like ODS (Fig. 3b). In addition to forming dimers with outer domains that are five and 11 subunits away, every EHEC H7 outer domain also pairs with an outer domain that is 27 subunits away (Fig. 3c). This long-range Sn−Sn+27 dimer results in an observable 8-start helix on the surface of the sheathed filament in addition to a 3-start (Fig. 3d) and Supplementary Fig. 5b).
Outer domain tetramerization of the *Achromobacter* flagellar filament. Like the EHEC H7, the *Achromobacter* ODS is also formed by interactions between flagellins that are 5, 11, and 27 subunits away (Fig. 3c). However, there are four flagellins in the *Achromobacter* ASU, with two up and two down conformations (Fig. 4a). The key distinction is that all of the EHEC H7 Sn:Sn +27 dimers form at a single radius (Fig. 3e) while the *Achromobacter* Sn:Sn +27 dimers occur at two different radii (Fig. 4b). Consequently, the *Achromobacter* outer domain exists in four conformations and the filament has four flagellins in the ASU with an axial rise and twist for the outer domains that is four times larger than that of the flagellar core (Table 1). This tetrameric symmetry is characterized by two 4-start helices that occur at different radii (Fig. 4c). This can be viewed as a reduction of the 8-start helix observed in the EHEC H7 outer domains (Fig. 3d and Supplementary Fig. 5b) to two 4-start helices occurring at different radii (Fig. 4c and Supplementary Fig. 5c).

Outer domain tetramerization of the EPEC H6 filament results in a seam. The power spectrum of the EPEC O127:H6 flagellar filament (Supplementary Fig. 1e) superficially looks similar to the tetrameric *Achromobacter* filament. However, the Bessel order of many of the layer lines must be different because the EPEC H6 outer domains cannot be reconstructed using the tetrameric flagellar symmetry of *Achromobacter*. We have determined that this is a result of a seam. The EPEC H6 filament ODS is created by an outer domain tetramer with most flagellins being in one of four flagellin outer domain conformations (Fig. 5a), but the details of this tetramerization are quite different from those in *Achromobacter*. Due to the presence of the seam, most but not all D2 domains are in one of these four conformations. In one dimer conformation, “D2 Dimer A”, domain D2 of one subunit forms a dimer with D2 from another flagellin that is 11 subunits away (Fig. 5b). In the other conformation, “D2 Dimer B”, flagellins that are 6-subunits away (Sn:Sn +6) form dimers with each other.

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Table 1 Helical symmetry parameters for *S. meliloti*, EHEC H7, *Achromobacter* sp. MFA1 R4, and EPEC H6.

| Organism                  | Core rise (Å) | Core twist (°) | Outer domain rise (Å) | Outer domain twist (°) | Outer domain symmetry |
|---------------------------|---------------|---------------|-----------------------|------------------------|-----------------------|
| *Sinorhizobium meliloti*  | 4.75          | 65.46         | 9.5                   | 130.92                 | Dimer                |
| EHEC O157:H7              | 4.8           | 65.4          | 9.6                   | 130.8                  | Dimer                |
| *Achromobacter*           | 4.725         | 65.4          | 18.9                  | −98.4                  | Tetramer             |
| EPEC O127:H6c             | 4.813         | 65.534        | N/A                   | N/A                    | Tetramer w/seam      |

*The core domain rise and twist are the helical parameters of core domains D0 and D1 for each filament.

*The outer domain symmetry indicates the symmetry used to reconstruct the full flagellar filament.

*Because of the seam in EPEC H6, there is no overall helical symmetry that can reconstruct the entire filament. However, the core (comprised of domains D0/D1) displays a canonical flagellar filament symmetry.
These two dimer conformations are easily observed in the 4.0 Å EPEC H6 map (Supplementary Fig. 7). While a large region of the ODS has very poor density, the resolution of another portion allows the creation of an ab initio atomic model of the complete flagellin structure. A 6.7 Å resolution volume was reconstructed asymmetrically where all flagellin subunits could be fitted into the density (Fig. 5d, e). Analysis of this low-resolution structure revealed the seam, which involves the lack of D2 dimer formation between two flagellin subunits (Fig. 5e). This seam is a consequence of a reduction of symmetry along the 9-start helices of a flagellar filament composed of dimeric flagellin such as EHEC H7. This results in two 4.5-start helices (Fig. 5d) that are discontinuous along a line indicated by the symbol "*" (Fig. 5e). The two outer domain subunits in the seam still form Sn:Sn+2 dimers, one blue and the other magenta, which are at the same radius.

Disruption of the EHEC H7 ODS-forming dimer in domain D4 impairs motility. The surface of the screw-like flagellar filament of S. meliloti and related species is thought to be an adaptation to swimming in highly viscous environments, while the function of the ODS is unknown. To investigate ODS function, we aimed to disrupt its formation by mutation of several key residues in the EHEC H7 Sn:Sn+2 dimer interface (Fig. 6a). Examination of the amino acid sequences of the prominent Sn:Sn+27 dimer in domain D2, the two-seam subunits form Sn:Sn+5 dimers with each other (Fig. 5f) similar to those forming the EHEC H7 and the Achromobacter ODSs.

Fig. 3 Structural details of the dimeric EHEC O157:H7 flagellar filament. a The two flagellin conformations of the EHEC H7 flagellar filament asymmetric unit. b Mesh-like sheath generated by interactions between four flagellin outer domains (blue, pink, yellow, and orange). c The EHEC H7 outer domains from subunit Sn interact with the outer domains of flagellins that are 5 (Sn+5), 11 (Sn+11), and 27 (Sn+27) subunits away. d The Sn:Sn+27 dimer generates prominent right-handed 3-start and left-handed 8-start helices in the sheath surrounding the core. e View of the EHEC H7 flagellar filament cut halfway through the filament. The arrows are pointing to two Sn:Sn+27 dimers, one blue and the other magenta, which are at the same radius.

Fig. 4 Structural details of the tetrameric Achromobacter flagellar filament. a The four flagellin conformations within the Achromobacter flagellar filament asymmetric unit. b Achromobacter outer domain interactions from subunits Sn:Sn+27 occur at two different radii. c The two different dimer conformations result in two left-handed 4-start helices on the surface of the Achromobacter flagellar filament.
Two double mutants were constructed: N319F N323F (FF) and N319R N323R (RR) and mutant flagellar filaments isolated. Cryo-electron micrographs revealed that the FF mutant filament (Supplementary Fig. 8a) had a disrupted surface compared to that of wild-type EHEC H7 (Supplementary Fig. 8b). This disruption was also apparent when comparing the low-resolution FF reconstruction (Supplementary Fig. 8c) to the low-pass-filtered wild-type H7 structure (Supplementary Fig. 8d). These differences between wild-type and mutant FF flagellar filaments were apparent in 2D class averages from Relion, where the wild-type H7 filaments displayed a characteristic 30 Å spacing corresponding to the D4 Sn+5 Dimer (Supplementary Fig. 9a) while the FF mutant filaments did not have this spacing (Supplementary Fig. 9b).

Soft agar (0.3%) motility assays revealed drastically reduced motility of cells with the FF and RR mutant flagellar filaments as compared to the wild-type H7 (Fig. 6c). To ensure that the impaired motility of the mutants is not the result of altered filament production, we performed western blot analysis on motile bacteria and culture supernatants. We detected comparable levels of flagellin in washed cell fractions of the wild type and the deletion strains with complementation plasmids (Supplementary Fig. 11). This is named “D2 dimer A”. Flagellin outer domains also form a symmetrical dimer between domain D4 in subunit Sn+5 with domain D4 from a flagellin 22 subunits away (Sn+17). The second EPEC H6 outer domain dimer, “D2 dimer B”, is created between Sn+5 and a flagellin six subunits away Sn+11. d Surface view of the EPEC H6 flagellar filament showing the fit of the outer domain models into the low-resolution map. The two dimer conformations are indicated by “A” and “B” and form two right-handed 4.5-start helices. e A surface view of the EPEC H6 flagellar filament, rotated by 180° around the filament axis from the view in (d), showing the seam, *, with the subunits forming the seam in gold. f Interactions between outer domain subunits (gold) along the seam. The two subunits in the seam are 5 subunits away from each other in the filament (Sn+Sn+5).

**Fig. 5 Structural details of the EPEC O127:H6 flagellar filament with a seam.** a The four main flagellin conformations of the EPEC H6 flagellar filament. b An EPEC H6 outer domain dimer is generated by a symmetrical interaction between outer domain D2 from flagellin Sn with domain D2 of a flagellin 11 subunits away, Sn+5. This is named “D2 dimer A”. Flagellin outer domains also form a symmetrical dimer between domain D4 in subunit Sn+5 with domain D4 from a flagellin 22 subunits away (Sn+17). c The second EPEC H6 outer domain dimer, “D2 dimer B”, is created between Sn+5 and a flagellin six subunits away Sn+11. d Surface view of the EPEC H6 flagellar filament showing the fit of the outer domain models into the low-resolution map. The two dimer conformations are indicated by “A” and “B” and form two right-handed 4.5-start helices. e A surface view of the EPEC H6 flagellar filament, rotated by 180° around the filament axis from the view in (d), showing the seam, *, with the subunits forming the seam in gold. f Interactions between outer domain subunits (gold) along the seam. The two subunits in the seam are 5 subunits away from each other in the filament (Sn+Sn+5).

**ODS flagellar filaments demonstrate non-canonical polymorphic transitions.** We used fluorescence microscopy to visualize labeled flagellar filaments on swimming wild-type EHEC H7 and FF mutant cells. Wild-type EHEC H7 cells exhibited different swimming behaviors depending on the number of flagellar filaments. Cells with one to two flagellar filaments had a tendency to be in a constant state of transitioning between waveforms and tumbled frequently (Supplementary Movie 1), while cells with more than two flagellar filaments exhibited long periods of straight swimming followed by robust tumbling or “double tumbling” where the filaments seem to take an extended period of time to return to the bundle causing the occurrence of two tumbles in a relatively short period of time (Supplementary Movie 2). Motile FF mutant cells entered periods of prolonged tumbling where the filaments exhibited an increased frequency of polymorphic switching (Supplementary Movie 3). Flagella from labeled wild-type EHEC H7, EHEC FF, and *Achromobacter* sp. MFA1 R4 frequently transitioned between the canonical normal and semi-coiled waveforms (Fig. 7a, Supplementary Movie 1, Supplementary Fig. 12). These same flagellar filaments also adopted a non-canonical intermediate waveform (Fig. 7a and Supplementary Fig. 12). The *Achromobacter* flagellar filaments appeared to primarily transition between these three waveforms as well as the semi-coiled form. The EHEC flagellar filaments also adopted the curly waveforms (Fig. 7b and Supplementary Fig. 12d). Images of fluorescently labeled, *E. coli* K-12 AW405 flagellar filaments, which do not possess an ODS, exhibited all the canonical flagellar waveforms as previously described and one of these filaments ever adopted the intermediate waveform (Fig. 7b and Supplementary Fig. 13).
The helical parameters of the intermediate waveform closely resembled those of the medium and unstable waveform in *Salmonella* flagella that was induced by mechanical force. The plot in Fig. 7c presents filament parameters, $\kappa$, versus twist, $\tau$, for each of the observed filament waveforms from wild-type EHEC O157:H7, *Achromobacter*, and *E. coli* K-12 AW405 compared to published values. The parameters used to calculate this plot are listed in Supplementary Table 2. A notable difference was the greater stability of the intermediate waveform in *Achromobacter* compared to *E. coli* K-12 outer domains using gECCO results in a model with a fold quite similar to the *S. typhimurium* flagellin outer domains and no structural similarity in the EHEC H7 flagellin (Supplementary Fig. 15). *E. coli* K-12 cells exhibited the highest average swimming velocity at about 25 $\mu$m/s (Table 2), which agrees with previous published values. Cells frequently displayed extended periods of straight swimming followed by very short tumbles with a duration of ~220 ms (Fig. 8b and Supplementary Movie 7). The K-12 wild type swam more slowly (Table 2) and stayed in the tumble mode for ~1500 ms (Fig. 8b and Supplementary Movie 9). 

The ODS prolongs *E. coli* tumbling. To compare the run-and-tumble motility behavior of the various EHEC strains to that of *E. coli* K-12 AW405 (K-12), we employed phase-contrast microscopy and recorded movies of swimming cells at 30 frames per second. The D0 and D1 domains of the *E. coli* K-12 flagellin exhibit over 90% sequence identity to the H7 flagellin (Fig. 8a). However, its outer domains show no significant sequence homology to H6 or H7 and do not form a sheath. In contrast, the K-12 outer domains align well and have 35% sequence identity with the *S. typhimurium* FlIC outer domains (Supplementary Fig. 14), a likely indicator of a conserved fold. Indeed, structure prediction of the *E. coli* K-12 outer domains using AlphaFold results in a model with a fold quite similar to the *S. typhimurium* flagellin outer domains and no structural similarity to the EHEC H7 flagellin (Supplementary Fig. 15). *E. coli* K-12 cells exhibited the highest average swimming velocity at about 25 $\mu$m/s (Table 2), which agrees with previous published values. Cells frequently displayed extended periods of straight swimming followed by very short tumbles with a duration of ~220 ms (Fig. 8b and Supplementary Movie 7). The K-12 wild type swam more slowly (Table 2) and stayed in the tumble mode for ~1500 ms (Fig. 8b and Supplementary Movie 9). 

To investigate whether the H7 flagellar filament is the cause of the prolonged tumbling duration, we complemented the *E. coli* K-12 Δflc deletion strain HCB5 with EHEC *flc* (FlIC1488 and the native K-12 Δflc strain (FlIC1488) from plasmid pTrc99a using the native K-12 outer domains, which underwent frequent polymorphisms. These frequent transitions suggest an inherent instability of the disrupted dimer interface (Supplementary Movie 6).
Discussion
Each of the four presented flagellar filament structures exhibits a different level of structural complexity: The *S. meliloti* and EHEC H7 structures are formed with outer domain dimers, while the *Achromobacter* and EPEC H6 structures are produced by outer domain tetramers. The effects of dimerization and tetramerization of flagellin outer domains have been seen before in diffraction patterns of flagellar filaments, but these studies were unable to determine the nature of the dimerization or tetramerization. Flagellar filaments without a seam were defined as helically perturbed, and ones with a seam as non-helically perturbed. In the light of our findings, the use of the term perturbation should be re-evaluated, because the generation of these structures involves a full 180° rotation of every other flagellin’s outer domain. In addition, the ODS we describe in this paper should not be confused with other flagellar sheaths which are either membranous or created by proteins other than flagellin.

A structural homology search of the individual flagellin outer domains from each structure using the Dali server yielded only a few potential structural homologs in unrelated proteins (Supplementary Table 3). However, none of these hits were convincing in terms of sharing an overall fold. Since the outer domains of the four structures formed dimers about a two-fold axis in strikingly similar manners (Supplementary Fig. 3), this led us to wonder if there was any commonality between the *S. meliloti* dimer site and that of the three ODS flagellar filaments’ D4 dimer site. Interestingly, the electrostatic potential of the EHEC H7 and *Achromobacter* D4 dimers along with the *S. meliloti* D2 dimer all have a negative charge at the dimer site (Supplementary Fig. 16a). In the case of the EHEC H7 dimer one aspartate at position 316 from each subunit is at the interface (Supplementary Fig. 16b) while for *S. meliloti* the negative charge is due to glutamates from each subunit (Supplementary Fig. 16c). It has been suggested that divalent cations are critical to the stability of the *S. meliloti* outer domains. We found no evidence of cation density being preset in these negatively charged pockets in any of these structures, but this does not necessarily exclude the possibility. Another possibility is that the dimer interactions are strengthened at lower pH values is a remote possibility that the EHEC H7 dimer site
functions as some sort of aspartic protease, but it is missing the classic Asp-Thr-Gly motif found in most aspartic proteases. The discovery of proteolytic flagellin outer domains might give some credibility to this possibility. The HIV-1 aspartic protease dimer (Supplementary Fig. 16d) bears some similarity to the negatively charged dimer sites in our models due to the interfacing aspartate residues from both subunits of the protease dimer. The surface areas of the S. meliloti, EHEC H7, Achromobacter, and EPEC H6 flagellins are unique from all other high-resolution flagellar filament structures because they form dimers or tetramers on the surface of the filament, which generate different helical lattices around their flagellar cores. These new outer domain interactions then alter the polymorphism of the flagellar filament in the form of the contracted normal waveform for the S. meliloti screw-like flagellar filament and the intermediate waveform for flagella with ODSs. The intermediate waveform with an average pitch of 1.8 µm and diameter of 0.45 µm adopted by the ODS flagellar filaments is strikingly similar to the previously discovered unstable right-handed “medium” waveform with a pitch of 1.9 µm and diameter of 0.43 µm. This medium waveform required the same magnitude of torque for the transition to curly or semi-coiled forms, while the torque needed for the transition from medium to normal was very small. Mutations in the EHEC H7 Sn:Sn filament, which generate different helical lattices around their flagellar cores, change the pitch of the filament. These new outer domain interactions then alter the polymorphism of the flagellar filament in the form of the contracted normal waveform for the S. meliloti screw-like flagellar filament and the intermediate waveform for flagella with ODSs. The intermediate waveform with an average pitch of 1.8 µm and diameter of 0.45 µm adopted by the ODS flagellar filaments is strikingly similar to the previously discovered unstable right-handed “medium” waveform with a pitch of 1.9 µm and diameter of 0.43 µm. This medium waveform required the same magnitude of torque for the transition to curly or semi-coiled forms, while the torque needed for the transition from medium to normal was very small. Mutations in the EHEC H7 Sn:Sn filament, which generate different helical lattices around their flagellar cores, change the pitch of the filament. These new outer domain interactions then alter the polymorphism of the flagellar filament in the form of the contracted normal waveform for the S. meliloti screw-like flagellar filament and the intermediate waveform for flagella with ODSs.
transition to curly. This is consistent with our observation that cells with the H7 flagellar filament undergo quick tumbling similar to cells with the H48 filament. We observed fewer EHEC H7 and Achromobacter flagellar filaments that adopted the curly waveform, which could suggest that the curly waveforms are disfavored in ODS filaments. The intermediate waveform of our ODS flagellar filaments is likely similar to the constricted normal waveform of flagellar filaments with screw-like surfaces, because both waveforms have shorter pitches and diameters than the normal waveform and are distinguishable from the semi-coiled and curly waveforms. The S<sub>1</sub>S<sub>1</sub>S<sub>2</sub>+<sub>5</sub>, S<sub>1</sub>S<sub>1</sub>S<sub>2</sub>+<sub>6</sub>, and S<sub>1</sub>S<sub>1</sub>S<sub>2</sub>+<sub>11</sub> dimers and the 3-3 helix formed on the surface likely allow for the formation of these unique shorter-pitch waveforms. Without the additional S<sub>1</sub>S<sub>1</sub>S<sub>2</sub>+<sub>27</sub> or S<sub>1</sub>S<sub>2</sub>+<sub>22</sub> dimer, such waveforms might be rather unstable in E. coli cells with bidirectional flagellar rotation that require flagellar polymorphism. A different behavior is observed for the unidirectional epithelium. It has been shown that the ODS filament, which could suggest that the curly waveforms are disadvantageous in environments with higher viscosity. In S. typhimurium mutant backgrounds these complexes could be stabilized by dimerization between the flagellar outer domains in the complex allowing for more robust TLR5-signaling. The outer domain dimers might also stabilize the flagellin subunits in detached flagellar filaments hindering the dissociation of the filament into monomers. A lower concentration of flagellin monomers might be advantageous during later stages as detached ODS flagellar filaments could possibly result in lower levels of TLR5 activation than canonical flagellar filaments. Flagellin can be the single most abundant protein produced by bacteria and is thus under intense selection. An example of such selection is the Macnab experiment: in stirred liquid culture, flagellar motility provides no advantage, spontaneous mutations in flagellar genes lead to the complete loss of flagellar filament in only 10 days, because bacteria that fail to synthesize flagellin have a slightly increased fitness. The outer domains required to produce ODS flagellar filaments are 50–100 amino acid residues larger than the E. coli K-12 and S. typhimurium flagellar filaments. Thus, producing these flagellar filaments with ODS would be even more energetically costly than producing flagellar filaments without a sheath. Given the intense selective pressure on their synthesis, flagellin oligomerization and the subsequent effect on motility may be viewed as a mechanism for specific adaptations to environmental niches.

Methods

Generation of EHEC H7 flagellin mutants and EHEC and K-12 complementation plasmids. The H7 FliC gene was deleted from EHEC 86–24 using lambda Red recombination as published. FliC was cloned into the pGEN-MCS vector using restriction digestion (NheI and HindIII) and ligation. Potential mutations were chosen based on changes in predicted stability calculated by the FoldX80 and all scripts written in MATLAB. Tumble durations were measured manually using TumbleScore scripts written in MATLAB. Tumble durations were measured manually using frame-by-frame playback of videos. Reported values are averages and standard deviations of at least 30 independent tumbling events.

EHEC O157:H7 soft agar motility assays. EHEC strain 86–24 was grown aerobically in LB overnight at 37 °C with shaking. The next day, cultures were diluted 1:100 into fresh LB and grown to mid-exponential phase (OD<sub>600</sub> 0.4–0.6). From these, a 1 μl aliquot of culture was stab inoculated into motility plates (LB with 0.3% agar). Plates were incubated for 7–24 h at 37 °C and halo diameter was measured.

Phase-contrast swimming behavior assays. EHEC strains were diluted from overnight LB broth cultures into fresh media with appropriate antibiotics and incubated at 37 °C in a roller drum to an OD<sub>600</sub> of ~1.5. E. coli K-12 strains were incubated in tryptone broth at 37 °C in a roller drum to an OD<sub>600</sub> of ~0.5. Cells were pelleted in round-bottom tubes for six minutes at 3000 g, the spent media removed, and cells were suspended in an equal volume of motility buffer (10 mM potassium phosphate, 10 mM lactate, and 70 mM sodium chloride, pH 7.0) in a roller drum at 20 rpm ~20 min. The centrifugation and suspension steps were repeated once prior to visualization. Videos were recorded at 30 fps using a Nikon Eclipse E600 phase-contrast microscope equipped with a custom Nikon camera from Imaging Source. Swimming velocities were quantified using TumbleScore scripts written in MATLAB. Tumble durations were measured manually using frame-by-frame playback of videos. Reported values are averages and standard deviations of at least 30 independent tumbling events.

Increased TLR5 activation seen by the ODS-forming H1, H6, and H7 E. coli flagellins when they are bound to TLR5. A crystal structure of the S. typhimurium flagellin FliC bound to TLR5 revealed a complex consisting of two TLR5:flagellin heterodimers, where domains D1 are bound by TLR5 and domains D2 of each flagellin are in close proximity (Supplementary Fig. 17). For ODS-forming EHEC H7 and EPEC H6 flagellins these complexes could be stabilized by dimerization between the flagellin outer domains in the complex allowing for more robust TLR5-signaling. The outer domain dimers might also stabilize the flagellin subunits in detached flagellar filaments hindering the dissociation of the filament into monomers. A lower concentration of flagellin monomers might be advantageous during later stages as detached ODS flagellar filaments could possibly result in lower levels of TLR5 activation than canonical flagellar filaments. Flagellin can be the single most abundant protein produced by bacteria and is thus under intense selection. An example of such selection is the Macnab experiment: in stirred liquid culture, flagellar motility provides no advantage, spontaneous mutations in flagellar genes lead to the complete loss of flagellar filament in only 10 days, because bacteria that fail to synthesize flagellin have a slightly increased fitness. The outer domains required to produce ODS flagellar filaments are 50–100 amino acid residues larger than the E. coli K-12 and S. typhimurium flagellar filaments. Thus, producing these flagellar filaments with ODS would be even more energetically costly than producing flagellar filaments without a sheath. Given the intense selective pressure on their synthesis, flagellin oligomerization and the subsequent effect on motility may be viewed as a mechanism for specific adaptations to environmental niches.

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Western blotting. Bacteria were grown and washed as described for swimming velocity and tumbling analyses. At each stage of the washing procedure, 10 μL of the corresponding sample was mixed with an equal volume of Laemmli buffer containing β-mercaptoethanol and samples were boiled for ten minutes prior to tank blot transfer as previously described. Blots were incubated in blocking buffer consisting of PBS with 5% milk for at least one hour at room temperature. Anti-flgC polyclonal antibody serum and horseradish peroxidase-linked goat anti-rabbit antibody were used at 1:10,000. Chemiluminescence signals were detected on ECL Hyperfilm.

Fluorescence labeling of flagellar filaments. Flagellar filaments were labeled using established methods3 using the Alexa Fluor™ 546 NHS Ester Protein Labeling Kit from Thermofisher Scientific. Overnight cultures of EHEC H7 and Achromobacter sp. MFA1 R4 cells were checked for motility using the DIC mode on a Zeiss Axioskop 2 Plus microscope. These cells were then pelleted at low centrifugation speeds of 1,200 × g for 5 min and washed three times by resuspension in 1× PBS and pelleting at the same low speed for 5 min. The final suspension volume of cells in 1× PBS was 500 μL and 50 μL of 1 M bicarbonate was added to the suspension. This mixture was then transferred to the tube containing the Alexa Fluor probe and rotated for 1 h at room temperature. After incubation at room temperature the steps were exactly the same however cells were grown to OD of 0.6 prior to labeling.

Asymmetric reconstruction of the EPEC H6 flagellar filament. The 4.5 Å resolution EPEC H6 core domain volume from helical reconstruction was filtered to 25 Å resolution and used as an input into the cryoSPARC Homogeneous Refinement program. A curved reconstruction of the H6 flagellar filament was obtained with core domain resolution around 3.5 Å and outer domains around 6 Å from ~270,000 particles. 3D variability analysis was performed, and the output was clustered. The particles were then subjected to CTF refinement. Using local refinement and a masking out of the core domains we achieved 4.3 Å resolution (FSC = 0.143 map/for) for the EPEC H6 outer domains. A large portion of the outer domain was on the inner most side of the symmetry poor density likely due to misalignment of particles. Using cryoSPARC’s 3D Variability analysis and solving for three different modes of heterogeneity we obtained several clusters (particles and their corresponding volumes) with differences in the overall quality of outer domains and one was chosen for further analysis. A single cluster from 60,000 particles showed low resolution (~8 Å) features corresponding to the outer domain dimers without the issue of non-uniform density in the inner curved region. Analysis of this region showed an apparent seam. Attempts to use the particles and volume from this 3D variability cluster for a reconstruction with extensive rotation and shift searches resulted in a final volume with no seam and very poor density in the region of the inner curved region of the filament (Supplementary Fig. 1a), and much larger physical area (83.78 µm × 83.78 µm) were obtained with 210 milliseconds between each image. Just prior to imaging, cells were diluted into 1.50 LB with or without 10%−tween.

Flagellar filament preparation. H7 flagellar filaments were prepared as published35. EPEC ICC526 (EPEC O127:H6 ΔdefA + ΔespA) was transformed with pBAD plasmid. To purify flagella, exponentially growing EPEC ICC526 culture and grown for 15 h at 30 °C was centrifuged at 10,000 × g for 15 minutes. The culture was then resuspended in 1× PBS and pelleted by centrifugation at 7000 × g for 5 min and the pellet was suspended in 500 μL of cold, 1 M Tris/HCl, 100 mM NaCl buffer (pH 6.5). Bacteria were deflagellated by passing multiple times through a 25 G needle until viscosity decreased. The deflagellated cells were pelleted by centrifugation at 10,000 × g for 15 minutes. The flagella in the supernatant were centrifuged at 10,000 × g at 4 °C to remove small debris. The resulting pellet was suspended in 500 μL of buffer. Purity of the flagella was analyzed by SDS PAGE.

Sinorhizobium meliloti flagellar filament purification was done essentially as described36 with slight modifications. S. meliloti wild-type strain RUT11/01 grown in TYC (0.5% tryptone, 0.3% yeast extract and 0.13% CaCl2 × 6H2O) was diluted to an OD600 of 0.05 in RB (6.1 mM KH2PO4, 3.9 mM KH2PO4, 1 mM MgSO4, 0.1 mM (NH4)2SO4, 0.1 mM CaCl2, 0.1 mM NaCl, 0.01 mM Na2MoO4, 0.001 mM FeSO4, 2 μg/gl biotin). Twenty Bromell plates (0.5% tryptone, 0.3% yeast extract and 0.13% CaCl2 × 6 H2O) were over-laid with 10 μL of the diluted culture and grown for 15 h at 30 °C to an OD600 of 0.6. Cells were harvested by centrifugation at 8000 × g for 15 minutes in 100 mM sucrose buffer (0.5 mM CaCl2, 0.1 mM EDTA, 20 mM HEPE [pH 7.2]). Flagella were sheared by agitation in a mixer at full speed for 20 seconds, separated from cells by centrifugation at 8000 × g for 7 min, 15,000 × g for 15 min, and 30,000 × g for 50 min at 4 °C. Purified flagella were sedimented at 87,000 × g for 2 h at 4 °C, washed once and suspended in 200 μL motility buffer. Purity of the flagella was analyzed by SDS PAGE.

Cryo-EM preparation. Flagella samples were prepared for cryoEM using established protocols13,16. Plunge freezing was done using a Vitrobot™ Mark II plunge freezer. Briefly, 3-4 μL of flagellar filament sample was applied to a lacey carbon grid. The droplet was blotted for 3.5 s with the blot force settings ranging from 3 to 6 and then plunged into liquid ethane.

Cryo-EM image acquisition. The image acquisition settings were the same as those previously published37, with data acquisition using either EPU (Thermo-Fisher Scientific) or cryoSPARC.

Image processing and helical reconstruction. For the Achromobacter sp. MFA1 R4 and EHEC H7 flagellar filament images processing and helical reconstruction were performed as published13,16 using the Spider46 and Reion 3D5 software packages. Motion correction was performed using MotionCorr29. The S. meliloti and EPEC H6 image processing and structural determination were performed using cryoSPARC37. Images of S. meliloti and EPEC H6 flagellar filaments were motion corrected using the Patch Motion Correction process and contrast transfer function (CTF) estimation was done using Patch CTF Estimation. Initial subsets of flagellar filament segments (500–1000) were manually picked and averaged with a processselected class averages were then used as inputs for both Template Picker and Filament Tracker. More quality flagellar filament images were selected using the Template Tracker function then Filament Tracker. The desired minimum separation between particles was determined based on the expected axial rise of the helical symmetry to be used during reconstruction. For S. meliloti a featureless cylindrical volume was used as the starting volume for reconstruction and an initial resolution reached 4.0 Å, and the helical symmetry converged to that shown in Table 1. Local CTF Refinement followed by helical reconstruction of these CTF refined particles resulted in a final volume with 3.5 Å resolution using the FSC = 0.143 map/criterion. For EPEC H6 the core domains were reconstructed with a monomeric symmetry and a resolution of ~4.5 Å was achieved. However, due to a seam in the outer domains, helical reconstruction could not reach high resolution for the full filament.

Model building. Model building for the flagellar filaments in this study was published for other flagellar filaments13,38. The corresponding density for an individual flagellin was isolated from the filament reconstruction using UCSF Chimera99. The D0/D1 model of a already deposited flagellin (PDB 5WJY) was fit into the corresponding region of each subunit’s density map and then the residues were mutated to the appropriate ones for each structure using Coot90. For the outer domains, the chains were traced manually in Coot and then refined using Rosetta CM21. Filament models were then generated in UCSF Chimera and refined and validated in Phenix using real-space refinement25. For refinement of the H6 flagellar filament, a homology model was initially generated using Swiss-Mode94. This was subsequently refined in coot and much of the model was manually rebuilt using coot and refined in phenix. For refinement of the H6 model to the high-resolution map, the model was fit into only the regions of good outer domain density in the map.

Achromobacter sp. MFA1 R4 structural determination. The Achromobacter sp. flagellar filament was a contaminant of a pili prep from Agrobacterium tumefaciens. Surprisingly, cryoEM images of the A. tumefaciens preparation revealed no pili at all but two very different flagellar filaments: thin flagellar filaments ~140 Å in diameter (yellow triangle Fig. 1c) with a canonical flagella power spectrum (Supplementary Fig. 1a), and much larger flagellar filaments (~220 Å diameter) with OD5 (red arrow Fig. 1c) and a tetrameric flagella power spectrum (Supplementary Fig. 1d). SDS-PAGE followed by silver staining was as published95 and identified numerous gel bands arising from many contaminants. Multiple bands were excised from the gel and submitted for mass spectrometry analysis at the University of Virginia Bioanalytical Analysis Facility. The gel pieces from the bands were transferred to a siliconized tube and washed in 200 μL 50% methanol. The gel pieces were dehydrated in acetonitrile, rehydrated in 30 μL of 10 mM dithiothreitol (DTT) in 0.1 M ammonium bicarbonate, and reduced at room temperature for 0.5 h. The DTT solution was removed, and the sample was alkylated in 30 μL 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The iodoacetamide was removed, and the gel pieces were dehydrated in 100 μL acetonitrile. The acetonitrile was removed, and the gel pieces rehydrated in 100 μL 0.1 M ammonium bicarbonate. The pieces were dehydrated in 100 μL acetonitrile, the acetonitrile removed, and the pieces completely dried by vacuum centrifugation. The gel pieces were rehydrated in 50 μL 0.1 M ammonium bicarbonate on ice for 30 min. Any excess enzyme solution was removed and 20 μL 50 mM ammonium bicarbonate was added. The sample was digested overnight at 37 °C and the peptides were extracted...
from the polycrylamide in a 100 µL aliquot of 50% acetonitrile/5% formic acid. This extract was evaporated to 9 µL for MS analysis.

The LC-MS system consisted of a Thermo Electron Q Exactive HF mass spectrometer with an Easy Spray ion source connected to a Thermo 75 μm × 15 cm C18 Easy Spray column. 1–3 µL of the extract was injected and the peptides eluted from the column by an acetonitrile/0.1 M formic acid gradient at a flow rate of 0.3 µL/min over 1.0 h. The nano spray ion source was operated at 1.9 kV. The digest was analyzed using the rapid switching capability of the instrument acquiring a full scan mass spectrum to determine peptide molecular weights followed by product ion spectra (10 HCD) to determine the amino acid sequence in sequential scans.

Mass spectrometry identified the presence of peptides belonging to A. tumefaciens flagelin as well as potential peptide hits for the flagelin from the sheathed flagellar filament from various strains of Achromobacter. A BLAST search of two peptide hits from the Mass Spec; FTANVRGLTQAAR and ISEQTFDNGVK, identified only two proteins in UniprotKB with 100% coverage in both sequences they were flagellen from Achromobacter sp. 27895STDS608615 and Achromobacter sp. MFAI R4. Achromobacter sp. 27895STDS608615 was a partial sequence in the UniprotKB database missing a small part of its C-terminal D0 sequence and its outer domain sequence was about 15 amino acids too large, while the Achromobacter sp. MFAI R4 was a better fit. Pure Achromobacter sp. MFAI R4 cells were then obtained, and the flagellar filaments were confirmed to be sheathed using negative stain TEM.

Modeling of the S. melloti flagellar filament. A sequence alignment of the four S. melloti flagellins is shown in Supplementary Fig. 12. Both flagellin subunits in the ASU correspond to a flagelin that is ~395 amino acids in length which would exclude either subunit being FlaC. FlaA has Met300 which corresponds to Ala298 in FlaD. Both subunits in the ASU show a large side-chain density at that position (no dihedral symmetry has been imposed on the outer domains) which could correspond to a methionine but not alanine (Supplementary Fig. 13a). Thus, we can exclude FlaD from being one of the main components of the flagellar filament in our images. There are several regions where side-chain density corresponding to the FlaA sequence would be difficult to explain with the FlaB sequence, such as those corresponding to FlaA residues Glu205 and Asn294 which correspond to Ala205 and Gly294 in FlaB (Supplementary Fig. 13b, c). Given that the densities of the two subunits in the ASU are nearly identical, it seems unlikely that the screw-like surface is formed specifically by a FlaA-FlaB heterodimer and most of the dimers must be formed by FlaA-FlaA homodimers. But it is still possible that some population of the segments used for reconstruction contained FlaB-FlaB homodimers or even FlaA-FlaB heterodimers that degrade the side-chain density of residues like Glu205 and Asn294 when averaged with the predominant FlaA-FlaB dimers.

AlphaFold predictions. The E. coli K-12 flagellin model was predicted using the AlphaFold option found in daily builds of UCSF ChimeraX. Multiple sequence alignments. Figures for sequence alignment were made using Jalview. All alignments were done using Clustal Omega.

Analysis of flagellar filament waveforms. Movies of fluorocently labeled bacterial flagellum were analyzed in Fiji. Achromobacter, Salmonella, and E. coli flagellum switching across multiple helical forms adopted by bacterial flagellar filaments. J. Mol. Biol. 425, 914–928 (2013).

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Author contributions
E.H.E., B.E.S., M.M.K., M.A.B.K., and R.C.S. designed the study and experiments. Achromobacter sp. MFA1 R4 flagellar filaments were provided by V.P.C. EHEC O157:H7 flagellar filaments were prepared by J.A.G. Initial EPEC O127:H6 flagellar filaments were prepared by A.P., T.R.D.C., and G.F. Additional EPEC O127:H6 flagellar filaments were prepared by S.C. and G.F. S. meliloti flagellar filaments were prepared by B.E.S. M.A.B.K. screened and prepared samples for cryoEM as well as performed the image processing and helical and asymmetric reconstructions. Atomic models were generated by M.A.B.K. F.W. assisted with cryoEM image processing and model building. A.B.S. and M.M.K. constructed the FF and RR mutants, performed soft agar motility assays, and prepared EHEC samples for analysis by negative stain TEM and cryoEM. R.C.S. performed the western blot analysis, soft agar motility assays, and the phase-contrast microscopy experiments to measure the velocity and tumbling of the various E. coli strains. M.A.B.K. and R.C.S. analyzed EHEC and E. coli K-12 cells tumbling. Flagellar filaments were labeled by M.A.B.K., and fluorescence microscopy experiments were done by M.A.B.K. and V.K. Figures were prepared by M.A.B.K. M.A.B.K., R.C.S., B.E.S., and E.H.E. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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