FliL ring enhances the function of periplasmic flagella

Shuaiqi Guoa,b,1, Hui Xuc,1, Yunjie Changa,b, Md A. Motalebc,2 and Jun Liua,b,2

Edited by Nicholas Taylor, Kobenhavns Universitet, Copenhagen, Denmark; received September 19, 2021; accepted January 28, 2022 by Editorial Board Member Thomas J. Silhavy

Bacterial flagellar motors are rotary machines that can power motility in various fluid and surface environments, including within hosts. Activation of the stator complex MotA/MotB is required for torque generation and motor rotation. During activation, the stator complex is expected to undergo an extensive conformational change to allow ions to flow through its transmembrane channels to generate torque. However, the detailed mechanism underlying stator activation remains poorly understood. Here, we use the Lyme disease–causing spirochete Borrelia burgdorferi as the model system to reveal the stator complex and its interaction with the FliL ring, using cryo-electron tomography and subtomogram averaging of flagellar motors from wild-type, ΔmotB, ΔfliL, and ΔfliLmotAB mutants. Upon recruitment of stator units to the motor, FliL oligomerizes from a partial ring into a full ring, which wraps around the MotB periplasmic linkers and stabilizes the stator complex in an extended, active conformation, thus enabling a continuous influx of ions to generate higher torque. Furthermore, we provide evidence that FliL can mediate the assembly of stator complexes around the motor, thereby regulating stator and motor function. Given that FliL and the stator complex are ubiquitous in flagellated bacteria, these mechanisms may be utilized by various bacteria to modulate torque and motility in response to changing environmental conditions.

molecular machine | motility | spirochete | mechanosensor | electron tomography

Many bacteria, including spirochetes, use flagella to swim and disseminate within diverse host environments to survive and cause disease. The flagellum consists of the motor, hook, and filament. The motor is a cell envelope–spanning nanomachine primarily composed of a rotor ring surrounded by multiple stator complexes that control motor rotation (1). The current prevailing view is that ion (H\(^+\) or Na\(^+\)) fluxes conducted through the stator complex within the inner membrane generate torque and drive the rotation of the switch complex (also called C ring) in the cytoplasm (2–5). In addition, the stator complex can serve as a mechanosensor in response to external load change and surface contact (6–8). In complex environments, such as viscous fluids within hosts and on various semisolid surfaces, additional stator complexes are recruited to the motor to generate sufficient torque to counter the high load caused by resistive forces (6, 7, 9–12).

Each stator complex is composed of two inner membrane–associated proteins: MotA and MotB (13, 14). Recent cryo-electron microscopy (cryo-EM) structures provided evidence that MotA and MotB assemble with a stoichiometry of 5:2 (3, 4). The MotA pentamer has a cone-shaped structure, with its narrower end enclosing the dimeric MotB in the inner membrane to form an ion channel (3, 4), while the wider end engages with the C ring to drive motor rotation, as observed by cryo-electron tomography (cryo-ET) (2, 15). MotB is a modular protein with a transmembrane region near its N terminus, a long flexible periplasmic linker (MotB\(_{\text{linker}}\)) with unknown structure, and a peptidoglycan-binding domain at the C terminus (MotB\(_{\text{PCG}}\)) (16). In the inactive state, the N-terminal modules of MotB form two wedges in trans to plug the MotA ion channel, while the C-terminal MotB\(_{\text{PCG}}\) is unthetered in the periplasmic space (17, 18). As the stator complex is recruited to the motor, MotB\(_{\text{PCG}}\) extends upward to bind the peptidoglycan of the cell wall (16, 19, 20). This large conformational change activates a continuous ion flow through the stator complex, generating torque to further drive the rotation of the C ring (2–4, 16, 19), thus achieving flagellar motility. How does the highly extended MotB dimer, immobilized to the peptidoglycan at one end, interact with the MotA pentamer at the other end to generate torque without introducing excessive torsional strain that may compromise stator assembly and function?

FliL, one of the less-characterized flagellar proteins, may provide the answer to this question. FliL is a ubiquitous, transmembrane protein expressed by all flagellated

Significance

How flagella sense complex environments and control bacterial motility remain fascinating questions. Here, we deploy cryo-electron tomography to determine in situ structures of the flagellar motor in wild-type and mutant cells of Borrelia burgdorferi, revealing that three flagellar proteins (FliL, MotA, and MotB) form a unique supramolecular complex in situ. Importantly, FliL not only enhances motor function by forming a ring around the stator complex in its extended, active conformation but also facilitates assembly of the stator complex around the motor. Our in situ data provide insights into how cooperative remodeling of the FliL–stator supramolecular complex helps regulate the collective ion flux and establishes the optimal function of the flagellar motor to guide bacterial motility in various environments.

Author affiliations: 1Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06536; 2Microbial Sciences Institute, Yale University, West Haven, CT 06516; and 3Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC 27834

Authors contributions: M.A.M. and J.L designed research; S.G., H.X., Y.C., and J.L. performed research; S.G., H.X., Y.C., M.A.M., and J.L. analyzed data; and S.G., M.A.M., and J.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. N.T. is a guest editor invited by the Editorial Board.

Copyright © 2022 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

5. G. and H.X. contributed equally to this work.
6. To whom correspondence may be addressed. Email: motalebm@ecu.edu or j.liu@yale.edu.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2117245119/-/DCSupplemental. Published March 7, 2022.
bacteria (21–23). The fliL gene is often proximal to the motA and motB genes of the stator in the same operon (24), and it can also be in a locus of neighboring genes (fliMN) that encode the proteins that are the building blocks of the flagellum (25). Loss of FlIL leads to a range of motility defects, such as instability of the flagellar rod and moderate to complete inhibition of swimming and swarming in various bacteria (8, 9, 21–24, 26–30). Although these studies suggest that FlIL modulates stator assembly and function, reports also have given contradictory results (8, 9, 22). More recently, a crystal structure of the periplasmic C-terminal domain of FlIL from Vibrio alginolyticus (VaFlILperi) shows a structural similarity to those in the stoma-
tin family (31), which typically oligomerize into ring-like structures to regulate the activity of various ion channels. In the crystal, VaFlILperi oligomerizes into a decameric ring, which was proposed to interact with the stator complex and regulate its torque generation (31). We therefore sought to determine the in situ structure and location of FlIL in Borrelia burgdorferi periplasmic flagella to gain a mechanistic understanding of how FlIL interacts with the stator complex and impacts motility.

B. burgdorferi is the causative agent of Lyme disease (32) and a model organism for studying spirochetes, a unique group of bacteria with distinct morphology and motility (33–35). B. burgdorferi has multiple internal flagella within the periplas-
mic space near each cell pole that can switch between counterclockwise and clockwise rotations to modulate bacterial motility in complex environments (33, 35, 36). Previous studies indicated that FlIL (BbFlIL) is important for the assembly of individual stator units around the B. burgdorferi flagellar motor (37, 38). Deletion of fliL in B. burgdorferi results in partial occupancies by the stator units and irregular orientation of the periplasmic flagellar filament, causing defects in swimming motility and abolishing swarming motility (37, 39). Although it was suggested that BbFlIL is localized between the stator and the spirochete-specific “collar”, the exact structure of BbFlIL and how it interacts with the stator complex to enhance motility have remained unknown.

Here, we use cryo-ET and subtomogram averaging to show in situ that BbFlIL forms a 10-nm circular structure, termed the BbFlIL ring, that surrounds the stator complex in its extended conformation. Interaction between the BbFlIL ring and MotB plays a crucial role in stabilizing the stator complex in its extended state. In addition, we demonstrate that the BbFlIL-stator complex is further enclosed by flagellar collar proteins, suggesting that an ordered, cooperative assembly is necessary for the proper localization and stability of individual stator complexes. Our data not only elucidate molecular mechanisms by which the FlIL ring helps recruit and stabilize the stator complexes in their active, extended conformation for optimal motor function but also provide an explanation for how the bacterial flagellar motor readily adapts to generate torque sufficient for bacteria moving through complex environments.

Results

The Stator Complex Is Surrounded by a Ring-Like Structure in the Intact Motor. Our recent cryo-ET studies have demonstrated that the spirochete-specific collar plays a critical role in recruiting and stabilizing stator complexes (38, 40). Comparative analyses of motor structures from B. burgdorferi wild-type (WT) and stator mutants revealed that each flagellar motor contains 16 stator complexes (2, 39). Here, we used focused refinement to determine the in situ structure of the WT stator complex at 20-Å resolution. Consistent with previously published results (2), each stator complex is composed of a cone-shaped structure embedded in the inner membrane, a long periplasmic linker, and a round periplasmic domain associated with the collar (Fig. 1 A–D). Notably, each periplasmic linker of the 16 stator complexes in the WT motor is surrounded by a ring-like structure (Fig. 1 B–D). Strikingly, both the ring-like structure and the stator complex are absent from a ∆motB mutant (Fig. 1 F–H), which is nonmotile (34, 39, 41, 42). A detailed comparison with the WT motor shows that only partial ring-like structures remain in the ∆motB motor (small patch in Fig. 1 G and H).

Because the ring-like structure is reminiscent of the VaFlILperi decameric ring found in the crystal structure (31) and because BbFlIL was previously suggested to associate with the stator complex in B. burgdorferi (37, 38), we speculated that the full and partial ring structures observed in WT and ∆motB motors are formed by BbFlIL. To test this hypothesis, we determined the motor structures in both ∆flIL and ∆flILmotAB triple-mutant strains (Fig. 1 I–L and SI Appendix, Fig. S1). In the ∆flIL map, the density corresponding to the ring structure surrounding the stator completely disappeared (Fig. 1 I–L), and those representing the stator complexes were also significantly diminished (Fig. 1 I and J). Furthermore, in the absence of the stator complex and FlIL, both the cone-shaped and ring-like structures observed in WT as well as the partial ring-like structure in the ∆motB mutant disappeared (SI Appendix, Fig. S2). Collectively, these results suggest that multiple FlIL proteins form the full ring around the stator complex in WT, while they assemble into a partial ring in the absence of the stator complex.

Spirochete-Specific Collar Facilitates the Assembly of BbFlIL and Stator. To better visualize the BbFlIL ring and stator complex in the intact motor in three dimensions (3D), we segmented the WT motor structure (Fig. 2 A and B). The BbFlIL ring is situated below MotBPGB in the periplasm and above the cone-shaped MotA anchored in the inner membrane (Fig. 2 A and B). The BbFlIL ring, with a diameter of ~10 nm, encloses the periplasmic linker of MotB (Fig. 2 A). The whole supramolecular complex of BbFlIL–MotA–MotB is ~19 nm in height and ~10 nm in diameter (Fig. 2 C). It is tightly associated with the spirochete-specific collar (Fig. 2 A and B), consistent with the previous reports that FlIL interacts with the collar proteins FlbB and FlcA (38, 40). Given that stator complexes are not observed in the absence of the collar, interactions between the collar and BbFlIL–MotA–MotB complex (SI Appendix, Fig. S3) appear to be important for recruitment and stabilization of the whole complex. Furthermore, a small patch remains associated with the collar in the ∆motB mutant, whereas it disappears in both the ∆flIL and ∆flILmotAB strains (Fig. 1 I–L and SI Appendix, Fig. S2 B and E). To better visualize the BbFlIL partial ring in the ∆motB mutant, we also segmented the ∆motB motor structure (Fig. 2 D and B). The BbFlIL patch is also tightly associated with the collar and not visible in its absence (38, 40), suggesting that the collar facilitates the assembly of the BbFlIL partial ring in the absence of the stator complex.

BbFlIL Forms a Decameric Ring in Complex with a Stator Unit. To further understand the molecular basis underlying BbFlIL assembly and interactions with the stator complex, we leveraged the recent crystal structure of VaFlIL (31) with our cryo-ET
maps and protein–protein interactions to build pseudoatomic models of $Bb$FliL. The VaFliL ring has an outer diameter of $\sim 10 \text{ nm}$ (31), consistent with the diameter of the $Bb$FliL ring. Even though $Bb$FliL$_{peri}$ shares only 9% amino acid sequence identity with VaFliL$_{peri}$ (SI Appendix, Fig. S4A), the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2) modeled the protein with high confidence (99%) that $Bb$FliL$_{peri}$ has a fold identical to that of VaFliL$_{peri}$ (Fig. 3A). Furthermore, the VaFliL ring fits well into the ring-shaped cryo-ET map observed between the stator units in $B. burgdorferi$ (SI Appendix, Fig. S4B). $Bb$FliL is therefore modeled as a decameric ring (Fig. 3B). The oligomer interfaces between individual $Bb$FliL monomers, which are primarily comprised of charged residues, were refined by the Rosetta protein–protein docking function (43). The $Bb$FliL$_{peri}$ ring interior is also mainly composed of polar and charged residues, with 10 pairs of inward-pointing positively charged residues (R89 and K103) forming a constriction of $\sim 36 \text{ Å}$ in diameter (Fig. 3C). The ring structure of $Bb$FliL$_{peri}$ is in excellent agreement with the cryo-ET map and surrounds the portion of the stator unit MotB that extends above MotA, which is embedded in the inner membrane (Fig. 3D).

In contrast to the decameric ring of $Bb$FliL in the WT motor, only four FliL$_{peri}$ units could be docked into the FliL partial ring in the $\Delta motB$ mutant (Fig. 3E). This result has key implications for the sequential assembly of the Fli–stator complex. As the stator complex is recruited to the motor, the FliL partial ring likely engages the stator complex before it can further oligomerize into a full ring to wrap around the stator complex in its extended, active conformation (Fig. 3D).

**Molecular Architecture of the Stator Complex in Extended Conformation.** The purified stator complex is $\sim 7 \text{ nm}$ in diameter and $\sim 9 \text{ nm}$ in height (3, 4), considerably shorter than the in situ structure of the stator complex observed in the WT motor. To better understand the overall architecture of the functional stator complex in the flagellar motor of $B. burgdorferi$, we performed homology modeling to generate structures for individual domains comprising the MotA and MotB subunits. We used the Phyre2 server to generate the $Bb$MotA structure (100% confidence), with MotA from *Clostridium sporogenes* (45% shared sequence identity with $Bb$MotA; Protein Data Bank [PDB]: 6YSF) as template (SI Appendix, Fig. S5A). $Bb$MotA forms the pentameric, cone-shaped structure (Fig. 4A and SI Appendix, Fig. S5B–E). Electrostatic potential maps show that the outer surface of the narrower end of $Bb$MotA is primarily hydrophobic, consistent with the observation that it is embedded in the inner membrane (SI Appendix, Fig. S5C). By contrast, the wider end of $Bb$MotA has more charged residues on its outer surface (SI Appendix, Fig. S5C), consistent with its need to interact with the C ring in the cytoplasm to drive motor rotation.

MotB is a modular protein that can span the peptidoglycan and inner membrane. The N terminus of $Bb$MotB ($Bb$MotB$_{N}$) has three distinct modules (SI Appendix, Fig. S6 A and B). Following a short cytoplasmic segment, a transmembrane helix (MotB$_{TM}$) dimerizes and complexes with MotA to form the ion channel in the inner membrane (SI Appendix, Fig. S6 B and D). Next, the highly conserved plug domain (MotB$_{plug}$) protrudes from the interior of the transmembrane MotA
Cryo-EM studies reveal that the MotBplug dimer wedges in trans conformation \((SI\ Appendix,\ Fig.\ S6\ B\ and\ D)\), blocking the stator ion channel. This clearly differs from the extended, active conformation found in the WT motor. With the C-terminal MotB\textsubscript{PGB} modeled as a dimer using the 2-Å crystal structure of PomB\textsubscript{PGB} (PDB: 3WPW) from \textit{V. alginolyticus} \cite{44} as template, the MotB\textsubscript{plug} points upward to allow the flux of ions through the stator complex (Fig. 4\ A and \textit{B}).
Unlike the well-folded domains at the N and C termini, the structure of the centrally located MotBlinker connecting MotBplug and MotBTM+plug had not been determined. The MotBlinker is expected to be highly flexible as the stator complex transitions from an inactive, compact conformation to an active, extended conformation (Fig. 4A and B). As speculated, no homologous structures are available to produce a reliable model for MotBlinker using the Phyre2 and I-TASSER servers (45, 46). We therefore used AlphaFold2, a recently developed machine learning–based tool, to predict the structure of full-length MotB (47). AlphaFold2 modeled MotBTM+plug and MotBplug of B. burgdorferi as having folds identical to those predicted by the Phyre2 server (SI Appendix, Fig. S6C). In addition, AlphaFold2 predicted that the MotBlinker has a centrally located short α-helix flanked by flexible loops (SI Appendix, Fig. S6C). Guided by the crystal structure (PDB: 3WPW) of the PomBGB dimer (16) and the cryo-ET map, we built a model of the MotB dimer in its extended conformation (Fig. 4A–C and SI Appendix, Fig. S6E). The MotB dimer was then inserted into the cone-shaped MotA pentamer to assemble the intact stator complex in its extended, active conformation (Fig. 4A and B).

Pseudoatomic Model of the FliL–Stator Complex in Active State. The MotBlinker amino acid residues that comprise the α-helix and loop immediately below it (N-terminal side) are primarily charged or polar (SI Appendix, Fig. S6B) and appear to interact with the interior of the BfFliL ring. The BfFliL ring interior has a cone shape, being wider at its opening near the inner membrane and becoming narrower toward the periplasmic space. As mentioned, the constriction of the interior of the BfFliL ring is ~36 Å in diameter, modeled with 10 pairs of inward-pointing arginine and lysine residues (Fig. 3C). This narrowest part of the FliL ring interior likely contacts the MotBlinker. Given that the interface between the MotBlinker and the BfFliL ring interior has a high content of polar and charged residues (SI Appendix, Fig. S6B and Fig. 3C), the two proteins likely interact through electrostatic or other polar interactions. As the BfFliL ring has a larger perimeter (~110 Å) than the MotA transmembrane region (~88 Å), there is space for ten BfFliL+TM helices to surround MotA embedded in the inner membrane (Fig. 4E). The unique architecture of the FliL–stator supramolecular complex appears ideal to stabilize the stator complex in its extended, active conformation (Fig. 4C and D).

Discussion

The flagellar stator complex generates torque to power rotation of the motor as ions flow across the inner membrane. Recent studies have significantly advanced our understanding of how the stator complex adjusts torque in response to changes in external load (6–8). FliL, one of the less-characterized flagellar proteins, plays crucial roles in modulating stator function and motility in some bacteria, particularly under high-load conditions (24, 31, 37). In this study, we sought in situ structural information to define the mechanistic details of how the stator complex is associated with FliL to enhance motor function in B. burgdorferi.

We demonstrated previously that deletion of FliL in B. burgdorferi results in motility defects (37) and a flagellar motor with fewer stator units (39). Here, we show that BfFliL in situ forms a ring-like structure around the stator complex of the B. burgdorferi WT flagellar motor. Importantly, assembly of the BfFliL ring provides a key support to maintain the MotB dimer in its upright, extended conformation, presumably keeping the stator transmembrane channel open for the flux of H+ ions required for torque generation. Our pseudoatomic models further indicate that BfFliL+TM helices form a circular,
Bb

fence-like structure embedded in the inner membrane around the stator complex (Fig. 4E), helping to properly position MotA while it rapidly drives the C ring rotation in a cogwheel-like manner. These data establish the structural rationale for how FliL indirectly modulates the interface between the stator complex and C ring, as indicated by biochemical data (24). In addition, individual BbFliL–stator complexes are embraced by the collar through protein–protein interactions (Fig. 5 A–C and Movie S1), as reported previously (38, 40). Together with BbFliL, the added level of reinforcement provided by the collar maintains a maximal number of active stator complexes around the motor (comparison shown in Fig. 5 B and C and Movie S1), thus generating sufficient torque to propel spirochetes through complex and highly viscous environments in vertebrate and tick hosts.

FliL shows remarkable structural similarity with stomatin proteins (31), which form oligomeric complexes to regulate various ion channels (48). Mechanosensing by the flagellar motor is intimately associated with the change in ion flow through its stator channels (21, 49). FliL has been implicated in the mechanosensing pathways in species such as Proteinus mirabilis and V. alginolyticus (31, 50, 51). However, FliL is not directly involved in mechanosensing in Escherichia coli (37). Therefore, the potential role of BbFliL in mechanosensing remains to be investigated. Our in situ study suggests that BbFliL can assemble into a partial ring or a full ring around the stator complex adjacent to the flagellar collar (Fig. 5 A and B). As the stator complex in its inactive conformation is recruited to the motor, ion flow and interaction with the C ring may trigger a local conformational change near the MotBplug, which then moves upward to unplug the transmembrane ion channel. This local conformational change in MotB is likely sufficient to initiate the interaction between solvent-exposed residues of MotBlinker and those in the interior of the BbFliL partial ring. This transient contact would facilitate the recruitment of additional BbFliL units to assemble around MotBlinker and form a full ring (Fig. 5 D, Left, and Movie S1), a more energetically favorable structure than the partial ring. We speculate that, as long as MotBPGB is anchored to the peptidoglycan and MotBlinker is held within the BbFliL ring, the transmembrane channel remains unplugged for continuous ion flux. Given that 16 FliL–stator supramolecular complexes can assemble around the motor, the BbFliL ring likely influences the collective ion flow through the motor to help maximize torque generation. Nevertheless, given that protein turnover and exchange are essential for the function of the bacterial flagellar motor (52–54), the stator–FliL ring association is likely dynamic. Remodeling of MotB from its extended conformation to a compact form may cause the FliL ring to disassemble back into a partial ring. The constant opening and closing of the FliL ring would allow the continuous replenishing of functional stator complexes to engage with the rotor, possibly as part of a regulatory mechanism to increase torque generation and prevent excessive proton leakage through defective stator ion channels. As such, the mechanistic model deduced from our in situ structural data provides insights into how the cooperative remodeling of the FliL–stator complex helps regulate the collective ion flux and establishes the full complement and optimal function of stator complexes to maximize torque generation.

Beyond providing structural support to the stator complex, FliL may protect the flexible MotBlinker from proteolysis by periplasmic proteases. As discussed, MotBlinker and MotBPGB are not present in the cryo-EM structures of the purified stator complexes (3, 4). This is likely due to the high degree of flexibility of the MotBlinker, but it is also possible that the floppy, solvent-exposed coils were rapidly proteolysed during the purification steps (a comparison of the active stator architecture in the presence and absence of the FliL ring is shown in Fig. 5 D). A solid FliL ring structure protecting the MotBlinker would help preserve stator structure and function. Indeed, V. fliLperi is highly resistant to trypsin digestion (31). It would be intriguing to test if the active, extended conformation of the stator complex can be preserved in the presence of FliL if the supramolecular complex is purified from the cellular membrane. This would make it possible to determine the structure of the

**Fig. 5.** Maximal torque generation by the flagellar motor requires proper assembly of the FliL–stator complex. (A) A top view of the ΔmotB motor. The FliL units that form partial rings are colored magenta, the collars are transparent, and the C ring subunits are colored cyan. (B) A top view of the WT motor. The 16 BbFliL rings surrounding the stators (MotB subunits colored orange) are colored magenta, the collars are transparent, and the C ring subunits are colored cyan. (C) A top view of the ΔfliL motor. In the absence of the BbFliL rings, the motor is surrounded by fewer stator complexes. (D) A model for the assembly of the FliL–stator supramolecular complex necessary for maximizing torque generation by the bacterial flagellar motor. Left of the schematics for the rotor, FliL units interact with themselves to form oligomers that will interact with the active stator units. Interactions between FliL oligomers and the stator units result in the cooperative assembly of the FliL–stator supramolecular complex around the rotor. Right of the schematics for the rotor, comparison between the stator in its active, extended conformation in the absence of FliL (Left) and stator in its inactive form. Double-headed arrows with a dotted line indicate MotBPGB binding to and dissociation from the peptidoglycan layer.
FliL–stator supramolecular complex at near-atomic resolution via in vitro methods, such as cryo-EM single-particle analysis and X-ray crystallography.

In addition to modulating stator function, FliL appears to play a global structural role in the flagella of diverse species. In the absence of FliL, the flagellar rod fractures when Salmonella enterica encounters swarming conditions (55). In B. burgdorferi, loss of FliL leads to abnormal flagellar filament orientation (37). Furthermore, both E. coli and Salmonella motors switch direction less frequently when FliL is not present (24). Our data indicate that B0FliL is a key scaffold protein that acts as a hinge between the stator complex and the rotor components of the flagellar motor in B. burgdorferi. Although the fliL gene is well conserved, the FliL protein shows relatively low shared sequence identity across species. This suggests that FliL interacts with other flagellar components in a species-specific manner. In B. burgdorferi, the B0FliL ring is intimately associated with MotB within its ring interior, while it is stabilized by the collar proteins on the outer surface. This conclusion is supported by the reported protein–protein interactions between FliL and MotA/MotB and the collar proteins FliB and FlcA (38, 40). In species such as Caulobacter, Salmonella, and Vibrio, which lack scaffolds complexes like the collar in B. burgdorferi, the in situ structures of the FliL–stator supramolecular complex remain to be discovered. Nevertheless, given the observation that B0FliL is involved in extensive protein–protein interactions, it is conceivable that FliL functions as a scaffold in other species to help stabilize the stators around the rotor and protect them against torsional stress generated during dynamic processes, such as switching rotational direction and encountering a drastic increase in external load. It is noteworthy that in a parallel study, we found a similar FliL ring in the flagellar motor of Helicobacter pylori (56). Further work is needed to understand how FliL in diverse species achieves the functions of stabilizing the flagella as well as modulating the stator ion channel to maximize torque generation. It will be interesting to determine whether FliL from other bacteria can form oligomeric complexes with shapes other than a ring and to identify species-specific interaction partners.

In summary, we report that FliL forms a decameric ring and interacts intimately with the stator complex in the B. burgdorferi flagellar motor. The in situ molecular architecture of the FliL–stator supramolecular complex reveals the specific role of FliL in enhancing the stator function and torque generation by stabilizing the stator complex in an extended, active conformation (Fig. 5 D). Given its conservation across flagellated bacteria, FliL is likely widely utilized to regulate motor function and bacterial motility in response to various complex and often viscous environments.

Materials and Methods

Bacterial Strains and Growth Conditions. A high-passage B. burgdorferi strain B31A was used as the WT clone throughout this study (57, 58). Constructions of B. burgdorferi Δflil (bb0279) and ΔmotB (bb0280) mutant strains were described elsewhere (37, 39). The B. burgdorferi triple-mutant ΔfliLΔmotAB strain was constructed as described below. B. burgdorferi cells were cultivated in liquid Barbour-Stoenner-Kelly (BSK-II) broth or agarose plates and incubated at 35 °C in a 2.5% CO2 incubator, as reported previously (59, 60). For culturing B. burgdorferi cells, 100 μg/mL streptomycin was included in BSK-II medium when required. E. coli strains were grown at room temperature or 37 °C in liquid Luria–Bertani (LB) broth or plated on LB agar (61, 62). When required, 100 μg/mL ampicillin, 0.2% glucose, and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were supplemented into LB medium.

Construction of B. burgdorferi ΔfliLΔmotAB Mutant Strain. Construction of the inactivation suicide plasmid and electroporation and plating of B. burgdorferi were as described earlier (42, 60, 63, 64). fliL (bb0279) and motB (bb0280) were inactivated, as reported previously (37, 39). Similarly, via chromosomal homologous recombination, a fliL-motB-motA triple-gene mutant strain (ΔfliLΔmotAB) was constructed. Streptomycin-resistant transformants were screened by PCR and further verified by immunoblotting.

Overexpression and Purification of Recombinant Proteins in E. coli. MotA, MotB, and FliL proteins possess transmembrane domains. When the transmembrane domains were deleted from each of the proteins and used for biochemical assays, FliL was found not to interact with MotA or MotB. We therefore expressed and purified full-length proteins with maltose-binding protein (MBP) tag and used those recombinant proteins in far-Western or affinity blot assays. In brief, DNA fragments harboring the MotA, MotB, and FliL full-length open reading frames were PCR (PCR) amplified from chromosomal DNA of B. burgdorferi B31 A using the primers PF MBPMotA_ BamHI (GGATCCGCGAAGTCTCTTTGCGGAAAGGAC) and PB MBPMotB_PstI (TAAT- TACTCGACTATGCTTCAATTCGTTTCAATGCTACTATTAAG), PF MBPMotB_BamHI (CTGGGACGATCCTGCTGATGGTGGATTGAAGGATCA), and PB MBFMotL_BamHI (CGTCGACGATCCTGCTAGTTACAAGGAC) and PCR MBPfliL_PstI (TAAT-TACTCGACTATGCTTCAATTCGTTTCAATGCTACTATTAAG) and cloned into plasmal pCMX (NEB) under NotI-BamHI or BamHI-PstI restriction sites to produce MBP-tagged MotA, MotB, and FliL. Similarly, the 1×FLAG (DYKDDDDK)-tagged FliL (FLAG–FliL) was constructed as a probe for far-Western blotting. A DNA fragment of full-length FliL fused with 1×FLAG tag coding sequence (GACTAACAGGACGATACGACCAGAC) at the C terminus was amplified by PCR with primers PF MBPMotA_BamHI and PR FLAG–FliL-PstI (GGCCTACGTCGACTATGCTTCAATTCGTTTCAATGCTACTATTAAG) and cloned into pCMX. All E. coli DH5α strains carrying the pCMX constructs for expressing full-length MotA, MotB, FliL, and FLAG–FliL were induced with 0.5 mM IPTG at room temperature, and purifications of recombinant proteins were performed with amylose resin (NEB) according to the manufacturer’s protocol. MBP-MCP5 was constructed and purified, as previously reported (65).

Affinity Blotting. Far-Western and affinity blot assays with recombinant proteins were performed as described previously (40, 65–68). In brief, 1 μg of purified recombinant proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were blocked in blocking solution (5% bovine serum albumin, 10 mM Tris, 150 mM NaCl, and 0.3% Tween 20, pH 7.4) with gentle shaking for 4 to 6 h at room temperature and incubated with purified FliL–FLAG at the concentration 2 μg/mL in blocking solution overnight. The membranes were washed three times with washing buffer (10 mM Tris, 150 mM NaCl, and 0.3% Tween 20, pH 7.4) and probed with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich), followed by ECL 2 detection as reported.

Cryo-ET Data Collection and Tomogram Reconstruction. Frozen-hydrated specimens of bacteria were prepared as described previously (69). In brief, various clones of exponentially grown B. burgdorferi cells were centrifuged individually at 5,000 × g for ~5 min, and the resulting pellets were suspended in phosphate-buffered saline. After adding 10-nm Aurion gold tracers (Fisher Scientific), we deposited 5 μL of the cell suspension onto freshly glow-discharged EM grids (Quantifoil). The grids were blotted with a filter paper (Whatman) and then quickly plunge-frozen in liquid ethane using a homemade plunger apparatus, as described previously (69). The frozen-hydrated specimens were then imaged using a 300 K Vitesse electron microscope (Thermo Fisher Scientific) equipped with a field emission gun, Volta phase plate (VPP), and a post-GIF direct electron detector (Gatan). SerialEM (70) was used to collect tilt series from −51° to 51° with 3° increments. The total dose of tilt series is ~80 e−/Å2. VPP was used to collect tilt series from WT and ΔmotB cells at defocus close to zero. Other data were collected at defocus ~3 μm without VPP. The number of tomograms used in this work for each strain is shown in Table 1.

MotionCor2 (71) was first used to align raw images. IMOD (72) was then used to align tilt series with gold markers. For the data collected without VPP, Gctf (73) was used to determine the defocus of each tilt image in the aligned stacks, and the “ctfphaseflip” function in IMOD was used for contrast transfer.
Table 1. Strains and cryo-ET data used in the manuscript

| Strain          | Camera | Pixel size | Tomogram No. | Subtomogram No. | Resolution estimated |
|-----------------|--------|------------|--------------|-----------------|---------------------|
| WT              | K2     | 2.7 Å      | 256          | 1,509           | 21 Å                |
| ΔmotB           | K2     | 2.7 Å      | 233          | 1,272           | 20 Å                |
| Δfil            | K3     | 2.7 Å      | 217          | 1,059           | 20 Å                |
| ΔmotBΔfil       | CCD    | 5.5 Å      | 512          | 3,573           | 34 Å                |
| ΔfilMotBΔmotA   | CCD    | 5.5 Å      | 306          | 1,015           | 35 Å                |

Function correction for the tilt images. Tomo3D (74) was used to generate tomograms by simultaneous iterative reconstruction technique (SIRT) or by weighted back-projection (WBP). SIRT reconstructions have higher contrast than those reconstructed by WBP. Thus, SIRT reconstructions were used for direct visualization of bacteria and their flagellar motors. By contrast, tomograms reconstructed by WBP retain high-resolution details better than those reconstructed by SIRT. Thus, WBP reconstructions were used for in situ structure determination of the flagellar motor by subtomogram averaging.

Subtomogram Analysis. Bacterial flagellar motors were visually identified and manually picked from the 6x binned SIRT tomograms, as these produce sufficient contrast (75). The number of the flagellar motors identified for each strain is shown in Table 1. Next, the subtomograms of flagellar motors were extracted from the 6x binned tomograms, and then the 3D software package (76, 77) was used for 3D alignment and classification to obtain the refined particle positions and remove junk particles. Afterward, the subtomograms were extracted from unbinned tomograms with the refined positions and were 4x binned to perform alignment and classification. This step was repeated with 2x binned subtomograms to further improve the resolution of the structure. Given that each flagellar motor has 16 stator-Fil complex symmetries, expansion usage was further refined in the structure of the stator-Fil complex. Fourier shell correlation coefficients were calculated by generating the correlation between two randomly divided halves of the aligned images to estimate the resolution of the final maps.

Modeling. On the basis of reported cryo-EM and X-ray crystal structures of MotA and FilJerm (PDB: 6AHQ), the B. burgdorferi MotA and FilJerm models were generated using the Phyre2 Server (45). Low-confidence regions of the resulting models were trimmed to retain the core correct topologies. The 2Bfil ring was constructed using the VaRIL ring (PDB: 6AHQ) as template. The interface between each 2Bfil subunit was refined by the protein-protein docking function of RosettaDock (43). The model for full-length MotB has been generated using Alphafold2 (47). Guided by the cryo-ET maps, the orientations of the MotB domains were corrected through the flexible loops predicted by Alphafold2. The models for MotA, MotB, and the FilJerm ring were placed into segmented WT and ΔmotB Δfil focus-reined cryo-ET maps and fitted using the ChimeraX “fit to map” function (78) and refined with the PHENIX real-space refinement (79).

Visualization. University of California San Francisco (UCSF) ChimeraX (80) and UCSF ChimeraX (78) were used for surface rendering of subtomogram averages, segmentation, molecular modeling, and making clips of molecular movies. The movie clips were edited and combined using Keynote.

Data Availability. All cryo-ET results are included in the article and/or supporting information.

ACKNOWLEDGMENTS. We thank Michael Manson, Seiji Kojima, Michi Homma, and Jennifer Aronson for critical reading of the manuscript. We thank Shening Wu for assisting with cryo-ET data collection. S.G., Y.C., and J.L. were partly supported by Grant RO1AI087946 from the National Institute of Allergy and Infectious Diseases (NIADD); S.G. is also supported by a CHIR fellowship from the Canadian Institutes of Health Research. H.X. and M.A.M. were supported by Grant RO1AI132818 from NIADD. Cryo-ET data were collected at the Yale Cryo-EM resources funded in part by the NIH Grant 15100D023603-01A1.
A novel gene inactivation system reveals altered periplasmic flagellar orientation in a Borrelia burgdorferi flh mutant. J. Bacteriol. 193, 3324–3331 (2011).

K. H. Moon et al., Sprochetes: flagellar collar protein FliB has astounding effects in orientation of periplasmic flagella, bacterial shape, motility, and assembly of motors in Borrelia burgdorferi. Mol. Microbiol. 102, 336–348 (2016).

Y. Chang et al., Structural insights into flagellar stator-rotor interactions. eLife 8, e48979 (2019).

K. H. Moon, X. Zhao, H. Xu, J. Liu, M. A. Motaleb, A tetra-tricopeptide repeat domain protein has profound effects on assembly of periplasmic flagella, morphology and motility of the Lyme disease spirochete Borrelia burgdorferi. Mol. Microbiol. 110, 634–647 (2018).

S. Z. Sultan et al., Motor rotation is essential for the formation of the periplasmic flagellar ribbon, cellular morphology, and Borrelia burgdorferi persistence within ixodes scapularis tick and murine hosts. Infect. Immun. 83, 1765–1777 (2015).

M. A. Motaleb et al., Borrelia burgdorferi periplasmic flagella have both skeletal and motility functions. Proc. Natl. Acad. Sci. U. S. A. 97, 10899–10904 (2000).

S. Lykvis, J. J. Gray, The RosettaDock server for local protein-protein docking. Nucleic Acids Res. 36, W231–W238 (2008).

S. Zhu et al., Conformational change in the periplasmic region of the flagellar stator coupled with the assembly around the rotor. Proc. Natl. Acad. Sci. U. S. A. 111, 13523–13528 (2014).

L. A. Kelley, S. Meulens, C. M. Yates, M. N. Wiss, M. J. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 10, 845–858 (2015).

J. Yang et al., The HASSER Suite: Protein structure and function prediction. Nat. Methods 12, 7–8 (2015).

J. Jumper et al., Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589 (2021).

J. Brand et al., A stomatin dimer modulates the activity of acid-sensing ion channels. EMBO J. 31, 3635–3646 (2012).

I. Hug, S. Desghande, K. S. Sprecher, T. Pfohl, U. Jenal, Second messenger-mediated tactile response by a bacterial rotary motor. Science 358, 531–534 (2017).

K. Cusick, Y. T. Lee, B. Youchak, R. Belas, Perturbation of FliL interferes with swarmer cell gene expression and differentiation. J. Bacteriol. 194, 437–447 (2012).

R. Belas, R. Suvanasuthi, The ability of Proteus mirabilis to modulate the response regulator CheY-P to the absence of FliL fractures the assembly around the rotor. Proc. Natl. Acad. Sci. U. S. A. 111, 7021–7026 (2014).

A. S. Toker, R. M. Macnab, Distinct regions of bacterial flagellar switch protein FlhF interact with FliG, FlfN and CheY. J. Mol. Biol. 273, 623–634 (1997).

T. Kariu et al., BB0323 and novel virulence determinant BB0328: Borrelia burgdorferi proteins that interact with and stabilize each other and are critical for infectivity. J. Infect. Dis. 211, 462–471 (2015).

H. Xu, J. He, J. Liu, M. A. Motaleb, BB0326 is responsible for the formation of periplasmic flagellar collar and assembly of the stator complex in Borrelia burgdorferi. Mol. Microbiol. 113, 418–429 (2020).

J. Liu et al., Intact flagellar motor of Borrelia burgdorferi revealed by cryo-electron tomography: Evidence for stator ring curvature and rotor/C-ring assembly flexion. J. Bacteriol. 191, 5026–5033 (2009).

D. N. Mastronarde, Automated electron microscopy: a new way to view images. Nat. Methods 13, 345 (2016).

M. C. Leake et al., Structural and functional analyses of the motor domain of the chemotaxis motor CheA of the pathogenic bacterium Borrelia burgdorferi. Mol. Microbiol. 75, 1346–1356 (2010).

K. Zhang, Gctf: Real-time CTF determination and correction. J. Struct. Biol. 152, 279–287 (2005).

J. D. Antani et al., Tomo3D 2.0: Automated electron cryo-microscopy methods for structure determination. J. Struct. Biol. 193, 110–116 (2016).

T. D. Goddard et al., UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).