Characterization of the Flagellar Collar Reveals Structural Plasticity Essential for Spirochete Motility

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ABSTRACT Spirochetes are a remarkable group of bacteria with distinct morphology and periplasmic flagella that enable motility in viscous environments, such as host connective tissues. The collar, a spirochete-specific complex of the periplasmic flagellum, is required for this unique spirochete motility, yet it has not been clear how the collar assembles and enables spirochetes to transit between complex host environments. Here, we characterize the collar complex in the Lyme disease spirochete Borrelia burgdorferi. We discover as well as delineate the distinct functions of two novel collar proteins, FlcB and FlcC, by combining subtractive bioinformatic, genetic, and cryo-electron tomography approaches. Our high-resolution in situ structures reveal that the multiprotein collar has a remarkable structural plasticity essential not only for assembly of flagellar motors in the highly curved membrane of spirochetes but also for generation of the high torque necessary for spirochete motility.

IMPORTANCE Many spirochetes cause serious human diseases. They are well recognized by their distinct morphology and motility. Spirochete motility is driven by a periplasmic flagellum, which possesses a unique collar essential for flagellar assembly and spirochete motility. Here, we discover two novel collar proteins in the Lyme disease spirochete Borrelia burgdorferi. We demonstrate, for the first time, that the collar is a multiprotein complex with a remarkable plasticity that enables the motor to accommodate the highly curved membrane of spirochetes and generate the high torque necessary for spirochete motility.

KEYWORDS molecular machine, motility, periplasmic flagella, plasticity, spirochete

Spirochetes are a group of bacteria that cause several serious human diseases, such as Lyme disease (Borrelia burgdorferi), syphilis (Treponema pallidum), periodontal disease (Treponema denticola), and leptospirosis (Leptospira interrogans). Spirochetes have a distinctive spiral or flat-wave morphology (1, 2). Enclosing the cell is a multilayered envelope including the outer membrane, peptidoglycan layer, and cytoplasmic membrane. The motility of spirochetes is unique among bacteria, as the whole cell body rotates without any external apparatus. Furthermore, this motility is crucial for host tissue penetration, virulence, and transmission of spirochetes (3–7). The periplasmic flagellum, which rotates between the outer membrane and peptidoglycan layer, is responsible for spirochete motility (1, 2). Each periplasmic flagellum is attached subterminally to one end of the cell poles and extends toward the other end. Spirochete species vary significantly in the number of periplasmic flagella and whether the flagella overlap in the center of the cell (8–12).

Like the external flagella of the model organisms Escherichia coli and Salmonella enterica, the periplasmic flagellum in spirochetes consists of a motor, hook, and...
The motor is a rotary machine responsible for the assembly and function of the periplasmic flagellum. Most components of the spirochetal flagellar motor have highly conserved counterparts in the external flagellar motor: the MS ring, C ring, rod, export apparatus, and stator (14–16). Uniquely, a spirochete-specific flagellar component—termed collar—not only contributes to the distinct spirochetal flagellar structures but also plays a role in recruiting 16 torque-generating stator units (9, 10, 12, 17–22), presumably enabling the increased torque required for spirochetes to swim through complex, viscous host environments (23). The collar structure also contributes to make the spirochetal flagellar motor considerably larger and more complicated than its counterparts in *E. coli* and *S. enterica* (15). However, how the collar supports the production of high torque by the spirochetal flagellar motor has remained poorly understood.

*B. burgdorferi* has emerged as an ideal model system for understanding the unique structure and function of periplasmic flagella (2, 24). At each cell pole, 7 to 11 periplasmic flagella wrap inward as a flat ribbon along the cell body and overlap in the middle of the cell (Fig. 1A) (11, 25). A combination of genetic and cryo-electron tomography (cryo-ET) approaches has enabled in situ visualization of *B. burgdorferi* flagellar motors at an unprecedented resolution, unveiling unique features of this complex machine (16, 24). Specifically, comparative analyses of the wild type, stator deletion mutant ΔmotB, and collar deletion mutant ΔflbB provided direct evidence that the collar is important for stator assembly, flagellar orientation, cell morphology, and motility in *B. burgdorferi* (13, 21, 22). The collar is a large complex consisting of the inner core and the outer, turbine-like structure. Three collar proteins have been identified in *B. burgdorferi* (20–22): FlbB (BB0286) appears to serve as the base of the collar structure (21), BB0236 is...
involved in collar assembly (22), and FlcA (BB0326) forms the turbine-like structure, directly interacting with the stator units (20). Given that the overall structure of the collar is 79 nm in diameter and ~20 nm in height, additional proteins are likely involved in collar assembly. Moreover, the large collar structure must be flexible to accommodate the highly curved membrane at the cell tip (13). How the collar assembles and contributes to stator assembly is essential for understanding the unique spirochete motility.

In this study, we identify two novel collar proteins, FlcB (BB0058) and FlcC (BB0624), each responsible for distinct portions of the collar. Together with studies of other collar proteins (20–22), our high-resolution in situ structural analyses of the B. burgdorferi flagellar motor provide a molecular basis for the assembly and flexibility of the periplasmic collar complex and its critical roles in the assembly of the stator complexes. Our results also highlight how the collar contributes to the distinct motility that allows spirochetes to swim through complex environments, such as inside ticks and vertebrate hosts.

**RESULTS**

**BB0058 and BB0624 are potential collar proteins.** To better understand collar assembly and function, we devised a subtractive bioinformatic approach to identify eight potential collar proteins (Fig. 1B). Each corresponding mutant was constructed and analyzed with respect to motility and morphology phenotypes (see Fig. S1 and S2 in the supplemental material). Two of these genes (bb0058 and bb0624) were ultimately identified as the genes encoding potential collar proteins for the following reasons. (i) \(\Delta bb0058\) and \(\Delta bb0624\) mutant cells exhibited rod-shaped morphology instead of the characteristic flat-wave morphology in wild-type (WT) spirochetes (Fig. 1C and E). \(\Delta bb0058\) mutant cells were significantly less motile than WT cells, whereas the \(\Delta bb0624\) mutant cells were completely nonmotile (Fig. S3). These mutants exhibited no polar effects on downstream gene expression. A complemented \(\Delta bb0058\) mutant in cis (bb0058 \(^+\)) was constructed as described previously (26), and it restored the morphology and motility phenotypes to WT levels (Fig. 1C and D and Fig. S3). (ii) Domain analysis data suggest that BB0058 possesses multiple tetratricopeptide repeat (TPR) domains, and both BB0058 and BB0624 possess a signal peptide at their N-terminal region that is likely required for their
export across the membrane (not shown in current work). (iii) Cryo-ET reconstructions of the cell tips indicate that the $D_{bb0058}$ and $D_{bb0624}$ cells possess approximately 40% and 34% fewer flagella than WT cells (Fig. 1G), respectively. In addition, the flagella in both $D_{bb0058}$ and $D_{bb0624}$ cells appear to show shorter lengths and abnormal orientations (Fig. 1F and G), with filaments extending toward their pole of origin instead of toward the other cell pole, as in WT cells. Similar shorter length and abnormal orientation phenotypes were also observed in our previously reported collar gene mutants (20).

**FlcB is a novel flagellar protein that contributes to the middle portion of the collar.** To determine whether BB0058 is involved in assembly of the collar complex, we used cryo-ET and subtomogram averaging to resolve the in situ structures of the flagellar motor in $D_{bb0058}$ and $bb0058^+$ cells. Compared to the WT motor (Fig. 2A and E), a bridge-like structure near the interface between the collar and the MS ring is absent from the $D_{bb0058}$ motor (Fig. 2B and F), but this structure is restored in the complemented $bb0058^+$ motor (Fig. 2C and G), suggesting that BB0058 is responsible for the formation of the bridge-like structure of the collar (Fig. 2D and H). We therefore renamed BB0058 periplasmic flagellar collar protein B (FlcB). Notably, in the spirochetal flagellar motor, 16 copies of this bridge-like structure form the FlcB ring directly above the MS ring (Fig. 2D and H). The FlcB ring does not directly interact with the stator complexes or the MS ring yet has a significant impact on flagellar rotation and bacterial motility.

**FlcC is a novel flagellar protein responsible for collar and stator assembly.** To identify specific roles of the BB0624 protein, we determined the in situ structure of the $D_{bb0624}$ motor by cryo-ET and subtomogram averaging, revealing that the top portion of the collar is absent (Fig. 3B). BB0624 is therefore a collar protein, renamed hereafter periplasmic flagellar collar protein C (FlcC). Furthermore, the densities corresponding to the stator complexes in the $D_{flcC}$ motor are considerably different from those in the WT motor, suggesting that FlcC directly impacts not only collar formation but also stator assembly. To estimate stator complex numbers in WT and these two new mutants, focused alignment and classification were utilized to analyze the stator densities. For the $D_{flcC}$ mutant, the class with stator density (Fig. 3C) accounts for ~40% of the total collar subunits, while the class without stator density (Fig. 3D) accounts for ~60%, indicating

**FIG 3** $D_{flcC}$ mutant cells show defects in the collar structure and have fewer stator units assembled in the motor. (A and B) A central section of the subtomogram average (16-fold symmetrized) of WT and $D_{flcC}$ flagellar motors, respectively. The top portion of the collar (indicated by a white arrow) is absent from the $D_{flcC}$ motor. (C and D) Class averages of the collar region (dashed box in panel B) with and without the density of the stator complex in the $D_{flcC}$ motor, respectively. (E and F) A tilted side view of the 3D rendering of WT and $D_{flcC}$ flagellar motors (with stator complexes), respectively. (G) A histogram showing stator occupancy in the WT, $D_{flcB}$, and $D_{flcC}$ motors, respectively. Refer to Materials and Methods section for details about the calculation of stator occupancy.
that stator occupancy in the Δflc motor is ~40%, considerably lower than in the WT (~96%) and ΔflcB motors (~94%). This result is consistent with immunoblotting data showing that the stator protein MotB is significantly reduced in the ΔflcC mutant compared to WT cells (Fig. S2C). Collectively, these results support the model that FlcC functions as a major collar protein directly involved in collar formation and stator assembly.

The molecular architecture of the collar reveals its intrinsic plasticity. Five collar proteins have been identified and characterized in B. burgdorferi: FlbB (21), BB0236 (22), FlcA (20), FlcB (BB0058), and FlcC (BB0624). To understand how these proteins assemble as the complex collar, we developed a sophisticated approach to analyze the collar structure in the absence of the stator complexes. First, we generated an asymmetric reconstruction of the ΔmotB motors (Fig. S4A and B) (13). The asymmetric reconstruction reveals 16 collar subunits and their associated membrane curvature (Fig. S4C), consistent with the observation that the motors are embedded in a highly curved membrane cylinder. Second, to determine the collar subunit structure at higher resolution, we extracted 16 collar subunits from each motor and performed three-dimensional (3D) classification and focused refinement (see Fig. S4D and S4E and Movie S1 in the supplemental material). Third, the high-resolution structure of the collar subunit was then mapped back to the asymmetric reconstruction of the ΔmotB motor structure to obtain a detailed overview of the collar complex (Fig. 4). The exact location of each collar protein was defined by comparing the high-resolution in situ structure of the ΔmotB motor with specific collar mutant structures reported in this study (Fig. 2 and 3) and previously (20–22) and analyzing the protein-protein interaction data (Fig. S5). In the large collar complex (79 nm in diameter) (Fig. 4A and B), FlcA is closely associated with the membrane and forms the turbine-like structure (Fig. 4). FlbB forms the base of the collar (Fig. 4B). FlcA is located on top of the collar structure.
(Fig. 4A, C, and D), and FlcB is located approximately in the middle of the collar complex (Fig. 4B). Although some components of the collar remain undefined, it is evident that the collar complex is composed of multiple different proteins, each contributing to a distinct portion of the highly modular, flexible architecture of the collar complex. Importantly, this highly modular architecture of the collar enables extensive remodeling to accommodate the curvature of the membrane cylinder, which is ubiquitous in spirochetes and other bacteria (Fig. 4C and D).

**DISCUSSION**

Spirochete motility is unique among bacteria, due to the location and distinct assembly of periplasmic flagella. It is increasingly evident that the periplasmic flagellum possesses a specialized multiprotein collar important for the assembly of periplasmic flagella and motility of spirochetes. Five spirochete-specific collar proteins in *B. burgdorferi*.
*B. burgdorferi* have been demonstrated to be involved in collar assembly. Given that these collar proteins are well conserved in spirochetes, their homologs are likely involved in collar assembly across diverse spirochetes (14, 15).

The ΔflcB and ΔflcC mutants produced shorter and irregular periplasmic flagella (Fig. 1G). We have also observed shorter and/or irregular flagella in our previously published ΔflbB, Δbb0236, and ΔflcA collar mutants and in the ΔfilC mutant (20–22, 27). Furthermore, some of the ΔmotB mutant’s periplasmic flagella are shorter than those of wild-type cells (4). While we do not fully understand why these mutants are more likely to have shorter flagella, we speculate that the assembly of periplasmic flagella is a highly coordinated process; therefore, any defects in the motor proteins may have a profound impact on overall morphology and motility.

One of the most remarkable features of *B. burgdorferi* is that multiple flagellar motors are embedded in the inner membrane cylinder in a highly organized pattern (Fig. 6A). As a result, the cell cylinder diameter varies remarkably, ranging from ~100 nm to ~300 nm (Fig. 6A). The collar must therefore be highly flexible to accommodate variable membrane curvatures (Fig. 6B and C; see also Movie S2 in the supplemental material). Indeed, our studies have clearly demonstrated that the collar has a highly modular architecture due to the highly coordinated assembly of multiple spirochete-specific proteins (including several transmembrane proteins). This modular architecture may be of key importance for facilitating the remarkable plasticity of the collar. Moreover, multiple collar proteins directly interact with the stator complexes. Therefore, the unique plasticity of the collar also facilitates the recruitment and stabilization of maximal numbers of stator proteins.
complexes around the motor even in highly curved membrane environments (Fig. 6B and C and Movie S2). That the entire flagellar motor remodels and adapts to accommodate variable membrane environments is crucial to generate the highest torques required to constantly drive the motility of spirochetes and benefit their distinct lifestyle.

In summary, we have identified and characterized multiple novel collar proteins in *B. burgdorferi*, providing a molecular basis for understanding the remarkable structural plasticity of this multiprotein spirochetal complex. The collar not only enables the assembly of the motor in the curved membrane of spirochetes but also provides a structural scaffold for stator recruitment and stabilization, both essential for the function of periplasmic flagella and motility in spirochetes. Identification of additional collar proteins based on the protein-protein interactions, along with high-resolution structural analyses, will provide further insights into how the structural plasticity of the collar is required for motility of spirochetes.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** High-passage *B. burgdorferi* strain B31-A was used as the wild-type (WT) clone throughout this study (28, 29). The \( \Delta \)bb0058 (ΔflcB) and \( \Delta \)bb0624 (ΔflcC) mutants and complementants \( \Delta \)bb0058 \( \Delta \)flcB strains were constructed as described below. \( \Delta \)bb0058 cells were cultivated in liquid Barbour-Stoenner-Kelly (BSK-II) broth or agarose plates and incubated at 35°C in a 2.5% CO\(_2\) incubator, as reported previously (30, 31). Antibiotics, when required, were included in the *B. burgdorferi* medium at the following concentrations: 200 \( \mu \)g/ml kanamycin and/or 100 \( \mu \)g/ml streptomycin. *Escherichia coli* cell strains were grown at room temperature or 37°C in LB broth or plated on LB agar (32, 33). When required, 100 \( \mu \)g/ml ampicillin, 100 \( \mu \)g/ml spectinomycin, 0.2% glucose, 80 \( \mu \)g/ml 5-bromo-4-choro-3-indoly-\( \beta \)-D-galactopyranoside (X-Gal), and/or 0.5 mM isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG) were added to LB medium as supplements.

**Bioinformatics.** Basic local alignment search tool (BLAST) (34, 35) was utilized to determine protein homologs from the sequence database. The lower the E-value (lower than 0), the more significant the score is. Signal peptides were predicted using SignalP 5.0 and Phobius programs (36–38). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42). Basic local alignment search tool (BLAST) (34, 35) was utilized to determine protein homologs from the sequence database. The lower the E-value (lower than 0), the more significant the score is. Signal peptides were predicted using SignalP 5.0 and Phobius programs (36–38). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42). Protein domains were analyzed using the Conserved Domain Database (39, 40) and Pfam (41, 42).

**Overexpression of recombinant proteins in *E. coli.* To express the *B. burgdorferi* BB0058 (FlcB) protein in *E. coli*, a DNA fragment harboring the BB0058 open reading frame (ORF) without the signal peptide region (1 to 20 amino acids [aa]) was PCR amplified from chromosomal DNA of *B. burgdorferi* B31-A cells using primers PF MBP0058_BamHI (CGTCGACGGATCCGATACTACAGCATTAGGACATTATC) and PR MBP0058_PstI (TTAATTCCTCGAGTTATCTTCTTTTAAGCAAGTAGGCTCG) (restriction sites are underlined) and cloned into the pMAL c5x (NEB Inc.) using BamHI and PstI restriction sites to produce the maltose-binding protein (MBP)-BB0058 protein. MBP-MotB was similarly generated. In brief, the coding sequence of MBP from pMAL c5x was fused to the 3′ end of the coding region of MotB without its transmembrane domain, aa 1 to 44 using PCR, and then cloned into pET28a (+) (Novagen Inc.). Similarly, 1-FLAG (DYKDDDDK)-tagged BB0624 (FlcC) and FlcA (BB0326) were constructed for affinity blotting. In brief, 1-FLAG tag coding sequence (GACTCAAAGCAGCTAGCAGAAG) was fused to the coding regions of BB0624 without the signal peptide region, aa 1 to 22 and the C terminus aa 360 to 931 of BB0624 using PCR amplification with primers PF MBP0624_BamHI (CGTCGACGGATCCAGGTCATCATCTTTTGCTAGGCTCCTGGGCGATTCATAGATGACATATGATTGATTTTTC) and PR MBP0624FLAG_PstI (TTAATTCCTCGAGTTATCTTCTTTTAAGCAAGTAGGCTCG) (restriction sites are underlined), respectively, which were then cloned into pMAL c5x (NEB Inc.). Expression of MBP, MBP-MCP5, -FlbB, -BB0236, -FlcA and FLAG-MotB, -FlbB, -BB0236, and -Flc were described elsewhere (20–22).

All *E. coli* strains were induced with 0.5 mM IPTG at room temperature, and purifications of recombinant proteins were performed using amylase resin for MBP-tagged proteins and HisPure Ni-nitriloacetic acid (NTA) resin for His-tagged proteins.

**SDS-PAGE, immunoblotting, and affinity blotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (31, 43, 44). Exponentially growing *B. burgdorferi* cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in the same buffer to process the preparation of cell lysate for SDS-PAGE. Immunoblotting with *B. burgdorferi* FlbA-, MotB-, FlbB-, FlgI-, and DnaK-specific antibodies (6, 21, 27, 45–47) was performed using Pierce enhanced chemiluminescence Western blotting substrate (Thermo Fisher Scientific). Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard. Unless specified, approximately 10 \( \mu \)g of cell lysates were subjected to SDS-PAGE.

Far Western or affinity blot assays were performed as described previously (20–22, 48, 49). In brief, 1 \( \mu \)g purified recombinant proteins was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in the blocking solution (5% skim milk, 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 then incubated with purified 1-FLAG-tagged protein at a 2-\( \mu \)g/ml concentration 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 then probed with monoclonal anti-FLAG M2 antibody (Sigma-Aldrich Co. LLC).
Construction of the bb0058 and bb0624 mutants and bb0058 complemented strain. Construction of the bb0058 (reb) and bb0624 (reb) inactivation plasmid, electroporation, and plating of B. burgdorferi were performed as described earlier (31, 43, 50, 51). bb0058 and bb0624 were inactivated individually using a promoterless kanamycin resistance cassette (PI-Kan), as reported in detail (50). The Δbb0058 mutant strain was complemented in cis by chromosomal integration using the pXLF14301 suicide vector as described previously (26, 52). In brief, the native promoter regions of the were performed as described earlier (31, 43, 50, 51).

The asymmetric reconstructed motor structure, using the representative reconstructions from WT and bbb0624 corresponding struct shell correlation (FSC) coefficients. The stator complex were considered as having no assembled stator units. The number of collar subunits complex were considered as having assembled stator units, while the class averages without density of the stator complex were considered as having no assembled stator units. The number of collar subunits within the intergenic region of bbb0045 and bbb0046. Multiple attempts to complement the Δbb00624 mutant in cis or in trans were unsuccessful, as it is well known that genetic manipulations in B. burgdorferi are challenging (21, 57–64).

Dark-field microscopy and swarm plate assays. Exponentially growing B. burgdorferi cells were observed using a Zeiss Axio Imager M1 dark-field microscope connected to an AxioCam digital camera to determine bacterial morphology, as described previously (30, 65). Swarm plate motility assay was also performed using our established protocol (30).

Cryo-ET data collection and tomogram reconstruction. Frozen-hydrated specimens were prepared as described previously (17). In brief, various clones of exponentially growing B. burgdorferi cells were centrifuged individually at 5,000 × g for ~5 min, and the resulting pellets were suspended in PBS to achieve a cell concentration of ~1 × 10^7/mL. After adding 10-nm gold marker solution, 5 µL of the cell suspension was placed on freshly glow-discharged (for ~25 s) holey carbon grids (Quantifoil Cu R2/1, 200 mesh). The grids were front blotted with Whatman filter paper and rapidly frozen in liquid ethane, using a homemade plunging apparatus as described previously (17). The grids were then imaged using a 300-kV electron microscope (Titan Krios; Thermo Fisher Scientific) equipped with a field emission gun, a Volta phase plate (VPP), and a post-Gatan imaging filter (GIF) direct electron detector (Gatan K2 Summit or K3 Summit). Serial EM was used to collect all tilt series (66). The defocus was set as close to 0 µm as possible for those tilt series collected with VPP, while the defocus was set ca. ~3 µm for those collected without VPP. A total dose of ~80 e^−/Å^2 is distributed among 35 (or 33) tilt images covering angles from ~−50° to 51° (or from −48° to 48°) with a tilt step of 3°.

All recorded images were first motion corrected using MotionCorr2 (67) and then stacked and aligned by IMOD (68). For the data collected with VPP, the aligned tilt series were directly used to reconstruct tomograms by weighted back-projection using IMOD or by simultaneous iterative reconstruction technique (SIRT) reconstruction using Tomo3D (69). For the data collected without VPP, Gctf (70) was used to determine the defocus of each tilt image in the aligned stacks, and the “ctfphaseflip” function in IMOD was used to do the contrast transfer function (CTF) correction for the tilt images. The tomograms were then reconstructed using IMOD or Tomo3D. The number of tomograms used in this work for each strain is shown in Table S1 in the supplemental material.

Subtomogram analysis. Bacterial flagellar motors were manually picked from the 6 × binned tomograms. The subtomograms of flagellar motors were analyzed by I3 software package (71, 72). Afterwards, the subtomograms were extracted from unbinned tomograms with the refined positions and further binned by 2 or 4 based on the requirement for alignment and classification.

(i) Focused refinement of collar region. Each flagellar motor has 16 collar subunits. After alignment for the whole motor structure, the regions around 16 collar subunits were first extracted from each motor, and then we refined the 3D alignment and applied 3D classification based on the density of the collar subunit to remove particles with bad contrast or large distortions to obtain the refined structures. The number of flagellar motor and collar subunits used for subtomogram averaging is shown in Table S1.

(ii) Measurement of stator occupancy. For the WT, ΔrebB, and ΔrebC motors, we first performed focused refinement to the collar region as described previously. Then 3D classification was applied to all collar subunits based on the density of the stator complex. The class averages with density of the stator complex were considered as having assembled stator units, while the class averages without density of the stator complex were considered as having no assembled stator units. The number of collar subunits with stator units was divided by the total number of collar subunits to calculate stator occupancy. Fourier shell correlation (FSC) coefficients were calculated by generating the correlation between two randomly divided halves of the aligned images used to estimate the resolution and to generate the final maps.

Three-dimensional visualization. UCSF Chimera (73) and ChimeraX (74) were used for 3D visualization and surface rendering of subtomogram averages of the whole motor or collar subunit. For the 3D surface views of the whole collar complex shown in Fig. 4 and 5, the surface view of each collar protein in the ΔmotB or WT strain was first segmented by ChimeraX and then fitted to the collar complex of the asymmetric reconstructed motor structure, using the “fitmap” command in ChimeraX. Segments of representative reconstructions from WT and Δreb cell tips were manually constructed using IMOD (68).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

MOVIE S1, MOV file, 2.8 MB.
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