Supplementary Information (SI)

Supplementary background on the bacterial flagellum

Architecture of the Flagellar Basal Body

The flagellar basal body is a membrane-spanning nanomachine that anchors the bacterial flagellum and converts electrochemical ion gradients into the high-speed rotation of an extracellular filament (Berg, 2003; Erhardt et al., 2010). High-resolution cryo-EM maps of intact Salmonella enterica basal bodies (2.2–3.7 Å) reveal a concentric, four-layer architecture comprising the LP-ring, rod, MS-ring, and C-ring, assembled from >170 protein chains of 13 different types (Johnson et al., 2021). The outermost LP-ring built from 26 copies each of FlgH (L-ring) and FlgI (P-ring) acts as a hydrophobic, lipid-anchored bushing embedded in the outer membrane and peptidoglycan, respectively; its invariant 26-fold symmetry correlates with discrete ∼13.8° stepping observed in partially energized motors, suggesting that the LP-ring provides a static interaction potential for rotation (Johnson et al., 2021). Nested inside the LP-ring, the axial rod serves as a rigid drive shaft assembled helically from FliE, FlgB, FlgC, FlgF, and three conformers of FlgG and is nucleated by the type III export gate whose FliP–FliQ–FliR core seeds ten protofilaments while FliR seeds an eleventh, thereby propagating a 5.5-subunit-per-turn lattice (Johnson et al., 2021).

At the inner membrane, the 34-mer MS-ring, composed solely of FliF, forms stacked RBM2 and RBM3 domains that both couple the export gate below and interface with the C-ring above while accommodating symmetry mismatch through local RBM2 deformation (Johnson et al., 2021). The cytoplasmic C-ring built from variable copies of FliG, FliM, and FliN functions as the rotor switch complex its FliG C-terminal ARM repeats dock MotA stator loops, transducing proton or sodium flux into torque (Deme et al., 2020; Santiveri et al., 2020). The resulting concentric symmetry mismatch (C₂₆ LP-ring: C₃₄ MS-ring: C₃²–₃⁶ C-ring) prevents gear-locking and minimizes friction while maintaining axial alignment (Johnson et al., 2021). Finally, a pentameric FlgD cap atop the distal rod operates as a stepped "revolver" that sequentially exposes binding sites for hook subunits, ensuring helical continuity between the rod and filament (Johnson et al., 2021).

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Torque generation

FliG constitutes the cytoplasmic C-ring, the central hub that interfaces with the MS-ring and stator complexes to generate bidirectional torque (Beeby et al., 2020). Its tripartite domain architecture enables this function. The FliG-N domain co-folds with the C-terminus of FliF via a split ARM-like motif, creating an interlocked rotor chassis with strict 1:1 stoichiometry. This interaction nucleates the C-ring assembly and provides membrane anchorage for the rotor complex (Beeby et al., 2020). The middle domain, FliG-M, forms the structural core of the rotor barrel while simultaneously binding FliM to bridge torque generation with chemotactic signaling cascades (Brown, 2002; Lee et al., 2010). Critically, the FliG-C domain hosts the conserved "torque helix," a lysine/arginine-rich amphipathic α-helix that functions as the stator's primary lever arm. Electrostatic surfaces on this helix guide MotA binding, while steric clashes during stator activation drive rotational power strokes (Tan et al., 2024).

Evolutionary adaptations demonstrate how FliG architecture enables torque amplification. Species such as ε-proteobacteria widen the C-ring diameter or incorporate additional FliG copies (up to 45 subunits), increasing the lever radius to achieve 3–4-fold higher torque than enteric bacteria like E. coli (Beeby et al., 2020). Directional switching is orchestrated through allosteric control of FliG-C. Phosphorylated CheY (CheY-P) binding to FliM/FliN induces a 180° rotation in the FliG-C torque helix (Sarkar et al., 2010). This reorients electrostatic complementarity with MotA, flipping motor rotation from counterclockwise (CCW) to clockwise (CW) in <1 ms (Figure 1), a switch critical for chemotactic reorientation (Yuan & Berg, 2008).

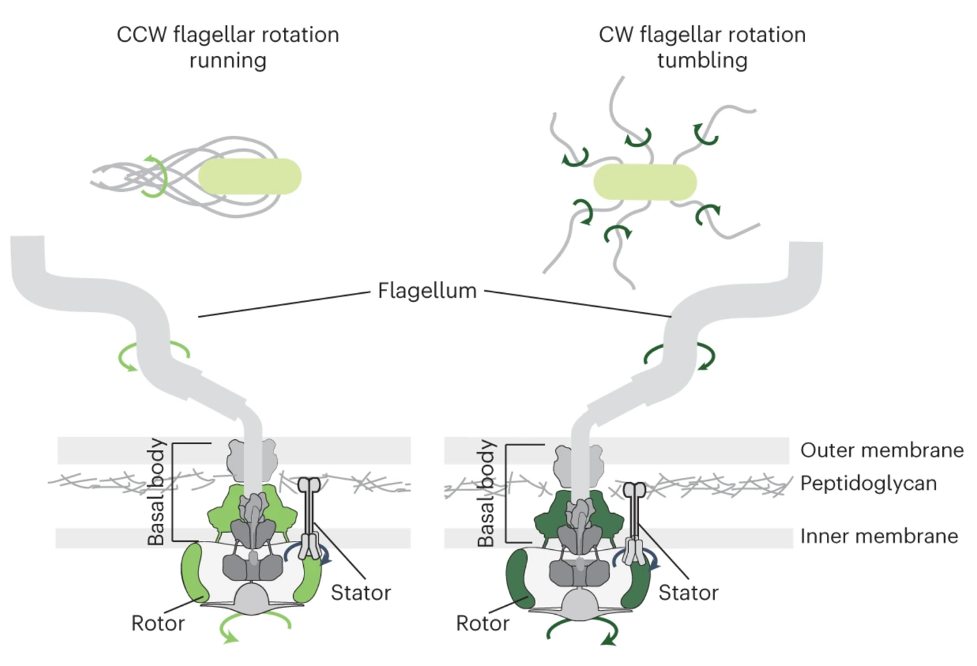


Figure 1: A structural overview of bacterial flagellum illustrating counterwise (CCW) rotation associated with smooth swimming and clockwise (CW) rotation associated with tumbling, highlights the primary structural components: the filament, hook, basal body, rotor (MS-ring and C-ring), and stator units (MotA–MotB complexes). The basal body spans the bacterial inner membrane, peptidoglycan layer, and outer membrane, facilitating torque transmission and directional switching (Johnson et al., 2024).

The rotor is surrounded by the stator layer, formed by multiple MotA₅MotB₂ complexes (Rieu et al., 2022; Tan et al., 2024; Yamaguchi et al., 2021). Each MotB dimer anchors to peptidoglycan via its OmpA-like periplasmic domain, while MotA pentamers create annular proton channels (Santiveri et al., 2020). Protonation of MotB-Asp32 induces a "kink-and-swivel" in adjacent MotA transmembrane helices (Nishihara & Kitao, 2015); conformational cycling delivers sequential electrostatic pushes to FliG-C torque helices, advancing the rotor in discrete 16° steps (Tan et al., 2024). High-torque motors (e.g., Paenibacillus) enhance power via continuous stator rings with additional MotA₅MotB₂ units, preserving the core FliF–FliG architecture (Rieu et al., 2022).

Despite divergent roles, their structural homology suggests an ancient evolutionary link. Comparative analyses reveal conserved α-helical folds in their cytosolic domains (Beeby et al., 2020), hinting at a shared ancestral protein. FliG directly generates rotational torque, while MgtE mediates Mg²⁺ uptake critical for cellular homeostasis and flagellar assembly.

**Supplementary information for FliG**

Conformational Cycling and Directional Switching

Directional switching is achieved through rapid allosteric remodeling of the C-ring triggered by chemotactic signals. Binding of phosphorylated CheY to FliM induces a pronounced expansion of the cylindrical C-ring and outward tilting of the torque helix, thereby inverting its electrostatic contacts with MotA and reversing rotational bias (Chang et al., 2020; Sarkar et al., 2010). Cryo-electron tomography and subtomogram averaging indicate that these conformational changes occur on sub-millisecond timescales, enabling swift motor reversals essential for chemotaxis (Johnson et al., 2024). Mutational studies further confirm that stabilizing hinge conformations in FliG locks the motor in a single rotational state, underscoring the mechanical basis of directional control (Guo & Liu, 2022). This mechanism highlights how dynamic remodeling of the amphipathic helix and C-ring architecture integrates chemotactic inputs to mediate bidirectional switching with high temporal precision (Minamino & Imada, 2015).

Evolutionary Adaptations for High-Torque Systems

FliG's modular domain architecture is readily re-tuned to meet species-specific biomechanical demands. Cryo-EM analysis of the thermophile Paenibacillus sp. TCA20 resolves a 45-subunit C-ring whose radially expanded FliG-M barrel increases the lever arm radius by ~40%, directly amplifying torque output (Onoe et al., 2025). Parallel adaptations in Campylobacter jejuni include charge-reinforcing substitutions at the FliG-C-MotA interface (e.g., Arg281→Lys in torque helix) and a widened periplasmic scaffold that templates a broader stator ring (Beeby et al., 2020). Earlier cryo-electron tomography across ε-proteobacteria confirmed that such FliG ring expansions are accompanied by proportionally denser stator belts (13–17 MotA₅MotB₂ units vs. 8–11 in E. coli), providing additional torque transfer points (Beeby et al., 2016). Critically, these lineage-specific optimizations occur while preserving the ancestral FliF–FliG-N anchor, highlighting FliG as an evolvable torque amplifier within a universally conserved structural framework (Johnson et al., 2024).

**Supplementary information on MgtE**

Roles in cellular magnesium homeostasis

Intracellular magnesium (Mg²⁺) concentrations are tightly regulated within a physiological range (0.5–2 mM) to sustain essential processes such as ATP hydrolysis, ribosomal function, and nucleic acid stability. The MgtE protein is central to this homeostasis, functioning as a Mg²⁺-selective channel, not a transporter or ATPase that gates open during intracellular Mg²⁺ depletion and closes upon repletion. This rapid, direct Mg²-sensing mechanism establishes a negative feedback loop for ionic control (Hattori et al., 2009). Structural studies reveal that Mg²⁺ binding at cytosolic sites (including an N-terminal ARM fold and tandem CBS domains) locks connecting helices, stabilizing a closed conformation and occluding the pore. Conversely, low Mg²⁺ disassembles these interdomain bridges, enabling pore opening for Mg²⁺ influx (Hattori et al., 2007, 2009). This allosteric gating ensures specificity for Mg²⁺ over similar divalent cations (e.g., Ni²⁺, Co²⁺).

Transcriptional regulation further refines Mg²⁺ homeostasis. In Bacillus subtilis, the Mg²⁺-Mg²-responsive M-box riboswitch suppresses MgtE mRNA expression when cytosolic Mg²⁺ exceeds ~1.5 mM. This dual-layer control fast channel gating and slower transcriptional modulation optimizes (Moomaw & Maguire, 2008) MgtE abundance and activity to cellular Mg²⁺ demands. Phenotypic evidence confirms MgtE’s physiological necessity, B. subtilis and E. coli MgtE mutants exhibit biphasic growth defects, showing hypersensitivity below 0.1 mM Mg²⁺ and toxic accumulation (>10 mM) in Mg²⁺-rich environments (>100 mM), reflecting impaired uptake/efflux coordination (Moomaw & Maguire, 2008).

Evolutionarily conserved across domains, mammalian MgtE homologs (SLC41 family) retain core transport functions despite structural divergence. SLC41A1, the best-characterized member, shares membrane topology with bacterial MgtE but lacks its cytosolic Mg²⁺-sensing domains (Kolisek et al., 2008). While it rescues Mg²⁺ uptake in transporter-deficient Salmonella, SLC41A1 primarily mediates Mg²⁺ efflux in mammalian cells, indicating functional adaptation. Its expression is upregulated in the kidney, heart, and colon during dietary Mg²⁺ deficiency, confirming conserved homeostatic roles (Kolisek et al., 2008).

Significance for molecular evolution

The relationship between FliG and MgtE is an example of modular protein evolution and exaptation. It exemplifies how complex molecular machines can evolve from scratch by repurposing existing structural elements (Beeby et al., 2020; Frenkel & Rodriguez, 1982). In this case, the ARM-repeat domain of MgtE likely served as an ancestral template. Through duplication and loss of membrane-spanning or ion-binding functions, this domain was co-opted into a new mechanical role within the flagellar motor, giving rise to the modern FliG protein (Snyder et al., 2009).

This structural repurposing, or tinkering, is consistent with the modular logic of molecular evolution, where domains are fused, lost, or reconfigured to meet new functional demands (Beeby et al., 2020). The evolution of FliG from a MgtE-like ancestor also directly counters arguments of “irreducible complexity” often invoked to challenge the evolutionary origin of flagella (Blair & Hughes, 2012). FliG’s homolog in a functionally unrelated transporter demonstrates that the flagellar motor’s core components could have evolved by gradually modifying pre-existing parts.

Orthologs, Paralogs, and Evolutionary Specialization

Molecular evolution distinguishes between orthologs (genes diverging after speciation that typically retain ancestral functions) and paralogs (genes diverging after duplication within a genome that often undergo functional divergence) (Koonin, 2005). The flagellar C-ring exemplifies paralog-driven complexity. FliM and FliN have established paralogs arising from domain shuffling events. Phylogenetic and structural evidence indicates FliM emerged through the fusion of an ancestral CheC-like phosphatase domain with a FliN-type SPOA (surface presentation of antigens) domain, incorporating into the flagellum after the bacterial root divergence (Beeby et al., 2020).

In contrast, FliG represents a distinct evolutionary lineage sharing only deep homology with MgtE through an ancient ARM-repeat ancestor, not paralogy with FliM/N (Lynch et al., 2017). This shared scaffold underwent radical functional specialization; for FliG, evolutionary pressures optimized the ARM repeat for rotary torque transmission, using its superhelical geometry for stator coupling and bidirectional switching (Beeby et al., 2020). In MgtE, the same fold was repurposed as a static Mg²⁺-sensing clamp, rigidifying to allosterically control pore occlusion via CBS domain engagement (Jin et al., 2021).

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