

# Chemotaxis signaling protein CheY binds to the rotor protein FliN to control the direction of flagellar rotation in *Escherichia coli*

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The direction of rotation of the *Escherichia coli* flagellum is controlled by an assembly called the switch complex formed from multiple subunits of the proteins FliG, FliM, and FliN. Structurally, the switch complex corresponds to a drum-shaped feature at the bottom of the basal body, termed the C-ring. Stimulus-regulated reversals in flagellar motor rotation are the basis for directed movement such as chemotaxis. In *E. coli*, the motors turn counterclockwise (CCW) in their default state, allowing the several filaments on a cell to join together in a bundle and propel the cell smoothly forward. In response to the chemotaxis signaling molecule phospho-CheY (CheY<sup>P</sup>), the motors can switch to clockwise (CW) rotation, causing dissociation of the filament bundle and reorientation of the cell. CheY<sup>P</sup> has previously been shown to bind to a conserved segment near the N terminus of FliM. Here, we show that this interaction serves to capture CheY<sup>P</sup> and that the switch to CW rotation involves the subsequent interaction of CheY<sup>P</sup> with FliN. FliN is located at the bottom of the C-ring, in close association with the C-terminal domain of FliM (FliM<sub>C</sub>), and the switch to CW rotation has been shown to involve relative movement of FliN and FliM<sub>C</sub>. Using a recently developed structural model for the FliN/FliM<sub>C</sub> array, and the CheY<sup>P</sup>-binding site here identified on FliN, we propose a mechanism by which CheY<sup>P</sup> binding could induce the conformational switch to CW rotation.

switching | cell motility | signal transduction | molecular motors

Many motile bacteria control the direction of their swimming by regulating the sense of flagellar rotation in response to sensory cues. In the well-studied enteric species *Escherichia coli* and *Salmonella* (*Salmonella enterica* serovar *typhimurium*), counterclockwise (CCW) rotation allows the several flagellar filaments on a cell to join in a bundle to propel the cell smoothly, whereas clockwise (CW) rotation of one or more flagella disrupts the bundle and causes the cell to tumble. In the absence of external stimuli, a cell executes smooth runs of about a second punctuated by brief tumbles that send it in a new, essentially random, direction; by delaying the switch to CW rotation in response to attractant stimuli, cells prolong runs that happen to be in a favorable direction and so bias their movement toward nutrients, temperatures, or other factors conducive to survival (1, 2).

The direction of flagellar rotation is regulated by a complex at the bottom of the basal body called the switch complex, constructed from the proteins FliG, FliM, and FliN (3). This assembly contains many copies of each protein—about 25 FliG, 35 FliM, and 140 FliN—and is therefore fairly large, having a total mass of about 4 MDa (4–8). In addition to controlling the sense of motor rotation, the switch complex is also essential for flagellar assembly and the generation of torque (3, 9).

Electron microscopic reconstructions in *Salmonella* have produced images of the flagellar basal body at resolution sufficient to reveal features the size of protein domains (Fig. 1) (10, 11). The switch complex corresponds to a fairly large (≈50 nm in diameter) drum-shaped feature at the bottom of the basal body, termed the C-ring for its location in the cytosol. In our current structural model of the C-ring (12–17), FliG is located at the top

where it accounts for two distinct lobes of electron density seen in the reconstructions (Fig. 1A). FliG is the rotor protein most closely involved in generation of torque, and its C-terminal domain, which we assign to the outer lobe, has been shown to interact with the stator protein MotA (18). The main body of the FliM protein is just below FliG where it forms the relatively thin side-wall of the ring (14), and FliN is at the bottom, organized as an array of ring-shaped tetramers (Fig. 1B and C) (17, 19). FliM contains a discrete C-terminal domain (FliM<sub>C</sub>) that is inserted between the FliN tetramers to form the lower part of the C-ring. Targeted cross-linking experiments showed that the switch from CCW to CW rotation is accompanied by a relative movement of FliN and FliM<sub>C</sub> (15).

In the cell, flagellar reversals are controlled by the signaling molecule phospho-CheY (CheY<sup>P</sup>), which promotes CW rotation (20). CheY<sup>P</sup> binds to a fairly well-conserved segment near the N terminus of FliM (sequence LSQAEIDALL, in the *E. coli* protein) (21, 22). A crystal structure showed this FliM segment folded into helical conformation and bound to CheY in a shallow cleft on a face nearly opposite the site of phosphorylation (23). Phosphorylation of CheY is believed to promote binding to the FliM N-terminal segment indirectly via a propagated conformational change (24–26). A recent NMR study gave evidence that CheY<sup>P</sup>, when bound to the N-terminal segment of FliM, can also interact with the FliM middle domain (FliM<sub>M</sub>) (27). Last, on the basis of mutational results, we proposed that CheY<sup>P</sup> might also interact with FliN in the vicinity of a strongly conserved hydrophobic patch (28). Mutations on this surface of FliN caused strongly CCW-biased motor rotation that could be offset, in most cases, by overexpression of CheY. The hypothesized binding of CheY<sup>P</sup> to FliN has not been confirmed directly.

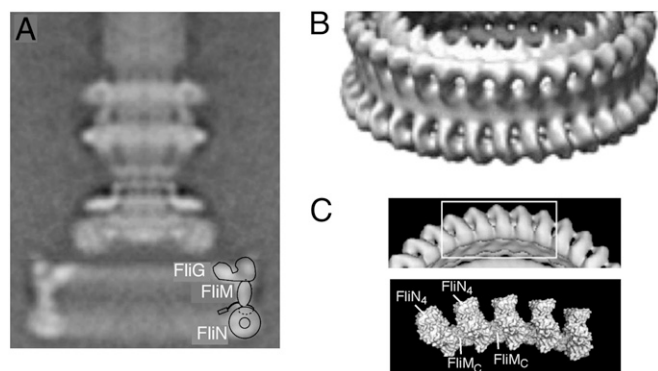
Whatever the binding target(s) for CheY<sup>P</sup>, switching must presumably involve movement of the C-terminal domain of FliG, which forms the interface with the stator, where the forces for motor rotation are produced or applied (18, 29). Thus, conformational changes occurring in the lower part of the C-ring, whether they originate primarily in FliN or FliM, must be transmitted “up” through FliM<sub>M</sub> to induce movement of FliG<sub>C</sub>. The C-terminal domain of FliG is linked to the rest of the protein by a segment containing a well-conserved Gly–Gly residue pair that could confer flexibility to allow switch-driven movement of FliG<sub>C</sub> (16). A mutational analysis showed that certain replacements in this segment confer a “rusty hinge” phenotype in which CCW–CW switching is slowed (30). Viable models for switching must also account for a very high degree of cooperativity; the transition to CW rotation depends very sharply on CheY<sup>P</sup> concentration, exhibiting a Hill coefficient greater than 10 (31). This cooperativity should enhance the responsiveness of the motor to small changes

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**Fig. 1.** Electron microscopic images of the flagellar basal body, from studies in *Salmonella* (10). (A) Single-particle reconstruction of the full basal body, including the LP-ring and a short segment of the rod. The structure is viewed from the side and has been axially averaged. The M5-ring is at the level of the cytoplasmic membrane; the C-ring is in the cytosol. The diameter of the C-ring is about 50 nm. A current working hypothesis for the locations of FliG, FliM, and FliN (12–17) is shown at the right of the C-ring. The two lobes of density at the top of the C-ring are both assigned to FliG, with the outer one corresponding to the C-terminal domain. [In a more fully detailed model, some of the FliM subunits are hypothesized to tilt inward to interact with the inner domain of FliG (13), but this feature is not important in the present context.] The inward-pointing extension on FliM represents the N-terminal segment that is known to interact with CheY<sup>P</sup>. (B) Detail from a higher-resolution reconstruction (11), showing rings of density at the bottom of the C-ring. [Reproduced with permission from Thomas D, DeRosier DJ (2001) (Copyright 2010, American Society for Microbiology).] (C) The appearance of the bottom of the C-ring as determined in the high-resolution reconstruction and the organization of FliN tetramers and FliM<sub>C</sub> domains at the bottom of the C-ring as deduced from cross-linking and mutational studies (15).

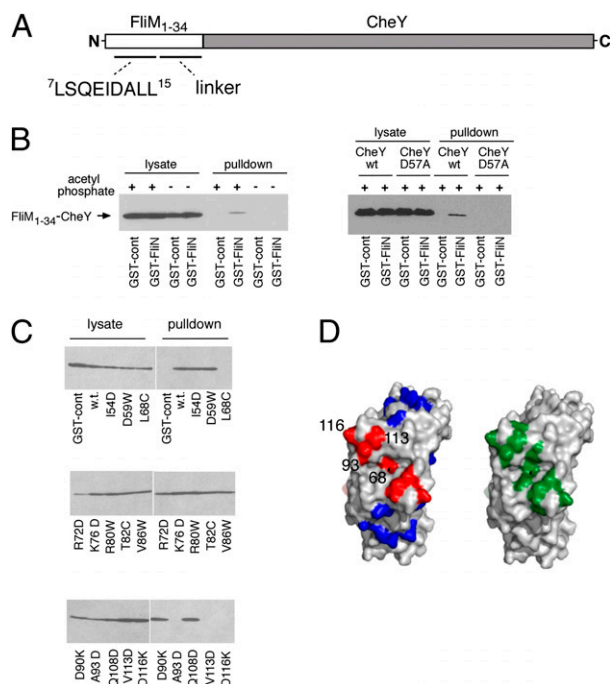
in cellular CheY<sup>P</sup> concentration and thereby increase the sensitivity of the chemotactic response.

The present study addresses the question of whether CheY<sup>P</sup> interacts with the rotor protein FliN and whether such an interaction has a role in flagellar direction switching. Using pull-down assays, we show that CheY<sup>P</sup> can bind to FliN, but this binding occurs only when CheY<sup>P</sup> is occupied by the N-terminal segment of FliM. Thus, in the normal setting, CheY<sup>P</sup> is expected to interact with FliN after first being captured by FliM. The binding determinants on both sides of the CheY–FliN interaction were mapped using mutations. FliN mutations that weakened the binding of CheY<sup>P</sup> were the same as those found previously to cause CCW motor bias (28), indicating that the CheY<sup>P</sup>–FliN interaction is important for motor direction reversal. The relative movement of FliN and FliM<sub>C</sub> that accompanies switching (15) is predicted to make the CheY-binding site on FliN more accessible in the CW than in the CCW state, providing a simple structural basis for coupling the binding of CheY<sup>P</sup> to a conformational change in the switch complex.

## Results

**CheY Interaction with FliN.** The switch from CCW to CW rotation is triggered by binding of the signaling protein CheY<sup>P</sup> to the motor. Although the FliM protein of the rotor has usually been considered the main player in switching, a mutational analysis gave evidence that FliN might also play a role, and identified a conserved hydrophobic patch as a probable target of action of CheY<sup>P</sup> (28). We looked for a CheY–FliN interaction using pull-down assays with a GST–CheY fusion construct but saw no evidence of binding either in the absence or presence of acetyl phosphate, a CheY-phosphorylating agent. In our current working model of the switch complex, the N-terminal segment of FliM that is known to bind CheY<sup>P</sup> lies near FliN. Accordingly, we hypothesized that CheY<sup>P</sup>

might bind first to this FliM segment and thereby gain binding determinants needed for interaction with FliN. This proposal was tested using a hybrid construct containing 34 residues from the N terminus of FliM fused to the N terminus of CheY (termed M<sub>34</sub>-Y). This construct contains the conserved CheY-binding segment of FliM as well as a less-conserved segment of about 15 residues that is predicted to have nonregular secondary structure and to function as a linker (Fig. 2A). Upon phosphorylation, the CheY part of this construct should be able to capture the FliM segment and so acquire binding determinants resembling those of FliM-bound CheY<sup>P</sup>. The binding of this fusion construct to FliN was tested using pull-down assays. Cells expressing the M<sub>34</sub>-Y fusion protein were mixed with cells expressing GST–FliN, lysed, and mixed with glutathione-Sepharose beads. Beads were washed and treated with glutathione to release GST–FliN and associated proteins, and samples were analyzed on immunoblots using a polyclonal anti-FliM antiserum that showed high sensitivity toward the FliM sequences present in the fusion protein. A binding interaction between the M<sub>34</sub>-Y construct and FliN was readily observed in the pull-down experiment, in the presence but not in absence of acetyl phosphate (Fig. 2B Left). Acetyl phosphate is believed to transfer its phosphoryl group to the physiologically relevant residue Asp-57 of CheY in a reaction facilitated by a nearby protein-bound Mg<sup>2+</sup> ion, and is in this sense specific (32). Conceivably, however, acetyl phosphate might react with other positions in the M<sub>34</sub>-Y construct to induce FliN binding by a mechanism that does not involve Asp-57. To substantiate the role of Asp-57, we carried out the same experiment but using a mutant protein with Asp-57



**Fig. 2.** (A) Schematic of the FliM<sub>1–34</sub>–CheY fusion construct. The part of the FliM sequence shown explicitly is well conserved across species and is known to bind to CheY<sup>P</sup> in helical conformation (23). (B Left) Pull-down assay with GST–FliN and the FliM<sub>1–34</sub>–CheY construct, in presence or absence of the phosphorylating agent acetyl phosphate. (B Right) Effect of the CheY mutation D57A. (C) Binding of the FliM<sub>1–34</sub>–CheY construct to FliN proteins with mutations in various surface positions. Acetyl phosphate was present in all samples. (D) Comparison of the CheY<sup>P</sup>-binding region on FliN with positions of previously characterized CCW-biased mutations. (Left) Results of the binding experiment (C) mapped onto the FliN structure (PDB ID code 1yab). Red, positions where mutations eliminated the binding; blue, positions where mutations did not affect binding. (Right) Positions of mutations in FliN that gave CCW motor bias, colored green (data from ref. 27; image made in PyMol).

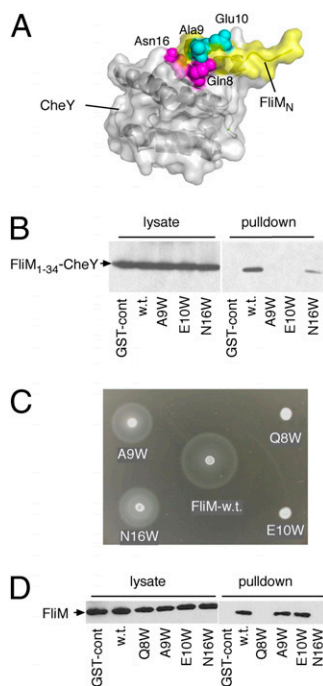
replaced by alanine. The D57A mutant protein did not bind to FliN, either in the presence or absence of acetyl phosphate (Fig. 2*B Right*).

**Mapping Regions Important for the Interaction.** To map the CheY-binding determinants on FliN, the pull-down experiment was repeated using GST-FliN proteins with nonconservative mutations at various surface positions. Of 14 FliN mutations studied, 10 showed no measurable reduction in binding. Binding to the M<sub>34</sub>-Y protein was eliminated by mutations in FliN residues Leu-68, Ala-93, Val-113, and Asp-116 (Fig. 2*C*). Mutations that weakened the binding lie in the vicinity of a conserved surface hydrophobic patch, and are the same as those shown previously to cause CCW motor bias (28) (Fig. 2*D*).

Because the FliM<sub>1-34</sub> segment was found necessary for the CheY-FliN interaction, we next tested whether this segment participates directly in the binding. In the crystal structure of a CheY-FliM segment complex (23), residues Ala-9 and Glu-10 of FliM are exposed on the surface where neither side-chain appears to contribute to the interaction with CheY (Fig. 3*A*). To determine whether these FliM residues are important for the interaction with FliN, they were individually replaced with the larger residue Trp, and binding was measured using the pull-down assay. Binding of the fusion construct to FliN was prevented by both mutations (Fig. 3*B*). To examine the effects of the A9W and E10W replacements in a more native setting, the same mutations were made in the full-length FliM protein, and function was measured using soft-agar plate assays. Chemotactic migration in soft agar was eliminated by the E10W replacement and reduced to about half of the wild-type

rate by the A9W replacement (Fig. 3*C*). Cells of the E10W mutant swam smoothly in liquid media, and the motors rotated exclusively CCW in a tethering experiment (in which filament stubs are attached to a coverslip to allow monitoring of motor rotation). To rule out an effect of the mutations on the interaction between the FliM segment and CheY, the FliM-CheY interaction was examined using a pull-down assay with GST-CheY. Binding of CheY to FliM in this assay depended on the presence of acetyl phosphate, as reported previously (33). In the pull-down experiment, the binding to CheY was not affected by the A9W or E10W mutations in FliM, consistent with the solvent-exposed positions of these residues in the crystal structure (23).

Two other Trp replacements (Q8W and N16W) were made in the FliM segment at more-buried positions that contact CheY and might stabilize the FliM-CheY<sup>P</sup> binding. Both of these mutations weakened the FliM-CheY<sup>P</sup> interaction significantly (Fig. 3*D*). In the soft-agar assay, chemotactic migration was eliminated by the Q8W mutation and decreased to about half of normal by the N16W mutation (Fig. 3*C*). Cells of the Q8W mutant swam smoothly in liquid media and exhibited only CCW motor rotation in a tethering experiment. The substantial function of the N16W mutant implies that this protein retains some ability to bind CheY<sup>P</sup> that could not be observed in the pull-down assay. This is not surprising, as the pull-down assay is fairly stringent in the sense that off-rates must be slow for binding to be observed. Consistent with the partial function of the N16W mutant, the M<sub>34</sub>-Y fusion construct containing this mutation also retained some ability to bind FliN in the pull-down experiment (where the FliM-CheY interaction can occur in *cis* within the M<sub>34</sub>-Y fusion; Fig. 3*B*).



**Fig. 3.** Effect of mutations in the segment of FliM that interacts with CheY. (A) Structure of the complex formed between CheY (light gray) and the FliM segment (yellow) (PDB ID code 1f4v) (23). The solvent-exposed residues Ala-9 and Glu-10 are colored cyan, and residues Gln-8 and Asn-16, which contribute to the FliM-CheY interface, are magenta (image made in PyMol) (B) Interaction of FliN with FliM<sub>1-34</sub>-CheY constructs with mutations in the FliM segment. The pull-down assay used GST-FliN and the FliM<sub>1-34</sub>-CheY fusion construct containing the mutations. (C) Effects of the FliM mutations on cell migration in soft agar. The Trp replacements were transferred into the full-length FliM protein for this experiment. (D) Effects of the FliM mutations on the interaction between the FliM segment and CheY, measured using a GST-CheY pull-down assay.

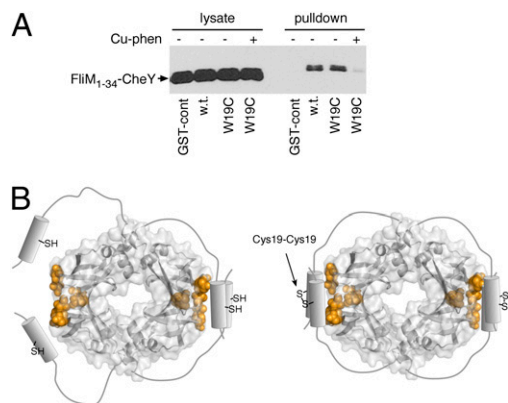
**Regulation of CheY Access by the N-Terminal Segment of FliN.** The function of the N-terminal part of FliN is uncertain, as this segment is not required for flagellar assembly and is to a large extent dispensable for motility and chemotaxis (34). A cross-linking study showed that in the FliN tetramer, the N-terminal segments of a FliN dimer interact with each other and with the hydrophobic patch of the other FliN dimer (19). Because CheY<sup>P</sup> also binds in the region of the hydrophobic patch, the N-terminal segments of FliN might be expected to prevent binding of CheY<sup>P</sup>. We hypothesized that these segments of FliN might regulate access of CheY<sup>P</sup>, possibly functioning to prevent CheY binding until the segments are displaced from the patch (upon installation of the FliN tetramer into the flagellum, for example). To test this, we introduced a Cys replacement at FliN residue 19, a position shown previously to allow efficient FliN-FliN cross-linking, and used Cuphenanthroline to induce disulfide bond formation. Binding of the M<sub>34</sub>-Y protein to the cross-linked FliN was tested using the pull-down assay. Binding of the M<sub>34</sub>-Y fusion protein was eliminated upon formation of the residue-19 cross-link in FliN (Fig. 4*A*).

## Discussion

Switch bias mutants were known to occur in FliN but at a low frequency relative to FliM or FliG (35, 36). This has been taken as evidence that FliN has a relatively small role in switching, but is also consistent with the protein having a crucial role—though one that involves comparatively few amino acid residues. The present findings show that FliN has a critical role in switching, interacting with CheY<sup>P</sup> to trigger or enable the switch to CW rotation. The interaction involves a conserved surface region on FliN where mutations were shown previously to cause CCW phenotype, and requires that CheY<sup>P</sup> be bound to the N-terminal segment of FliM. Accordingly, we propose that the N-terminal segment of FliM serves to capture CheY<sup>P</sup>, and that switching is triggered by the subsequent interaction of this FliM-bound CheY<sup>P</sup> with FliN.

Mutations in the N-terminal segment of FliM had effects suggesting that the segment contributes directly to the interaction with FliN; replacements on the exterior-facing part of the FliM segment permitted normal binding of the segment to CheY<sup>P</sup> (Fig. 3*D*)





**Fig. 4.** Effect of cross-linking through the N-terminal segment of FliN on the binding of FliN to the FliM<sub>1-34</sub>-CheY construct. Residue Trp-19 of FliN (in the GST-FliN fusion) was replaced with Cys. In the samples indicated, Cu-Tris [1,10-phenanthroline] was added to induce disulfide formation.

but weakened binding of the M<sub>34</sub>-Y fusion protein to FliN (Fig. 3B). In the context of full-length FliM, the E10W replacement eliminated chemotactic migration in soft-agar plates (Fig. 3C), and tethered cells displayed the fully CCW bias expected of motors unable to respond to CheY<sup>P</sup>. The retention of some chemotactic function in the A9W mutant might appear at odds with the pronounced defect in FliN binding, but note that the pull-down experiment involves binding between two separate proteins, whereas motor switching in the present scenario depends only on interaction between the FliM-tethered CheY and the FliN units nearby in the motor. Given the constrained nature of the CheY-FliN interaction, even a partial defect such as that in the A9W mutant might be taken to indicate a substantial weakening of the binding. In addition to Ala-9, the FliM residues Ala-13 and Leu-14 also lie on the exterior of the segment, and together with two nearby residues of CheY (Ile-96 and Ala-99) form a surface hydrophobic patch that might interact with the hydrophobic patch on FliN. In this scheme, the FliM segment would become sandwiched between CheY<sup>P</sup> and its target on the flagellar switch, a possibility first noted in the structural study of the CheY-FliM peptide complex (23). Further structural studies will be needed to define the FliN-CheY interaction in fuller detail.

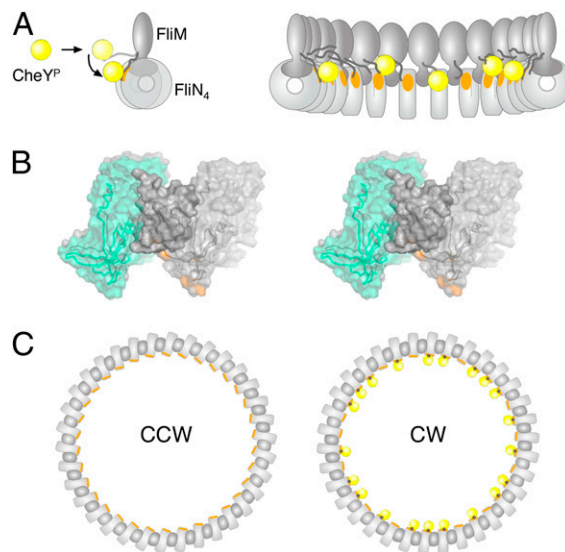
Because the N-terminal segment of FliN has been shown to lie near the hydrophobic patch, it might be expected to occlude the CheY-binding site (19) (Fig. 4B). Although this segment of FliN was present in the constructs used in pull-down experiments and did not prevent the CheY-FliN interaction, when the N-terminal segments were stably associated through a disulfide crosslink, the binding of CheY<sup>P</sup> was decreased. We suggest, therefore, that the N-terminal segment could function to modulate the binding of CheY<sup>P</sup> to FliN, possibly blocking the binding of CheY<sup>P</sup> to any FliN-FliM complexes not yet installed in motors. Once FliM-FliN<sub>4</sub> complexes are installed in the motor, the segment could be displaced to expose the site for interaction with CheY<sup>P</sup>.

The CheY<sup>P</sup>-binding site identified here overlaps closely with the binding site identified previously for FliH, a protein that functions in the export process needed for assembly of the exterior parts of the flagellum (28). The N-terminal segments of FliN might therefore serve also to prevent premature interactions with FliH. Further, if FliH and CheY<sup>P</sup> compete for overlapping binding sites on FliN, we might expect some interplay between flagellar assembly and switching.

The capture of CheY<sup>P</sup> through the initial FliM<sub>N</sub>-CheY interaction would, besides producing the determinants for binding FliN, also ensure that CheY<sup>P</sup> is present at a high “local concentration” in the vicinity of FliN. In a system such as the flagellar

switch that involves the cooperative action of many CheY<sup>P</sup> molecules, it might be particularly important to hold many copies of CheY<sup>P</sup> poised nearby. A local concentration effect appeared important in the CheY<sup>P</sup>-FliM<sub>M</sub> interaction reported in a recent NMR study (27). This interaction was seen most clearly when the N-terminal and middle parts of FliM were present in the same polypeptide, so that CheY<sup>P</sup> was tethered in the vicinity of the FliM<sub>M</sub> domain through its interaction with FliM<sub>N</sub>. An interaction between CheY and FliM<sub>M</sub> appears compatible with the CheY-FliN interaction described here, in the sense that both types of interaction could occur within a single motor (and both can be accommodated in the current working model for switch-complex organization). Further work will determine whether the CheY<sup>P</sup>-FliM<sub>M</sub> interaction is, like the FliN-CheY interaction, essential for the switch to CW rotation.

Cross-linking experiments showed that the lower portion of the C-ring is formed from an alternating array of FliN tetramers and FliM C-terminal domains, and that the switch from CCW to CW rotation is accompanied by relative movement at one of the FliN-FliM<sub>C</sub> interfaces (15). The CheY<sup>P</sup>-binding site identified here on FliN is predicted to lie near the adjacent FliM<sub>C</sub> domains and to become somewhat more accessible upon switching to the CW state (Fig. 5). Accordingly, we propose that the interaction of CheY<sup>P</sup> with FliN directly induces the relative movement of FliN and FliM<sub>C</sub>, and that this is the initiating event in the conformational switch to the CW state. Given their close proximity in the emerging structural model, a direct interaction between CheY and FliM<sub>C</sub> also appears possible and might also contribute to the energetics



**Fig. 5.** Model of CheY<sup>P</sup>-induced flagellar motor switching. (A *Left*) CheY<sup>P</sup> (yellow) interacts initially with the N-terminal segment of FliM, which is flexible enough to allow subsequent binding to a site on FliN (orange) in the vicinity of the hydrophobic patch. Only the FliM and FliN proteins of the switch are shown; FlgB would be at the top (Fig. 1). (*Right*) View showing multiple FliM-FliN units in the lower part of the C-ring, and the binding of multiple CheY<sup>P</sup> molecules. (B) Relationship of FliN<sub>4</sub> and FliM<sub>C</sub> units in the bottom of the C-ring, as determined from cross-linking and mutational analysis (15). One FliN<sub>4</sub>-FliM<sub>C</sub>-FliN<sub>4</sub> unit is shown, in stereoview. Altered yields of certain cross-links upon switching (15) indicated that motor reversal is accompanied by a movement along one of the FliN<sub>4</sub>-FliM<sub>C</sub> interfaces, shown here by the two locations for the left-hand FliN<sub>4</sub> unit (CW state, gray; CCW state, cyan). The CheY<sup>P</sup>-binding site on FliN is colored orange. (C) Hypothesis for switching in all of the FliN<sub>4</sub>-FliM<sub>C</sub> units, shown in top view. CheY<sup>P</sup> molecules are yellow and binding sites are orange; the black dot signifies the N-terminal segment of FliM sandwiched between CheY<sup>P</sup> and FliN. These segments would attach to the FliM middle domain, by a flexible linker (A).

of switching. We note, in this context, that a previous study of the binding interactions of various FliM deletion constructs gave evidence for a direct interaction between CheY and FliM<sub>C</sub> (33). Because switching will presumably alter the relationship between rotor and stator, the movements initiated near the bottom of the C-ring must be transmitted “up” through FliM<sub>M</sub> to affect FliG<sub>C</sub>. The nature of the movements in FliM<sub>M</sub> and FliG<sub>C</sub> are presently unknown. The origins of cooperativity are likewise not yet clear, although important factors are likely to be the substantial number of FliM<sub>C</sub> and FliN<sub>4</sub> units (and CheY-binding sites) present and the close association among these elements within the C-ring.

In summary, the present findings show that flagellar motor reversal depends on an interaction between the CW signal CheY<sup>P</sup> and the rotor protein FliN. The interaction with FliN should occur subsequently to CheY<sup>P</sup> capture by the N-terminal domain of FliM. The proximity of the CheY<sup>P</sup>-binding site to the FliN–FliM<sub>C</sub> interface suggests a simple mechanism by which CheY<sup>P</sup> binding could induce relative subunit movements that initiate the process of switching.

## Experimental Procedures

**Plasmids and Strains.** The *E. coli* strains and plasmids used are listed in Table 1. Procedures for DNA manipulation were as described previously (37). The gene encoding the FliM<sub>1–34</sub>–CheY fusion was cloned into the IPTG-inducible expression plasmid pTBM30 (Ap<sup>R</sup>) (38), yielding pKP235. The FliM<sub>1–34</sub>–CheY (D57A) mutant protein was obtained by site-directed mutagenesis using the QuikChange (Qiagen) procedure, yielding pKP453. The GST–FliN mutant variants were cloned in plasmid pHT96 (34), also under an IPTG-inducible promoter. All binding experiments were carried out in the  $\Delta$ *flhDC* strain (RP3098), a gift from J.S. Parkinson (Salt Lake City, UT). The *flhDC* genes are required for expression of all other flagellar operons (39, 40), and so this strain expresses no flagellar genes from the chromosome. Swimming motility in soft agar and in liquid were assayed as described previously (37), using the *fliM*-deletion strain DFB228 transformed with wild-type or mutant plasmids. To monitor the direction of motor rotation in mutant strains, cells were tethered to coverslips using anti-flagellin antibody using procedures described previously (37). Swarm plates contained TB, 0.27% agar, appropriate antibiotic (s), and IPTG at concentrations of 0, 40, and 100  $\mu$ M, for assay of FliM function at different levels of expression.

**GST Fusion Coprecipitation Procedure.** Coprecipitation experiments were carried out essentially as described (33, 41) with minor modifications. In experiments to probe interactions of FliM<sub>1–34</sub>–CheY fusion with FliN, the RP3098 strain was transformed with a plasmid expressing the GST–FliN fusion or its mutant variants, or a plasmid expressing the FliM<sub>1–34</sub>–CheY fusion. Negative-control experiments used GST, expressed from plasmid pHT100 (41).

Cells were cultured overnight at 32 °C in 40 mL LB containing appropriate antibiotics and 400  $\mu$ M IPTG. Cells expressing GST–FliN and its mutant variants were mixed with cells expressing FliM<sub>1–34</sub>–CheY, pelleted, and resuspended in

**Table 1. *E. coli* strains and plasmids used in this study**

| Strain or plasmid | Relevant genotype or property  | Source                   |
|-------------------|--|--------------------------|
| DFB228            | <i>fliM</i> -null strain   | (30)                     |
| RP3098            | <i>flhDC</i> deletion strain; expresses no chromosomal flagellar genes         | Gift from J.S. Parkinson |
| pTBM30            | Ptac expression vector; Ap <sup>r</sup>  | (31)                     |
| pHT100            | GST-only expression vector; Km <sup>r</sup>                                    | (30)                     |
| pHT96             | GST–FliN expression vector; Km <sup>r</sup>                                    | (30)                     |
| pKP235            | FliM <sub>1–34</sub> –CheY fusion in pTBM30 expression vector; Ap <sup>r</sup> | This study               |
| pKP453            | FliM <sub>1–34</sub> –CheY(D57A) in pTBM30 expression vector; Ap <sup>r</sup>  | This study               |

PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) containing 100  $\mu$ L of lysozyme (5 mg/mL in 50% glycerol), 10  $\mu$ L APMSF (4-amidinophenylmethanesulfonyl fluoride), 60  $\mu$ L of 1 M MgCl<sub>2</sub>, and 100  $\mu$ L of either water (nonphosphorylating conditions) or 0.5 M acetyl phosphate (final concentration, 40 mM). After 1 h on ice, cells were disrupted by sonication. Debris was pelleted (16,000  $\times$  g, 30 min, 4 °C), and 50  $\mu$ L of the supernatant was saved for use in estimating the amount of FliN present before addition of affinity beads. The rest (~1 mL) was transferred to a clean tube, mixed with 150  $\mu$ L of a 50% slurry of glutathione-Sepharose 4B (Pharmacia) prepared according to the manufacturer's directions, and incubated for 1 h at 4 °C with gentle rotation to allow binding. The beads were pelleted (14,000  $\times$  g, 1 min), washed with 1 mL of PBS, and repelleted. Supernatant was removed, and GST–FliN and associated proteins were released from the beads by addition of 50  $\mu$ L elution buffer [50 mM reduced glutathione in 50 mM Tris–HCl (pH 8)] for 10 min at room temperature with gentle rotation. Beads were pelleted and the supernatant was collected for analysis by SDS/PAGE and immunoblotting using anti-FliM antibody, which showed high sensitivity toward the FliM segment present in the fusion protein (and not toward CheY alone). Experiments with mutant variants were done in the same way, except always under phosphorylating conditions and with wild-type proteins as control. In the experiment with Cys introduced at position 19 of FliN in the GST–FliN fusion, disulfide cross-linking was induced using Cu-phenanthroline, prepared as described (19). Pull-down experiments with the disulfide cross-linked GST–FliN used the same protocol as above.

**SDS/PAGE and Immunoblotting.** Protein samples were separated on 8.5% SDS/PAGE minigels (Bio-Rad Mini-PROTEAN system) and transferred to nitrocellulose using a semidry apparatus (Bio-Rad). Rabbit polyclonal antibody against FliM was prepared as described (37) and was used at 2,000-fold dilution. Bands were visualized using the SuperSignal West Pico Luminol system (Pierce) and X-ray film (Kodak).

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