Mutational Analysis of the Flagellar Rotor Protein FliN: Identification of Surfaces Important for Flagellar Assembly and Switching†

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FliN is a component of the flagellar switch complex in many bacterial species. The crystal structure is known for most of FliN, and a targeted cross-linking study (K. Paul and D. F. Blair, J. Bacteriol. 188:2502–2511, 2006) showed that it is organized in ring-shaped tetramers at the bottom of the basal body C ring. FliN is essential for flagellar assembly and direction switching, but its precise functions have not been defined. Here, we identify functionally important regions on FliN by systematic mutagenesis. Nonconservative mutations were made at positions sampling the surface of the protein, and the effects on flagellar assembly and function were measured. Flagellar assembly was disrupted by mutations in a conserved hydrophobic patch centered on the dimer twofold axis or by mutations on the surface that forms the dimer-dimer interface in the tetramer. The assembly defect in hydrophobic-patch mutants was partially rescued by overexpression of the flagellar export proteins FliH and FliI, and coprecipitation assays demonstrated a binding interaction between FliN and FliH that was weakened by mutations in the hydrophobic patch. Thus, FliN might contribute to export by providing binding sites for FliH or FliH-containing complexes. The region around the hydrophobic patch is also important for switching; certain mutations in or near the patch caused a smooth-swimming chemotaxis defect that in most cases could be partially rescued by overexpression of the clockwise-signaling protein CheY. The results indicate that FliN is more closely involved in switching than has been supposed, possibly contributing to the binding site for CheY on the switch.

Bacterial flagella are assembled in a precisely choreographed process that involves more than 25 proteins (Fig. 1) (16, 23). The membrane-embedded MS ring is formed first, from about 25 copies of the FlIF protein. This is followed by assembly of the C ring onto the cytoplasmic face of the MS ring and of the flagellar export apparatus in the membrane within the MS ring (12, 17, 32, 42). This apparatus transports the protein subunits that form the exterior structures (the rod, hook, and filament) into a central channel in the flagellum, through which they move to their sites of assembly (14, 24). Once the export apparatus is assembled and functioning, these exterior structures are added in a proximal-to-distal sequence (Fig. 1) (17, 23, 34, 35). The flagellar export apparatus is related to the type III secretion systems that function in export virulence factors in many bacterial pathogens (1).

The C ring is part of the export apparatus, FlIG, FlIM, and FliN, each present in many copies (approximately stoichiometries are 25 FlIG, 35 FlIM, and >100 FliN) (5, 33, 39, 47, 48). The FlIG-FlIM-FliN complex is essential for flagellar assembly, rotation, and clockwise/counterclockwise (CW/CCW) switching and is usually termed the switch complex (45, 46). FlIG is involved most directly in rotation (10, 21) and is known to interact with the stator protein MotA (49) and the MS ring protein FlIF (8, 15, 18, 29). Because MotA and FlIF are membrane proteins, FlIG must presumably be located in the upper (membrane-proximal) part of the C ring. FliM does not function as directly in torque generation (21) but has a large role in switching (31, 46) and contains a binding site for the CW-signaling protein phospho-CheY (CheYP) (25, 41, 44). The precise function(s) of FliN has not been determined. It is the most abundant component of the C ring and is essential for both flagellar assembly and normal rotation: fliN null mutants are nonflagellate (36), while point mutants show either aberrant CW/CCW switching or subnormal rotation (10).

Vogler and coworkers found that temperature-sensitive fliN mutants are unable to grow flagellar filaments at the restrictive temperature and accordingly suggested that FliN may have a role in flagellar export (43). Consistent with this, homologs of FliN occur in a number of type III secretion systems that function to export virulence factors (and which are not thought to have any role in motility) (14, 24, 36). It is possible that FliN has only an indirect role in export, because it is needed for assembly of the C ring, which might in turn be necessary for assembly of the export apparatus. A more direct role in export seems likely, however, given the occurrence of FliN homologs in type III secretion systems. In the type III secretion systems of Yersinia pestis and Shigella flexneri, homologs of FliN were found to interact with homologs of the export protein FlIH (11, 13). If the analogous interactions also occur in the flagellar system, this could explain the requirement for FliN in flagellar assembly.

Although some fliN point mutations are known to cause aberrantly CW or CCW motor bias, such switch bias mutations are less common in FliN than in FlIM or FlIG (10, 31). FliN might have a relatively small role in switching, or it might play an important role that involves only a small part of the protein. FliN has also been implicated in torque generation; certain fliN point mutations allow flagellar assembly but impair rotation (10). Mutants with this mot phenotype are not entirely defec-

† Supplemental material for this article may be found at http://jb.asm.org.
tive in torque generation, because they were partially rescued by overexpression of the mutant FliN protein or by overexpression of FliM (21).

The crystal structure is known for most of FliN, and it shows a saddle-shaped dimer formed largely from $\beta$-strands (3) (Protein Data Bank [PDB] accession numbers 1yab and 1o6a). Using analytical ultracentrifugation and targeted cross-linking, we showed that FliN is organized in tetramers with a ring-like shape (3, 30). We proposed (30) that the FliN tetramers are located at the bottom of the C ring, where electron microscopic reconstructions (6, 38) show a bulge with the appropriate shape and dimensions. En face views of the basal body show...

FIG. 1. (Top) Sequence of events in flagellar assembly. For simplicity, only the major stages in assembly are shown. Proteins that form the axial structures exterior to the cytoplasmic membrane (the rod, hook, and filament) are actively transported into the central channel of the flagellum by an export apparatus at the base. Protein components of the export apparatus are indicated. FliJ is believed to be the chaperone for rod and hook proteins; FlgN, FlgS, and FliT are chaperones for more distal components. The outer membrane (OM), peptidoglycan layer (PG), and inner membrane (IM) are indicated. (Bottom) Approximate locations of rotor and stator proteins in the basal body. Although FliN is believed to have a role in export, it is located in the lower (membrane-distal) part of the C ring.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or property</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFB223</td>
<td>fliN null strain</td>
<td>36</td>
</tr>
<tr>
<td>RP3098</td>
<td>$\Delta$flhDC; expresses no chromosomal flagellar genes</td>
<td>J. S. Parkinson</td>
</tr>
</tbody>
</table>

- **Plasmids**
  - pTM30: P$_{tn}$ expression vector; Ap'; parent of pHT39
  - pLS4: fliN in pAlter-1; Ap'
  - pSB4: fliN codons 58 to 137 cloned in pGEM7
  - pKG116: Vector for salicylate-inducible expression; Cm' (36) J. S. Parkinson
  - pDFB92: P$_{tn}$ flim-fliN; Cm'
  - pDFB66: Para cheY; Cm'
  - pJY5: Parent of pKP59; encodes His$_6$ tag and TEV protease cleavage site; Ap' (This work)
  - pKP54: fliHI in pKG116
  - pKP59: fliI in pJY5
  - pKP61: fliHI in pKG116
  - pKP62: fliDST in pKG116
  - pKP63: fliI in pKG116
  - pKP66: fliDST in pKG116; Km' EcoRI cassette replaces Cm' cassette
  - pKP67: fliI in pKG116
  - pKP68: fliI in pKG116
  - pKP71: fliI in pKG116 (This work)
34-fold subunit structure, with the number of subunits varying somewhat from specimen to specimen (39, 47). If FliN corresponds to the bulge at the bottom of the C ring as our evidence suggests, then the C ring should contain about this number of FliN tetramers.

Here, we have used the FliN crystal structure to guide a systematic mutational study of the protein. Mutations were made at many positions distributed over the surface of FliN, and the effects on flagellar assembly and function were measured. In support of the ring-shaped tetramer model, flagellar assembly and function were affected by mutations on the surface that is predicted to form the dimer-dimer interface. A conserved hydrophobic patch on FliN was found to be important for flagellar assembly and CW/CCW switching. Nonconservative mutations near the center of this patch prevented flagellar assembly, and this assembly defect was partially rescued by overexpression of the flagellar export proteins FliH and FliI. Copurification assays showed that FliH interacts with FliN through the hydrophobic patch. Thus, FliN might assist flagellar assembly by providing docking sites for FliH and associated proteins.

Mutations in a region adjoining the hydrophobic patch affected switching; cells assembled flagella but swam smoothly, indicating a CCW motor bias. The chemotaxis defect in most of these mutants was partially rescued by CheY overexpression. Previous studies have highlighted the importance of FliM for CW/CCW switching and of an N-terminal segment of FliM for binding to CheY (2, 19, 25, 41, 44). Our results indicate that regions of FliN are also critical for switching, either stabilizing the CW state of the switch or contributing to the binding site for CheY.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** The *Escherichia coli* strains and plasmids used are listed in Table 1. LB, TB, and SB media were prepared as described previously (30, 37). LB medium was used for most culture growth and transformations, TB medium for assays of motility in liquid, and SB medium for high-level expression. Ampicillin, chloramphenicol, and kanamycin were used at 125 μg/ml, 50 μg/ml, and 50 μg/ml, respectively, in liquid media and at 50 μg/ml, 12.5 μg/ml, and 12.5 μg/ml, respectively, in swarm plates. Isopropyl-β-D-thiogalactopyranoside (IPTG) was prepared as a 0.1 M stock in water and used at the concentrations indicated in the figures.
Mutagenesis and assays of mutant function. Single-stranded DNA preparation and site-directed mutagenesis were performed according to the Alterted Sites procedure (Promega) with fliN cloned in plasmid pLS4 (21). The mutations were confirmed by sequencing, and then the mutated genes were cloned into plasmid pH739 (36) to allow controlled expression of the mutant fliN genes from the tac promoter. Swarming in soft agar, swimming in liquid, and flagellation were measured as described previously (37), using the fliN deletion strain DBF223 (36) transformed with wild-type pH739 or mutant variants of pH739. Swim plates contained TB, 0.27% Bacto Agar, appropriate antibiotics, and IPTG at various concentrations between 0 and 400 μM, giving FliN expression levels ranging from slightly below to well above the wild-type level (36). Flagella were stained using the wet mount procedure of Heimbrook et al. (9).

His-binding affinity chromatography. FliN with an N-terminal eight-His tag (His-FliN) was expressed from plasmid pKP59 in cells of the ΔΔBDC strain RP3098, a gift of J. S. Parkinson. The fliN/D genes are required for expression of all other flagellar operons (20, 22), so this strain expresses no flagellar genes from the chromosome. Wild-type or mutant FliN was expressed from plasmid pH739 (36) in strain RP3098. Cells were grown at 37°C in 500 ml of SB medium containing ampicillin to an optical density at 600 nm of 0.7 and then induced with 400 μM IPTG for 3 hours. Cells of the two cultures (one expressing His-FliN and the other expressing FliN or its mutant variants) were mixed, collected by centrifugation, and then resuspended in 20 ml of binding buffer (50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). Cell pellets were lysed by using a French pressure cell, and the lysate was centrifuged (23,000 × g, 45 min) to pellet the membranes. The supernatant was applied to a Ni ion affinity column (Ni-nitrilotriacetic acid agarose; QIAGEN), and the column was washed in the following buffers: 50 ml of binding buffer; 50 ml of elution buffer containing 20 mM imidazole; and then 30 ml each of elution buffers containing imidazole at concentrations of 50 mM, 100 mM, 200 mM, and 400 mM. To analyze the fractions for FliN content, the samples were run on sodium dodecyl sulfate-polyacrylamide gels (12%) (mini gels), and then proteins were transferred to nitrocellulose using a semidry transfer apparatus (Bio-Rad Trans-Blot SD), as described previously (30). Rabbit polyclonal antibody against FliN was detected using the Super Signal West PicoLuminol system (Pierce) and X-ray film. Bands were quantified by video densitometry using the public domain image-processing program NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

The crystal structure is known for residues 68 to 154 of Thermotoga maritima FliN (3), corresponding to residues 51 to 137 of the E. coli protein. The present mutational analysis used the protein of E. coli. Nonconservative replacements were made at 30 positions on the protein surface (Fig. 2). Multiple replacements were made at some positions, and some of the Cys replacements made for the recent cross-linking study (30) were also included when they were the least conservative replacement available at a given position. The positions and phenotypes of the mutations are summarized in Table 2 and are displayed on the structure in Fig. 2. FliN forms a stable tetramer (3) with the shape of a doughnut (30). Such a doughnut shape is observed in one of the FliN crystal structures (PDBe accession number 1yab) (3), and we adopted this as a structural framework for interpreting the mutations.

Most regions on the surface of FliN are evidently not critical for flagellar assembly or function: The mutational replacements at most positions (19 of 30) allowed swarming at 50% or better of the wild-type rate (Table 2 and Fig. 2). Mutations that strongly disrupted FliN function clustered in particular regions on the protein, as detailed below.

**Mutations that affect assembly.** Two mutations (V111D and V112D) prevented flagellar assembly, and two others (R70D and V113D) caused cells to assemble fewer flagella than normal (typically one or two). The V111D, V112D, and V113D mutations are in the hydrophobic patch. The R70D mutation, which exhibited a milder defect than the others in swarming assays, falls on a different surface (Fig. 2).

Vogler et al. (43) reported that FliN temperature-sensitive mutants are unable to regrow flagellar filaments at the restrictive temperature, indicating a role for FliN in flagellar export. In a report describing the FliN structure, we noted that the hydrophobic patch is a conserved feature of FliN and suggested that it might provide docking sites for export substrate or for substrate-chaperone complexes (3). Interactions have previously been demonstrated between homologs of FliN and

### TABLE 2. FliN mutations and their phenotypes

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Residue(s) in T. maritima</th>
<th>Swarming rate (IPTG)</th>
<th>Note(s)</th>
</tr>
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<tr>
<td>I54D</td>
<td>L71</td>
<td>1.16 (10–25)</td>
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<tr>
<td>L56D</td>
<td>L73</td>
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<td>n</td>
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<td>L74</td>
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<td>n</td>
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<td>M75</td>
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<td>n</td>
</tr>
<tr>
<td>D98W</td>
<td>D76</td>
<td>1.01 (10)</td>
<td>n</td>
</tr>
<tr>
<td>I60D</td>
<td>f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L68C</td>
<td>L85</td>
<td>0.15 (10)</td>
<td>CCW</td>
</tr>
<tr>
<td>W19C/L68C</td>
<td>–/-L85</td>
<td>0.10</td>
<td>CCW</td>
</tr>
<tr>
<td>I54C/L68C</td>
<td>L71/L85</td>
<td>0.17</td>
<td>CCW</td>
</tr>
<tr>
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<td>R87</td>
<td>0.34 (50)</td>
<td>Flac+/–; n</td>
</tr>
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<td>R89</td>
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<td>n</td>
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<td>E97</td>
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<tr>
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<td>D107</td>
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<td></td>
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<tr>
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<td>V128</td>
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<td>V113D</td>
<td>V130</td>
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<tr>
<td>V113C</td>
<td>V130</td>
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<tr>
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<td>I31</td>
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<td>CCW; y</td>
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<td>I31</td>
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<td>n</td>
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<td>E133</td>
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<td>ccw; y</td>
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<td>n</td>
</tr>
<tr>
<td>E130K</td>
<td>E147</td>
<td>0.90 (10)</td>
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<td>L151</td>
<td>1.25 (25)</td>
<td>n</td>
</tr>
<tr>
<td>S136C</td>
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</tr>
<tr>
<td>S136W</td>
<td>N153</td>
<td>1.11 (10–25)</td>
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*The swarming rates are relative to the maximum rate of the wild-type control in the same experiment. The numbers in parentheses are the IPTG concentrations (micromolar) that gave optimal swarming of each mutant; in cases where swarming varied little with induction level, no concentration is given. Flac+, flagellate; Flac+/–, flagellate and motile but with fewer filaments than for the wild type; Flac−, nonflagellate; Mot–, immotile; CCW, motile but smooth swimming; ccw, smoother than normal swimming but occasional tumbles; n, altered dependence of swarming on the FliN expression level (Fig. 5); m, swarming improved by overexpression of FlIM and some swarming also observed after prolonged incubation (Fig. 3); y, swarming helped by overexpression of CheY; h, swarming helped by overexpression of FlIH plus FliI.

**Note:** no residue in T. maritima.
homologs of the export protein FliH in the type III secretion systems of *Y. pestis* (11) and *S. flexneri* (13). FliH is an export-related protein that binds and regulates FliI, the ATPase that provides energy for flagellar export (4, 27). FliH also interacts with FliJ, a chaperone for certain flagellar export substrates (26). To test for a possible role of the hydrophobic patch in binding FliH or other export proteins, we examined the effects of overexpressing FliH, FliI, and FliJ, together or in various subsets, in the *fliN* mutants. The mutants defective in motility or switching were not helped by overexpression of all three proteins (FliH, FliI, and FliJ). By contrast, the nonflagellate hydrophobic-patch V111D and V112D mutants showed a detectable improvement in swarming motility (Fig. 3). Experiments using subsets of the proteins showed that both FliH and FliI were necessary for the enhanced swarming, but FliJ was not (Fig. 3). Two other chaperones of flagellar export, FliS and FliT, were also tested, together with the flagellar cap protein FliD whose gene is in the same operon. FliS, FliT, and FliD together did not rescue the nonflagellate mutants and did not augment the effects of FliH and FliI (Fig. 3).

The FliH/FliI overexpression effect suggests that the hydrophobic patch might assist assembly by interacting with FliH and/or FliI. To look more directly for binding interactions, we used a copurification assay. Cells expressing His-tagged FliH and cells expressing wild-type or mutant FliN were mixed, lysed, and Ni-nitrilotriacetic acid beads were used to isolate His-FliH and associated proteins. Immunoblots were used to detect FliN in the partially purified material. Wild-type FliN was coisolated with His-FliH at a readily detectable level. FliN proteins with the hydrophobic-patch mutations V111D and V112D were coisolated at much lower levels (Fig. 4). By contrast, the FliH-FliN interaction was not greatly weakened by the I60D mutation in the hypothesized dimer-dimer interface or by the CCW-bias mutation A93D, V113D, or V114D (Fig. 4). The FliN-FliH interaction did not require any other flagellar proteins, because the coisolation experiments used a *fliDC* strain that expresses no flagellar genes from the chromosome.

**Mutations giving immotile but flagellated (mot) phenotype.** The I57D and I60D mutants were flagellate but practically immotile, with only one cell in thousands showing weak motility when observed in the microscope. Residues 57 and...

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**FIG. 3.** Effects of overexpression of flagellar and chemotaxis proteins in the FliN mutant strains. Cells of strain DFB223 (*ΔfliN*) were transformed with a plasmid encoding the indicated mutant variant of FliN (or wild-type FliN as a control) and an additional plasmid(s) to direct overexpression of the indicated proteins (Y, CheY; H, I, J, D, S, and T, FliH, FliI, FliJ, FliD, FliS, and FliT, respectively; cont, control). IPTG (10 μM) was used to induce expression of *fliN*; other *fli* genes were expressed from the salicylate promoter, using induction with 10 μM sodium salicylate. The CheY gene was expressed from the arabinose promoter; the plates shown contained 1 mM arabinose. Fresh transformants were picked and transferred to plates containing tryptone and 0.27% agar, and the plates were incubated at 32°C for 24 h. The D116K mutant was aberrantly smoothly swimming but retained some chemotactic ability; the effects of CheY overexpression (CheY o/p) in this mutant are shown in the bar graph. This experiment used 0.5 mM arabinose to induce CheY overexpression. Error bars indicate the standard deviations for three measurements. w.t., wild type.

**FIG. 4.** Copurification of FliN with His-FliH and effects of FliN mutations. His-FliH and FliN (or its mutant variants) were expressed in cells of strain RP3087 (*ΔflhDC*). Cell lysates were loaded on a Ni affinity column, and the column was washed with increasing concentrations of imidazole. Samples prior to loading on the column are shown at the top, the flowthrough fractions are shown in the middle, and fractions eluted with 50 mM imidazole are at the bottom. “FliN only” indicates a negative control in which the cells expressed wild-type FliN but no His-tagged FliH. w.t., wild type.
60 are on the hypothesized dimer-dimer interface (Fig. 2). A previous mutational study in *Salmonella enterica* serovar Typhimurium (10) yielded three other FliN mot mutations near the dimer-dimer interface in residues 61, 75 and 78 (see Fig. S1 in the supplemental material). Those mutations conferred an immotile phenotype at normal expression levels (10) but showed some swarming when FliM was overexpressed (21). To determine whether the I57D and I60D mutants behaved similarly, we introduced a second plasmid that expressed wild-type FliM. When FliM was overexpressed in the cells, the swarming rates of both mutants improved to about 25% of the wild-type rate, and in the microscope many cells were observed to swim (Table 2; swarm plates not shown).

**Mutations that affect switching.** Several flin mutations caused cells to swim smoothly, indicating an aberrantly CCW motor bias. In some cases, the nonchemotactic mutants also produced fewer flagella than normal (Table 2), but all were capable of vigorous swimming. Most of the CCW-bias mutations occurred on the margins of the hydrophobic patch (Fig. 2). An asparagine replacement of the hydrophobic-patch residue Val111 also gave a CCW-bias phenotype, in contrast to the aspartate replacement at this position which, as noted above, prevented assembly. A smooth-swimming, nonchemotactic phenotype was also observed for one mutation near the center of the hydrophobic patch (L68C), a Cys replacement made originally for the targeted cross-linking study (30). Residue 68 is largely buried, so this mutation might perturb the overall protein conformation rather than directly altering a surface important for switching.

Because the nonchemotactic mutants showed an aberrantly CCW motor bias, we next tested the effects of overexpressing the CW-signaling protein CheY in the flin mutants. As expected, the nonflagellate mutants and the immotile mutants were not helped by overexpression of CheY. Three of the motile, nonchemotactic mutants (A93D, V113D, and V114D mutants) showed markedly improved swarming when CheY was overexpressed (Fig. 3). Two other CCW mutants, V111N and L68C mutants, showed no improvement.

**Mutations with altered dependence on FliN level.** Several FliN mutations allowed swarming at rates comparable to that of the wild type, but at expression levels different from the optimum for the wild-type protein. Data for nine such mutations are shown in Fig. 5. In most cases, a higher level of induction was needed for optimal swarming, and swarming at

![FIG. 5. Mutations that alter the dependence of swarming rate upon expression level of FliN. Swarming rates versus induction levels are shown for the mutants that allowed close to wild-type function at some expression level. Wild-type (w.t.) controls are shown for comparison. Variability in wild-type swarming rates reflects experimental variations in agar concentration, which did not alter the IPTG dependence of either the wild type or the mutants.](http://jb.asm.org/)
very high levels of expression was better than that of cells overexpressing wild-type FliN. In two cases, the optimal level of expression was unchanged from the level for the wild type overexpressing wild-type FliN. In two cases, the optimal level of expression was marked for positions in the upper half of the tetramer, and symmetry-related positions in the lower half are colored but not numbered. The L56D mutation is also in this category but is not pictured because it is near the dimer-dimer interface. The positions of four mutations that conferred the mot phenotype in a screen of spontaneous mutants of Salmonella (10, 21) and that are not in the region of the dimer-dimer interface are shown, in magenta. The function of these mutants was also improved by overexpression of either the mutant FliN or FliM (21). (See Fig. S1 in the supplemental material for locations of other mutations that occur near the dimer-dimer interface.) The hydrophobic patch is shown for reference in green; this patch is formed from the side chains of residues 68, 93, 111, 113, and 118 (see also reference 3).

Dominance. To measure dominance of the swarming-defective FliN mutations, plasmids expressing the FliN variants with swarming rates less than 50% of normal were transformed into wild-type cells, and swarming rates were measured at various levels of induction. Most of the mutant FliN proteins impaired swarming of wild-type cells to some extent. Dominance was strongest for the mutations that give CCW motor bias (V111N, L68C, A93D, V113D, and V114D) (Fig. 7).

DISCUSSION

Most of the FliN mutations did not substantially affect flagellar assembly or function, identifying sizable regions of the protein that are unlikely to engage in critical, nonredundant interactions. Functionally critical regions of FliN include the hydrophobic patch and its immediate surroundings and the dimer-dimer interface containing residues 57 and 60 (Fig. 2).

We hypothesized previously that the hydrophobic patch on FliN might assist flagellar assembly by binding to protein complexes involved in export (3). Support for this idea comes from studies demonstrating an interaction between FliN homologs and FliH homologs in the type III secretion apparatus of Y. pestis (11) and S. flexneri (13). The FliH/FliN overexpression experiment (Fig. 3) gives some indication that a FliN-FliH interaction occurs and is functionally important. The pull-down experiment provides more direct evidence for the FliN-FliH interaction and implicates the hydrophobic patch in particular (Fig. 4). A similar conclusion regarding the FliN-FliH interaction was reached in a recent study by Gonzales-Pedrajo et al. (7), also using pull-down assays.

The precise function of the FliN-FliH interaction in flagellar export is not yet known. Export might be facilitated by localization of substrate-chaperone complexes; Thomas and coworkers (40) showed that substrate-chaperone complexes can be localized to the membrane through their interaction with FliN. The FliN-FliH interaction may increase the rate of substrate delivery by targeting FliH-containing complexes to the vicinity of the export apparatus. Alternatively, FliN might influence export steps more directly, for example by modulating the stability or ATP hydrolysis activity of the FliH-FliN complex.

The doughnut model for FliN organization is based on a targeted cross-linking study that identified the surface of FliN-
FliN contact within the tetramer (30). The mutational data support this model for the tetramer; both the I57D and I60D replacements on the hypothesized dimer-dimer interface caused severe motility impairments. The I57D and I60D mutants showed improved swarming when FliM was overexpressed. This was also true of three mot mutants of Salmonella that map in the region of the dimer-dimer interface (10, 21) (see Fig. S1 in the supplemental material). This rescue by overexpression prompted us to suggest previously that the mot mutants of FliN are defective in some aspect of switch-complex assembly, rather than in torque generation per se (10, 21). The structural model for the FliN tetramer allows a more specific interpretation; the mot mutations near the dimer-dimer interface most likely destabilize the tetramer or alter its shape so that assembly is disfavored. If FliM and FliN act cooperatively in assembly of the C ring, as a number of studies suggest (25, 29, 41), then overexpression of FliM might partially offset the effects of FliN mutations that destabilize or distort the tetramer.

Although we cannot yet specify the exact site(s) of FliM binding on the surface of FliN, the mutational data from this and previous studies (10, 21) provide clues to the region(s) that might be involved. Weakened binding to FliM might be expected to increase the level of FliN needed for optimal motility. This behavior was seen in several of the mutants characterized here, most of which fell on the face containing the well-conserved residue Val86 (Fig. 6). Mutations in Val86 altered the dependence on the FliN level in different ways, depending on the replacement. This surface is a likely candidate for interaction with FliM. Other parts of FliN might also contact FliM. One mutation showing altered level dependence (R134D) occurs in a different region, near four mutations that gave immotile phenotype in Salmonella (10, 21) (Fig. 6). Binding at two sites on FliN would enable FliM to bridge between adjacent FliN tetramers, an arrangement that would stabilize the C ring and that could account for the cooperative action of FliM and FliN in forming the ring.

Switch bias mutations were known to occur in FliN but at a lower frequency than in FliM or FliG (10, 31). This could indicate that FliN has a relatively small role in switching or, alternatively, that just a small region of the protein is involved. In light of the FliN structure and the present mutational data, we suggest that FliN has a crucial role in switching and that this involves a region on the margin of the hydrophobic patch. Three site-directed mutations in this region gave a strong defect in several of the CCW mutants was substantially rescued by overexpression of CheY, the signaling protein that, when phosphorylated, interacts with the motor to promote CW rotation (Fig. 3). CheYp is known to bind to a segment near the amino terminus of FliM (2, 19, 41). Our results would be accounted for if CheYp binds to FliN also, at a site near the hydrophobic patch. A CheY-FliN interaction has so far not been detected but does not appear to be ruled out by experiments carried out to date. An alternative possibility is that FliN mutations alter the intrinsic bias of the motor (i.e., the relative stabilities of the CCW and CW states). Further experiments are needed to distinguish these possibilities. In either case, the present results highlight the importance of FliN for CW/CCW switching and are consistent with the previous structure-based proposal that switching might involve conformational changes within the FliN tetramer (30).

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