JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 223–231 Vol. 178, No. 1
0021-9193/96/$04.00+0

Torque Generation in the Flagellar Motor of *Escherichia coli*: Evidence of a Direct Role for FliG but Not for FliM or FliN

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Received 1 August 1995/Accepted 27 October 1995

Among the many proteins needed for assembly and function of bacterial flagella, FliG, FliM, and FliN have attracted special attention because mutant phenotypes suggest that they are needed not only for flagellar assembly but also for torque generation and for controlling the direction of motor rotation. A role for these proteins in torque generation is suggested by the existence of mutations in each of them that produce the Mot$^-$ (or paralyzed) phenotype, in which flagella are assembled and appear normal but do not rotate. The presumption is that Mot$^-$ defects cause paralysis by specifically disrupting functions essential for torque generation, while preserving the features of a protein needed for flagellar assembly. Here, we present evidence that the reported *mot* mutations in *fliM* and *fliN* do not disrupt torque-generating functions specifically but, instead, affect the incorporation of proteins into the flagellum. The *fliM* and *fliN* mutants are immotile at normal expression levels but become motile when the mutant proteins and/or other, evidently interacting flagellar proteins are overexpressed. In contrast, many of the reported *fliG mot* mutations abolish motility at all expression levels, while permitting flagellar assembly, and thus appear to disrupt torque generation specifically. These mutations are clustered in a segment of about 100 residues at the carboxyl terminus of FliG. A slightly larger carboxyl-terminal segment of 126 residues accumulates in the cells when expressed alone and thus probably constitutes a stable, independently folded domain. We suggest that the carboxyl-terminal domain of FliG functions specifically in torque generation, forming the rotor portion of the site of energy transduction in the flagellar motor.

Many motile bacteria are propelled by thin helical filaments that are each driven at the base by a rotary motor embedded in the cell membrane (reviewed in references 1, 20, 25). The filament/motor organelle is called a flagellum. In contrast to eukaryotic molecular motors, which use nucleoside triphosphates as an energy source, bacterial rotary motors are powered by the transmembrane gradient of protons (19, 21) or, in some species, sodium ions (14). The mechanism by which bacterial flagellar motors convert the chemical energy of the ion gradient into the mechanical energy of rotation is not yet understood.

Approximately 50 genes are needed for the assembly and operation of the flagella of *Escherichia coli* or *Salmonella typhimurium* (20). Among these, only a few encode products thought to be directly involved in the process of torque generation. Genes whose products might participate in torque generation have been identified in extensive mutant screens as those giving the Mot$^-$ phenotype, in which flagella are assembled but do not rotate. The presumption has been that *mot* mutations affect torque-generating activities specifically, while preserving the features of a protein needed for flagellar assembly. By using this criterion, five proteins that could have direct roles in torque generation have been identified in *E. coli* and *S. typhimurium*. These are MotA, MotB, FliG, FliM, and FliN (7, 13, 35).

MotA and MotB together form a transmembrane proton channel (2, 3, 10, 27, 33). The precise functions of FliG, FliM, and FliN are less clear; mutational analysis and intergenic suppression studies suggest that they are associated in a "switch complex" essential for flagellar assembly, torque generation, and controlling the direction of motor rotation (34, 35). Analyses of the abundance of *mot* alleles of each gene suggested that FliM has only a small role in torque generation (26), whereas FliG and FliN are more extensively involved (15). In recent studies concerned with the effects of underexpression or mutation of FliM or FliN, we obtained evidence that FliM and FliN might not be closely involved in torque generation (29, 30). This view would appear inconsistent with the occurrence of *mot* mutations in *fliM* and *fliN*, if those mutations specifically affect torque-generating functions.

We reported previously that underexpression of FliM or FliN impairs motility more than flagellation, thus mimicking the Mot$^-$ phenotype to some degree (29, 30). This observation suggests that the Mot$^-$ phenotype of certain *fliM* or *fliN* mutants might be caused by reduced binding of the mutant proteins to their sites in the flagellum, rather than by alteration of residues that directly participate in torque generation. In cases such as this the phenotype might depend upon the level of expression of the mutant proteins and/or other proteins with which they interact in the flagellum.

In this work, we examine more closely the cause of the Mot$^-$ phenotype in certain *fliM*, *fliN*, and *fliG* mutants. We made numerous *mot* mutations in these genes in *E. coli*, corresponding to a representative subset of the *mot* mutations of *S. typhimurium* reported by Sockett et al. (26) and Irikura et al. (15). The effects of overexpressing the mutant proteins, alone or together with other wild-type switch complex proteins, were then examined. The results indicate that *mot* mutations in *fliM* and *fliN* do not abolish torque generation specifically but affect the binding of proteins to their sites in the flagellum. In contrast, many mutations in *fliG* abolish torque generation specifically. On the basis of these results, we suggest that FliG participates closely in torque generation, whereas FliM and FliN do not. FliG appears to be organized into at least two separable domains: the amino-terminal two-thirds contains all deter-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or property(ies)	Source or reference
E. coli		
RP437	Wild type for motility and chemotaxis	J. S. Parkinson
RP526	*mutD* mutator	J. S. Parkinson
DFB190	*fliM* null	30
DFB223	*fliN* null	29
DFB225	*fliG* null	This work
BL21-DE3	T7 RNA polymerase gene in the chromosome, used for overexpression from pAED4 derivatives	28
S. typhimurium TR6579	Restriction deficient	J. Roth
Plasmids		
pACYC184	Cm^r, pSC101 origin, parent of pHT93, pDFB94a, pDFB95, and pDFB96	New England Biolabs
pAED4	Ap^r, vector for overexpression from the T7 promoter, parent of pSL22	6
pAlter-1	Vector for site-directed mutagenesis	Promega
pDFB72	*Ptac-fliM lacI*^{q} Ap^r	30
pDFB92	*Ptac-fliMN lacI*^{q} Ap^r	29
pDFB94a	*Ptac-fliM lacI*^{q} Cm^r	This work
pDFB95	*Ptac-fliN*^{a} *lacI*^{q} Cm^r	This work
pDFB96	*Ptac-fliMN lacI*^{q} Cm^r	This work
pHT39	*Ptac-fliN*^{a} *lacI*^{q} Ap^r	This work
pHT41	*fliM* in pAlter-1	30
pHT53	*Ptac-fliG lacI*^{q}	This work
pHT92	*Ptac-fliN lacI*^{q} Ap^r	29
pHT93	*Ptac-fliN lacI*^{q} Cm^r	This work
pJT101	Source of *fliG*	P. Matsumura
pLS4	*fliN* in pAlter-1	29
pMAK705	Temperature-sensitive origin of replication, parent vector for chromosomal replacements	11
pMAKGdel	pMAK705 derivative containing a *fliG* in-frame deletion	This work
pSL22	*fliG* expression vector derived from pAED4	This work
pSL27	*fliG* in pAlter-1	This work
pSL38	*Ptac-fliG* (N-terminal 245 codons) *lacI*^{q} Ap^r	This work
pSL39	*Ptac-fliG* (C-terminal 126 codons) *lacI*^{q} Ap^r	This work
pTBM30	*Ptac* expression vector, parent of pHT39, pHT53, pSL38, pSL39, pDFB72, pDFB92, and pHT92	22

[a] With additional residues encoded by the vector polylinker fused to the amino terminus (see Materials and Methods for details).

minants needed for flagellar assembly, whereas the carboxyl-terminal one-third is needed specifically for torque generation.

MATERIALS AND METHODS

Strains, media, and plasmids. The strains and plasmids used are listed in Table 1. Media for culture growth, transformations, plasmid isolation, and swarming and motility assays were described previously (30). Cells were cultured at 37°C for purposes such as plasmid propagation and at 32°C for motility and swarming assays. Ampicillin (AP) was included in liquid media at 100 μg/ml when appropriate. In cells carrying both Ap resistance and chloramphenicol resistance plasmids, AP and chloramphenicol were used at 50 and 20 μg/ml, respectively. Prior to transformation of *E. coli* plasmids into the *S. typhimurium fliM* or *fliN* mutants, the plasmids were propagated in the restriction-defective, modification-competent *S. typhimurium* TR6579. Routine manipulations of DNA were done according to the methods of Sambrook et al. (24).

For some experiments, very high-level expression of FliN was achieved by using plasmids that encoded a translational fusion of expression vector sequences to *fliN* (pHT39 or pDFB95; Table 1). The parent expression vector was pTBM30 (22), and the additional amino acids fused to the amino terminus of FliN were MLQDPGTAQ. The additional amino acids do not impair FliN function; plasmids expressing this fusion protein complement the *fliN* null strain DFB223 to wild-type swarming at an appropriate low level of induction (ca. 25 μM IPTG [isopropyl-β-D-thiogalactopyranoside]; data not shown).

***fliG*-null strain construction.** The source of the *fliG* gene was plasmid pJT101, a gift of P. Matsumura. An in-frame deletion in *fliG* was made by digestion with *Eco*47III and *Hin*cII. The *fliG* in-frame deletion, as well as *fliF* and *fliH* flanking sequences, were subcloned into pMAK705 (11), a gift of S. Kushner. Plasmid pMAK705 encodes chloramphenicol resistance and a temperature-sensitive origin of replication that allows for the transfer of sequences into the chromosome, as described by Hamilton et al. (11). The pMAK705 derivative encoding deleted *fliG* (pMAKGdel) was transformed into the wild-type strain RP437. Clones containing a *fliG* in-frame deletion in the chromosome were obtained as described previously (30). The presence of the deletion at the appropriate chromosomal locus was confirmed by PCR amplification of genomic DNA using primers complementary to sequences flanking *fliG*.

Site-directed mutagenesis and dideoxy sequencing. Single-stranded-DNA preparation and site-directed mutagenesis were carried out according to the Altered Sites procedure (Promega, Madison, Wis.). Mutations were confirmed by dideoxy sequencing using reagents from U.S. Biochemical (Cleveland, Ohio).

Flagellation, motility, and swarming. Flagellum staining and counting and soft-agar swarm assays were carried out as described previously (30). For microscopic observations of motility, overnight cultures grown in tryptone broth (TB) and an appropriate antibiotic were diluted 100-fold into fresh media containing various concentrations of IPTG and cultured for 4 h at 32°C. Motility was observed with a phase-contrast microscope.

Swarm plates contained tryptone broth, approximately 0.28% Bacto agar, and IPTG at the concentrations indicated in the figure legends. One microliter of each mid-log phase culture was spotted onto swarm plates, and the plates were incubated at 32°C. The incubation times and the strains used for each overexpression experiment are indicated in the figures.

Overexpression and purification of FliG and preparation of anti-FliG antiserum. The *fliG* gene was inserted into the T7 expression vector pAED4 (6), and the resulting plasmid was transformed into strain BL21-DE3 (28). Cells were cultured at 37°C in 1 liter of Luria broth containing 200 μg of AP per ml to an optical density (600 nm) of 0.5, induced with 2 mM IPTG, grown for an additional 4 h, and collected by centrifugation at 4°C. The cells were resuspended in 200 ml of 50 mM Tris-Cl (pH 8)–50 mM NaCl–5 mM EDTA, and lysozyme was added to 0.5 mg/ml. After 30 min on ice, cells were disrupted by sonication and treated with DNase I (0.01 mg/ml) and $MgCl_2$ (10 mM) for 30 min on ice. Cell membranes and inclusion bodies containing FliG were pelleted by centrifugation (23,000 × *g*, 30 min), resuspended in 60 ml of 1% Triton X-100–1 mM EDTA, and sonicated. The washed inclusion bodies were pelleted by centrifugation (20,000 × *g*, 20 min) and frozen for later use. Thawed inclusion bodies were dissolved in 6 ml of urea buffer (7.3 M urea, 50 mM Tris [pH 8]), and dithioth-

reitol was added to 15 mM. The solution was centrifuged to remove insoluble material (10,000 × *g*, 20 min), and the supernatant was slowly added to 300 ml of ice-cold 50 mM Tris (pH 8)–1 mM dithiothreitol–0.1 mM EDTA. FliG was precipitated by addition of solid ammonium sulfate to 70% saturation and pelleted by centrifugation (13,000 × *g*, 10 min). The pellet was dissolved in urea buffer and diluted into urea-free buffer as before, concentrated by ultrafiltration to a volume of 60 ml, centrifuged (27,000 × *g*, 20 min) to remove precipitated material, and concentrated to a volume of 10 ml. The sample was loaded onto an ion-exchange column (Q-Sepharose) equilibrated with 50 mM Tris (pH 8)–0.1 mM dithiothreitol–0.1 mM EDTA and eluted with a continuous 0 to 1 M NaCl gradient. Fractions containing FliG were pooled. At this stage FliG was more than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue. The purified FliG was used to raise anti-FliG antibodies in rabbits (HRP Inc., Denver, Pa.). Preabsorption of antibody and immunoblotting were done as described previously (30).

Random *fliG* mutagenesis. A plasmid bearing the wild-type *fliG* gene (pHT53) was mutagenized by passage through the *mutD* mutator strain RP526, a gift of J. S. Parkinson. Mutagenized plasmids from single colonies were isolated and transformed into the *fliG* null strain DFB225. The motility of the transformants was tested by the soft-agar swarm assay. Immotile clones were stained by using a wet-mount protocol (12) to determine whether they produced flagella. Plasmids were isolated from mutants that were well flagellated but immotile. Mutations were then screened for dominance by transforming the plasmids into the wild-type strain RP437. Plasmids that impaired swarming of the wild-type strain were isolated and sequenced.

Expression of FliG domains. PCR was used to clone the amino-terminal 245 codons or carboxyl-terminal 126 codons of *fliG* into plasmid pTBM30. Reactions included 1 ng of plasmid pHT53 as the template, 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 50 mM KCl, 100 pmol of appropriate upstream and downstream primers, 250 μg of bovine serum albumin, and 0.5 U of *Taq* polymerase (Stratagene) in a volume of 10 μl. Amplification was done for 35 cycles in a Rapid-Cycle Thermocycler (Idaho Technologies). The resulting PCR products were then cloned into plasmid pTBM30. The resulting plasmids (pSL38, encoding the amino-terminal 245 residues of FliG, and pSL39, encoding the carboxyl-terminal 126 residues) were transformed into the *fliG* null strain DFB225. An overnight culture of each transformant was grown in TB containing 100 μg of AP per ml. Cultures were diluted 100-fold into fresh medium containing AP and 2 mM IPTG and grown for 4.5 h at 32°C. Immunoblots of whole-cell extracts were performed as described elsewhere (30).

Isolation of flagellar basal bodies from *fliG* mutants. The *fliG*-null strain DFB225 was transformed with derivatives of plasmid pSL27, which encoded wild-type *fliG* or mutant F234S or I306S, or with plasmid pSL38, which encodes the amino-terminal 245 residues of FliG. Cells were cultured and flagellar basal bodies were isolated by the methods of Zhao et al. (36), with one exception. Prior to cell lysis, cultures were divided into two aliquots. One aliquot was lysed overnight at 4°C as called for in the published procedure, and the other was lysed for only 30 to 90 min. The procedure was then completed. Samples of the soluble fraction and the basal-body fraction were electrophoresed on SDS-polyacrylamide gels, and immunoblots were performed with the anti-FliG antiserum.

TABLE 2. *fliM*, *fliN*, and *fliG* mutations of *E. coli* characterized in this study

Mutation	Amino acid change[a]
fliM	
M1	F131C
M2	F131L
M3	F131S
M4	G132D
M5	G133D
M6	T147I
M7	T147P
M8	T149K
M9	L250Q
M10	L250R
M11	L272R
fliN	
N1	P61L
N2	I75S
N3	L78P
N4	L100P
N5	G103V
N6	Y104N
N7	L105Q
fliG	
G1	P126L
G2	Q127H
G3	T131P
G4	del167–169
G5	A197E
G6	A198E
G7	A215P
G8	F234S
G9	L237Q
G10	Q246Term
G11	L249P
G12	V252E
G13	L257P
G14	K271E
G15	del280–285
G16	del292–295
G17	I306S

[a] Most of the mutational changes are based on those originally reported for *S. typhimurium* by Sockett et al. (26) for *fliM* and Irikura et al. (15) for *fliG* and *fliN*.

RESULTS

The amino acid changes in a large number of *fliM*, *fliN*, and *fliG mot* mutants of *S. typhimurium* were determined by Sockett et al. (26) and Irikura et al. (15). The *E. coli* mutants studied here were made by using those amino acid changes as a guide and include all except one of the 12 reported *fliM mot* mutants, 7 *fliN* mutants that include examples in each codon altered in the reported *fliN mot* alleles (excluding frameshifts), and 13 *fliG* mutants that represent about three-fourths of the codons altered in the reported *fliG mot* alleles. The amino acid changes in the mutants studied here are listed in Table 2.

Conditions that make the *fliM* mutants motile. All of the *E. coli fliM* mutants exhibited severe motility defects, as reported for *S. typhimurium* by Sockett et al. (26). We noted previously (30) that cultures of some *fliM* mutants contain a small number of motile cells, which implies that the mutations do not fully abolish torque generation. It was suggested that these mutations might affect interactions between FliM and other flagellar components, thus reducing the amount of FliM and/or the other components incorporated into the flagellum.

If the defects in FliM affect protein-protein interactions, then their phenotypes might depend upon the level of expression of the mutant FliM proteins or of other flagellar proteins with which FliM interacts. To test the effects of increasing the level of the mutant FliM proteins, they were moderately overexpressed in an *E. coli fliM*-null strain and motility was tested in a soft-agar swarming assay. The swarming behavior of the mutants at three levels of expression of the mutant FliM proteins is shown in Fig. 1A to C.

When the mutant FliM proteins were moderately overexpressed, mutants M9, M10, and M11 began to swarm, albeit slowly. Slow swarming of these mutants at somewhat lower FliM levels was reported previously (30). The swarms were much denser than a wild-type control (not shown), indicating that motility was not normal. In the microscope, many cells of mutant M9, M10, or M11 were motile, but tumbling, suggesting that the flagellar motors had an abnormal directional bias.

The other eight *fliM* mutants tested failed to swarm when the mutant proteins were overexpressed. Some of the *fliM* mutations might hinder the installation of another flagellar component; a likely candidate is FliN, which appears to interact with FliM (29). To see if any of the defects in FliM affect the incorporation of FliN into the flagella, wild-type FliN was overexpressed along with the mutant FliM proteins by using a compatible second plasmid (pHT93; Table 1). Swarms of strains moderately overexpressing both the mutant FliM pro-

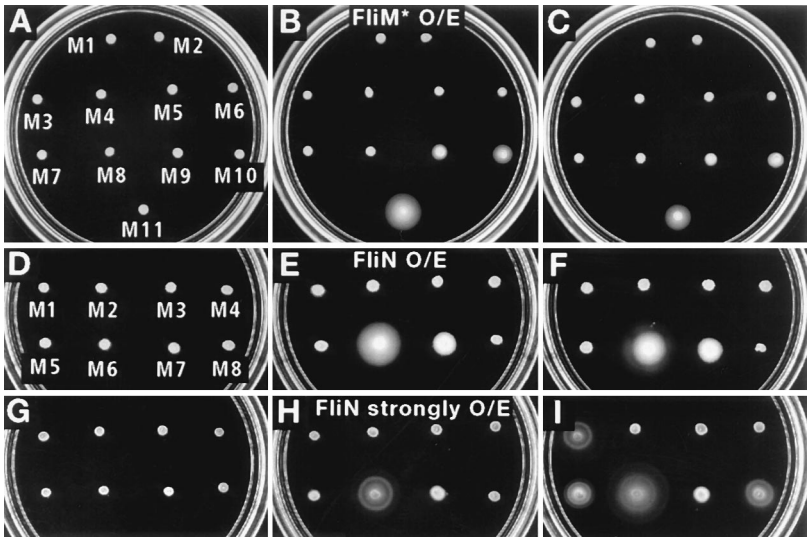

FIG. 1. Effects of different overexpression (O/E) protocols on motility of the *E. coli fliM* mutants. Amino acid changes in the mutants are listed in Table 2. (A to C) Effects of overexpressing the mutant (asterisk) FliM proteins. The strains were DFB190 (*fliM* null) transformed with derivatives of pDFB72 that carried the mutant *fliM* genes, expressed from the *tac* promoter. Plates were incubated for 13 h at 32°C. (D to F) Effects of moderately overexpressing wild-type FliN in *fliM* mutants 1 to 8. The strains were DFB190 transformed with derivatives of pDFB72 that expressed the mutant *fliM* genes and with pHT93 that expressed wild-type *fliN*. Plates were incubated for 22 h at 32°C. (G to I) Effects of very high-level expression of FliN in the *fliM* mutants 1 to 8. The strains were DFB190, transformed with pDFB72 derivatives expressing the mutant *fliM* genes and pDFB95, which expresses very high levels of a normally functioning FliN variant with some nonnative residues fused to its amino terminus (see Materials and Methods). Plates were incubated for 10 h at 32°C. IPTG concentration, 0 (A, D, and G), 100 (B, E, and H), or 500 (C, F, and I) μM.

teins and wild-type FliN are shown in Fig. 1D to F. Two of the *fliM* mutants (M6 and M7) swarmed at significant rates when provided with additional FliN. Five others (M1, M2, M4, M5, and M8) showed an increased tendency to form trails (trails indicate that a small fraction of the cells can swim). To examine the effect of furnishing very high levels of FliN, a different plasmid (pDFB95) was introduced into the mutants. This plasmid directs overexpression of a FliN variant with nine nonnative amino acids fused to its amino terminus. The additional amino acids are encoded by the polylinker of the expression vector used and do not impair the function of the protein (see Materials and Methods). At high levels of induction (250 to 2,000 μM IPTG), the additional FliN allowed *fliM* mutants M1, M5, M6, and M8 to swarm well and mutant M7 to swarm slowly (Fig. 1G to I). Mutants M2, M3, and M4 did not swarm, but the colony edges developed a soft-edged appearance indicative of motility, and motile, tumbling cells of these mutants were observed in the microscope. Also, we note that mutants M2 and M3 have defects in the same codon as mutant M1, which swarmed well.

Conditions that make the *fliN* mutants motile. Point mutations in seven codons in *fliN* are reported to give a Mot^- phenotype in *S. typhimurium* (15). Corresponding mutations were made in the *E. coli fliN* gene (Table 2) and studied at various levels of expression from plasmid pHT92, which uses the *tac* promoter. At an expression level shown previously to give good swarming with the wild-type gene (25 μM IPTG) (29), all of the mutants were well flagellated, as reported previously for *S. typhimurium* (15), and most were immotile. Mutants N4 and N5 swarmed surprisingly well (Fig. 2B), probably because of the somewhat higher-level expression of the FliN proteins in our strains than in the *S. typhimurium* mutants, which contain single chromosomal copies of *fliN*. When the FliN proteins were expressed at an even higher level, swarming of mutant N5 was further improved. One mutant (N7) swarmed poorly, yet some motile cells were observed in the microscope; the motility was aberrantly smooth. The other four *fliN* mutants did not swarm and were not motile in liquid culture, even when the mutant FliN proteins were overexpressed.

To see if the phenotype of the *fliN* mutations depended on the level of FliM, wild-type FliM was overexpressed along with the mutant FliN proteins by using derivatives of plasmid pDFB92 that encoded wild-type *fliM* and the mutant *fliN* genes, both under control of the *tac* promoter. The results are shown in Fig. 2D to F for the five *fliN* mutants that did not swarm when the mutant FliN proteins alone were overexpressed. All five mutants swarmed, albeit slowly, when wild-type FliM was overexpressed along with the mutant FliN. Similar results were obtained when the mutant FliN proteins were overexpressed from plasmid pHT92 and wild-type FliM was overexpressed from a compatible second plasmid (pDFB94a; data not shown).

Motility of the *fliM* and *fliN* mutants of *S. typhimurium*. The *mot* mutations in *E. coli* studied here were originally isolated and characterized in *S. typhimurium* (15, 26, 35). To see if motility could also be restored to the original *S. typhimurium* mutants, they were studied by using overexpression protocols analogous to those described above. First, *S. typhimurium* strains harboring chromosomal *fliM* or *fliN mot* mutations, provided by R. Macnab, were transformed with plasmids encoding wild-type *E. coli fliN* or *fliM*, respectively. Swarming was scored at several levels of induction of the wild-type *E. coli fli* genes. The results were similar to what was observed with the *E. coli* mutants. The *S. typhimurium fliM* mutants M1, M2, and M4 to M8 swarmed, some slowly and some well, when the *E. coli* FliN protein was overexpressed (Fig. 3A to C). Mutant M3 did not swarm, but motile cells were observed in the microscope; we note that mutant M3 is changed in the same codon as mutants M1 and M2, which swarmed. One of the reported *fliM mot* mutants of *S. typhimurium* (H106P [26]) was not made in *E. coli* but was tested in this overexpression experiment. It also

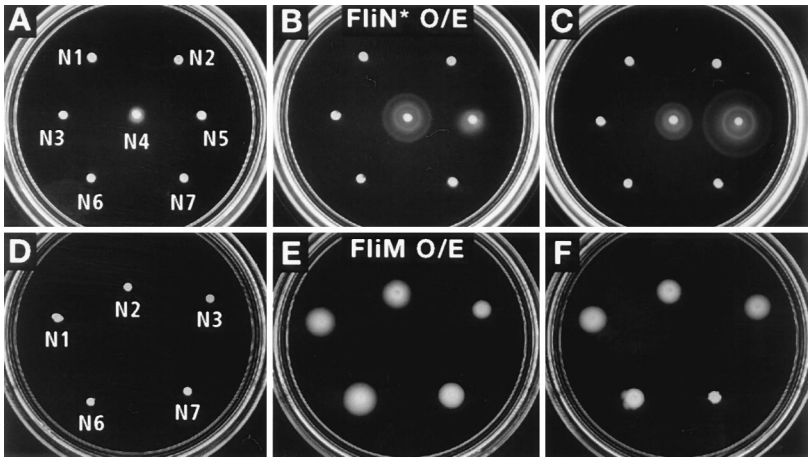

FIG. 2. Effects of different overexpression (O/E) protocols on the *E. coli fliN* mutants. The amino acid changes in the mutants are listed in Table 2. (A to C) Effects of overexpressing the mutant FliN proteins. The strains were DFB223 (*fliN* null) transformed with derivatives of pHT92 that expressed the mutant (asterisk) variants of *fliN*. IPTG concentration, 0 (A), 25 (B), or 100 (C) μM. The plates were incubated for 11 h at 32°C. (D to F) Effects of overexpressing both the mutant FliN proteins and wild-type FliM in the five mutants that did not become motile when only FliN was overexpressed. The strains were DFB223 transformed with derivatives of pDFB92 that expressed the mutant *fliN* genes and wild-type *fliM* from the *tac* promoter. IPTG concentration, 0 (D), 100 (E), or 200 (F) μM. Plates were incubated for 24 h at 32°C.

swarmed when *E. coli* FliN was overexpressed (data not shown).

The *S. typhimurium fliN* mutants also resembled their counterparts in *E. coli*: mutants N1, N2, N3, N6, and N7 formed dense swarms when the *E. coli* FliM protein was overexpressed (Fig. 4A to C). Although mutant 3 swarmed very slowly, motile cells were seen in the microscope.

As was true in *E. coli*, some of the *S. typhimurium fliM* and *fliN* mutants were not helped by overexpression of the other protein (FliN or FliM, respectively). Those *S. typhimurium* strains, bearing the *fliM* or *fliN* mutations on the chromosome, were transformed with plasmids encoding the same mutations, except in the *E. coli fliM* or *fliN* genes. Motility was assayed at various levels of expression of the plasmid-borne mutant genes. When the mutant *E. coli* proteins were overexpressed in *S. typhimurium* strains harboring the same mutations on the chromosome, motility was significantly improved (Fig. 3D to F and 4D to F). Thus, the *fliM* and *fliN* mutants of both species can be made motile by appropriate overexpression protocols.

Overexpression protocols do not improve motility of the *fliG mot* mutants. Point mutations in 17 codons in *fliG* are reported to give a Mot$^-$ phenotype (15). A subset of these mutations was made in the *E. coli fliG* gene by site-directed mutagenesis (Table 2); additionally, one missense mutation (K271E) reported to give a Mot$^-$ phenotype was isolated by random mutagenesis. Together, these mutations represent about three-fourths of the *fliG* codons reported to produce the Mot$^-$ phenotype in *S. typhimurium*. Three small in-frame deletions of *fliG* that give a Mot$^-$ phenotype in *S. typhimurium* were also made. The *fliG* mutants were characterized by using procedures like those described above for *fliM* and *fliN*. When the mutant FliG proteins were overexpressed from plasmid pHT53 in a *fliG* null strain, none was motile at any expression level tested. At the highest level tested, 500 μM IPTG, FliG was expressed at about 40 times the wild-type level as determined by immunoblots (not shown). One of the mutants (P126L) formed trails on swarm plates, indicating that a very small fraction of the cells were motile; the other mutants remained completely immotile. To see if additional FliM and FliN could improve the motility of the *fliG* mutants, the mutant FliG proteins were expressed constitutively from plasmid pSL27, at about twice the wild-type level as determined by immunoblots, and wild-type FliM and FliN were overexpressed from a compatible second plasmid (pDFB96). None of the mutants

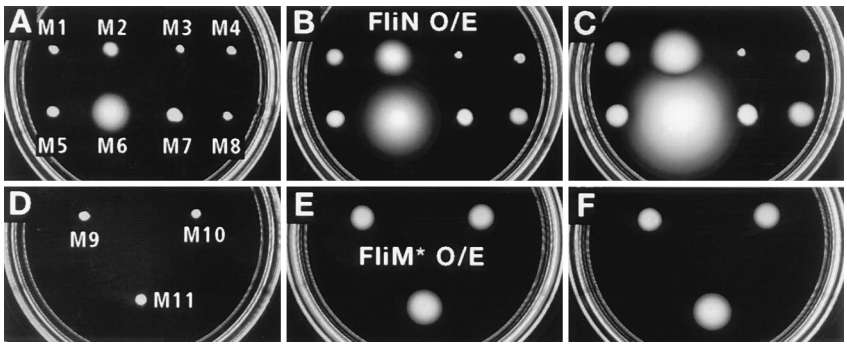

FIG. 3. Effects of overexpression (O/E) protocols on the *S. typhimurium fliM* mutants (see Table 2). (A to C) Effects of overexpressing *E. coli fliN* in the *S. typhimurium fliM* mutants. The *S. typhimurium* mutants were transformed with pHT39 (Table 1). Incubation temperature, 32°C; incubation period, 14 (A and B) or 18 (C) h; IPTG concentration, 0 (A) or 100 (B and C) μM. (D to F) Effects of overexpressing the mutant (asterisk) FliM proteins in the three *fliM* mutants that did not become motile when FliN was overexpressed. Each *S. typhimurium* mutant was transformed with a pDFB72 variant that encoded the same mutation, except in the *E. coli fliM* gene. Plates were incubated for 14 h at 32°C. IPTG concentration, 0 (D), 100 (E), or 500 (F) μM.

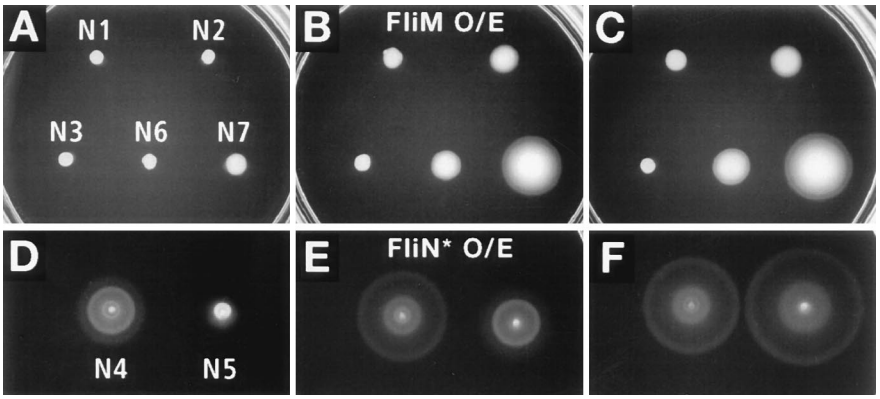

FIG. 4. Effects of overexpression (O/E) protocols on the *S. typhimurium fliN* mutants (see Table 2). (A to C) Effects of overexpressing *E. coli fliM* in five *S. typhimurium fliN* mutants. The strains were transformed with pDFB72 (Table 2). IPTG concentration, 0 (A), 200 (B), or 500 (C) μM. Plates were incubated for 12 h at 32°C. (D to F) Effects of overexpressing the mutant (asterisk) FliN proteins. The *S. typhimurium fliN* mutants were transformed with derivatives of pHT92 that encoded the same mutations, except in *E. coli fliN*. IPTG concentration, 0 (D), 50 (E), or 100 (F) μM. Plates were incubated for 7 h at 32°C.

swarmed (data not shown). Finally, the effects of overexpressing the mutant FliG proteins and also overexpressing wild-type FliM and FliN were examined. Motility was not improved for any of the *fliG* mutants (Fig. 5).

Flagellation of the *fliG* mutants. Certain *fliM* mutations greatly reduce flagellation in *E. coli* but not in *S. typhimurium* (30). To see if interspecies differences also occur with the *fliG* mutants, each of the *E. coli fliG* mutants was stained, and the flagella were counted. The results are summarized in Table 3. In most cases flagella were abundant, as reported for these mutations in *S. typhimurium*. However, a subset of the *E. coli fliG* mutants had very few flagella (≤0.2 flagellum per cell versus approximately 5 flagella per cell in a wild-type control), indicating that flagellar assembly was affected. The *mot* mutations that greatly decreased flagellation were in the amino-terminal two-thirds of the protein (up to codon 215). The mutations in the carboxyl-terminal one-third of the protein had much smaller effects on flagellation, allowing assembly of between 1.6 and 2.5 flagella per cell.

Stability of separated carboxyl-terminal and amino-terminal domains. A random screen for dominant *fliG mot* mutants was carried out as described in Materials and Methods. Among several dominant *mot* mutations, this screen yielded one with a termination codon in place of codon 246. The mutant reverted rapidly, so subsequent characterization employed plasmid pSL38 (Table 1), which encodes only the first 245 residues of FliG, and therefore cannot revert. Immunoblots using polyclonal anti-FliG antiserum confirmed that a protein of approximately 24 kDa accumulated when the truncated form of FliG was expressed (Fig. 6). The mutant was well flagellated (2.7 flagella per cell on average; Table 3), implying that the amino-terminal fragment contains all the determinants needed for flagellar assembly. A slightly smaller FliG molecule of 225 residues was also tested. Although it accumulated when expressed in the *fliG* null strain, it did not support flagellar assembly (data not shown).

PCR was used to clone the complementary part of *fliG*, encoding a carboxyl-terminal fragment of 85 residues, into plasmid pTBM30. This protein fragment was not detected on immunoblots. A plasmid expressing a 126-residue carboxyl-terminal fragment was then constructed. This directed expression of a 15-kDa protein that was readily detected on immunoblots (Fig. 6). All of the *fliG* mutations that gave the clear Mot^- phenotype (well flagellated but immotile under all conditions tested) are contained within this domain.

Presence of *fliG mot* mutant proteins in purified flagellar motors. Zhao et al. (36) recently reported that mutations near the carboxyl terminus of *fliG* can disrupt the normal association of FliG with the motor, as evidenced by the absence of the mutant FliG proteins in preparations of flagellar basal bodies. This observation is surprising because FliG is required for flagellar assembly (15, 19a), and the mutants with changes in the carboxyl-terminal domain of FliG are well flagellated. To determine whether FliG proteins with mutations in the carboxyl-terminal domain can associate with the flagellum, we isolated flagella from strains expressing the mutant proteins by using the procedure of Zhao et al. (36). The mutant *fliG* genes were expressed from plasmid pSL27, which produces FliG constitutively at about twice the wild-type level. FliG in the basal-body preparations was detected by immunoblots. For two missense mutants tested, F234S and I306S, FliG was found in the basal-body preparations in amounts comparable to that for a wild-type control (Fig. 6). Significant proteolysis of the mutant proteins was observed if the samples were left to lyse overnight at 4°C, a step in the published protocol (36) (blots not shown). When the 245-residue amino-terminal fragment of

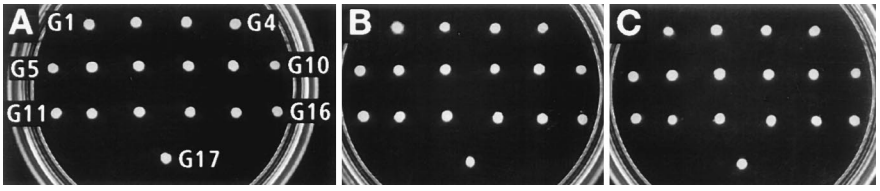

FIG. 5. Effects of overexpressing the mutant FliG proteins together with the wild-type FliM and FliN proteins. The strains were DFB225 (*fliG* null), transformed with derivatives of pHT53 expressing the mutant *fliG* genes and with pDFB96 expressing wild-type *fliM* and *fliN*. All of the *fli* genes were expressed from the *tac* promoter. IPTG concentration, 0 (A), 50 (B), or 500 (C) μM. Plates were incubated for 23 h at 32°C.

TABLE 3. Flagellation of *E. coli fliG* mutants

Mutation[a]	No. of flagella/cell[b]
P126L	0.0
Q127H	0.2
T131P	0.0
del167–169	0.0
A197E	0.2
A198E	0.2
A215P	0.1
F234S	2.0
L237Q	1.6
Q246Term[c]	2.7
L249P	2.2
V252E	2.3
L257P	2.0
K271E	2.2
del280–285	1.9
del292–295	1.5
I306S	2.5

[a] Except for Q246Term, the *fliG*-null strain DFB225 transformed with derivatives of plasmid pHT53, which encoded the *fliG* mutations, cultured in 10 μM IPTG was used.

[b] The flagella on 50 cells were counted. Wild-type *fliG* (on plasmid pHT53), 5.0 flagella per cell.

[c] pSL38, which is identical to pHT53 except that it lacks *fliG* sequences downstream of the termination codon, and therefore cannot revert, was used.

FliG was expressed in the cells, it was also found in the isolated flagella (Fig. 6). Thus, the carboxyl-terminal domain of FliG is not needed for incorporation of the protein into the flagellum or for subsequent steps in assembly.

DISCUSSION

The nature of *mot* mutants of *fliM* and *fliN*. The mutations studied here were first isolated in *S. typhimurium*, in which they cause essentially complete paralysis of the flagella (15, 26). Because the mutations cause paralysis, their existence has usually been taken to indicate that the proteins in question have some role in torque generation. The implicit assumption is that the mutated protein is present in the flagellum but unable to carry out a function needed specifically for motor rotation. Recent experiments concerned with the effects of underexpressing wild-type FliM (30) or FliN (29) suggested that the paralyzed phenotype might be produced in a different way, by defects that hamper the installation of proteins in the flagellum.

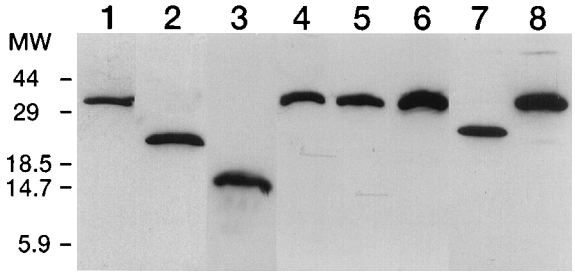

FIG. 6. Immunoblots of FliG, mutant variants of FliG, and FliG fragments. Lane 1, wild-type FliG protein expressed from plasmid pHT53 in the *fliG*-null strain DFB225. Expression was induced with 1 mM IPTG. The band at 38 kDa is not seen for the FliG-null strain (not shown). Lane 2, ca. 24-kDa amino-terminal fragment of FliG that accumulates when the first 245 residues of FliG are expressed. The FliG fragment was expressed from pSL38, which uses the same promoter and translational start site as pHT53, induced with 1 mM IPTG. Lane 3, ca. 15-kDa fragment that accumulates when 126 carboxyl-terminal residues of FliG are expressed. The fragment was expressed from pSL39, induced with 2 mM IPTG. Lane 4, wild-type FliG in flagella isolated by the method of Zhao et al. (36). Lane 5, FliG mutant protein F236S in isolated flagella. Lane 6, FliG mutant protein I308S in isolated flagella. In lanes 4 to 6 the proteins were encoded by derivatives of plasmid pSL27, which express FliG or its mutant variants constitutively at about twice the wild-type level. Lane 7, 24-kDa amino-terminal fragment of FliG in isolated flagella. The fragment was encoded by plasmid pSL38, which expresses FliG at a level slightly lower than that of the wild type under the conditions used (no IPTG). Lane 8, wild-type FliG in isolated flagella, for comparison with the FliG fragment in lane 7. FliG expression and flagellar isolation were as in lane 7. MW, molecular weight (in thousands).

All of the *fliM* and *fliN mot* mutants studied here became motile to some extent either when the mutant protein itself was overexpressed or when both the mutant protein and another, wild-type switch complex protein were overexpressed. These results are not expected if the mutations specifically abolish torque-generating activities of proteins present in the motor, because that is a qualitative defect and should not be cured by supplying additional protein. The results obtained here for the *fliM* and *fliN* mutants are what is expected for defects that alter occupancy, because in that case increased expression could compensate for the defects by increasing the concentration of a needed part and promoting its incorporation into the flagellum. We conclude that the *fliM* and *fliN* mutations studied here all impede the installation of proteins into the flagellum and do not specifically abolish torque-generating activities of proteins present in the structure.

In principle, overexpression might restore motility by augmenting the activity of proteins that function away from the motor rather than by driving assembly of the motor toward completion. Two considerations make this alternative unlikely. First, it is known that FliG functions in association with the basal body MS ring (8), and in vitro binding studies in our laboratory have demonstrated that FliG, FliM, and FliN all bind to each other (30a). All three proteins are therefore likely to function in a complex attached to the basal body, rather than free in the cytoplasm. Secondly, when motility is restored by overexpression, the cells sometimes have an abnormal run/tumble bias, a defect which suggests that mutant components are present in the motors.

When the *fliM* and *fliN* mutants are made motile by overexpression, many swarm slowly, owing to either an abnormal run/tumble bias or motility that is weaker than the wild type. This imperfect restoration of function is not surprising, because alterations that interfere with the installation of motor parts are likely also to affect the geometry of the parts once installation has been "forced" by overexpression. Although FliM is probably not located at the site of torque generation, it clearly is important for controlling the direction of motor rotation (26, 32). It has been suggested (26, 30) that FliM functions to position a component(s) at the site of torque generation, in one of two arrangements corresponding to either clockwise or counterclockwise rotation. The incorporation of aberrant FliM molecules into the motor is therefore likely to lead to abnormal motility. The same applies to FliN, because FliN interacts with FliM (29) and also with FliG (30a).

Nothing can be proven conclusively by the failure to isolate a particular class of mutations. The mutations studied here were isolated by Yamaguchi and coworkers in quite extensive screens, however, which involved hundreds of independent nonswarming isolates (35). It is therefore significant that all of the *fliM* and *fliN* mutants that fit the Mot^- classification appear defective in aspects of assembly rather than torque generation per se. On the basis of the present results and previous underexpression studies (29, 30), we suggest that neither FliM nor FliN is a direct participant in torque generation. The previous mutational studies of *S. typhimurium* also suggested only a

small role for FliM in torque generation (26) but a larger role for FliN (15).

Interaction between FliM and FliN. Evidence for an interaction between FliM and FliN in the flagellar motor was reported previously (29). Additional evidence for this interaction comes from the observation that certain mutant defects in FliM or FliN can be partly reversed by overexpression of the other protein. In FliM, the mutations that can be suppressed in this way are clustered in a segment spanning residues 131 to 149 (Fig. 1 and Table 2). In FliN they are found in several places in the sequence, with several clustered near residue 100 (Fig. 2 and Table 2). These segments might form the surfaces on FliM and FliN that make contact, or they might affect the interaction less directly, by maintaining the proteins in conformations that facilitate their installation into the flagellum.

Participation of FliG in torque generation. The abundance of torque-abolishing mutations in FliG suggests that it could have a large direct role in torque generation. This puts it in a class distinct from FliM and FliN and more like the motility proteins MotA and MotB, in which many mutations give a clear Mot^- phenotype that persists over a wide range of expression levels (1a). MotA and MotB function together as a transmembrane proton channel (2, 3, 10, 27, 33) and might form the stator, or nonrotating portion of the flagellar motor (4, 5, 10). The precise role of FliG is unclear. It is located on the cytoplasmic surface of the basal-body MS ring (8, 23) and, so, is probably part of the rotor of the flagellum. Because FliG appears to be a direct player in torque generation, we suggest that it is located at the interface between rotor and stator, constituting the rotor portion of the site of torque generation. Consistent with this hypothesis are the observations that a mutation in *motB* can suppress an in-frame deletion in *fliG* (34) and that mutations in *fliG* can suppress certain mutations in *motB* (10).

The clustering of torque-abolishing mutations in FliG is striking. All such mutations are found in a carboxyl-terminal segment of about 95 residues. A carboxyl-terminal fragment of 126 residues is stable and thus appears to be an independently folded domain. This domain evidently functions primarily in torque generation. It is unlikely that all of the residues altered in the *mot* mutants have direct roles in torque generation, however. Most of the mutations involve changes in hydrophobic side chains or the gain or loss of proline residues and could cause structural changes that displace torque-generating residues from their proper location in the motor. The fact that two of the mutant proteins, F234S and I306S, show increased susceptibility to proteolysis suggests that their conformations are different from that of the wild-type protein.

Some indication that carboxyl-terminal segments of FliG are dispensable for assembly came previously from Irikura et al. (15), who reported three in-frame deletions in this part of FliG, together covering 25 residues, that give a Mot^- phenotype (Table 2). Although the carboxyl-terminal domain of FliG appears to function mainly in torque generation, changes in it can also have some effect on flagellar assembly, as evidenced by the relatively minor decreases in flagellation in the carboxyl-terminal *mot* mutants (Table 3) and by the existence of a missense mutation in codon 266 of *S. typhimurium fliG* that produces a nonflagellate phenotype (15).

Interspecies differences in flagellation. As seen previously with certain *fliM* mutants (30), a subset of the *fliG* mutants that do not interfere with flagellation in *S. typhimurium* do reduce flagellation in *E. coli*. These interspecies differences are not yet fully explained. Flagellar assembly begins with the FliF protein, which forms the basal-body MS ring. When *S. typhimurium* FliF is overexpressed, it spontaneously associates to form MS rings, each containing about 25 copies of the protein (31). We have overexpressed the FliF protein of *E. coli* and found that it accumulated mostly in monomeric form (21a), suggesting that it is less predisposed to assemble into MS rings than its *S. typhimurium* counterpart. Efficient assembly of MS rings in *E. coli* might therefore require other proteins in addition to FliF, such as FliG and FliM, and accordingly be more sensitive to mutations in those proteins. Whatever the cause of the less-robust assembly in *E. coli*, it is a useful feature: the extremely poor flagellation of some of the *E. coli* mutants allows us to conclude that defects in the amino-terminal two-thirds of FliG do not affect torque generation specifically.

Comparison with ultrastructural studies. Electron microscopic analyses of motor quaternary structure are consistent with the proposal that FliG is near the MotA/B stator complexes. Francis et al. (8, 9) showed that FliG is located near the cytoplasmic face of the MS ring, probably extending from the edge of this ring to a radius of approximately 15 nm. Khan et al. (17) showed that a circular array of membrane-bound particles, likely to be MotA and MotB, surrounds the MS ring, also at a radius of about 15 nm. Thus, FliG could approach the MotA/B channels closely.

Recently, gentler isolation procedures have allowed purification of flagella that contain large, cytoplasmically located structures not observed previously (9, 16, 18). These ring- (9) or bell-shaped (16, 18) structures extend approximately 17 nm into the cytoplasm and have a maximum diameter of about 45 nm (9). This structure probably contains the FliM and FliN proteins, because basal bodies that have it can be decorated with antibodies against FliM or FliN whereas those that lack it cannot (9). Immunoblots of flagellar proteins also showed that FliM and FliN are not present in preparations that lack the cytoplasmic structure (9, 36). Zhao et al. (36) observed that the cytoplasmic structure was not present in basal bodies isolated from *fliM* and *fliN mot* mutants of *S. typhimurium*, in agreement with the present suggestion that *mot* mutations in *fliM* and *fliN* affect aspects of assembly. The cytoplasmic structure was also absent, or present at a much lower frequency, in basal-body preparations from most *fliG mot* mutants. Interestingly, in one *fliG* mutant the cytoplasmic feature was retained in a significant fraction (about 30%) of the basal bodies (36). This mutant is altered in codon 236, in the putative motility domain, in accordance with the suggestion that *mot* mutations in this part of FliG affect primarily torque generation and do not seriously hamper assembly.

Other results of Zhao et al. (36) contrast with those obtained in the present study. They observed that mutations near the carboxyl terminus of FliG caused the protein to disappear from the basal-body preparations and, on this basis, suggested that the carboxyl-terminal part of FliG might be needed for folding or stability of the protein. We detected these mutant variants of FliG at levels similar to those of wild-type FliG controls in similarly prepared basal-body samples. As the authors note (36), proteolysis of the mutant FliG proteins may have prevented their detection; we observed some proteolysis of FliG in our samples, especially those with mutations in the carboxyl-terminal domain. In any case, it is clear that the carboxyl-terminal domain is not essential for folding of the protein or its incorporation into the flagellum, because a truncated variant of FliG that lacks about 90 carboxyl-terminal residues can support flagellar assembly and is easily detectable in flagellar basal-body preparations.

Structural studies of the carboxyl-terminal domain of FliG should be valuable for understanding its precise function in torque generation. We have purified the carboxyl-terminal

FliG fragment and are characterizing it further to see if it will be suitable for structural studies.

ACKNOWLEDGMENTS

We thank S. R. Kushner, R. M. Macnab, P. Matsumura, J. S. Parkinson, and J. Roth for strains and plasmids, K. Matz for preparation of the figures, and R. M. Macnab and P. Renfranz for comments on the manuscript.

This work was supported by grant MCB-9117785 from the National Science Foundation. S.B. and X.W. received support from the Hughes Undergraduate Research Program (Department of Biology, University of Utah), sponsored by the Howard Hughes Medical Institute. The Protein-DNA Core Facility at the University of Utah receives support from the National Cancer Institute (5P30 CA42014).

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