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Regulated Underexpression and Overexpression of the FliN Protein of *Escherichia coli* and Evidence for an Interaction between FliN and FliM in the Flagellar Motor

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The FliN protein of *Escherichia coli* is essential for the assembly and function of flagella. Here, we report the effects of regulated underexpression and overexpression of FliN in a *fliN* null strain. Cells that lack the FliN protein do not make flagella. When FliN is underexpressed, cells produce relatively few flagella and those made are defective, rotating at subnormal, rapidly varying speeds. These results are similar to what was seen previously when the flagellar protein FliM was underexpressed and unlike what was seen when the motility proteins MotA and MotB were underexpressed. Overexpression of FliN impairs motility and flagellation, as has been reported previously for FliM, but when FliN and FliM are co-overexpressed, motility is much less impaired. This and additional evidence presented indicate that FliM and FliN are associated in the flagellar motor, in a structure distinct from the MotA/MotB torque generators. A recent study showed that FliN might be involved in the export of flagellar components during assembly (A. P. Vogler, M. Homma, V. M. Irikura, and R. M. Macnab, J. Bacteriol. 173:3564–3572, 1991). We show here that approximately 50 amino acid residues from the amino terminus of FliN are dispensable for function and that the remaining, essential part of FliN has sequence similarity to a part of Spa33, a protein that functions in transmembrane export in *Shigella flexneri*. Thus, FliN might function primarily in flagellar export, rather than in torque generation, as has sometimes been supposed.

Many bacteria swim by using flagella, helical propellors driven by a reversible rotary motor in the cell membrane (reviewed in references 5, 21, 25, and 32). Rotation is powered by a transmembrane gradient of ions, usually protons (23) but sometimes sodium ions (19). In *Escherichia coli*, the assembly and function of flagella require about 50 genes. Among these, *fliG*, *fliM*, and *fliN* have attracted special attention because they are involved in multiple processes, including flagellar assembly, switching between the clockwise and counterclockwise directions of motor rotation, and possibly torque generation (20, 34, 41, 42). Certain mutations in these genes can be phenotypically suppressed by mutations in each of the others (41), suggesting that FliG, FliM, and FliN function in a complex, termed the switch-complex to reflect its role in controlling the direction of rotation.

Irikura et al. (20) recently reported that several point mutations in *fliN* give a paralyzed or *mot* phenotype, in which the flagella appear normal but do not rotate. Since those mutations affect motor rotation specifically, their existence has been taken to indicate that FliN functions in torque generation. Relatively few mutations in *fliN* affected clockwise-counterclockwise switching, suggesting that FliN is not very important for determining motor direction. Its role in assembly is not clear, since it is not known with certainty whether the null phenotype is nonflagellate (*fla*) or flagellate but paralyzed (*mot*); gross disruptions of *fliN* can cause either defect, depending upon their location within the gene (20).

Previously, insight into the function of the motility proteins MotA and MotB was obtained by underexpressing the proteins and examining the effects on flagellar motor performance. Specifically, it was shown that both MotA and MotB function as components in multiple, independent torque generators within the flagellar motor (2, 7). Electron microscopic evidence suggests that MotA and MotB are arranged in a ring surrounding the flagellar basal body (22); other evidence shows that they function together (16, 35, 40) as a proton-conducting channel (3, 4) that is probably part of the stator or nonrotating part of the motor (6, 8, 9, 11). More recently, the effects of FliM underexpression were reported (37). Underexpressing that protein had effects quite different from what was seen with MotA or MotB, suggesting a different location for FliM, probably on the rotor.

Here, we report the effects of underexpression and overexpression of the *E. coli* FliN protein in a *fliN* null strain constructed for this purpose. Cells that lack FliN do not make flagella. When FliN is underexpressed, cells make comparatively few flagella and those made are defective, rotating at subnormal, fluctuating speeds. These effects are similar to what was seen previously with FliM and suggest that FliN and FliM function together, in a complex distinct from the MotA/MotB torque generators. A variety of additional experiments are described which support the suggestion that FliM and FliN interact in the flagellar motor.

To see if truncated forms of the FliN protein retain any function, two small deletions in the *fliN* gene were made and characterized. Approximately 50 residues from the amino terminus of the FliN protein are dispensable; a truncated protein lacking those residues functioned well in both flagellar assembly and rotation. The truncated FliN protein is small (ca. 80 residues), and through much of its length it exhibits similarity to Spa33, a protein involved in export of antigens in *Shigella flexneri* (31, 38). The relationship of FliN to Spa33 suggests that the main function of FliN might be in flagellum-specific export (39) rather than in motor rotation. These results are discussed with reference to a hypothesis for the location and function of FliN in the flagellar motor.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or property	Source or reference
RP437	Wild type for motility and chemotaxis	J. S. Parkinson
RP7947	*pcnB1 zad-981*::*mini-kan*; reduced plasmid copy number	24
DFB223	*fliN* null strain	This work
DFB190	*fliM* null strain	37
DFB232	*fliM fliN* null strain	This work
BL21-DE3	T7 RNA polymerase gene in chromosome; used for overexpression from pAED4 derivatives	36
pMAK705	Temperature-sensitive origin of replication; parent vector for chromosomal replacements	18
pHT70	pMAK705 derivative containing *fliN* with in-frame deletion	This work
pAED4	Vector for overexpression from T7 promoter; parent of pHT26	12
pHT26	*fliN* expression vector derived from pAED4	This work
pTBM30	P*tac* expression vector; parent of pDFB72 and pHT39	J. S. Parkinson
pDFB57	P*lac-fliN lacI*^{q}	This work
pHT92	P*tac-fliN lacI*^{q}	This work
pDFB72	P*tac-fliM lacI*^{q}	37
pDFB92	P*tac-fliMN lacI*^{q}	This work
pAlter-1	Vector for site-directed mutagenesis	Promega
pLS4	*fliN* in pAlter-1	This work
pACYC184	Cm^r; parent of pDFB95, pJL13	New England Biolabs
pDFB95	P*tac-fliN lacI*^{q} in pACYC184	This work
pJL13	*motA motB* in pACYC184	J. S. Parkinson

MATERIALS AND METHODS

Strains and media. The *E. coli* strains and plasmids used are listed in Table 1. Media for culture growth, transformations, plasmid isolations, and motility assays were described previously (37). Ampicillin was used at 100 μg/ml unless specified otherwise. Isopropyl-β-D-thiogalactopyranoside (IPTG) was prepared as a 0.1 M stock in water and used at the concentrations indicated in the figures. Cells were cultured at 37°C for routine purposes, such as plasmid isolation, and at 32°C for assays of swarming motility or for tethering. Routine manipulations of DNA, such as transformation and plasmid purification, employed the methods of Sambrook et al. (29).

Null strain construction. The source of the *fliN* gene for this work was plasmid pJM2 (26), a gift of P. Matsumura (University of Illinois). An in-frame deletion in *fliN* was made by digestion with *Eag*I and *Eco*RV, followed by treatment with mung bean nuclease to blunt the *Eag*I site and replacement of the excised sequences with a 10-nucleotide linker containing an *Eco*RI site (New England Biolabs). The presence of the desired deletion was confirmed by dideoxy sequencing (30). The *fliN* gene containing the deletion and flanking sequences of *fliM* and *fliO* were subcloned into pMAK705 (18), a gift of S. Kushner (University of Georgia). Plasmid pMAK705 encodes chloramphenicol resistance and has a temperature-sensitive origin of replication that allows transfer of sequences onto the chromosome by a regimen of temperature shifts, as described by Hamilton et al. (18). The pMAK705 derivative encoding deleted *fliN* (pHT70) was transformed into strain RP437 (wild type for motility and chemotaxis), and cells with a chromosomally integrated copy of the plasmid were selected on LB plates containing chloramphenicol (34 μg/ml) at 44°C. Integrants were colony purified and cultured without selection at 30°C for several days with dilution into fresh medium daily to promote resolution of the plasmid sequences. Several chloramphenicol-sensitive clones were tested for motility; all nonmotile clones could be complemented by the *fliN* gene on plasmid pHT92. One of these was chosen for further study. The presence of the deletion at the appropriate chromosomal locus was verified by PCR amplification of genomic DNA with primers homologous to sequences flanking *fliN*.

Overexpression and purification of FliN. The *fliN* gene was subcloned into expression vector pAED4 (12) to allow overexpression from the T7 promoter. The resulting plasmid was transformed into strain BL21-DE3 (36), which contains a chromosomal copy of the T7 RNA polymerase gene under control of the *lac* promoter. Cells were cultured at 37°C in LB containing 200 μg of ampicillin per ml to an optical density at 600 nm of 0.6 to 0.8, induced with 0.4 mM IPTG, grown for ca. 3 h more, and collected by centrifugation (4,000 × *g*, 10 min, 4°C).

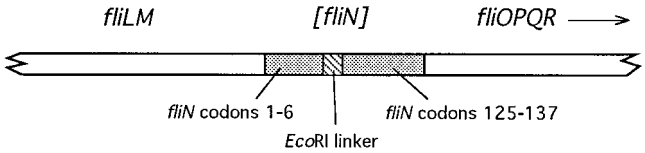


FIG. 1. In-frame deletion in *fliN* used to make the null mutant. Nucleotides 21 to 372 in *fliN* were removed by digestion with *Eco*RV, *Eag*I, and mung bean nuclease and replaced by a 10-nucleotide *Eco*RI linker.

Subsequent steps were done on ice and are described for an initial culture volume of 2 liters. Cells were resuspended in 40 ml of 50 mM Tris (pH 8)–50 mM NaCl–5 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride. Lysozyme was added to 0.6 mg/ml, and after 30 min on ice, cells were disrupted by sonication (Branson 450, power 6, 50% duty cycle, 1.5 min) and treated with DNase I (0.01 mg/ml) for 30 min on ice. Cell membranes and insoluble material were pelleted by centrifugation at 23,000 × *g* for 30 min. Most of the FliN was found in the supernatant, indicating that the protein did not form inclusion bodies despite its high concentration in the cells. Solid $(NH_4)_2SO_4$ was added to 30% saturation, and precipitated protein was collected by centrifugation (13,000 × *g*, 10 min). Pellets were resuspended in column buffer (50 mM Tris [pH 8], 1 mM EDTA, 0.5 mM phenylmethylsufonyl fluoride) and passed through a gel filtration column (Sephadex G-150; Pharmacia, Piscataway, N.J.) equilibrated with the same buffer. FliN eluted at an apparent molecular mass of about 60 kDa. Fractions containing FliN were combined, concentrated by ultrafiltration, and loaded onto an anion-exchange column (Q-Sepharose; Pharmacia) equilibrated with column buffer. The column was washed with the same buffer containing a 0 to 0.8 M gradient of NaCl. After this column step, the FliN was about 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue; the principal contaminant was lower-molecular-mass material believed to be the products of proteolysis.

Amount and distribution of FliN in cells. Purified FliN was used to raise anti-FliN antibodies in rabbits (HRP Inc., Denver, Pa.). The antiserum was purified by preabsorption against total cellular proteins from the *fliN* null strain linked to CNBr-activated Sepharose 4B (Pharmacia) in accordance with the manufacturer's instructions. Following this treatment, the antiserum was specific for FliN, as judged by immunoblots of proteins from the wild-type and *fliN* null strains. Immunoblotting and FliN quantification were done as described previously (37). The density of gel bands was related to the mass of FliN present by constructing a standard curve based on known amounts of purified FliN electrophoresed on the same gel. FliN concentration was determined spectrophotometrically by using an extinction coefficient of 8,250 M^{-1} cm^{-1} at 280 nm (17). To convert the amount of FliN to a number of molecules per cell, cells were counted in a Petroff-Hausser counting chamber (Hausser Scientific). To determine relative abundances of FliN in the membrane and cytoplasmic fractions, cells were lysed by treatment with lysozyme-EDTA and sonication, and membranes were separated from cytoplasm by centrifugation at 16,000 × *g* for 10 min at 4°C.

Flagellation and swarming. Staining and counting of flagella and measurement of swarming rates in soft agar were carried out as described previously (37). Plots of swarm diameter versus time were fitted to a line, and the slopes are reported in millimeters per hour.

Motor torque and rotation rate. *E. coli* cells were washed into motility medium and tethered by their flagellar filaments in a flow cell (1) as described previously (3). Cells were videotaped, and their rotation was analyzed by frame-by-frame analysis of video recording as described previously (37).

RESULTS

A *fliN* null mutant. To construct a *fliN* null mutant, an in-frame deletion was made in the *fliN* gene carried on a plasmid and the disrupted gene was transferred into the chromosome by homologous recombination. Of 137 codons in the *fliN* gene, 116 were removed by the deletion (Fig. 1). Integration at the correct locus was verified as described in Materials and Methods.

Cells carrying the *fliN* deletion did not produce the FliN protein, as evidenced by immunoblots with anti-FliN serum (Fig. 2). Interestingly, the FliM protein also was affected, being reduced to less than one-half of its wild-type level (as determined by video densitometry) upon disruption of *fliN*. When the wild-type *fliN* gene was reintroduced on a plasmid (pHT92; Table 1), the FliN protein reappeared and FliM also was restored to its normal level (Fig. 2), showing that the loss of FliM

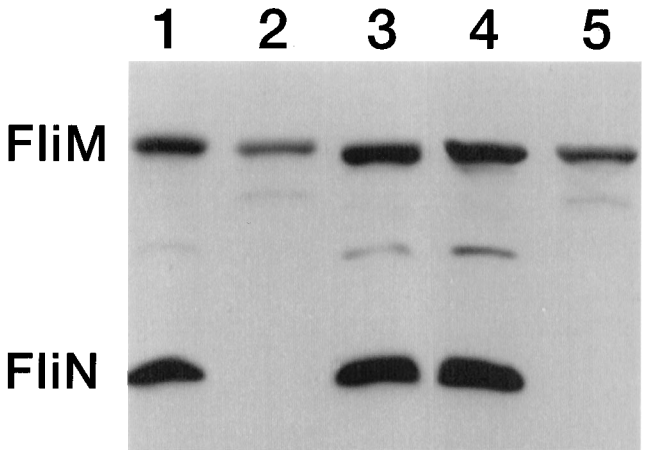


FIG. 2. Immunoblots of proteins in the *fliN* null mutant and other strains. Lanes: 1, strain RP437, wild-type for flagellation and motility; 2, strain DFB223, the *fliN* null strain; 3, strain DFB223(pHT92), cultured in 5 μM IPTG to induce expression of FliN at close to the wild-type level; 4, strain DFB190(pDFB72), a chromosomal *fliM* null strain that expresses *fliM* from the *tac* promoter on a plasmid, cultured in 10 μM IPTG to induce FliM expression at close to the wild-type level; 5, strain DFB232(pDFB72), a chromosomal *fliM fliN* double null mutant containing a plasmid that expresses *fliM* from the *tac* promoter, cultured in 10 μM IPTG to induce expression of FliM. Bands between FliM and FliN are breakdown products of FliM.

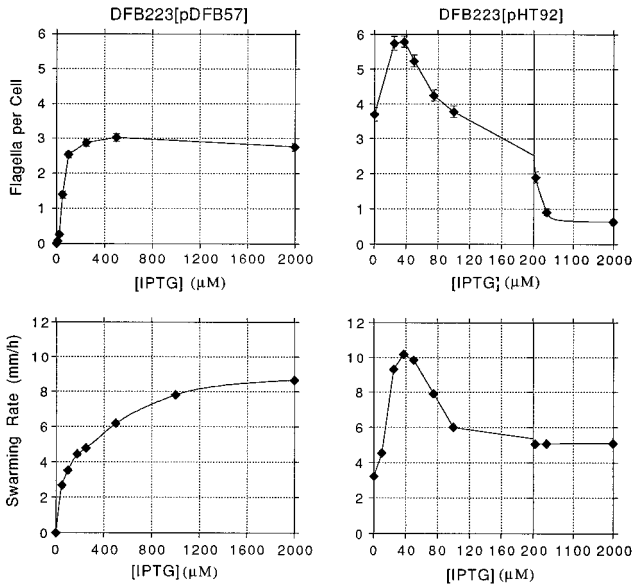


FIG. 3. Numbers of flagella per cell and rates of swarming in soft agar of cells expressing FliN at various levels. To allow expression of FliN over a wide range, *fliN* null strain DFB223 was used with the two plasmids indicated, which express FliN at different levels (see the text). The top panels show numbers of flagella per cell for the two strains as a function of IPTG concentration. Flagella were stained and counted as described in Materials and Methods. The values plotted are averages (± the standard error of the mean) obtained by counting the flagella on ≥100 cells. In a parallel experiment, the wild-type strain had an average of 2.4 ± 0.07 flagella per cell (200 cells). The bottom panels show swarming rates of the same strains in soft agar as a function of IPTG concentration. The values reported are averages of two determinations that differed by an average of 3% and not more than 10%. In a parallel experiment, the swarming rate of the wild type was 10.6 ± 0.2 mm/h ($n = 6$).

was due to the absence of the FliN protein rather than any unintended disruption of the *fliM* gene. FliN could influence FliM levels either by stabilizing the protein or by enhancing transcription of the *fliL* operon, which encodes *fliM*, *fliN*, and several other genes. To test for a transcriptional effect, FliM levels were measured in cells of a *fliM fliN* double null strain harboring plasmid pDFB72, which expresses *fliM* from a different promoter (P*tac*). The amount of FliM in this strain was compared with that in a strain harboring the same plasmid but with only *fliM* deleted. When *fliM* was expressed from the *tac* promoter, the results were the same; the FliM level was reduced in the strain with *fliN* deleted (Fig. 2, lanes 4 and 5). FliN must therefore act posttranscriptionally to affect the level of FliM.

Deletion of *fliN* caused the cells to become nonflagellate, as judged from the absence of flagella on >100 cells stained by a wet-mount procedure (see Materials and Methods). In principle, this might reflect a requirement for either FliN or FliM in flagellar assembly, since the levels of both proteins are reduced by the *fliN* deletion. Cells moderately underexpressing FliM still produce flagella (37), however, so the absence of flagella must reflect a requirement for FliN in flagellar assembly. We conclude that the null phenotype of *fliN* is nonflagellate.

Effects of altered expression of FliN. The effects of under- and overexpression of FliN were studied in the null background by using regulatable plasmids, one (pDFB57) that uses the *lac* promoter and the native *fliN* ribosome-binding site to allow low-level expression and another (pHT92) that uses the *tac* promoter and a stronger ribosome-binding site to express the protein at higher levels. At various levels of induction of each of the plasmids, flagella were counted and swarming rates in soft agar were measured. When plasmid pDFB57 was used, both flagellation and swarming were negligible in the absence of IPTG but increased sharply upon induction (Fig. 3). At 100 μM IPTG and above, the number of flagella per cell matched the wild-type levels but swarming remained slower than that of the wild type. Thus, at these levels of FliN expression, swarming and flagellation were not strictly correlated, with the swarming rate being more severely affected.

When pHT92 was used to express FliN at higher levels, more complex results were obtained. The number of flagella per cell showed a maximum at 25 to 40 μM IPTG, where it surpassed that of the wild type, and then decreased at higher levels of induction (Fig. 3). The swarming rate also had a maximum, matching that of the wild type at 40 μM IPTG and falling off at lower or higher levels. Overexpression of FliN thus impairs both flagellation and motility. Overexpression of FliM also has been reported to impair motility (10, 37) and, to a much lesser degree, flagellation (37).

Quantification and localization of FliN. To express these effects in more quantitative terms, the amounts of FliN in wild-type cells and in cells induced to make the protein at various levels were measured by using immunoblots. A typical wild-type cell was estimated to contain about 10,000 molecules of FliN (±4,000, $n = 2$). As expected from the measurements of swarming and flagellation, plasmid pDFB57 underexpressed FliN, at levels ranging from below the threshold of detection to one-third that of the wild type. Plasmid pHT92 expressed FliN at levels ranging from less than one-fifth to 40 times that of the wild type. When wild-type cells were separated into cytoplasmic and membrane fractions, most of the FliN was found in the cytoplasm but a significant fraction (ca. 10%) was found with the membranes. Interestingly, localization of FliN to the membrane depended upon FliM: in the *fliM* null strain, none of the FliN was found in the membrane fraction (Fig. 4).

Performance of flagellar motors in cells underexpressing FliN. At very low levels of FliN, few flagella are produced, whereas at slightly higher levels, many flagella are produced but evidently perform poorly (Fig. 3; pDFB57 induced with ca. 250 μM IPTG). To examine more closely the performance of

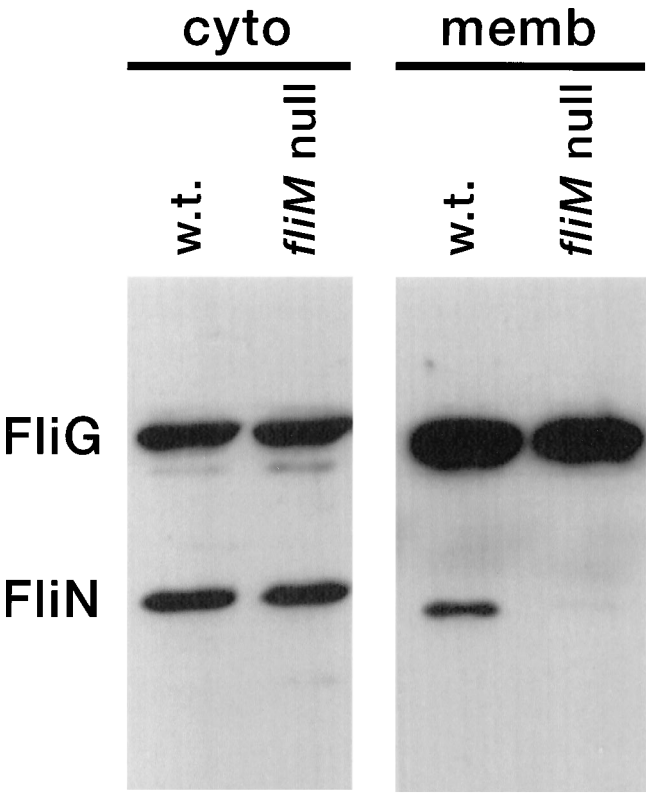


FIG. 4. Localization of the FliN protein in wild-type (w.t.) cells and in a *fliM* null mutant. Cells were fractionated, and FliN was detected by using immunoblots as described in Materials and Methods. Equal amounts of total cellular protein were loaded in each lane; another flagellar protein (FliG), whose localization is evidently not affected by deletion of *fliM*, also was probed to serve as an internal standard. In the wild-type strain, the FliN found in the membrane fraction (memb) represents approximately 10% of the total. In the *fliM* null strain, the amount of FliN found in the membrane fraction was negligible. cyto, cytoplasmic fraction.

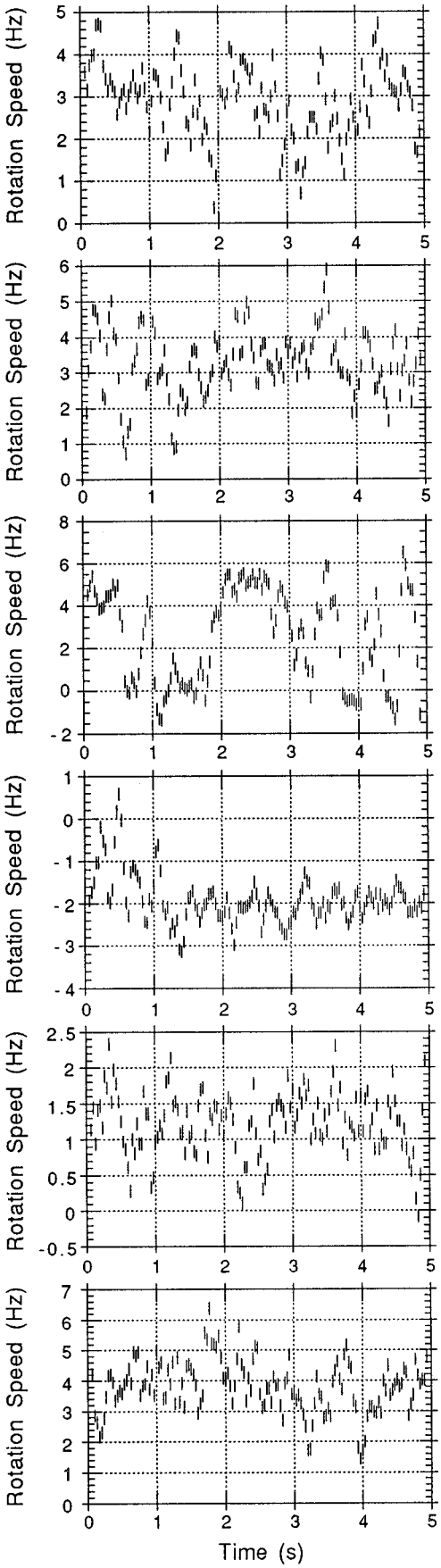
individual flagellar motors in cells underexpressing FliN, cells of strain DFB223(pDFB57) were cultured in 250 μM IPTG, tethered to coverslips by their flagellar filaments, and videotaped. Immunoblots (not shown) indicated that at this level of induction of *fliN*, the FliM protein was present at close to normal levels, so any performance defects should be due to the shortage of FliN alone. Frame-by-frame playback of the videotapes was used to determine rotation speeds. When FliN was underexpressed, many motors turned at subnormal, fluctuating speeds. Several examples are shown in Fig. 5; for comparison, note that wild-type cells typically rotate at 10 to 15 Hz. The rapid, large-amplitude fluctuations in speed are similar to what was observed previously when FliM was underexpressed (37), suggesting that similar defects result when FliN or FliM is in limited supply.

Co-overexpression of FliN and FliM. The underexpression effects, the decreased level of FliM in the *fliN* null strain, and the altered localization of FliN in a *fliM* null strain all suggest that FliM and FliN interact and possibly function together in a complex. Binding between FliM and FliN could account for the motility impairment that occurs when either protein is overexpressed, because one of the proteins present in excess could sequester the other and prevent its incorporation into the flagellum. This hypothesis was tested by overexpressing the FliM and FliN proteins together. Strain DFB190(pDFB72) is a *fliM* null strain containing a plasmid that expresses *fliM* from the *tac* promoter. This strain swarms well in 50 μM IPTG but is impaired at higher levels of induction (37). When *fliM* was expressed from another plasmid (pDFB92) that uses the same promoter and ribosome-binding site but, in addition, expresses *fliN*, motility was much less impaired at high levels of induction (Fig. 6). Other, slightly different protocols gave a similar result: when cells overexpressing *fliM* from pDFB72 were transformed with a second, compatible plasmid that supplies additional *fliN*, motility was greatly improved (data not shown). When *fliM* was overexpressed constitutively from another plasmid (pHT41), swarming was impaired, but swarming was improved by introduction of a second plasmid encoding *fliN* behind the *tac* promoter. In this case, the improvement in swarming depended to some degree upon the induction of *fliN* expression by IPTG (data not shown).

Immunoblots were used to quantify FliM and FliN in cells overexpressing these proteins, either singly or together. In principle, additional FliN might alleviate the effects of FliM overexpression by somehow reducing the amount of FliM present in the cell. Co-overexpression of FliN did not decrease the level of FliM, however (blot not shown). Similarly, overexpression of FliM could impair motility by reducing the amount of FliN present. In cells overexpressing FliM, the amount of FliN was somewhat reduced (by ca. 25%; blot not shown) relative to that in the wild type, but this relatively small reduction in FliN cannot, by itself, account for the severe motility impairment observed.

Since overexpression of FliM impairs motility much more than flagellation (10, 37), it was suggested (10) that FliM might act by binding to MotA or MotB, proteins needed for motility but not flagellar assembly. To test that possibility, a compatible plasmid encoding both *motA* and *motB* (pJL13; Table 1) was introduced into the FliM-overexpressing strain and swarming rates were measured at various levels of induction. Additional MotA and MotB did not alleviate the effects of FliM overexpression (Fig. 7). Thus, the motility impairment does not appear to be due to binding of FliM to MotA or MotB.

Analysis of amino-terminal truncations of FliN. Mutations in *fliN* have been reported to cause a variety of defects, including loss of flagella, paralysis of flagella, or assembly of flagella that rotate at normal speeds but are abnormally biased in either the clockwise or counterclockwise direction (42). The nucleotide changes in several *fliN* mutations were recently determined (20). Among the mutations classified as *mot* (because they cause paralysis) were some frameshifts that should cause translation to terminate after codon 55. Since the present results show that the null phenotype of *fliN* is nonflagellate, the frameshift mutants evidently express a variant of the FliN protein that can function in flagellar assembly. Irikura et al. (20) suggested that following termination due to the frameshift, translation of FliN might reinitiate somewhere within the gene to produce a shortened form of FliN that retains some function. The Met-58 codon is a likely site of reinitiation, being preceded by a sequence that matches the Shine-Dalgarno consensus (33) in several positions. To determine if translation can begin within the gene and give rise to a stable FliN fragment, a segment of the *fliN* gene containing codons 34 through 137 was subcloned into pGEM7, producing pSB4 (Table 1). Plasmid pSB4 complemented the *fliN* null strain to good motility and swarming in soft agar (Fig. 8). Immunoblots with anti-FliN serum showed the appearance of a protein of about 7 kDa (Fig. 8), consistent with the initiation of translation at Met-58. A smaller *fliN* segment containing codons 71 through 137 was also tested, but it failed to complement the *fliN* mutant (data not shown). We conclude that a FliN fragment, probably consisting of amino acids 58 to 137, is expressed and is sufficient



for both flagellar assembly and rotation. The *S. typhimurium* mutations previously classified as *mot* were chromosomal (20) and might produce less of the FliN fragment than the plasmid used here, which could account for the nonmotile phenotype seen in those experiments.

Homology of FliN to a protein involved in transport. The dispensable segment at the amino terminus of FliN is poorly conserved among the FliN proteins from *E. coli*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, and *Caulobacter crescentus* (alignment not shown). When a homology search was carried out with only the well-conserved, essential part of FliN (residues 58 to 137), a homology was found that had escaped detection in our earlier searches with the full sequence. The essential part of FliN has homology to the carboxy-terminal part of Spa33 (31, 38), a protein that functions in the presentation of certain antigens on the cell surface of *S. flexneri*, probably as part of a transmembrane export apparatus. The sequence alignment of the FliN fragment with the corresponding part of Spa33 is shown in Fig. 9. While not very strong, the homology spans much of the sequence of the essential part of FliN.

DISCUSSION

The null phenotype. In a previous study of *fliN* mutants, the null phenotype could not be assigned with certainty because some gross disruptions of the gene abolished flagellation while others caused only paralysis (20). A paralyzed null phenotype would not be unexpected, since many *fliN* mutations have been reported to cause paralysis, and at one time the gene was known as *motD* (28). The present results demonstrate that the null phenotype is nonflagellate. The frameshift mutations previously classified as *mot* do not produce null mutants but express a carboxy-terminal fragment of FliN because of initiation of translation within the gene, probably at codon 58. The resulting phenotype evidently depends upon the level of expression: when expressed from the chromosome, the FliN fragment permits flagellar assembly but not rotation (20); when overexpressed from a plasmid, it permits both assembly and function.

Interaction between FliN and FliM. When the *fliN* gene is deleted, much of the FliM protein is lost from the cells. A similar effect was seen with motility proteins MotA and MotB, in which case deletion of *motA* reduced the level of MotB (40). That result is part of a growing body of evidence that the MotA and MotB proteins interact (16, 35, 40). The present result similarly suggests that FliM and FliN interact and that this interaction is important for the stability and/or proper folding of FliM. The interaction between FliM and FliN appears also to be important for the localization of the latter protein; a fraction of FliN is associated with the membranes in wild-type cells but not in cells lacking FliM.

The overexpression effects also suggest that FliN functions in association with FliM. Overexpression of FliM impairs motility (10, 37) and, to a lesser degree, flagellation (37). The motility impairment can be largely reversed by overexpression of FliN along with FliM (Fig. 6), which implies that excess FliM acts, at least in part, by reducing the amount of FliN that is freely available. The main effect of FliM overexpression is to impair motility while permitting flagellar assembly, which is

FIG. 5. Rotation speed versus time of tethered cells underexpressing FliN, determined by frame-by-frame analysis of video recordings. The strain was DFB223(pDFB57), cultured in Tryptone broth containing 250 μM IPTG to induce expression of FliN. Speed is plotted at intervals of 1/30 s, corresponding to the video frame rate.

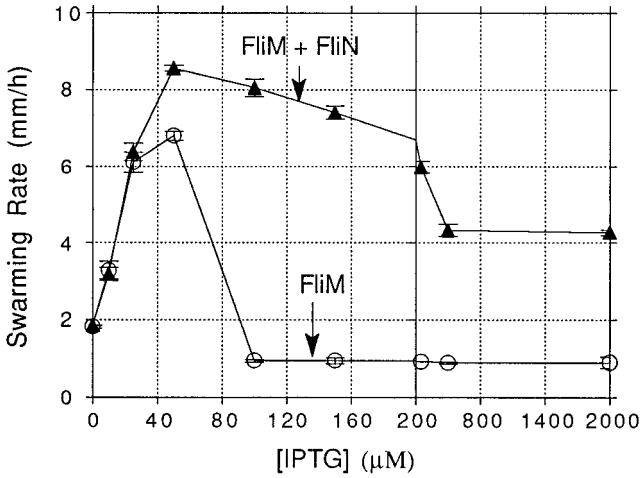


FIG. 6. Swarming rates of cells overexpressing FliM or both FliM and FliN at various levels. The strain overexpressing FliM was DFB190(pDFB72), and the strain overexpressing both FliM and FliN was DFB190(pDFB92). The values shown are averages of three determinations (± the standard error of the mean).

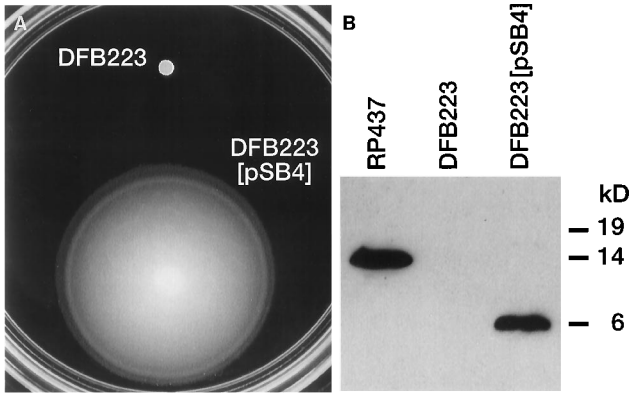


FIG. 8. (A) Complementation of the *fliN* null strain by a plasmid that encodes a fragment of the *fliN* gene. Plasmid pSB4 is a derivative of pGEM7 that contains codons 37 to 137 of *fliN* (see the text). Following inoculation with 1 μl of 100-fold-diluted saturated cultures, the plate was incubated at 32°C for 22 h and photographed. The strain containing pSB4 swarms at approximately one-third of the wild-type rate. (B) Immunoblot showing the appearance of a ca. 7-kDa FliN fragment in the strain complemented by pSB4. Lanes: 1, proteins of the wild-type strain; 2, proteins of the *fliN* null strain; 3, proteins of the *fliN* null strain containing plasmid pSB4. The blot was probed with anti-FliN serum.

also the effect of moderate underexpression of FliN (Fig. 3). These results can be explained by postulating that FliM and FliN can form a complex apart from the flagellum and that FliM and FliN can each bind to flagella that lack the full complement of the other protein. According to this hypothesis, excess FliM would impair motility by binding to FliN and preventing its incorporation into the flagellum. When FliN is overexpressed, it has an analogous effect, blocking installation of FliM and impairing both motility and flagellation (Fig. 3).

At the highest levels of FliM overexpression, motility is not fully restored by co-overexpression of FliN, which suggests that other, unspecified proteins might also interact with FliM or FliN. Obvious candidates include the switch complex protein FliG (14), and the FliO, FliP, FliQ, and FliR proteins, whose functions are unknown but whose genes are cotranscribed with *fliN*. We have co-overexpressed FliO, FliP, FliQ, and FliR along with FliN; motility was not improved but was made even worse (unpublished results). We are presently investigating different protocols for co-overexpression of all three switch complex proteins together. Since overexpression of FliM affects motility more than flagellation, it has been suggested that FliM might act by binding to the motility proteins MotA and MotB (10). However, additional MotA and MotB did not alleviate the effects of overproducing FliM, a failure that is consistent with the previous suggestion (37) that FliM is part of a structure distinct from the MotA/MotB torque generators.

Using purified proteins, Oosawa et al. (27) detected no interactions between FliN and FliM, FliG, or FliF. It is possible that strong binding of FliN to its sites in the flagellum requires additional proteins that were not present in those experiments. The abundance of FliN in the cell suggests that its affinity for motor sites could be quite low. Francis et al. (15) have reported the isolation of flagellar basal structures that contain FliN; the protein was lost unless comparatively gentle treatments were used. Alternatively, it is possible that certain associations require cofolding of the proteins and thus cannot be reconstituted by using separately isolated components.

Underexpression effects. Many of the effects of underexpression of FliN are similar to what was seen previously with FliM (37). The subnormal motor torque in cells underexpressing FliN implies that in a normal motor, the protein is present in multiple copies that can function independently to some degree. The reasoning is as stated previously for FliM: if each motor contained only a single FliN molecule or contained multiple molecules but could function only with the full complement present, then the motors should rotate normally or not at all. The speed fluctuations seen here are like those seen when FliM was underexpressed and unlike what was seen in underexpression experiments with MotA or MotB (2, 7). We

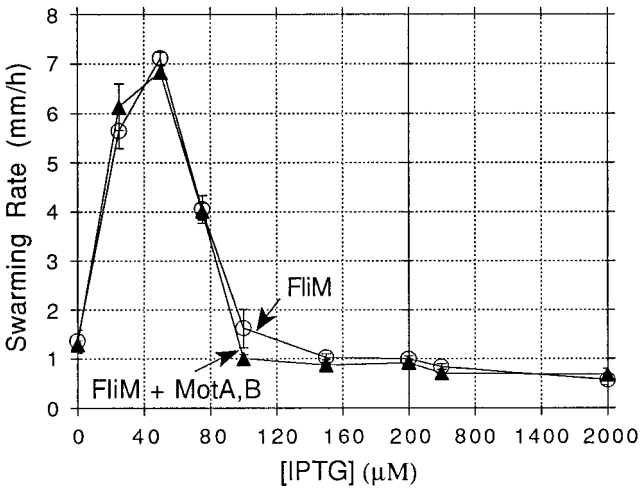


FIG. 7. Swarming rates of cells overexpressing FliM alone or together with MotA and MotB. The values plotted are averages of three determinations (± the standard error of the mean). The FliM-expressing strain was DFB190(pDFB72, pACYC184); the strain overexpressing FliM, MotA, and MotB was DFB190 (pDFB72, pJL13).

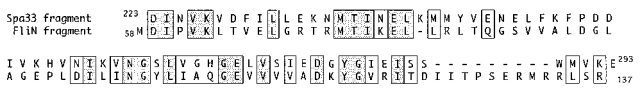


FIG. 9. Sequence alignment of the carboxy-terminal 80 residues of FliN with the carboxy-terminal part of *S. flexneri* Spa33 (31, 38). Identical residues are boxed and shaded; similar residues are boxed.

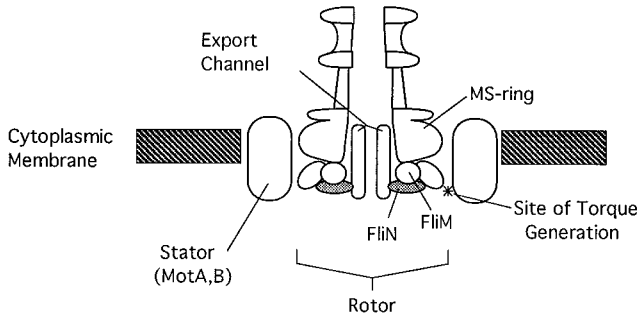


FIG. 10. Hypothesis for the location and function of FliN in the flagellar motor. A longitudinal cross-section through a flagellar basal body is shown. Protein shapes are schematic only. FliN is suggested to be a component of the rotor, making contacts with FliM and with one or more other proteins whose identities are not specified but which are suggested to be components of the flagellum-specific export apparatus. In this model, FliN would have a role in the export of flagellar components during assembly. The protein is pictured away from the interface between the rotor and stator and thus would have only an indirect role in torque generation. While only two copies of FliN are shown, more are likely to be present in a fully assembled flagellum. The rotor component at the site of torque generation is not specified but might be FliG (15, 16, 27).

conclude that like FliM, FliN is part of a structure distinct from the MotA/MotB torque generators.

Underexpression of FliN impairs motility more than flagellation, to a certain degree mimicking the *mot* (paralyzed) phenotype. It is therefore possible that some *fliN* mutations classified as *mot* do not affect the torque-generating machinery directly but produce a *mot* phenotype by decreasing the binding of FliN to sites in the flagellum, by destabilizing the protein or reducing its affinity for the sites. Mutations of that kind should produce phenotypes that depend on expression level. We are presently examining the effects of moderate overexpression of mutant variants of FliN, alone or in combination with other flagellar proteins. Initial results support the idea that *mot*-like mutations of *fliN* do not affect the process of torque generation per se but act by decreasing the binding of proteins to their sites in the flagellum.

The essential parts of FliN. The first 57 residues of *E. coli* FliN are evidently dispensable for function. This segment is poorly conserved between species, whereas most of the rest of the protein is well conserved. The function of the amino-terminal segment is unclear but might be to enhance the solubility of the protein, since a large fraction of the residues are polar. The essential part of FliN is small and thus might be suitable for structural studies using nuclear magnetic resonance. We have overproduced and purified the FliN fragment but found it to be much less soluble than the full-length protein, aggregating even at modest concentrations.

Although the homology of *E. coli* FliN to *S. flexneri* Spa33 is not very strong overall, through much of the essential part (residues 58 to 137), the similarity appears to be significant (Fig. 9). Spa33 is thought to function as part of an apparatus for the export of certain proteins across the cell membrane of *S. flexneri* (31, 38). Flagellar assembly also involves an export process, by which many components are exported to their proper locations through a channel in the middle of the organelle. FliN is among the proteins identified by Vogler et al. (39) as having a possible role in this process. Another connection betwen the *spa* genes and the genes of the *fliL* operon was noted by Dreyfus et al. (13); *fliP*, a gene cotranscribed with *fliN*, encodes a protein homologous to Spa24, another *S. flexneri* protein involved in export. Thus, the resemblance of FliN to Spa33 most likely reflects the involvement of both in some aspect of transmembrane export. Segments of FliN that are most unlike Spa33 and might be specialized for motility functions include residues 79 to 99 and 125 to 137. The former segment is well conserved among the known FliN sequences and might have a specific role in flagellar assembly or function, while the latter is poorly conserved.

Hypothesis for FliN function. A hypothesis for the location and function of FliN is shown in Fig. 10. We suggest that FliN is located on the rotor of the flagellar motor, where it contacts FliM and possibly one or more proteins involved in flagellar export. Those proteins might include the hydrophobic, presumably membrane-bound proteins FliO, FliP, FliQ, and FliR; these proteins have not been assigned specific roles, but their hydrophobic character and the homology of FliP to a protein engaged in export (13) suggest that they might form a transmembrane pore through which flagellar components are exported. If several copies of FliN are present in a mutually reinforcing structure, such as a ring, then a partial complement of FliN could be unstable, causing the observed fluctuations in speed.

In Fig. 10, FliN is pictured where it could contact components of the export apparatus, fairly close to the axis of the flagellum but at some distance from the interface between the rotor and stator, where torque is generated. Nevertheless, *mot* or paralyzed *fliN* mutants are abundant (20). Two considerations can reconcile the present hypothesis with the genetic data. First, FliN might have an indirect but essential role in motor function, acting through its contacts with FliM or other proteins to maintain the position of components at the rotor-stator interface (Fig. 10). Mutations in FliN that affect this positioning could then disrupt torque generation. Alternatively, as stated above, it is possible that *mot*-like alleles of *fliN* do not affect torque-generating activities, as has been assumed, but instead reduce the occupancy of binding sites in the flagellum for FliN, FliM, or other proteins. The hypothesis makes a number of predictions concerning the interaction between FliN and other components that we are presently testing by characterizing interactions among the switch complex proteins in vitro.

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