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Regulated Underexpression of the FliM Protein of *Escherichia coli* and Evidence for a Location in the Flagellar Motor Distinct from the MotA/MotB Torque Generators

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The FliM protein of *Escherichia coli* is essential for the assembly and function of flagella. Here, we report the effects of controlled low-level expression of FliM in a *fliM* null strain. Disruption of the *fliM* gene abolishes flagellation. Underexpression of FliM causes cells to produce comparatively few flagella, and most flagella built are defective, producing subnormal average torque and fluctuating rapidly in speed. The results imply that in a normal flagellar motor, multiple molecules of FliM are present and can function independently to some degree. The speed fluctuations indicate that stable operation requires most, possibly all, of the normal complement of FliM. Thus, the FliM subunits are not as fully independent as the motility proteins MotA and MotB characterized in earlier work, suggesting that FliM occupies a location in the motor distinct from the MotA/MotB torque generators. Several mutations in *fliM* previously reported to cause flagellar paralysis in *Salmonella typhimurium* (H. Sockett, S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab, J. Bacteriol. 174:793–806, 1992) were made and characterized in *E. coli*. These mutations did not cause flagellar paralysis in *E. coli*; their phenotypes were more complex and suggest that FliM is not directly involved in torque generation.

Many species of bacteria swim by rotating helical filaments, each driven at the base by a reversible rotary motor (1) that utilizes the transmembrane proton gradient as an energy source (reviewed in references 5, 19, 25, and 31). The complete organelle is called a flagellum. In *Escherichia coli* or *Salmonella typhimurium*, assembly and operation of a flagellum require the products of about 50 genes (5, 25). Three flagellar genes, *fliG*, *fliM*, and *fliN*, are of special interest because mutations in them have been reported to disrupt function in several different ways, producing either a nonflagellate, flagellate but paralyzed, or nonchemotactic phenotype, depending upon the allele (42). Certain mutations in these genes can be suppressed by mutations in each of the others, suggesting that their products function together in a complex (41); this complex has been termed the switch complex because the FliG, FliM, and FliN proteins appear to be involved in switching between the clockwise (CW) and counterclockwise (CCW) directions of motor rotation.

Mutations that cause paralysis without obstructing flagellar assembly, called *mot* because they affect motility specifically, can provide clues to the mechanism of torque generation. The existence of *mot* mutations in a protein suggests that it has a role in torque generation: these mutations presumably alter residues involved in some aspect of motor rotation while preserving any features needed for flagellar assembly. Since mutations with the *mot* phenotype have been reported in all three switch complex proteins, it has been supposed that they are all involved, to various degrees, in torque generation. A recent extensive analysis of *fliM* mutants suggested that FliM has a relatively small role in torque generation and a much larger role in controlling motor direction since many mutations affected CW-CCW switching (33). Additional evidence for a role in direction control comes from in vitro binding studies which demonstrated that FliM can bind to the chemotaxis signaling protein CheY when CheY is phosphorylated (40). Phospho-CheY causes the motor to switch to the CW direction, probably by triggering a conformational change in FliM and/or the other switch complex proteins. Recently, Oosawa et al. presented evidence for binding of FliM to the basal-body MS ring in vitro (26). Beyond this, little is known concerning the specific functions of FliM in flagellar assembly and function. Also, much remains to be learned concerning the stoichiometries and locations of the switch complex proteins in the flagellum.

Previously, some insight into the functions of motility proteins MotA and MotB of *E. coli* was obtained by expressing them at a low level and measuring the effects on motor performance. In cells underexpressing MotA or MotB, the motors rotated at slow but relatively stable speeds. When the level of MotA or MotB was increased in these cells, the torque (as judged by rotation speed) increased in a series of eight well-defined steps (3, 7). On the basis of these results, it was suggested that MotA and MotB are components in several independently functioning torque generators in the motor. Analogous experiments with the switch complex proteins have not been done.

Here, we report the effects of underexpression of the FliM protein in a *fliM* null strain of *E. coli*. Underexpression of FliM causes a decrease in the number of flagella per cell. The flagella that are made rotate at subnormal speeds, implying that a normal, fully assembled flagellar motor contains multiple copies of FliM that can function independently to some degree, each contributing a fraction to the total motor torque. Moreover, in motors that contain less than the normal complement of FliM, the speed fluctuates rapidly, suggesting that binding or positioning of the FliM molecules is unstable when relatively few copies are present. This behavior is unlike the more stable rotation seen when MotA or MotB is underexpressed and suggests that FliM is part of a structure distinct from the MotA/MotB torque generators.

motA and *motB* torque restoration experiments (3, 7) used

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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
RP5232	Δ*cheY*	J. S. Parkinson
JC7623	*recBC sbcBC*	23
DFB190	*fliM* null strain	This work
DFB191	Δ*cheY* in *fliM* null	This work
BL21-DE3	T7 RNA polymerase gene in chromosome; used for expression of pAED4 derivatives	35
pAED4	Vector for overexpression from T7 promoter	11
pHT28	*fliM* expression vector derived from pAED4	This work
pAlter-1	Vector for site-directed mutagenesis	Promega
pHT41	*fliM* in pAlter-1	This work
pCAT	Source of *cat* gene	Promega
pHT16	*cat* gene disruption of *fliM*; used to make *fliM* null strain	This work
pTBM30	P*tac* expression vector; parent of pDFB72	J. S. Parkinson
pDFB63	P*lac-fliM lacI*^{q}; weak ribosome-binding site	This work
pDFB72	P*tac-fliM lacI*^{q}; strong ribosome-binding site	This work

mutants with paralyzed flagella. For analogous experiments with *fliM*, we made several mutations in *fliM* which, on the basis of earlier studies on *S. typhimurium*, were expected to give the paralyzed phenotype. We did not observe a fully paralyzed phenotype with any of these *fliM* mutations. All permitted flagellar rotation under some circumstances, and many caused a significant reduction in the number of flagella. A screen of randomly generated mutants also did not yield *fliM* alleles with the fully paralyzed phenotype. The difficulty of isolating fully paralyzed, flagellated alleles suggests that FliM does not have a direct role in torque generation. These results are discussed with reference to a hypothesis for the function and location of FliM in the motor.

MATERIALS AND METHODS

Strains and media. The *E. coli* strains and plasmids used are listed in Table 1. LB broth was used for routine culture growth, transformations, and plasmid isolations and contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl. Tryptone broth (TB) was used in all experiments in which motility was assayed, and it contained (per liter) 10 g of tryptone and 5 g of NaCl. Swarm plates contained TB, 0.3% agar, and an antibiotic when appropriate. Motility medium contained 10 mM potassium phosphate (pH 7), 67 mM NaCl, 10 mM sodium lactate, 0.1 mM EDTA, and 1 μM methionine. 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. For assays of motility or torque, cells were cultured at 32°C and harvested at mid-exponential phase.

The methods of Sambrook et al. (29) were used for plasmid isolation, transformation, and recombinant DNA manipulations. Site-directed mutations were made by using the oligonucleotide-directed Altered Sites procedure (Promega, Madison, Wis.). Mutations were confirmed by dideoxy sequencing (30) with reagents from U.S. Biochemical (Cleveland, Ohio).

Null strain construction. The source of the *fliM* gene for this work was plasmid pCK210 (9), a gift of D. Koshland. The *fliM* gene on a plasmid encoding ampicillin resistance was disrupted by replacing most of the coding sequences with a copy of the *cat* gene encoding chloramphenicol resistance, as illustrated in Fig. 1. The *cat* gene was taken from plasmid pCAT (Promega) by amplification with the PCR to separate the gene from transcriptional termination sequences found just downstream. The plasmid containing the disrupted *fliM* gene was transformed into strain JC7623 (23), which propagates plasmids poorly, by using ampicillin to select plasmid recipients. The transformants were repeatedly streaked onto LB plates containing chloramphenicol (20 μg/ml), but no ampicillin, to promote transfer of the *cat* gene into the chromosome and loss of the plasmid. Several nonmotile, ampicillin-sensitive clones were isolated that could be complemented by the *fliM* gene on a plasmid. The presence of the expected chromosomal disruption was verified by a Southern blot and by PCR amplification of genomic DNA with primers that flank the site of the insertion. The disrupted *fliM* gene was then moved into a wild-type background (strain RP437) by P1 transduction with the *cat* gene as a selectable marker. Complementation tests (see below) indicated that the mutation was not polar.

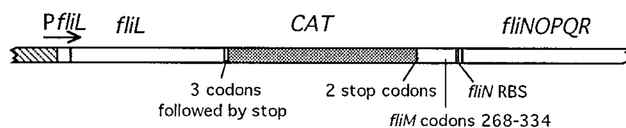

FIG. 1. Gene disruption used to make the *fliM* null strain. The *cat* gene was inserted in place of most of the coding sequences of *fliM* as shown, with two stop codons at the 3′ end to prevent formation of a fusion protein. The ribosome-binding site (RBS) of *fliN* was left intact, as indicated.

Overexpression and purification of FliM. The *fliM* gene was inserted into expression vector pAED4 (11) to allow overexpression from the T7 promoter. The resulting plasmid, pHT28, was transformed into strain BL21-DE3 (35) to allow overexpression. The purification of FliM followed a scheme similar to that of Oosawa et al. (26), with the following modifications. Cells were cultured at 37°C in 1 liter of LB containing 200 μg of ampicillin per liter to an optical density at 600 nm of 0.5, induced with 0.4 mM IPTG, grown for 3.5 h more, and then collected by centrifugation at 4°C. The cells were resuspended in 200 ml of 50 mM Tris (pH 8)–50 mM NaCl–5 mM EDTA with lysozyme and EDTA added to 0.5 mg/ml and 10 mM, respectively. After 30 min on ice, cells were disrupted by sonication and treated with DNase I (0.01 mg/ml) and $CaCl_2$ (10 mM) for 30 min on ice. Cell membranes and inclusion bodies containing FliM 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 dithiothreitol was added to 15 mM. The solution was centrifuged to remove insoluble material (10,000 × *g*, 20 min), and the supernatant was added slowly to 300 ml of ice-cold 50 mM Tris (pH 8)–1 mM dithiothreitol–0.1 mM EDTA. FliM was precipitated by addition of solid $(NH_4)_2SO_4$ to 24% 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 about 60 ml, centrifuged (27,000 × *g*, 20 min) to remove precipitated material, and concentrated to a volume of 10 ml. (Further concentration caused the FliM to precipitate.) The protein 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 gradient of NaCl. Fractions containing FliM were pooled. At this stage, the FliM was more than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue.

Preparation of anti-FliM antibody and immunoblotting. Purified FliM was used to raise anti-FliM antibodies in rabbits (HRP Inc., Denver, Pa.). The crude antiserum was purified by preabsorption against total cellular proteins from the *fliM* null strain linked to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, N.J.) in accordance with the manufacturer's instructions. Immunoblotting was carried out in accordance with the ECL Western blotting (immunoblotting) procedures (Amersham, Arlington Heights, Ill.). Films were scanned with a video densitometer and the program NIH-Image (27). The integrated optical density measured in this way was related to the mass of FliM present by constructing a standard curve based on known amounts of purified FliM electrophoresed on the same gel. FliM concentration was determined spectrophotometrically by using an extinction coefficient of 19,180 M^{-1} cm^{-1} at 280 nm (16). To estimate the amount of FliM per cell, cells were counted in a Petroff-Hausser counting chamber (Hausser Scientific). To facilitate comparison of different cultures, a standard curve relating cell number to total cellular protein was constructed. Total protein concentrations were measured with the bicinchoninic acid assay (32). To determine relative abundances of FliM 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.

Anti-FliN serum was prepared essentially as described for anti-FliM serum. Details of FliN purification and antiserum preparation are described in a separate communication.

Flagellation and swarming. Flagella were visualized by a wet-mount stain (17). Fields of well-stained cells were located, and numbers of flagella were estimated by counting the flagella on at least 100 cells, when possible including all of the cells within a field.

Rates of swarming in soft agar were measured by inoculating swarm plates with 1 μl of a 1/100 dilution of a fresh saturated TB culture and incubating the plates at 32°C. Swarms began to grow after about 8 h; their diameters were measured at intervals of 1 to 1.5 h for the following 6 to 10 h (depending on the

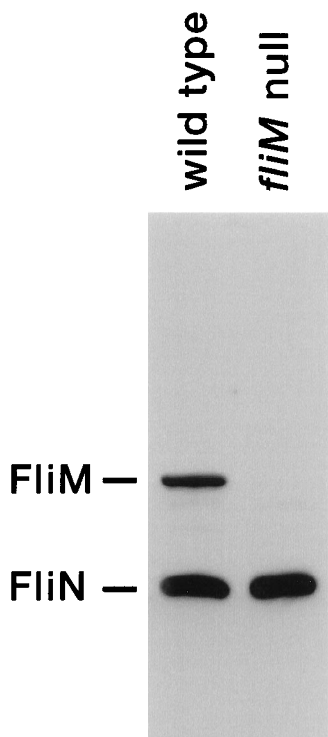

FIG. 2. Immunoblot of proteins in the wild-type and *fliM* null strains. Equal amounts of total cellular protein were loaded onto each lane, and immunoblotting was done with a mixture of anti-FliM and anti-FliN sera as described in Materials and Methods. Positions of the FliM and FliN proteins are indicated.

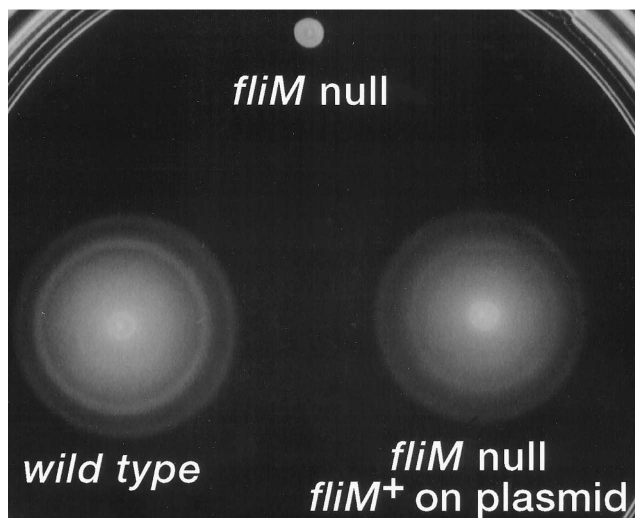

FIG. 3. Swarming in soft agar of *fliM* null strain DFB190 and complementation by plasmid pDFB72, encoding wild-type *fliM*. Wild-type strain RP437 is shown for comparison. Cells were cultured to saturation at 32°C in TB, and 1 μl of a 100-fold dilution was spotted onto a swarm plate containing 25 μM IPTG to induce FliM expression. The plate was photographed after incubation at 32°C for approximately 15 h.

rate). When swarming occurred at a rate clearly different from zero, plots of diameter versus time were well fitted by a line (average R value, 0.993). The slopes are reported in millimeters per hour.

Motor torque and rotation rate. Cells were washed into motility medium and tethered by their flagellar filaments in a flow cell (2) as described previously (4). The temperature was maintained at 32°C with a temperature controller that held the flow cell in a copper block (Physitemp, Clifton, N.J.). Cells were videotaped, and their sizes and radii of gyration were measured on a video screen with a ruler calibrated against an objective micrometer. Cell rotation speeds were estimated by using the single-frame advance feature of the video recorder used (JVC BR-S601MU) and counting the video frames that elapsed during individual revolutions. At least five cell revolutions were used to estimate average speed. Cell speed, size, and radius of gyration were used to compute motor torque by using published formulas for the drag coefficients of a cylinder (37, 38).

The behavior of some tethered cells was analyzed at high time resolution by measuring the cell angle from the video screen with a hand-held protractor. The angles measured on each video frame were entered into a computer and used to compute rotation speeds and to generate plots of speed versus time. To reduce noise due to Brownian motion and to imprecision in the angle measurements, the data presented were subjected to a three-point smoothing procedure, giving an effective time resolution of 0.1 s.

RESULTS

The null phenotype of *fliM*. A chromosomal null mutant of *fliM* was needed to serve as the background for controlled expression of the *fliM* gene carried on plasmids. To construct the null mutant gene, a plasmid-borne copy of *fliM* was disrupted by replacing most of the coding sequences with the gene encoding chloramphenicol acetyltransferase (*cat*), and the *cat* gene and flanking sequences were transferred into the chromosome by homologous recombination. The resulting chromosomal disruption of *fliM* is illustrated in Fig. 1. Insertion into the appropriate locus was verified as described in Materials and Methods. Immunoblots with polyclonal anti-FliM antibodies showed that the FliM protein was absent from the mutant (Fig. 2). The ribosome binding site near the start of *fliN* was left intact to allow normal expression of *fliN* and other downstream genes in the *fliL* operon; immunoblots with antibodies against FliN confirmed that it was expressed at normal levels (Fig. 2). Since FliN expression was normal, we concluded that expression of the genes further downstream in the operon (*fliOPQR*) also should not be affected.

Disruption of the *fliM* gene caused the cells to become nonflagellate, as judged by the complete absence of flagella on >100 cells stained by using a wet-mount procedure and by the inability of the cells to swim in liquid medium or swarm on soft agar plates (Fig. 3). Thus, the null phenotype of *fliM* is nonflagellate, as suggested previously by Sockett et al. (33), who observed a nonflagellate phenotype of several frameshift mutations and small deletions in *fliM*.

Complementation of the null strain. The wild-type *fliM* gene was introduced into the null strain on either of two plasmids, both using IPTG-inducible promoters to express the gene. One plasmid (pDFB63; Table 1) lacked a strong ribosome-binding site and expressed FliM at relatively low levels, while the other (pDFB72; Table 1) had a strong ribosome-binding site and expressed FliM at somewhat higher levels, including levels in excess of the wild-type level. At an appropriate level of induction, plasmid pDFB72 restored normal swarming to the null mutant (Fig. 3), further demonstrating that the *cat* insertion did not disrupt the expression of other essential flagellar genes.

Effects of FliM underexpression on flagellation and swarming. To examine the effects of altered FliM expression on the production of flagella, cells of the null strain harboring the *fliM* plasmids were cultured in various concentrations of IPTG and their flagella were counted. The average number of flagella per cell is plotted as a function of the inducer concentration in Fig. 4 for expression from both of the *fliM* plasmids. When FliM was expressed from pDFB63, flagella were not produced in the absence of IPTG (no flagella were seen on 100 cells) but became numerous as the IPTG concentration was increased. At low levels of induction, the number of flagella increased approximately linearly with increasing IPTG concentrations, with no indication of a threshold for the onset of flagellar synthesis. At 500 μM IPTG and above, the number of flagella

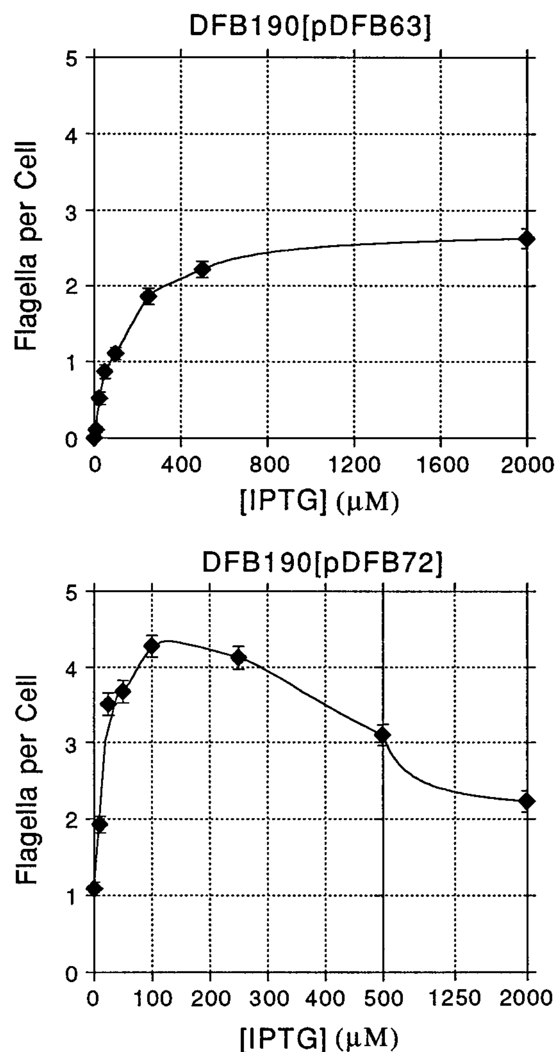

FIG. 4. Number of flagella per cell in strains expressing FliM at various levels. To enable expression of FliM over a wide range, two strains containing two different *fliM* plasmids were used, as indicated. Cells were cultured to saturation in TB containing ampicillin at 100 μg/ml, diluted 100-fold in TB-ampicillin containing the indicated concentrations of IPTG, and cultured for 4.5 h at 32°C. The data plotted are averages (± the standard error of the mean) obtained by counting the flagella on 100 cells. In parallel experiments, the wild-type strain had an average of 2.6 flagella per cell (±0.1; 200 cells).

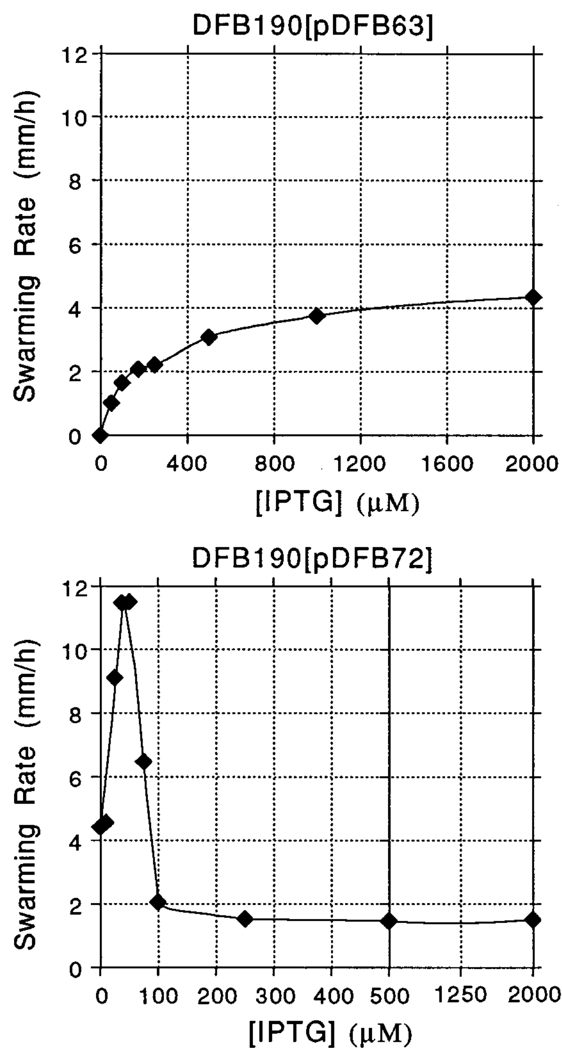

FIG. 5. Swarming rates in soft agar of cells expressing FliM at various levels. Swarming was measured as described in Materials and Methods. The data plotted are averages of two determinations that differed by an average of 3% and not more than 12%. In parallel experiments, the wild-type strain swarmed at 10.6 ± 0.2 mm/h (n = 6).

approached that measured in parallel experiments with wild-type cells (2.6 flagella per cell). When plasmid pDFB72 was used to express FliM at somewhat higher levels, more complex results were obtained. The number of flagella per cell at first increased upon induction with IPTG, from about one in the absence of the inducer to about four at 100 μM IPTG, where it exceeded the number seen on wild-type cells. At higher levels of induction, the number of flagella per cell decreased significantly but never approached zero.

To examine the effects of altered FliM expression on motility, swarming rates in soft agar were measured. When FliM was expressed at low levels from pDFB63, the swarming rate was negligible in the absence of the inducer and increased upon induction to a speed about half that of the wild-type strain (Fig. 5). When pDFB72 was used to express FliM, the swarming rate increased at first with increasing induction, matching the wild-type rate near 30 μM IPTG and slightly outperforming the wild type at 50 μM IPTG. At higher levels of induction, the swarming rate decreased sharply. At inducer concentrations of 100 μM and above, cells were well flagellated but swarmed very slowly and also swam poorly when observed under a microscope. Clegg and Koshland previously reported that FliM overproduction impairs motility (9).

FliM quantification. Immunoblots were used to quantify the FliM protein in the wild type and in cells induced to express the protein at various levels. By using known amounts of purified FliM to calibrate the immunoblots, the amount of FliM in wild-type cells was estimated. Each cell of the wild type contained approximately 1,400 molecules of FliM (±200; n = 2). When the cells were lysed, most of the FliM (ca. 1,100 molecules) was found in the cytoplasmic fraction. As expected from the measurements of swarming and flagellation, plasmid pDFB63 underexpressed FliM, at levels ranging from 1/100 to 1/2 that of the wild type. Plasmid pDFB72 expressed FliM at levels ranging from one-sixth to four times that of the wild type (immunoblots not shown).

Motor torque in cells underexpressing FliM. Underexpres-

sion of FliM evidently causes cells to produce fewer than normal flagella. The few flagella produced might each be fully functional or might exhibit defects such as reduced torque caused by the shortage of FliM. To examine the performance of individual motors in cells underexpressing FliM, cells were tethered to coverslips by their flagella and videotaped. Cell sizes and average rotation speeds were measured from the videotapes and used to compute motor torques by using published formulas to estimate the drag coefficients of the cells. Histograms of motor torques at four levels of FliM expression are presented in Fig. 6. To simplify the measurements of rotation speed, a *cheY* deletion mutant that rotates its motors unidirectionally was used; the data shown are for cells rotating exclusively CCW. Similar torques were measured when cells that contain CheY and can rotate their motors in both directions were used. Wild-type cells exhibited an average torque of 1.4×10^{-11} dyne cm, similar to that reported previously (1.2×10^{-11} to 1.3×10^{-11} dyne cm; reference 3). The broad range of torques observed is also similar to what was reported previously; the source of this variance is not clear, but it might reflect variation in the proton motive force of the cells or the number of MotA/MotB torque generators (3, 7) installed in the motors.

In cells underexpressing FliM, most of the flagellar motors had subnormal torque, sometimes only 1/10 that of the wild type, corresponding to rotation at only 1 to 2 Hz. The average motor torque increased as the level of FliM was increased, suggesting that the defect is due to the shortage of FliM. The simplest interpretation of these results is that a fully functional motor contains multiple copies of FliM that each contribute a fraction to the total torque, and in cells that underexpress the protein, each motor contains less than the normal amount of FliM and so generates subnormal torque.

The rotation of cells underexpressing FliM was analyzed more closely by frame-by-frame analysis of selected video recordings. Speed-versus-time plots for cells expressing FliM at three levels are shown in Fig. 7. In cells underexpressing FliM at either a very low level [DFB190(pDFB63) cultured in 25 μM IPTG] or a moderately low level (the same strain in 100 μM IPTG), most motors turned relatively slowly. (For comparison, the rate is typically 10 to 15 Hz in the wild type.) Furthermore, the rotation speed of most cells fluctuated rapidly. When fluctuations were observed, the time-averaged motor torque was less than normal. Thus, the speed fluctuations, like the low average speed, appear to be caused by the shortage of FliM. At a FliM level closer to that of the wild type, most motors turned steadily and at normal speeds (Fig. 7, right-hand column of panels). Some of the cells underexpressing FliM rotated slowly yet were steady in time. For example, while the speed of cell 4 (from the top) in the left-hand column of panels in Fig. 7 appears to fluctuate, a plot of speed versus cell angle (Fig. 8) shows that the variation is due to an angular dependence of speed rather than fluctuations with time. The data for cell 6 are also plotted in this way for comparison; the rotation speed of that cell did not vary periodically with angle but fluctuated with time. The angular dependence of the speed of cell 4 is most likely due to interactions with the coverslip that impose greater drag on the cell body at certain orientations.

The underexpression experiments show that flagellar motors can be built that contain less than the normal complement of FliM molecules. To determine if additional FliM molecules can be incorporated into a motor that contains relatively little FliM, cells underexpressing FliM were tethered in a flow cell and exposed to 2 mM IPTG to induce synthesis of additional FliM. The average torque of these cells was quite low initially and did not improve measurably during 1 h of incubation at

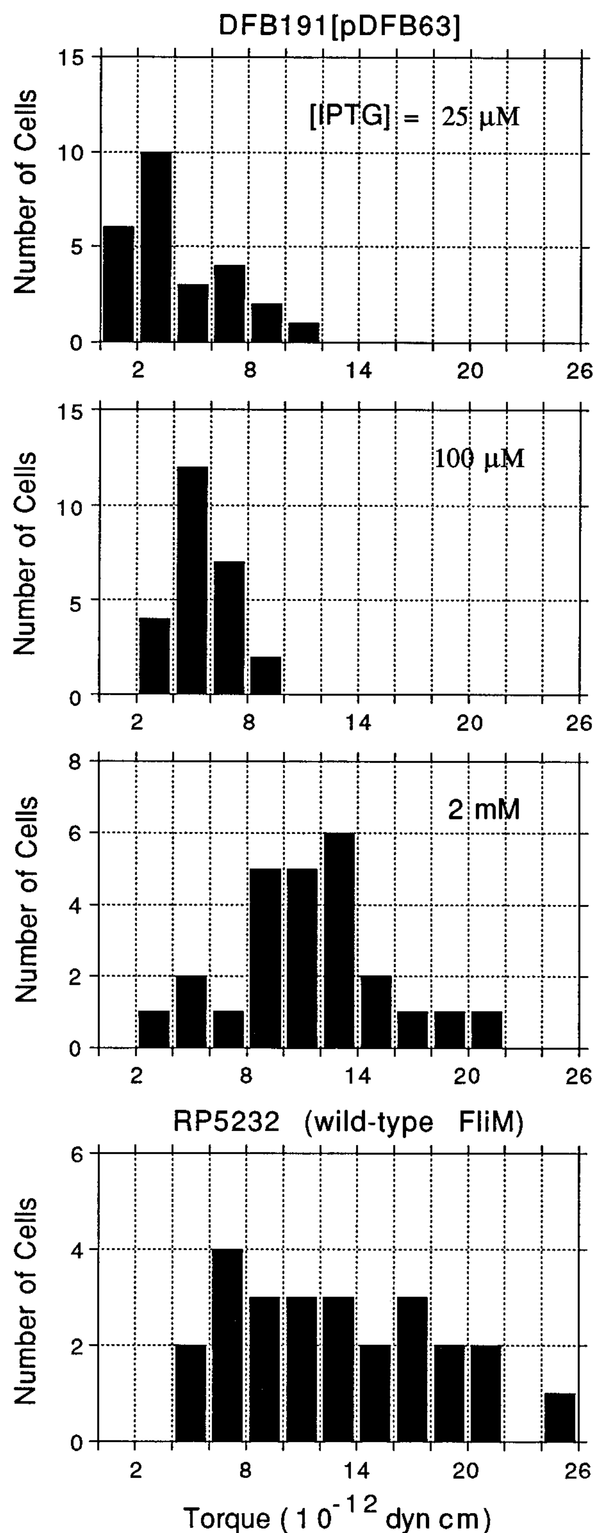

FIG. 6. Histograms of motor torques in a strain underexpressing FliM at three levels and a strain wild type for *fliM*. All strains contained a chromosomal *cheY* deletion to simplify measurements of rotation speed. Cells were tethered to coverslips by their flagellar filaments and videotaped, and motor torques were computed as described in Materials and Methods.

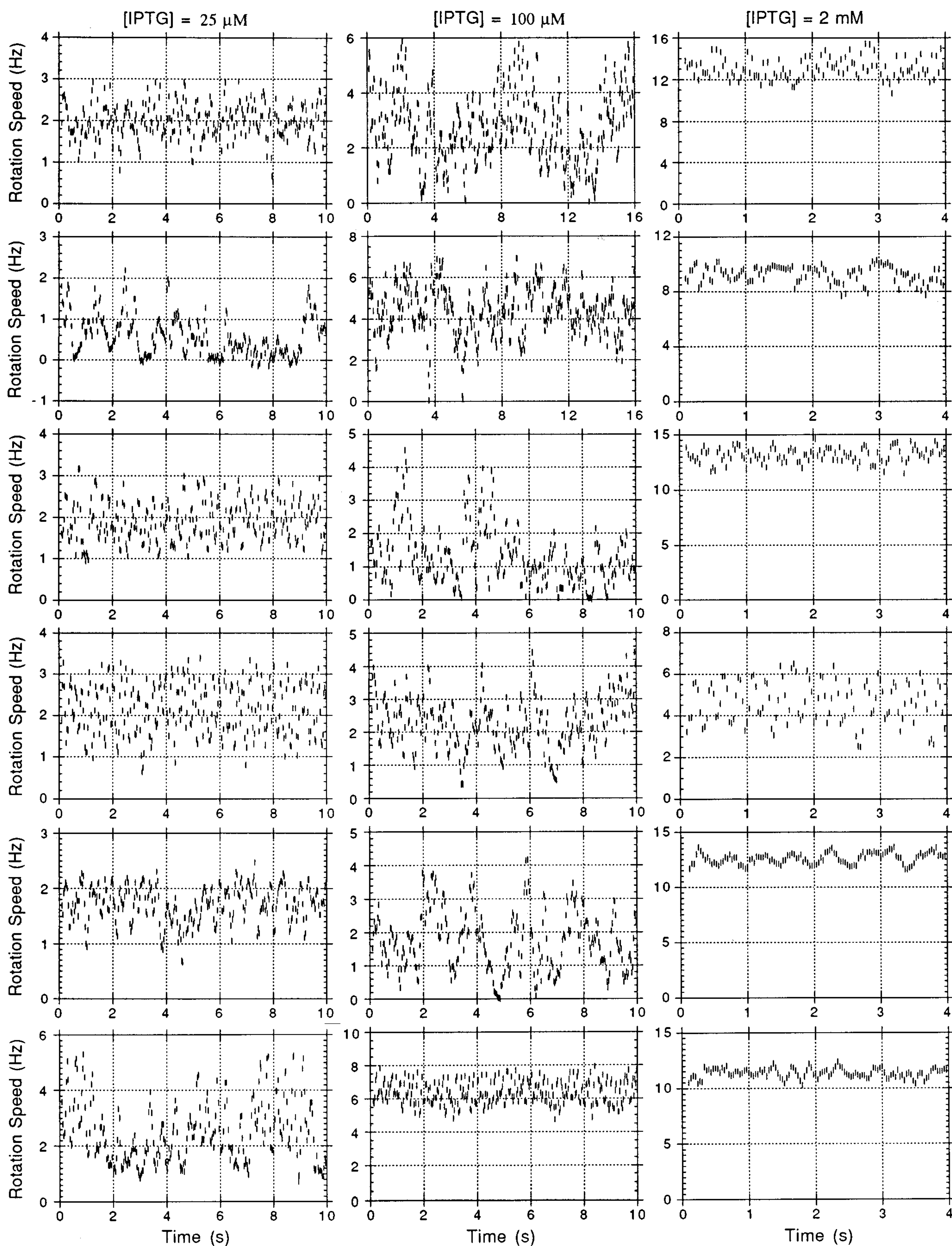

FIG. 7. Rotation speeds versus time of tethered cells expressing FliM at three levels, obtained by frame-by-frame analysis of video recordings. Strain DFB191(pDFB63) was cultured in TB containing ampicillin and IPTG at the concentrations indicated. Each panel corresponds to a different cell. Speed is plotted at intervals of 1/30 s, the video frame rate.

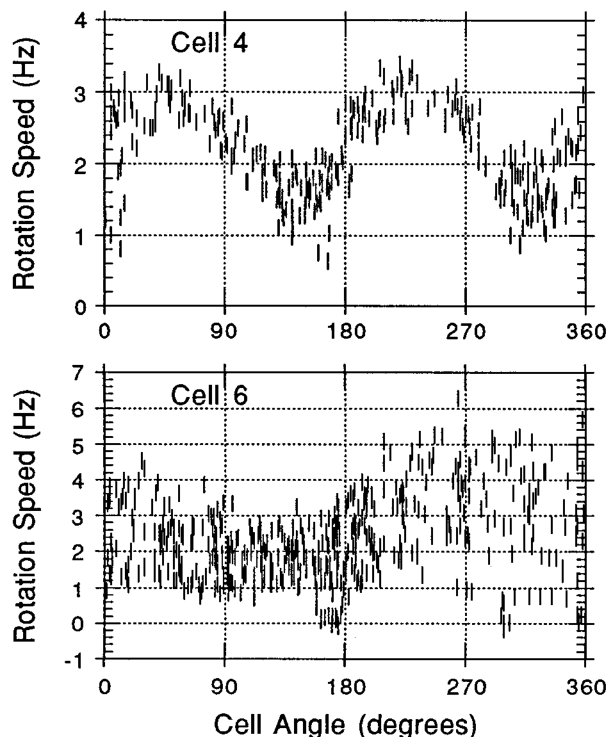

FIG. 8. Rotation speed plotted as a function of tethered cell angle for two cells underexpressing FliM. The data shown are for cells 4 and 6 (from the top) in the left-hand (25 μM IPTG) column of panels in Fig. 7.

TABLE 2. Numbers of flagella per cell in *fliM* mutants

fliM mutation	No. of flagella/cell[a]		
	E. coli[b] (25 μM IPTG)	*E. coli* (50 μM IPTG)	*S. typhimurium*[c]
F-131→C	0.02	0.06	2.7
F-131→L	0.04	0.04	2.5
F-131→S	0.00	0.02	3.0
G-132→D	0.04	0.00	3.1
G-133→D	0.08	0.16	3.1
T-147→I	0.00	0.06	3.4
T-147→P	0.02	0.10	3.8
T-149→K	0.04	0.10	2.9
L-250→Q	3.9	4.9	4.0
L-250→R	3.5	3.2	4.1
L-272→R	3.3	4.5	2.4
None (wild-type control)	3.7	3.1	

[a] The flagella on 50 cells were counted.
[b] The mutations were expressed from plasmid pDFB72 in *E. coli* DFB190 cultured in the concentrations of IPTG indicated to induce expression of *fliM.*
[c] The *S. typhimurium* mutants are among those reported by Sockett et al. (33); these mutations are chromosomal.

32°C in a medium containing 2 mM IPTG and nutrients to support protein synthesis (10% TB, 90% motility medium; reference 3).

Response to CheY. All of the frame-by-frame recordings shown here are for cells that lack the chemotaxis protein CheY. None of the cells lacking CheY reversed direction in the underexpression experiments. In contrast, in cells underexpressing FliM but otherwise wild type for chemotaxis (i.e., possessing CheY), the motors reversed frequently, spending a significant fraction of time rotating both CW and CCW. This was true even for motors with grossly subnormal torque. Thus, motors that contain less than the normal complement of FliM are able to respond to CheY by reversing direction.

Characterization of *mot* alleles of *fliM*. Several *fliM* mutants of *S. typhimurium* have been reported to have a nonmotile but flagellated (also called *mot*) phenotype (33). By using the nucleotide changes reported in the *S. typhimurium* mutants as a guide, 11 site-directed mutations (all but 1 of those classified as *mot* in the *S. typhimurium* study) were made in the *fliM* gene of *E. coli*. To score flagellation and motility, the mutated *fliM* genes were transferred from the plasmid used to make them (pHT41; Table 1) to plasmid pDFB72, which allows regulated expression, and introduced into the null strain. The mutant strains were characterized at induction levels shown above to give optimal swarming and flagellation with the wild-type gene (25 and 50 μM IPTG). Although the mutants were defective to various extents in swimming and swarming, none strictly fit the *mot* classification. Eight of the mutants had very few flagella (Table 2), while three had numerous flagella but also swarmed at a significant rate in soft agar (Fig. 9) and contained motile cells when examined under a microscope. The poorly flagellated mutants did not swarm on soft agar plates but, after relatively long incubation, gave rise to trails surrounding the point of inoculation (Fig. 9). Trails are produced when a small fraction of the cells in a population acquire the ability to swim but cannot pass this trait on to their daughters and so leave behind immotile cells that grow into microcolonies. When the poorly flagellated, trail-forming mutants were cultured in liquid and examined under a microscope, most of the cells were immotile but in several instances a few motile cells were seen. Thus, these *fliM* mutations do not abolish flagellar rotation completely.

To see if the different phenotypes observed in *E. coli* and *S. typhimurium* were caused by the use of different methods for scoring flagellation and motility, *fliM mot* mutant strains of *S. typhimurium*, provided by R. M. Macnab, were characterized by using the same procedures. In contrast to many of their *E. coli* counterparts, the *S. typhimurium* mutants were all well flagellated, as reported by Sockett et al. (33) (Table 2). Like their *E. coli* counterparts, however, many of the *S. typhimurium* mutants formed trails on soft agar plates (Fig. 9), implying that a subpopulation of the cells could swim. When the mutants were cultured in liquid and examined under a microscope, motile cells of all but one of the mutants were seen at various abundances (F-131→S; motile cells of two other mutants changed at this codon were seen). Thus, while many of the *fliM* mutations affect flagellation differently in the two species, they permit flagellar function in a fraction of the cells in both species.

In the previous assays of *S. typhimurium* motility, cells were cultured at 37°C, while in the present assays, cells were grown at 32°C, a temperature conductive to better motility in *E. coli*. To see if the motility observed in the mutants was caused by use of the lower temperature, the *S. typhimurium* mutants were also assayed at 37°C. Motility was generally worse at the higher temperature. Motile cells were more difficult to find in cultures of most mutants and were not found in the F-131→C, F-131→S, G-132→D, and T-149→K mutants. All of the mutants that produced trails on soft agar plates at 32°C also did so at 37°C, however, implying that motile cells were still present at the higher temperature (Fig. 9).

Paralyzed alleles of *fliM* were also sought by random mutagenesis of the *fliM* gene on a plasmid by using either hydroxylamine or passage through a *mutD* mutator strain (10). While many nonswarming mutants were isolated (approximately 100

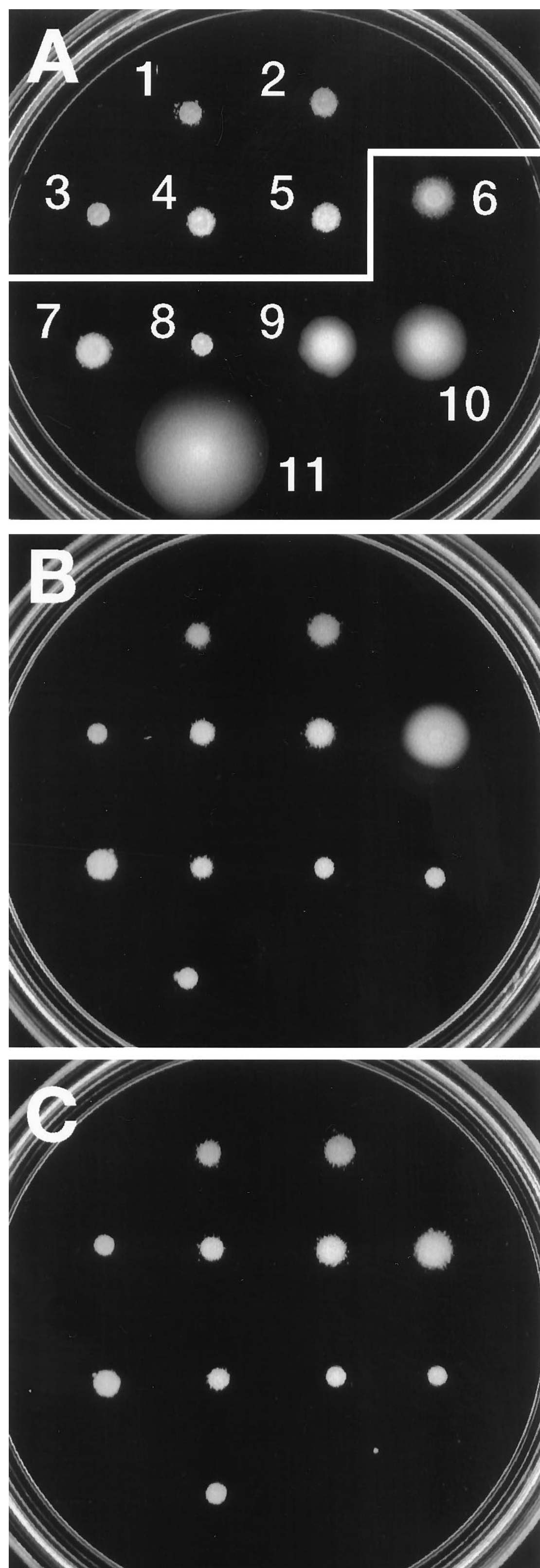

FIG. 9. Swarming behavior on soft agar of *fliM mot* mutants. The mutations are among those reported by Sockett et al. (33) in *S. typhimurium*. Amino acid changes in the mutants are as follows: 1, F-131→C; 2, F-131→L; 3, F-131→S; 4, G-132→D; 5, G-133→D; 6, T-147→I; 7, T-147→P; 8, T-149→K; 9, L-250→Q; 10, L-250→R; 11, L-272→R. (A) Swarming behavior of *E. coli fliM* mutants. The mutant *fliM* genes were expressed from plasmid pDFB72 in *fliM* null strain DFB190. The plate contained 50 μM IPTG to induce FliM expression, a level that gives good swarming with the wild-type gene (Fig. 5). The plate was incubated at 32°C for 39 h (mutations F-131→C to G-133→D) or for 29 h (mutations T-147→I to L-272→R); the incubation time was extended for some mutants to allow the trails to become more prominent. (B) Swarming behavior of *S. typhimurium fliM* mutants at 32°C. The *S. typhimurium* strains (a gift of R. M. Macnab) contained chromosomal mutations in *fliM*. The plate was photographed after incubation for 18 h. (C) Swarming behavior of *S. typhimurium fliM* mutants at 37°C. The plate was photographed after incubation for 18 h.

with each mutagen), none had the flagellated, fully paralyzed phenotype.

DISCUSSION

Previously, Sockett et al. found that severe mutations, such as frameshifts and partial deletions, in the *fliM* gene of *S. typhimurium* produced a nonflagellate phenotype (33). The *cat* replacement described here is clearly a null mutation of *fliM* and confirms that the null phenotype is nonflagellate, implying that FliM is required for flagellar assembly. Detailed studies of the partial flagellar structures produced in various mutants showed that FliM is involved at a fairly early step in assembly, after construction of the basal-body MS ring but before completion of the rod (22, 36).

Underexpression effects. In cells that underexpress FliM, relatively few flagella are built and most have significantly reduced torque relative to the wild type. If a fully functional motor contained only a single FliM molecule or contained several molecules that could function only with the full number present, then subnormal torque should not be observed because a given motor would rotate at its normal wild-type speed or not at all. The subnormal torque seen when FliM is in short supply therefore suggests that a fully functional motor contains multiple FliM molecules that can function independently to some degree. According to this hypothesis, subnormal torque results when a motor contains some FliM molecules but less than the normal complement.

Many motors also showed rapid fluctuations in speed when FliM was underexpressed. Large-amplitude, rapid fluctuations of this kind have not been documented before in *E. coli*, although smaller speed variations have been seen (12). The speed fluctuations are evidently related to the shortage of FliM, but the precise cause is not clear. At least two mechanisms can be imagined. In a motor that contains less than the normal complement of FliM, the molecules present (or domains thereof) might be abnormally free to move, causing them to function intermittently as they sample different positions in the motor. Alternatively, when few FliM molecules are present they might bind to the motor weakly, dissociating and reassociating rapidly. There are no data allowing discrimination between these possibilities. Underexpression of FliM did not have identical effects on all motors; while most motors fluctuated in speed, some turned slowly but more steadily. This suggests that when only a small number of FliM molecules are present in the motor they can be installed in different ways, some more stable than others. Alternatively, it is possible that other motor components also are affected. Some motors could lack copies of a second protein in addition to FliM, exhibiting more marked speed fluctuations, while others lack only FliM and rotate more steadily.

Regardless of their cause, the speed fluctuations seen here are in striking contrast to what was observed previously with MotA or MotB. When the Mot proteins were underexpressed, cells turned at subnormal but steady speeds, accelerating in clear steps as the level of protein was increased (3, 7). In some

instances, fluctuations were seen, but they were slower than those seen here and discrete speed levels could still be resolved (3). Those results suggested that MotA and MotB are present in multiple torque generators that, to a good approximation, operate independently. The less stable behavior seen here suggests that FliM is part of a structure in which the components are not fully independent but rely on mutual reinforcement to function stably. MotA and MotB are believed to constitute part or all of the stator (the nonrotating part of the motor) (6, 8, 15, 34) and are most likely arranged in a circle around the basal-body MS ring (21). We suggest that the contrasting behavior seen with FliM reflects a different location, probably on the rotor. This suggestion is consistent with recent biochemical and ultrastructural studies: Oosawa et al. reported evidence of an interaction between FliM and FliF, the protein which forms the MS ring (26). In electron micrographs of basal structures isolated by using comparatively gentle procedures, Francis et al. (14) and Khan et al. (20) have reported the presence of a large new feature, called the C ring, attached to the MS ring. Structures that contain the C ring also contain the FliM protein (14).

Implications for flagellar assembly. A wild-type cell contains approximately 1,400 FliM molecules, a surprisingly large number given that each cell has only a few flagella. Roman et al. (28) found that the switch complex protein FliG is also present in surprisingly large amounts (ca. 3,700 molecules per cell). Most of the FliM was found in the cytoplasm following disruption of the cells, as observed previously in minicells or maxicells overproducing the protein (9). Despite the sizable pool of FliM molecules, the protein is evidently a limiting resource in flagellar assembly, because when the amount of FliM is increased above the wild-type level the number of flagella increases. This implies that the affinity of FliM for sites in the motor is not very high or that only a fraction of the FliM is competent for incorporation into the motor.

Motors that contain relatively little FliM could either be assembled directly, meaning that relatively little FliM is incorporated at all stages, or be produced by the dissociation of some FliM from motors built initially with the full complement. Two observations favor the former, direct-assembly pathway. First, motors that generate normal torque are usually quite stable, not showing the speed reductions that would be expected if FliM could dissociate readily from a complete motor. Second, the number of flagella per cell increases in an approximately linear fashion as the amount of FliM is increased from a low level (Fig. 4). If several copies of FliM were needed at some step in assembly, the increase would be sigmoidal. We suggest that while some FliM is absolutely required for flagellar assembly, the requirement is flexible with respect to amount so that a small number of FliM molecules can suffice.

Francis et al. (14) reported that flagellar structures containing the C ring also contain the FliM and FliN proteins and calculated that about 100 copies of each protein would be needed if they alone formed this large structure (assuming equal numbers of each). If an intact C ring contained that much FliM, then our results, specifically, the linear increase in the number of flagella with increasing expression of FliM at low levels, would imply that assembly requires only a very small fraction of the C ring. Another possibility is that FliM does not form the bulk of the C ring but serves to attach the C ring to the basal body, a function that could require relatively few FliM molecules. Electron microscopic examination of the basal structures produced in cells that underexpress FliM might help to settle this point.

Overexpression effects. Clegg and Koshland reported previously that overexpression of FliM causes cells to swim more slowly, without reducing the number of flagella (9). We have confirmed that motility is impaired and also found that too much FliM reduces the number of flagella from the maximum value seen at a lower level of induction. Excess FliM probably acts by binding to another flagellar protein(s) and preventing its normal incorporation into the motor. The protein affected has not been identified. The nature of the impairment suggests that underexpression of the protein in question should reduce motility more than flagellation. Possible candidates include MotA, MotB, and the other switch complex proteins FliG and FliN.

The nature of *mot* mutant forms of *fliM*. On the basis of reports that certain mutations in *fliM* give a paralyzed phenotype in which flagella are built but fail to rotate, it has been suggested that FliM is involved to some extent in the process of torque generation (e.g., reference 42). Several mutations in the *E. coli fliM* gene were made which, on the basis of an earlier study of *S. typhimurium*, were expected to give a paralyzed phenotype. None of the mutants had a fully paralyzed phenotype. Most had very few flagella, indicating a defect in assembly. More importantly, the motility impairment was not complete in any of the *E. coli fliM* mutants characterized here. Even when flagella were rare, a small fraction of the cells were able to swim, as evidenced by trails on soft agar plates and, in some cases, a few motile cells in liquid culture. In three mutants with abundant flagella, motility was strong enough to support fairly rapid swarming through soft agar.

The *E. coli* mutants that exhibited heterogeneous behavior (trails) were poorly flagellated. Evidently, those mutations make flagellar assembly improbable but do not abolish function in the flagella that are built. All of the mutant FliM proteins were seen at near-normal levels on immunoblots (not shown), implying that they are efficiently expressed and are not proteolyzed. Thus, a simple shortage of FliM is not the cause of the poor flagellation. It is likely that these mutations weaken an interaction, needed for flagellar assembly, between FliM and another component. We have examined the effects of increased FliN expression on the phenotypes of these mutants and found that several of the *fliM* mutants became motile when wild-type FliN was supplied in excess (unpublished results). This suggests that some of the *fliM* mutations affect the installation of FliN in the motor, possibly by weakening a FliM-FliN interaction.

Several of the *fliM* mutations had very different effects in *E. coli* and *S. typhimurium*, nearly abolishing flagellation in only the former species. This result is surprising because the flagella of these species are often assumed to be identical in essential respects and contain some components that are interchangeable. The cause of this interspecies difference is not clear. It is not due to differing requirements for FliM in the two species, because the *E. coli* mutants were poorly flagellated across a wide range of expression levels (data not shown). If the mutations affect protein-protein interactions as suggested, then it might reflect differing requirements for a protein that interacts with FliM during flagellar assembly. Regardless of the exact cause, the interspecies difference constitutes additional evidence that the *fliM* mutations do not simply affect activities needed for torque generation, because those activities should be similar in the two species. It bears emphasis that the mutations do not fully abolish torque generation in either species; like their *E. coli* counterparts, the *S. typhimurium* mutants produced trails on soft agar plates.

These *fliM* alleles were originally identified in a mutant screen by Yamaguchi et al. in which a very large number of nonswarming *S. typhimurium* isolates were characterized (42).

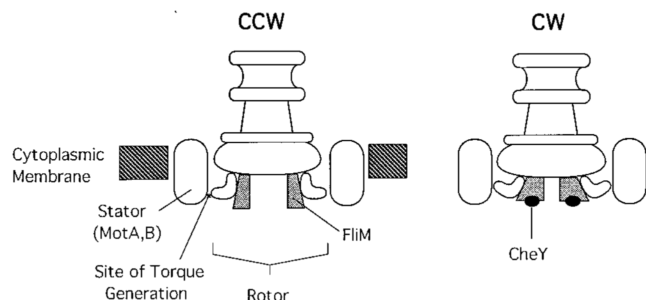

FIG. 10. Hypothesis for the location and function of FliM. The drawing represents a flagellar basal body; shapes indicated for the proteins are schematic. Torque is generated by interactions between the stator, consisting of MotA and MotB, and an unspecified component on the rotor, possibly FliG (13, 15, 26, 39). The present results suggest that FliM is present in multiple copies in the flagellar motor but does not directly contact the site of torque generation. Only two FliM molecules are shown in this view, but an intact motor is likely to contain more. The attachment of FliM to the MS ring is suggested by the in vitro binding studies of Oosawa et al. (26). While FliM is not at the site of torque generation, it might function to position a component that is, in one of two different configurations that correspond to CW or CCW rotation. Binding of CheY phosphate to FliM (40) would promote transition into the CW state, as shown.

The fact that these mutations do not abolish torque generation thus implies that fully paralyzed mutants, if they exist, are rare. Our inability to isolate any fully paralyzed *fliM* mutants of *E. coli* by random mutagenesis, using either hydroxylamine or a *mutD* strain, also indicates that they are rare.

A hypothesis for the location and function of FliM. On the basis of the scarcity of mutations with the *mot* phenotype, Sockett et al. (33) suggested that FliM might have only a small role in torque generation. We found that these *mot* mutations do not fully abolish motor rotation, further distancing FliM from a direct role in torque generation. Nevertheless, FliM is important for controlling the direction of rotation and therefore must influence events at the site of torque generation. We suggest that FliM is not located at the site of torque generation but acts to position other proteins that are, holding them either in a configuration that produces CCW rotation or, when phospho-CheY is bound, in a different configuration that produces CW rotation. This hypothesis is illustrated in Fig. 10. Our conclusions are similar to those of Irikura et al. (18), differing mainly in the attachment of FliM to the MS ring, a feature suggested by the in vitro binding studies of Oosawa et al. (26). Steady rotation could depend on most or all of the FliM sites being occupied if the FliM molecules were present in a ring or other structure that is most stable when the full complement is present. The component that is at the site of torque generation and is positioned there by FliM might be FliG (13, 15, 26, 39). The model thus requires that FliM and FliG interact. We have obtained two kinds of evidence in support of this suggestion. (i) When a glutathione-*S*-transferase fusion to FliG or FliM is purified by using glutathione affinity columns, the other protein (FliM or FliG) is coisolated. (ii) Fluorescence spectra of mixtures of FliG and FliM, compared with the spectra of the individual proteins, show nonadditivity, which indicates that these proteins interact (unpublished results).

The hypothesis predicts that paralyzed alleles of *fliM* will be rare because FliM is removed from the site of torque generation. While very uncommon, they might exist. The hypothesis allows that certain mutations in FliM could alter the position of the torque-generating component(s) so much that rotation ceases while preserving other interactions needed for flagellar assembly.

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