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Deletion Analysis of the FliM Flagellar Switch Protein of *Salmonella typhimurium*

ANNE S. TOKER, MAY KIHARA, AND ROBERT M. MACNAB*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520-8114

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The flagellar switch of *Salmonella typhimurium* and *Escherichia coli* is composed of three proteins, FliG, FliM, and FliN. The switch complex modulates the direction of flagellar motor rotation in response to information about the environment received through the chemotaxis signal transduction pathway. In particular, chemotaxis protein CheY is believed to bind to switch protein FliM, inducing clockwise filament rotation and tumbling. To investigate the function of FliM and its interactions with FliG and FliN, we engineered a series of 34 FliM deletion mutant proteins, each lacking a different 10-amino-acid segment. We have determined the phenotype associated with each mutant protein, the ability of each mutant protein to interfere with the motility of wild-type cells, and the effect of additional FliG and FliN on the function of selected FliM mutant proteins. Overall, deletions at the N terminus produced a counterclockwise switch bias, deletions in the central region of the protein produced poorly motile or nonflagellate cells, and deletions near the C terminus produced only nonflagellate cells. On the basis of this evidence and the results of a previous study of spontaneous FliM mutants (H. Sockett, S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab, J. Bacteriol. 174:793–806, 1992), we propose a division of the FliM protein into four functional regions: an N-terminal region primarily involved in switching, an extended N-terminal region involved in switching and assembly, a middle region involved in switching and motor rotation, and a C-terminal region primarily involved in flagellar assembly.

The chemotactic behavior of *Salmonella typhimurium* and *Escherichia coli* is produced by controlled changes in the direction of rotation of the motors that drive bacterial flagella (17, 18). During counterclockwise rotation, the helical flagellar filaments form a bundle that propels the cell forward in a smooth swimming motion; during clockwise rotation, the bundle flies apart and the smooth swimming is interrupted by erratic tumbling that randomly reorients the cell (17, 18). A complex of three proteins, FliG, FliM, and FliN, constitutes a switch that controls the direction of flagellar rotation (11, 27). Information about the environment, received by cell surface receptors, is relayed by chemotaxis proteins through the cytoplasm to the switch complex. In particular, it is believed that the chemotaxis protein CheY (when phosphorylated) binds to the switch and produces a clockwise rotational bias (1, 2, 13, 15, 26).

FliG was the first of the three switch proteins to be localized in the flagellar structure: it was found to be attached to the cytoplasmic face of a membrane-embedded structure known as the basal-body MS ring (Fig. 1) (4). The external hook and filament structures are attached to the MS ring through the basal-body rod. Electron microscopy also revealed the presence of a structure termed the cytoplasmic ring (C ring) extending from the MS ring out into the cytoplasm (5, 9, 29). Immunoblotting and immunoelectron microscopy indicated that the C ring is composed of the switch proteins FliM and FliN. In this cytoplasmic location, the switch complex is accessible to chemotaxis proteins, such as CheY, with which it is thought to interact.

The detailed roles of the three switch proteins in flagellar assembly, motor rotation, torque generation, and switching are not known. Extensive screens of spontaneous switch protein mutants have revealed that each protein can give rise to four different mutant phenotypes: nonflagellate (Fla^-), paralyzed (Mot^-), switch biased to counterclockwise (Che^- [CCW]), and switch biased to clockwise (Che^- [CW]) (8, 22). Of the three proteins, FliG gave rise to the greatest number of paralyzed mutants and FliM gave rise to the greatest number of switch-biased mutants. These results suggested that FliG may be predominantly involved in motor rotation and torque generation and that FliM may be predominantly involved in switching. In particular, Sockett et al. (22) suggested that FliM might be the primary target for CheY binding; subsequent biochemical studies demonstrated a FliM-CheY interaction in vitro (25). It has been shown that overexpression of mutant FliM or FliN (but not FliG) proteins that produce a paralyzed phenotype enables these proteins to support motor rotation (14); these data suggest that FliM and FliN mutant proteins causing paralysis are defective in association with the flagellar structure rather than in torque generation. It is therefore possible that only FliG may be directly involved in torque generation.

Different lines of evidence indicate that relative to FliG, FliM and FliN are the more labile components of the flagellar structure (5, 10, 29). Mot^- mutations in any of the three switch proteins result in essentially total loss of FliM and FliN (and only partial, if any, loss of FliG) from isolated flagellar basal bodies (29); mutations in FliG significantly reduce association of FliM and FliN with the MS ring (28). Finally, a mutant strain of *S. typhimurium* has been isolated that contains an in-frame fusion between FliM and FliN (10); although this mutant is flagellate and somewhat motile, electron microscopy revealed that the mutant C rings were much more labile than those of the wild type.

Interactions among the three switch proteins have been demonstrated genetically and biochemically (19, 20, 24, 27). In particular, mutations in each of the three switch complex proteins have been discovered that suppress, in an allele-specific fashion, mutations in the other two (27); these suppression

* Corresponding author. Phone: (203) 432-5590. Fax: (203) 432-9782. Electronic mail address: robert_macnab@quickmail.yale.edu.

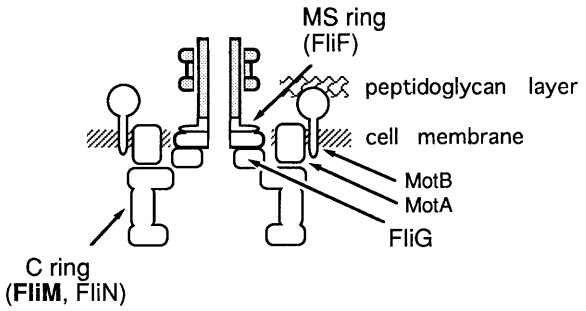

FIG. 1. Diagram of the extended flagellar hook-basal-body of *S. typhimurium*, showing the switch complex (composed of FliG, FliM, and FliN), which forms the C ring structure and is mounted on the cytoplasmic face of the MS ring. FliG is shown as directly attached to the MS ring, and FliM and FliN are shown as located further toward the cytoplasm. The presumed locations of the MotA and MotB proteins, involved in transmembrane proton conduction and torque generation, are also shown.

data are evidence of physical interaction among the three proteins. In vitro studies have demonstrated interactions between FliG and FliM (19, 20, 24), FliM and FliN (20, 24), and FliG and FliN (24). Finally, there is in vitro evidence that FliG and FliM (but not FliN) interact with the MS-ring protein, FliF (21).

Although the exact regions of each switch protein that interact with the others have not yet been mapped out, there are some clues pointing to the important surfaces of interaction among them. Because the mutant containing the in-frame fusion between FliM and FliN (in which the C terminus of FliM is fused to the N terminus of FliN) is flagellate and retains some degree of motility (10), it seems likely that in wild-type cells, the C terminus of FliM interacts with the N terminus of FliN. Similarly, because a mutant containing an in-frame fusion of the MS-ring protein, FliF, to FliG (in which the C terminus of FliF is fused to the N terminus of FliG) is flagellate and retains almost wild-type motility (4), it is likely that the N terminus of FliG normally interacts with the C terminus of FliF.

Here, we present a systematic mutational study of the FliM switch protein. We have constructed a series of 34 mutant FliM proteins, each lacking a different 10-amino-acid region. We describe the phenotype associated with each mutant protein, the ability of each mutant protein to interfere with the motility of wild-type cells, and the effect of additional FliG or FliN protein on the function of selected FliM mutant proteins. This study permits certain conclusions about the structure and function of the FliM protein itself and about its interactions with the other switch proteins, FliG and FliN.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DFB190 (*fliM*::Cm), a gift from David Blair, has been described (23). It contains a chromosomal replacement of the *fliM* gene with a chloramphenicol acetyltransferase cassette and is nonflagellate. *E. coli* RP437, the parent of DFB190, is wild type for motility and chemotaxis (21a). XL-1 Blue (Stratagene, La Jolla, Calif.) was used as the recipient in cloning experiments. pUC18 and pUC19 were used as cloning vectors. Plasmid pAMH5 (7, 11), containing a *fliLMNOP* insert of *S. typhimurium* DNA, was used as a template for PCR mutagenesis.

Antibodies. Polyclonal rabbit antibodies directed against FliG, FliM, and FliN were a generous gift from K. Oosawa and S.-I. Aizawa, Teikyo University, Utsunomiya, Japan.

Construction of FliM scanning deletion mutants. Primers were synthesized with a model 393 DNA/RNA synthesizer (Applied Biosystems, Foster City, Calif.). Outside primer A was complementary to a sequence in *fliL* upstream of the putative ribosome binding site for the *fliM* gene; this primer created a *Bam*HI site in *fliL*. Outside primer B was complementary to the sequence at the junction between *fliM* and *fliN*; this primer created a *Hin*dIII site in *fliN*. A series of internal, mutagenic primers (MutA and MutB primers) were designed to delete 30-bp regions of the *fliM* gene. Each MutA primer was exactly complementary to the sequence immediately downstream of the desired deletion; each MutB primer was partly complementary to the sequence immediately upstream of the desired deletion and partly complementary to the 5′ end of the corresponding MutA primer. This method is a variant of standard recombinant PCR methods (6). First, two half-PCRs were carried out in an MJ MiniCycler (MJ Research, Watertown, Mass.), one reaction with primers A and MutB and the other reaction with primers MutA and B. The product of each half-reaction was gel purified from a 1% Tris-acetate-EDTA agarose gel with the QIAquick gel extraction kit (Qiagen Inc., Chatsworth, Calif.). The gel-purified products were then mixed together with primers A and B, and a second round of PCR was performed. The most C-terminal deletion mutant protein (no. 34) was constructed by only a single round of PCR: the template was amplified with primer A and a B-direction primer, containing a *Hin*dIII site and a stop codon, that bound 30 bases upstream of the 3′ end of *fliM*. In addition, PCR was used to amplify the wild-type *fliM* gene. This PCR involved mixing the two outside primers, A and B, with the template, pAMH5.

Cloning and purification of deletion mutant plasmids. Scanning deletion mutant alleles were cloned by digesting each overlap PCR product or the wild-type PCR product with *Bam*HI and *Hin*dIII and ligating it into *Bam*HI- and *Hin*dIII-digested pUC18. Competent XL-1 Blue cells were transformed with the ligated plasmids and plated onto Luria plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). White colonies were selected for small-scale plasmid preparations, which were carried out with the QIAprep Spin Plasmid Miniprep kit (Qiagen). Plasmids containing an insert of the correct size were identified by digestion with *Bam*HI and *Hin*dIII. Mutant clones were sequenced with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) in the region of the deletion to ensure that no mutations other than the desired deletion had been introduced. Several independent clones of each mutant plasmid were selected and tested for function to safeguard against the possibility that the observed phenotype was the product of a mutation outside the sequenced region.

Swarm plate assay. DFB190 or RP437 cells transformed with control or mutant plasmids were spotted onto soft tryptone agar plates (0.35% Bacto Agar, 1% tryptone, 0.7% NaCl) containing 50 μg of ampicillin per ml and were incubated at 30°C for up to 24 h. Colonies that appeared null on the swarm plates were examined under a dissecting microscope to check for the presence of small trails or for subtle changes in colony morphology not visible to the naked eye. Swarm phenotypes were ranked on a scale from 0 (not swarming) to 7 (swarming of wild-type RP437 cells). This scale was chosen to correlate with the scale developed for measurements of motility in liquid medium.

Motility in liquid medium. DFB190 or RP437 cells transformed with control or mutant plasmids were grown in liquid medium (Luria broth containing 50 μg of ampicillin per ml) at 30°C with vigorous shaking. Control cultures were checked periodically by high-intensity dark-field microscopy (16) until maximum flagellation and motility were observed. At this point, all cultures were examined under the high-intensity dark-field microscope to determine levels of motility and flagellation. The motility of transformants was ranked on a scale of 0 (nonmotile) to 7 (motility of wild-type RP437 cells) because seven easily distinguishable and reproducible phenotypic classes were observed. All rankings were made by a single observer (A.S.T.).

Immunoblotting. Cellular levels of the mutant proteins (and of wild-type FliG, FliM, and FliN) in DFB190 or in RP437 were examined on immunoblots. Protein samples were prepared for immunoblotting by growing cells at 30°C with vigorous shaking until cells attained optimal flagellation and motility. Optical density measurements (at a λ of 600 nm) of all cultures were then taken to standardize the amounts of protein used for immunoblotting. Cells were spun down and resuspended in protein sample buffer. Sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis gels were run on a Hoefer (San Francisco, Calif.) Mighty Small II apparatus, and the proteins were transferred to nitrocellulose membranes with a Hoefer Transblot apparatus. The membranes were then probed with polyclonal antibodies raised against FliG, FliM, or FliN. The antibodies were detected by enhanced chemiluminescence with an ECL immunoblotting detection kit (Amersham International, Little Chalfont, United Kingdom). Coomassie-stained gels were run in parallel to confirm that loading was uniform from lane to lane.

Construction of p(ΔM)G and p(ΔM)N plasmids, containing deletion alleles of *fli*M plus wild-type *fli*G or *fli*N. p(ΔM)G and p(ΔM)N plasmids were constructed by adding a wild-type copy of *fliG* or *fliN* into selected deletion mutant plasmids and into the control plasmid pUCM. Clones of *fliG* or *fliN* in pUC19 were digested with *Hin*dIII and *Nde*I to excise *fliG* or *fliN*. Simultaneously, plasmids containing mutant alleles of *fliM* were cut with the same enzymes. Fragments were gel purified, ligated, and used to transform XL-1 Blue cells which were then plated onto Luria plates containing 50 μg of ampicillin per ml. Colonies were screened by preparing plasmid DNA and digesting it with *Hin*dIII and *Nde*I to identify plasmids containing an insert of the correct size. The resulting plasmids contained *fliG* or *fliN* immediately downstream of the mutant *fliM* gene.

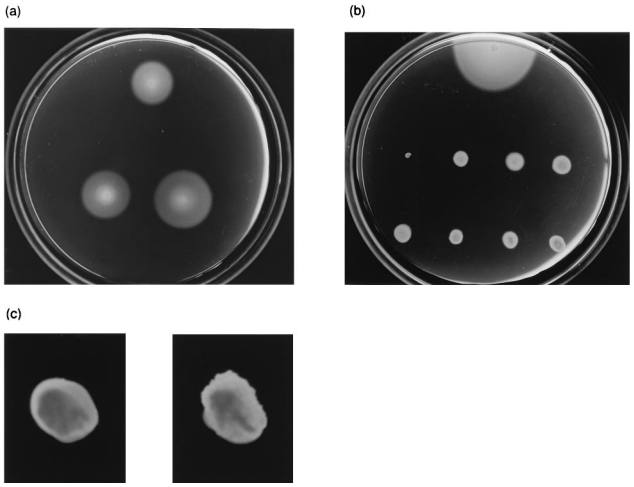

FIG. 2. Soft tryptone plates showing swarm phenotypes of DFB190 transformants containing control plasmids or selected deletion mutant plasmids. (a) The top row shows transformants containing pUCM, and the bottom row shows transformants containing deletion mutant plasmid 33 (left) or deletion mutant plasmid 34 (right). The photograph was taken after 9 h at 30°C. (b) The top row shows transformants containing pUCM, the middle row shows transformants containing (from left to right) pUC18 or deletion mutant plasmids 1 to 3, and the bottom row shows transformants containing deletion mutant plasmid 4, 5, 23, or 24 (left to right). The photograph was taken after 14 h at 30°C. (c) Transformants containing deletion mutant plasmid 26 or 18 (left and right, respectively). The photograph was taken after 14 h at 30°C with a macro lens.

RESULTS

Mutant phenotypes. Wild-type FliM protein contains 334 amino acids. Using mutagenic PCR, we constructed 34 deletion mutant versions of the protein, each lacking a different 10-amino-acid segment: mutant protein 1 lacked residues 2 to 10 (codon 1 was retained as the start codon), mutant protein 2 lacked residues 11 to 20, and so on, up through mutant protein 33 (lacking residues 321 to 330). Mutant protein 34 lacked residues 325 to 334; this deletion overlaps with the deletion in mutant protein 33.

Each mutant allele of the *fliM* gene was cloned into pUC18. In addition, a control plasmid, pUCM, that contained the wild-type *fliM* gene was constructed. Mutant and control plasmids were used to transform an *E. coli fliM* null strain, DFB190 (*fliM*::Cm), for characterization of phenotype. DFB190 transformants were tested for swarming ability by spotting them onto soft tryptone agar plates. They were examined for flagellation and motility in liquid medium by high-intensity dark-field microscopy, which permits visualization of individual flagellar filaments (16).

The control plasmid pUCM restored good swarming and swimming to DFB190 cells (Fig. 2a). However, these transformants did not swarm as rapidly as wild-type RP437 cells, their swarms had one less swarm ring than RP437 cells, and they were somewhat less vigorous in liquid medium (data not shown). These phenotypes are perhaps the result of moderate overexpression of the FliM protein (3); the pUCM plasmid produced approximately two to three times the chromosomal level of FliM (see Fig. 4).

DFB190 cells transformed with the mutant plasmids produced a number of different phenotypes on swarm plates and in liquid medium. The transformants could be roughly classified into six different groups, based on their swarming and swimming phenotypes.

Group 1 (wild-type). Both on swarm plates (Fig. 2a) and in liquid medium, DFB190 transformants containing mutant plasmid 33 or 34 were indistinguishable from transformants containing control plasmid pUCM. Like wild-type RP437 cells, all of these transformants had about six to eight filaments per cell.

Group 2 (smooth swimming). DFB190 transformants containing mutant plasmid 1, 3, 4, or 5 produced small, dense swarms (Fig. 2b). In liquid medium, these transformants appeared reasonably vigorous and well flagellated (six to eight filaments per cell) but swam smoothly without tumbling.

Group 3 (poor motility). DFB190 transformants containing mutant plasmid 2, 23, or 24 produced small, dense swarms (Fig. 2b) similar to those produced by transformants containing plasmid 1, 3, 4, or 5. In liquid medium, however, the group 3 transformants were easily distinguishable from those of group 2: although the group 3 transformants were well flagellated, they were not very motile. In many cases, they swam slowly and failed to form a stable flagellar bundle.

Group 4 (slight swarming). DFB190 transformants containing mutant plasmid 25 or 26 produced colonies that, when viewed under the dissecting microscope, showed a small amount of spreading; thus, at least some cells were motile to some degree (Fig. 2c, left panel). In liquid medium, these transformants appeared to be well flagellated but essentially nonmotile; only occasional cells showed some evidence of motility.

Group 5 (paralyzed). DFB190 transformants containing mutant plasmid 18 or 19 produced colonies that showed no swarming at all, even under the dissecting microscope (Fig. 2c, right panel). In liquid medium, these transformants appeared to be well flagellated but nonmotile. Transformants containing mutant plasmid 18 had six to eight filaments per cell; those containing mutant plasmid 19 had four to six filaments per cell.

Group 6 (nonflagellate). The largest group of mutant plasmids (no. 6 to 17, 20 to 22, and 27 to 31) totally failed to restore flagellation to DFB190 cells. Transformant colonies appeared to be totally nonswarming even under the dissecting microscope, and no motile or flagellated cells were seen in liquid medium. DFB190 transformants containing mutant plasmid 32 produced colonies that, as observed under the dissecting microscope, gave rise to small trails. The presence of trails indicates that at least a few cells must have been flagellated and somewhat motile. The number of motile cells, however, must have been extremely small, because no motile or flagellated cells were seen in liquid medium by dark-field microscopy.

The swarming ability, motility in liquid medium, and presence or absence of flagella associated with each deletion mutant protein are summarized in Fig. 3. Both swarming and swimming ability have been ranked on a scale of 0 (indicating no swarming or swimming ability) to 7 (indicating swarming or swimming ability of wild-type RP437 cells). As this figure shows, the DFB190 transformants did not, on the whole, exhibit subtly varying degrees of flagellation: they were essentially either well flagellated or nonflagellate.

Cellular amounts of mutant FliM proteins. Immunoblotting was used to determine the cellular levels of each of the FliM mutant proteins. These levels were compared with each other and with levels of chromosomally encoded wild-type FliM.

DFB190 cells transformed with each mutant plasmid (and with control plasmid pUCM) were grown in liquid medium at 30°C with vigorous shaking. RP437 cells were used to determine chromosomal FliM levels. After dark-field microscopy revealed that cells had attained maximum motility and flagellation, the cultures were harvested for immunoblotting. Representative examples of immunoblots are shown in Fig. 4. The mutant proteins exhibited various mobilities and (as expected) generally ran slightly faster than the wild-type protein.

Immunoblotting revealed that the amount of wild-type FliM

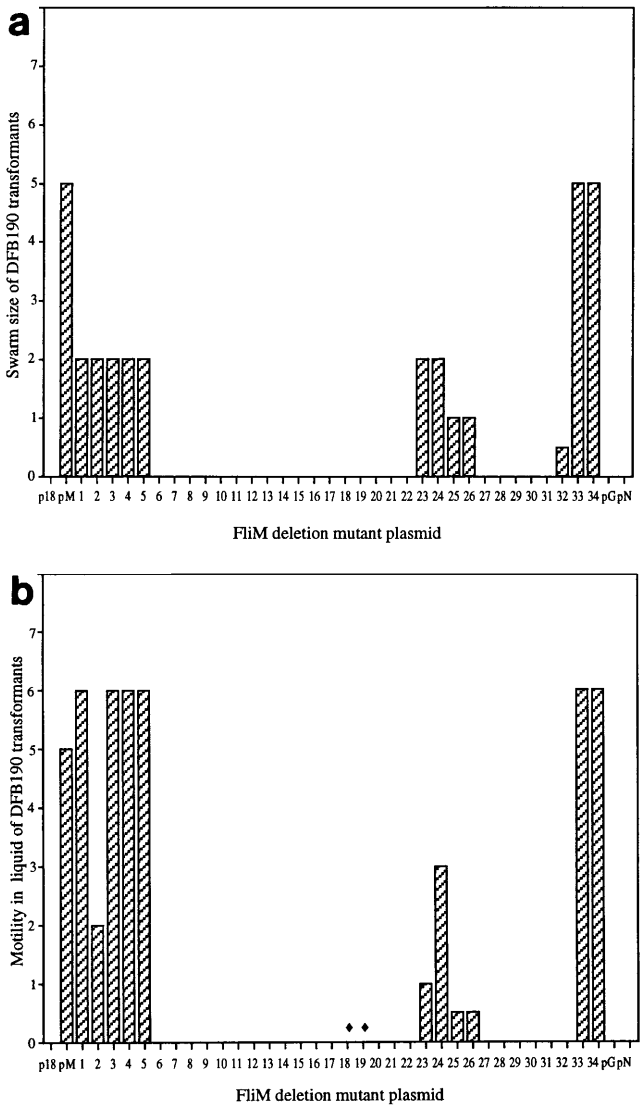

FIG. 3. Summary of swarming and swimming abilities of DFB190 transformants containing each deletion mutant plasmid. The horizontal axis shows the deletion mutant plasmids and four control plasmids: pUC18 (p18), pUCM (pM), pUCG (pG), and pUCN (pN). Transformants that were nonmotile but well flagellated are indicated by diamonds; all other nonmotile transformants were nonflagellate. All motile transformants were flagellated at wild-type levels.

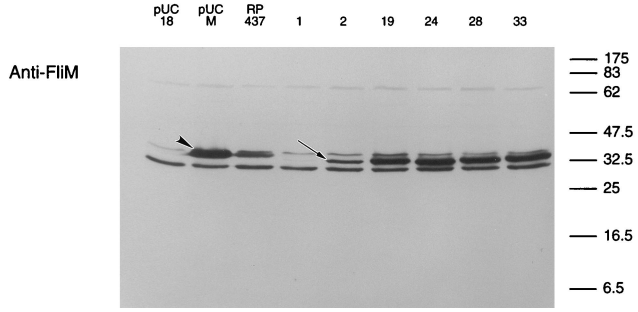

FIG. 4. Immunoblot of RP437 cells and DFB190 transformants containing pUC18, pUCM, and the indicated mutant plasmids. The membrane was blotted with anti-FliM antibodies. The arrowhead indicates the wild-type FliM protein. The arrow indicates mutant protein 2. Mutant proteins 19, 24, 28, and 33 can be seen at various apparent molecular masses close to the apparent molecular mass of mutant protein 2. Molecular mass markers (in kilodaltons) are indicated at the right.

protein produced by the pUCM plasmid was about two to three times as high as that produced by the chromosomal copy of the gene. These relative levels are not surprising: although pUC18 is a high-copy vector, motility and expression experiments were carried out under noninducing conditions (i.e., no IPTG was added).

In addition, the blots showed that most of the mutant proteins were present in approximately the same amount as the wild-type protein produced from pUCM; the mutant phenotypes thus were not the result of insufficient mutant protein expression.

Certain mutant proteins, however, appeared to be produced at lower levels than the rest. Mutant proteins 2 to 5 were detected in about two- to threefold-lower amounts than the other mutant proteins, and mutant proteins 1 and 9 were at most barely detectable. These results were highly reproducible in repeated experiments. The fact that cells transformed with mutant plasmid 1 were well flagellated and quite motile, however, indicated that these transformants produced sufficient amounts of protein to support flagellation and motility. It is possible that mutant protein 1 was difficult to detect because it was unstable when not incorporated into flagellar structure. It is also possible that the N-terminal sequence deleted from mutant protein 1 contributes heavily to the epitopes against which the polyclonal antibody is directed. The apparent lower expression level of N-terminal mutant proteins 2 to 5 is consistent with the hypothesis that the N-terminal region of the protein is important for recognition by the polyclonal antibody.

Only mutant protein 9 appeared to be both nonfunctional and not clearly detectable. It is possible, therefore, that the inability of this protein to support flagellation was the result of an inadequate expression level or of instability.

Dominance tests. We wished to see whether the FliM mutant proteins could inhibit the swimming or swarming of wild-type cells. RP437, the wild-type (*fliM*$^+$) parent of DFB190, was transformed with each mutant plasmid or with control plasmid pUCM or pUC18. Immunoblots revealed that mutant protein levels in RP437 cells were similar to those in DFB190 cells (data not shown).

Control and mutant transformants were tested for swarming on soft tryptone agar and observed under a dark-field microscope. Although these transformants varied in their swarming and swimming abilities, no dominance effects on flagellation levels were observed: all transformants were as well flagellated as untransformed RP437 cells. Selected examples of dominance swarm test results are shown in Fig. 5. The dominance phenotypes on swarm plates and in liquid medium are summarized in Fig. 6.

(i) Dominance effects on swarming. Plasmid pUCM inhibited RP437 swarming significantly (Fig. 5 and 6a). Twelve mutant plasmids (no. 2, 6, 7, 10 to 12, 14, 15, 21, 25, 33, and 34) inhibited swarming to the same degree as pUCM, 4 mutant plasmids (no. 3 to 5 and 24) inhibited swarming to a greater degree than pUCM, and 18 mutant plasmids (no. 1, 8, 9, 13, 16 to 20, 22, 23, and 26 to 32) inhibited swarming to a smaller degree. In no instance did RP437 transformants containing a mutant plasmid swarm as well as RP437 transformants containing the pUC18 vector alone; in other words, every mutant plasmid (and plasmid pUCM) inhibited RP437 swarming to some extent.

(ii) Dominance effects on motility in liquid medium. RP437 transformants containing mutant or control plasmids were also examined in liquid medium under a dark-field microscope

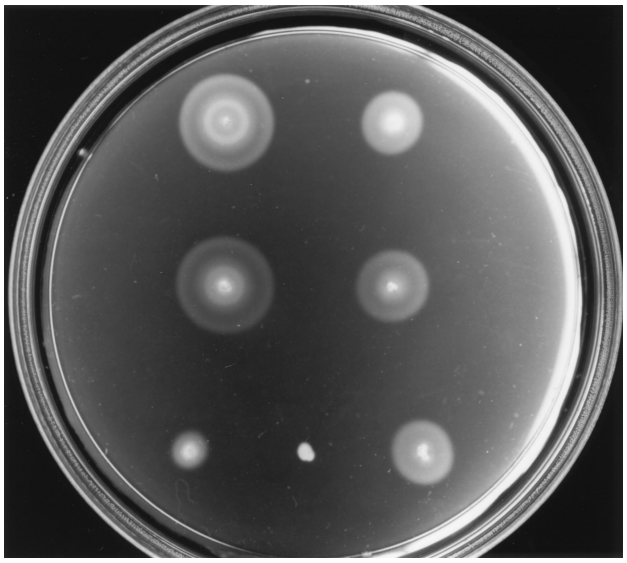

FIG. 5. Dominance experiments on a soft tryptone plate; shown are swarm phenotypes of RP437 cells transformed with control plasmids or selected mutant plasmids. The top row shows transformants containing pUC18 (left) or pUCM (right), the middle row shows transformants containing mutant plasmid 16 (left) or 2 (right), and the bottom row shows transformants containing mutant plasmid 3, 4, or 25 (left to right). The photograph was taken after 6.5 h at 30°C.

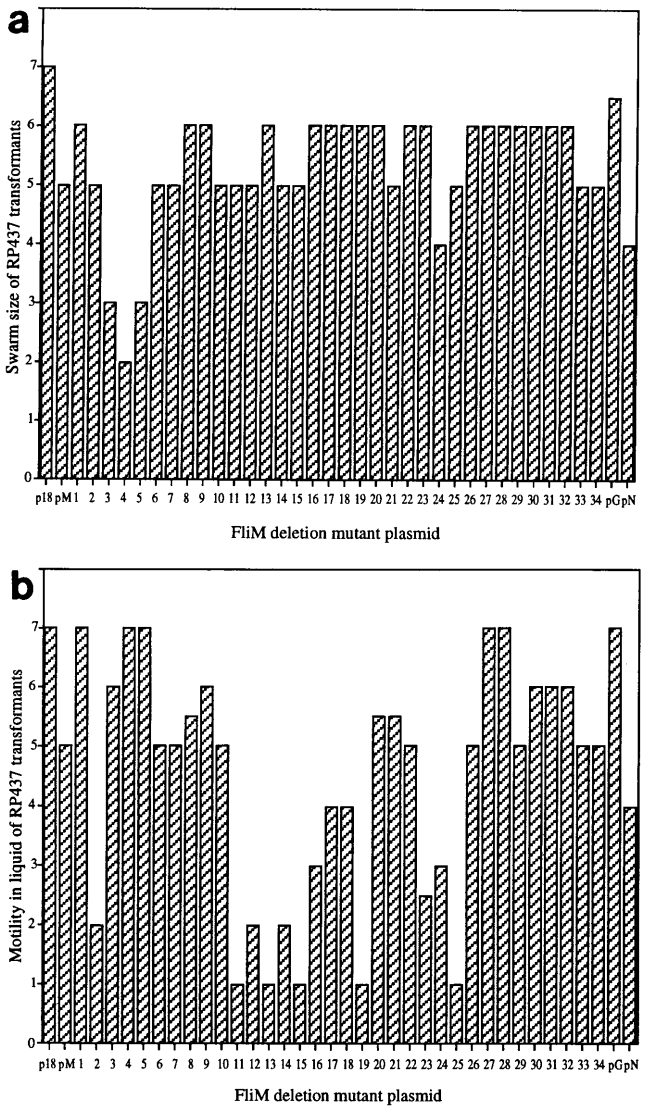

FIG. 6. Summaries of dominance data showing swarming ability and motility in liquid medium of RP437 transformants containing control or mutant plasmids. All transformants were flagellated at wild-type levels. Abbreviations are as in Fig. 3.

(Fig. 6b). Transformants containing pUCM were noticeably less vigorous than those containing pUC18. In 11 cases (with plasmids 6 to 8, 10, 20 to 22, 26, 29, 33, and 34), transformants swam approximately as well as those containing pUCM. In eight cases (with plasmids 2, 11 to 15, 19, and 25), transformants swam much more poorly than those containing pUCM; these transformants were barely motile. In five cases (with plasmids 16 to 18, 23, and 24), transformants swam more poorly than those containing pUCM but retained some motility. In five cases (with plasmids 3, 9, and 30 to 32), transformants swam more vigorously than those containing pUCM but slightly less vigorously than those containing pUC18. Finally, in five cases (with plasmids 1, 4, 5, 27, and 28), transformants swam as vigorously as those containing pUC18.

Mutant plasmid 4 was unusual in two respects. First, it inhibited RP437 swarming more than any other mutant plasmid (Fig. 5 and 6a). Second, it did not produce better swarming in the wild-type than in the mutant background (Fig. 3a and 6a).

It is interesting that several mutant FliM proteins (no. 11 to 19) appeared to inhibit motility in liquid medium significantly more than they inhibited swarming on soft tryptone. We attribute this effect to the selective pressure for good swarming applied to cells on soft tryptone agar plates; there is no comparable selective pressure in liquid medium.

Motility and swarming ability of cells transformed with p(ΔM)G and p(ΔM)N plasmids. Selected plasmids (no. 1 to 5, 8, 13, 15, 18, 19, 23 to 26, 28, and 32) were modified by the addition of a wild-type copy of either *fliG* [to create p(ΔM)G plasmids] or *fliN* [to create p(ΔM)N plasmids]. The additional gene was inserted into the mutant plasmid directly downstream of the mutant *fliM* gene. Control plasmids pUCMG and pUCMN were constructed by addition of wild-type *fliG* or *fliN* to pUCM. These plasmids were used to transform DFB190, and the resulting transformants were examined for swarming ability and motility. Selected examples of results of these swarm tests are shown in Fig. 7. The swarming and swimming results are summarized in Fig. 8.

DFB190 transformants containing control plasmid pUCMG swarmed slightly less well than those containing plasmid pUCM (Fig. 7a and 8a). DFB190 transformants containing plasmid pUCMN swarmed substantially better than those containing pUCM (Fig. 7b and 8c); in fact, they swarmed as well as wild-type RP437 cells. Addition of *fliG* or *fliN* to mutant plasmids mirrored the effects observed for the control plasmids: overall, addition of *fliG* diminished (or did not affect) swarming and motility, while addition of *fliN* improved (or did not affect) swarming and motility. Flagellation levels of both control and mutant transformants, however, were unaffected by the addition of *fliG* or *fliN*.

(i) Swarming of DFB190 transformants containing p(ΔM)G mutant plasmids. Addition of *fliG* to mutant plasmids 2, 4, 5, and 23 to 26 (to create plasmids 2G, 4G, 5G, and 23G to 26G) produced their ability to restore swarming to DFB190 cells. Transformants containing mutant plasmid 2G, 4G, 5G, 23G, or 24G produced smaller swarms than those produced by transformants containing the original mutant plasmid (Fig. 7a and

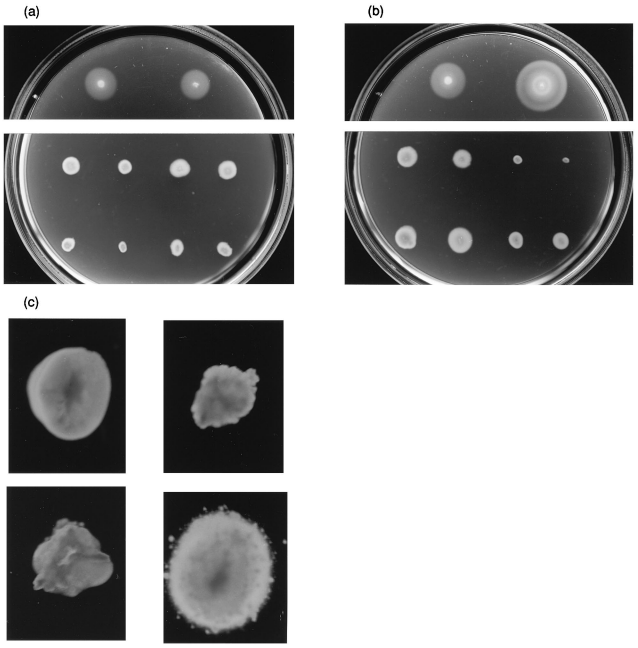

FIG. 7. Soft tryptone plates showing effects of additional FliG or additional FliN on swarming of DFB190 transformants containing control plasmids or selected mutant plasmids. (a) The top row shows transformants containing pUCM (left) or pUCMG (right). The photograph was taken after 8 h at 30°C. The middle row shows transformants containing mutant plasmid 4, 5, 23, or 24 (left to right). The bottom row shows transformants containing mutant plasmid 4G, 5G, 23G, or 24G (left to right). The photograph was taken after 20.5 h at 30°C. (b) The top row shows transformants containing pUCM (left) or pUCMN (right). The photograph was taken after 8 h at 30°C. The middle row shows transformants containing mutant plasmid 1, 23, 25, or 26 (left to right). The bottom row shows transformants containing mutant plasmid 1N, 23N, 25N, or 26N (left or right). The photograph was taken after 20.5 h at 30°C. (c) Transformants containing mutant plasmid 25 or 25G (left side, top and bottom, respectively) and 19 or 19N (right side, top and bottom, respectively). The photographs were taken after 20.5 h at 30°C with a macro lens.

8a). Transformants containing mutant plasmid 25 or 26 produced colonies that appeared to be smooth and round under a dissecting microscope. Transformants containing plasmid 25G or 26G, however, produced colonies that appeared to be completely null under the dissecting microscope (Fig. 7c, left panel). Addition of *fliG* to mutant plasmids 1, 3, 8, 13, 15, 18, 19, 28, and 32 did not affect their ability to restore swarming to DFB190 cells (Fig. 8a).

(ii) Motility in liquid medium of DFB190 transformants containing p(ΔM)G mutant plasmids. The addition of *fliG* to mutant plasmids 2 to 5, 23, and 24 decreased their ability to restore motility to DFB190 (Fig. 8b): cells generally appeared to be less vigorous, and in each case more were swimming poorly or not swimming at all. Addition of *fliG* to mutant plasmids 1, 8, 13, 15, 18, 19, 25, 26, 28, and 32 had no observable effect on their ability to restore swimming to DFB190 cells.

(iii) Swarming of DFB190 transformants containing p(ΔM)N mutant plasmids. Addition of *fliN* to mutant plasmids 18, 19, and 23 to 26 improved their ability to restore swarming to DFB190 cells (Fig. 7b and 8c). Transformants containing plasmid 18 or 19 did not swarm, even as viewed under the dissecting microscope. Transformants containing plasmid 18N or 19N, however, produced colonies that appeared to be round when viewed under the dissecting microscope (Fig. 7c, right panel). Transformants containing plasmids 23N to 26N produced slightly larger, dense swarms than transformants containing plasmids 23 to 26.

Addition of *fliN* to mutant plasmids 1 to 3, 5, 8, 13, 15, 28, and 32 did not affect their ability to restore swarming to DFB190 cells (Fig. 8c).

(iv) Motility in liquid medium of DFB190 transformants containing p(ΔM)N mutant plasmids. Addition of *fliN* to mutant plasmids 1 to 3, 5, 18, 19, and 23 to 25 improved their ability to restore motility to DFB190 cells (Fig. 8d). In particular, there was a striking improvement in the motility of transformants containing plasmid 2N, 23N, or 24N relative to that of transformants containing 2, 23, or 24. Addition of *fliN* to these plasmids produced transformants that were uniformly vigorous and motile, while transformants containing the original mutant plasmids were poorly motile. Transformants containing plasmid 1N, 3N, or 5N were even more vigorous than transformants containing plasmid 1, 3, or 5. Transformants containing plasmid 25N swam very poorly but slightly better than those containing plasmid 25. Finally, while transformants containing plasmid 18 or 19 appeared to be completely paralyzed, transformants containing plasmid 18N or 19N showed some limited swimming ability.

Addition of *fliN* to mutant plasmids 8, 13, 15, 26, 28, and 32 did not affect the swimming ability of DFB190 cells transformed with these plasmids (Fig. 8d).

Interestingly, in one instance, addition of *fliN* to a mutant plasmid actually decreased its ability to restore swarming and swimming to DFB190 cells: transformants containing mutant plasmid 4 swarmed and swam better than those containing plasmid 4N (Fig. 8c and d).

FliG, FliM, and FliN protein levels in cells transformed with p(ΔM)G and p(ΔM)N plasmids. Selected DFB190 transformants containing the p(ΔM)G and p(ΔM)N plasmids were immunoblotted with polyclonal antibodies directed against FliG, FliM, and FliN to test levels of production of these proteins. Five control plasmids were used: pUCM, pUCMG, pUCMN, pUCG (containing the wild-type *fliG* gene cloned into pUC19), and pUCN (containing the wild-type *fliN* gene cloned into pUC19). Protein levels in transformants containing these control plasmids or mutant plasmid 2, 2G, 2N, 24, 24G, or 24N were compared. Immunoblotting with anti-FliG antibody (Fig. 9a) indicated that the levels of FliG present in transformants containing pUCG, pUCMG, 2G, or 24G were approximately five times the chromosomal level of FliG. Immunoblotting with anti-FliN antibody (Fig. 9b) indicated that FliN levels in transformants containing pUCN, pUCMN, 2N, or 24N were significantly higher than the chromosomal levels of FliN, which were not detectable. Finally, it was clear from immunoblots with anti-FliM antibody (Fig. 9c) that the levels of wild-type and mutant FliM protein were not affected by the presence of additional FliG or FliN (compare the FliM level in transformants containing pUCM with that in transformants containing pUCMG or pUCMN, compare the FliM level in transformants containing plasmid 2 with that in transformants containing plasmid 2G or 2N, and compare the FliM level in transformants containing plasmid 24 with that in transformants containing plasmid 24G or 24N).

DISCUSSION

Context for interpretation of deletion mutant data. Our deletion study of FliM consisted of three sets of experiments: characterization of mutant phenotypes in the null background, characterization of mutant phenotypes in the wild-type background, and effect of additional FliG or FliN on mutant phenotypes in the null background. (Figure 10 shows a summary of these data and the spontaneous-mutant data.) In interpreting the results of each set of experiments, we have relied on the

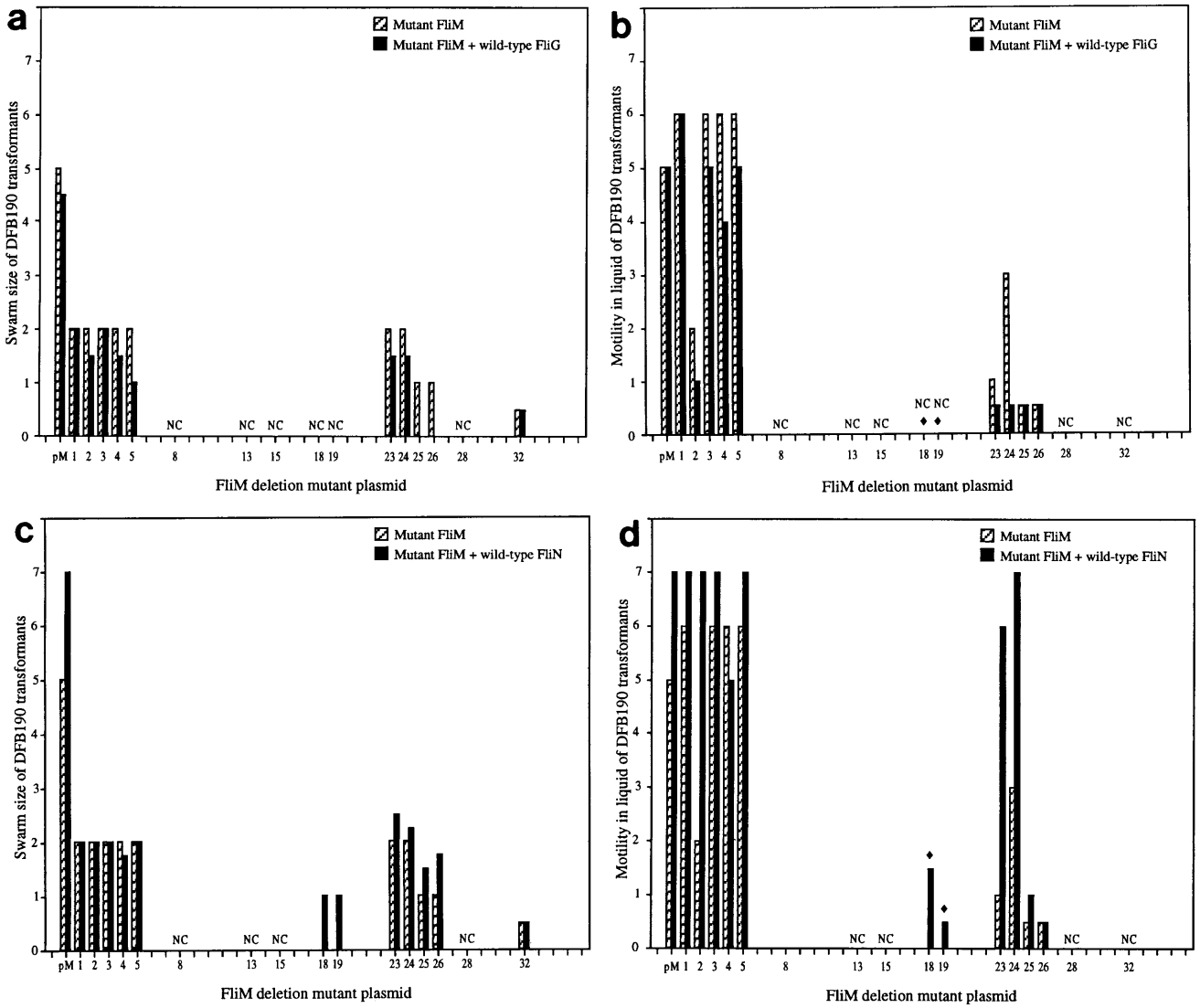

FIG. 8. Summaries of effect of additional FliG on swarming and swimming of DFB190 cells transformed with control plasmids and selected deletion mutant plasmids and summaries of effect of additional FliN on swarming and swimming of DFB190 cells transformed with control plasmids and selected deletion mutant plasmids. Transformants that were nonflagellate with or without additional FliG or FliN are labelled NC to indicate "not changed." Transformants that were well flagellated but nonmotile with or without additional FliG are indicated both by NC and by diamonds. Transformants that were well flagellated but nonmotile before the addition of FliN are indicated by diamonds.

following assumptions derived in part from our own observations in this study and in part from previous studies of flagellar switch protein interactions.

(i) Mutant phenotypes in the null background. We have assumed that a 10-amino-acid deletion constitutes damage to the FliM protein severe enough that any functions surviving the deletion are not principally carried out by the region in which the deletion is located. We have not assumed that if a particular deletion mutant protein is unable to restore flagellation to the null strain, the deleted region is important only for flagellation; it might be important also for motor rotation or switching.

In interpreting data involving mutants unable to restore flagellation to the null strain, we have considered the possibility that the three-dimensional structures of these proteins have been altered to such an extent that they no longer resemble that of the wild-type protein. In this case, it would not be accurate to infer that the deleted regions are essential for flagellation—rather, the data would indicate only that these regions are essential for proper folding of the protein. Nonetheless, on the basis of two principal pieces of evidence, we have assumed that the majority of the mutant proteins are not dramatically misfolded. First, virtually every mutant protein that produced a nonflagellate phenotype in the null strain was easily detectable on immunoblots and was present in an amount comparable to that of the wild-type protein; these proteins were therefore not misfolded or unfolded to the extent that they became abnormally susceptible to proteolysis. Second, a majority of these mutant proteins (all but seven) produced dominant effects in the wild-type background and must therefore have substantially resembled wild-type protein. We therefore have assumed that if a particular deletion prevents the protein from supporting flagellation in the null strain, the deleted residues are required for flagellar assembly rather than for proper folding of the protein.

(ii) Mutant phenotypes in the wild-type background (dominance experiments). In many instances, mutant FliM proteins inhibited swarming or swimming of wild-type cells more than

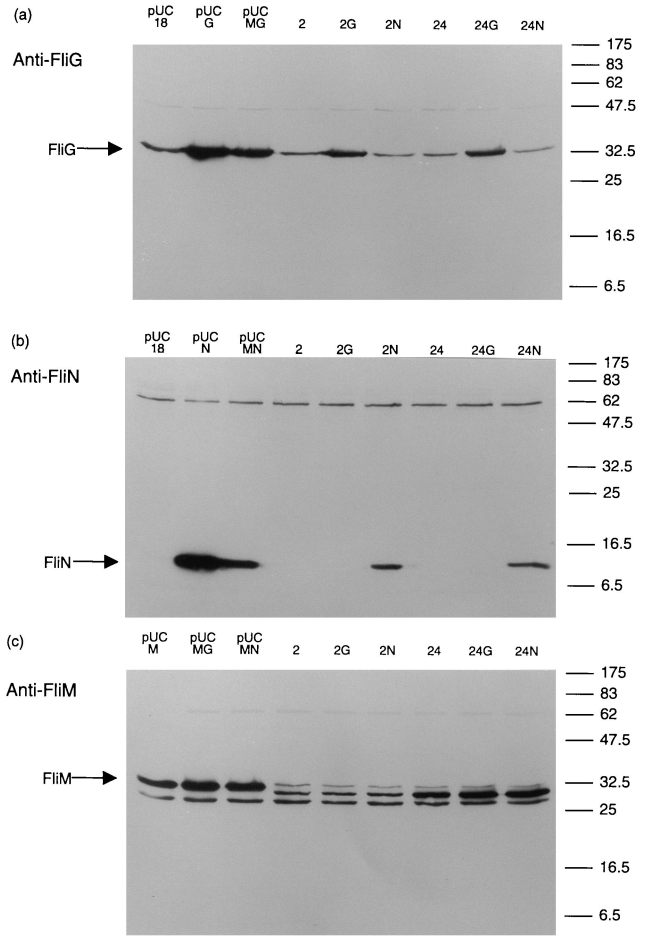

FIG. 9. Immunoblots of DFB190 cells transformed with control and selected mutant plasmids, as indicated, containing additional FliG or FliN. In each case, some molecular mass markers (in kilodaltons) are indicated at the right. In panels a and b, wild-type FliG and FliN proteins are apparent and are indicated at the left. In panel c, wild-type FliM protein is indicated at the left, and mutant proteins 2 and 24 can be seen at an apparent molecular mass slightly lower than that of wild-type FliM.

wild-type FliM expressed from control plasmid pUCM. We believe it is reasonable to assume that these dominance effects were produced by incorporation of mutant FliM proteins into the flagellar structure. In these cases, the phenotype of a mutant FliM protein in the null background was generally reflected in its phenotype in the wild-type background. Several mutant proteins that conferred only poor motility on the *fliM* null strain substantially reduced the motility of wild-type cells; similarly, other mutant proteins that produced a switch-biased phenotype in the null strain produced the same bias in the wild-type strain. We do not believe that these dominant mutant proteins inhibited wild-type function by sequestering some other flagellar protein—it is unlikely that a mutant protein would be more successful than the wild-type protein in binding to or sequestering some other flagellar component.

We have also assumed that the observed dominance phenotypes were produced by a combination of wild-type and mutant FliM molecules in the flagellar structure: phenotypes of the mutant proteins in the wild-type and null backgrounds, although similar in several instances, were not identical.

Finally, we have concluded that the dominance effects reflect the ability of a mutant protein to interact with wild-type copies of FliM. We have based this interpretation on the observation that several mutant proteins, although unable to support flagellation in the null strain, were nonetheless able to interfere with the motility of wild-type cells. In other words, these mutant proteins were capable of incorporation into the flagellar structure only in the presence of wild-type FliM and therefore were probably capable of interaction with wild-type FliM. This line of reasoning is consistent with previous indications that FliM associates with itself (20, 24).

(iii) Effect of addition of FliN on mutant phenotypes in the null background. We have interpreted this set of experiments in light of several lines of evidence indicating that the ratios of FliM to FliN in the cell and in the flagellar structure are critical for proper function. First, Kihara et al. found that addition of FliN to a FliM-FliN fusion mutant substantially improved its motility (10). Second, Lloyd et al. showed that addition of FliN to paralyzed FliM mutants (each containing a single point mutation in FliM) could restore some degree of motility (14). Third, we observed that addition of the *fliN* gene to the control plasmid pUCM or to several of the deletion mutant plasmids improved the ability of these plasmids to restore swarming and swimming to the *fliM* null strain. Fourth, we found that transformation of wild-type cells with plasmid pUCM or pUCN noticeably decreased wild-type function. Although we cannot yet explain the exact mechanism for this effect, we have assumed that it reflects some type of interaction between FliM and FliN. We therefore have concluded that if additional FliN improved the function of a FliM deletion mutant protein, that protein is capable (at least to some extent) of interaction with FliN.

(iv) Effect of addition of FliG on mutant phenotypes in the null background. Additional FliG decreased the function of several mutant proteins in the null background. We observed, furthermore, that addition of the *fliG* gene to control plasmid pUCM slightly decreased its ability to restore swarming to the null strain and that transformation of the pUCG plasmid into wild-type cells slightly decreased their swarming ability. These data suggest that the ratio of FliG to FliM in the cytoplasm and in the flagellar structure is important for proper motility. We do not, however, have a more detailed explanation for this effect that is convincing and therefore have not used it as a basis for our conclusions.

Overall characteristics of the FliM protein. Three main regions of the *S. typhimurium* FliM protein permit deletion of 10-amino-acid segments without the loss of ability to restore flagellation to the null mutant: the first is the N-terminal portion (amino acids 1 to 50), the second is a central region (amino acids 171 to 260), and the third is the C-terminal portion (amino acids 321 to 334). Regions in which the protein cannot tolerate 10-amino-acid deletions tend to be predominantly hydrophobic; those in which 10-amino-acid deletions are tolerated tend to be more hydrophilic (11, 12). One possible interpretation of this observation is that many of the hydrophobic regions are required for self-association of FliM or for its association with other proteins in the flagellar structure; if some of these interactions are critical to flagellar assembly, their disruption by deletion of 10-amino-acid stretches would be expected to block the assembly process.

The N terminus (residues 1 to 50) is important for switching but not essential for interactions with FliN or other FliM molecules. (i) Switching. Four of the five N-terminal mutant proteins (no. 1, 3, 4, and 5) restored wild-type flagellation and vigorous motility to the null strain. This region therefore does not appear to be essential either for flagellar assembly or for motility. However, these mutant proteins produced a counterclockwise switch bias in transformed cells, which consequently

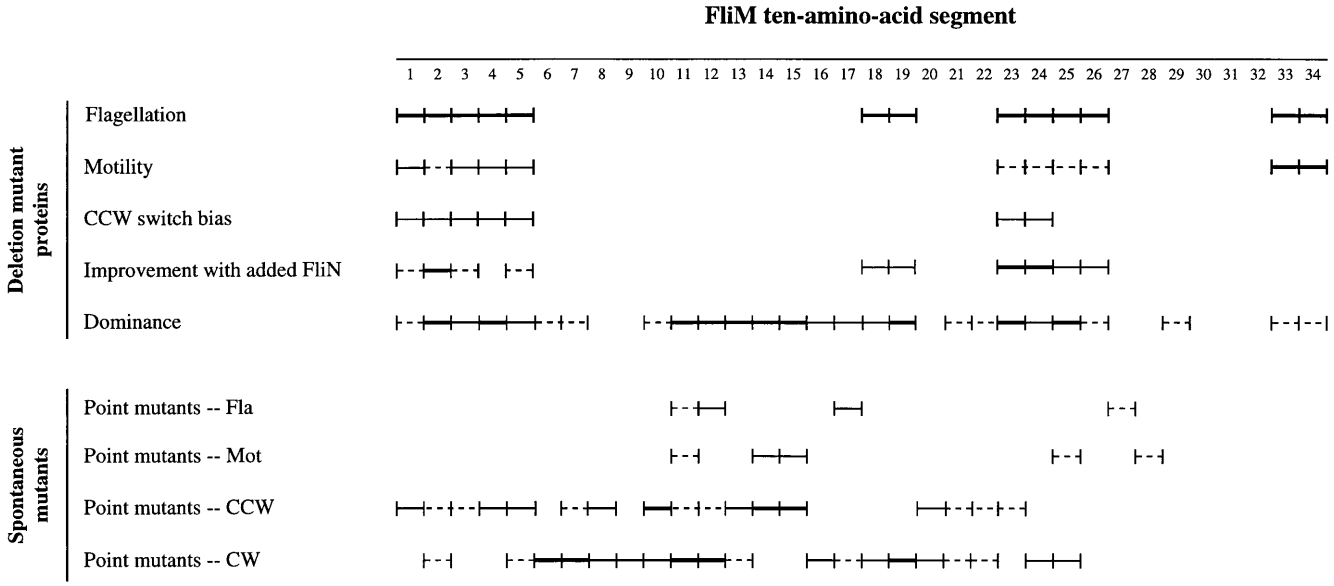

FIG. 10. Summary of deletion analysis and spontaneous-mutant study. The FliM protein is indicated by a horizontal bar at the top and is divided into 10-amino-acid segments. Characteristics of the deletion mutant proteins and spontaneous mutants are listed at the left. Each deletion mutant protein is represented by a horizontal bar spanning the deleted region and bounded at each end by a vertical bar. The degree to which the deletion mutant proteins exhibit each characteristic is indicated by the appearance of the horizontal bar: a dashed line indicates a small degree, a thin solid line indicates an intermediate degree, and a thick solid line indicates a large degree. Swarming and swimming data have been combined: for example, a mutant that is not dominant on swarm plates but is substantially dominant in liquid medium is represented by a thick solid line, indicating substantial dominance. The number of spontaneous mutations in a given 10-amino-acid segment is represented by a dashed line (indicating one position mutated), a thin solid line (indicating two or three positions mutated), or a thick solid line (indicating four or more positions mutated); frameshift and nonsense mutations have not been included. CCW, counterclockwise; CW, clockwise.

exhibited poor swarming on soft tryptone plates and smooth swimming as observed under the dark-field microscope. The N terminus thus appears to be primarily involved in switching.

The previous investigation of spontaneous FliM suppressors of CheY and CheZ (22) lends support to this conclusion. In that study, 21 spontaneous point mutations conferring a switch bias were found in the region between amino acid 1 and amino acid 50; of these, 19 produced a counterclockwise bias.

(ii) FliM self-association. Mutant proteins 3, 4, and 5 substantially interfered with swarming of wild-type cells; mutant protein 2 substantially interfered with wild-type motility in liquid medium. In each case, the phenotype of wild-type cells transformed with these mutant plasmids was similar, but not identical, to the phenotype of *fliM* null cells containing these plasmids: mutant proteins 3, 4, and 5 produced a switch bias and mutant protein 2 produced a motor rotation defect in wild-type cells. These dominance phenotypes were, most likely, produced by a combination of mutant and wild-type FliM molecules in the flagellar structure. We therefore conclude that mutant proteins 2 to 5 were capable of interaction with wild-type FliM molecules and that the N-terminal region of the molecule (different parts of which were missing from each of these mutants) is not essential for self-association of wild-type FliM molecules.

(iii) Interactions with FliN. Addition of a wild-type copy of the *fliN* gene to mutant plasmids 1 to 3 and 5 substantially improved their ability to restore motility to the null strain. Thus, mutant proteins 1 to 3 and 5 are able to interact with FliN, and the N-terminal region of FliM is not essential for the interaction.

Addition of the *fliN* gene to mutant plasmid 4 actually decreased its ability to restore motility to the null strain; no other mutant protein exhibited a decrease in function in the presence of additional FliN. Mutant protein 4, however, was unusual in another respect: it showed the greatest dominant effect of any mutant protein and was thus able to compete very successfully with wild-type FliM for installation into the motor. These results suggest that the affinity of mutant protein 4 for the flagellar structure is greater than that of wild-type FliM. If the effect of additional FliN is to increase the affinity of the deletion mutant proteins for the flagellar structure, it seems reasonable that additional FliN would not improve (and might decrease) the function of a mutant protein that already showed an affinity for the structure greater than that of the wild type.

The FliM extended N-terminal region (residues 51 to 100) is important for assembly and switching and possibly for FliM self-association. (i) Assembly. Mutants 6 to 10 were all unable to restore flagellation to the null strain. They were also unable to interfere with swarming or swimming of wild-type cells: they were not dominant. These mutant proteins are thus not capable of incorporation into the flagellar structure, even in the presence of wild-type FliM. These data suggest that the region from amino acid 51 through amino acid 100 is essential for flagellar assembly and possibly also for FliM self-association.

(ii) Switching. The prior spontaneous-mutant data (22) revealed a large cluster of switch-biased point mutations in this region; it is not surprising, however, that the more severe disruption (deletion of 10-amino-acid segments rather than point mutations) produced a more severe loss of function.

The FliM central region (residues 101 to 260) is important for motor rotation and switching. (i) Motor rotation and switching. Deletions in the central region gave rise to six examples of proteins that supported flagellation but produced motility defects in the null strain. Four of these mutant proteins (no. 23 to 26) gave rise to transformants that were poorly motile, and the remaining two (no. 18 and 19) produced transformants that were completely paralyzed. Seven other mutant proteins (no. 11 to 17) containing deletions in this region were

unable to support flagellation in the null background but substantially inhibited the motility of wild-type cells; these proteins therefore were also defective with respect to motor rotation. Finally, all but one of the spontaneous point mutations that produced a paralyzed phenotype occurred in this central region of the protein (22).

A number of spontaneous point mutations affecting switch bias were also found in this central region (22). A growing body of evidence indicates that FliM serves as an intermediary between the cytoplasmic switch regulator CheY in its phosphorylated state (22, 25, 26) and FliG (which is thought to be the switch protein primarily involved in torque generation [14, 22]). It therefore seems likely that mutations in the FliM protein in a region that is necessary for the interaction with FliG could produce either a switch-biased or motor-impaired phenotype. We therefore suggest that the FliM central region is involved in FliM-FliG interactions.

(ii) FliM self-association. A number of the central region mutant proteins (11 to 17 and 20 to 22) were unable to restore flagellation to the null strain. Most of these mutants (no. 11 to 17), however, exhibited dominance in the wild-type background. Thus, at least in the presence of wild-type FliM, most of these mutants were capable of incorporation into the flagellar structure. Therefore, the residues deleted from these mutant proteins are not absolutely required for flagellar assembly.

(iii) Interactions with FliN. Addition of a wild-type copy of the *fliN* gene to mutant plasmids 18, 19, and 23 to 25 improved (in some cases, dramatically) the ability of these plasmids to restore motility to the null strain. The residues deleted from these mutants are therefore not essential for interaction with FliN.

The C-terminal region (residues 261 to 320) is important for assembly and for FliM self-association. Mutant proteins 27 to 32 were essentially unable to support flagellation and were not dominant in the wild-type background. Most likely, these mutant proteins were unable to interact with each other or with wild-type FliM. This region thus appears to be critical for FliM self-association and flagellar assembly.

The prior spontaneous-mutagenesis study produced only four mutations in this region (a frameshift mutation, a nonsense mutation, and two missense mutations); three of the four mutants were nonflagellate (22). These data thus support the conclusion that this region is heavily involved in flagellar assembly.

The C terminus (residues 321 to 334). C-terminal mutant proteins 33 and 34 restored wild-type motility and switching to the null strain. This result is consistent both with the prior spontaneous-mutant study, which failed to find any spontaneous mutations in this region (22), and with another study in which a fully functional FliM mutant protein was found to lack the eight C-terminal residues and to possess instead a few residues of frameshifted sequence (11).

A model of switch protein interactions. The results of this study and of the prior spontaneous-mutant study (22) suggest that the different functions FliM must carry out (assembly, motor rotation, and switching) are separable to some extent and can be localized to different regions of the protein (Fig. 11). Mutations producing a particular phenotype tended to be clustered: mutations in the N-terminal region (residues 1 to 50) gave rise to mutants exhibiting a counterclockwise switch bias, mutations in the extended N-terminal region (residues 51 to 100) gave rise to nonflagellate deletion mutants and switch-biased point mutants, mutations in the central region (residues 101 to 260) gave rise to motor-impaired deletion mutants and switch-biased point mutants, mutations in the C-terminal region (residues 261 to 320) gave rise to nonflagellate mutants, and, finally, mutations in the C terminus (residues 321 to 334) gave rise to mutants exhibiting wild-type function.

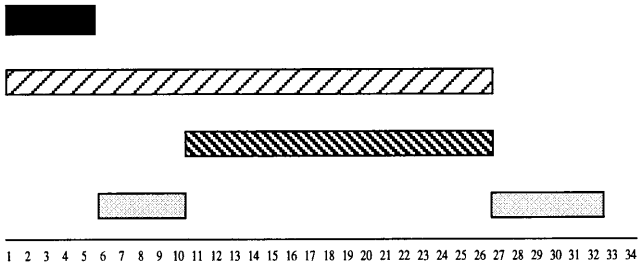

FIG. 11. Model of functional regions of the FliM protein (see text). The horizontal axis shows 10-amino-acid segments of the protein. Functional regions are indicated by coded bars above the FliM sequence (■, CheY-FliM interface; ▨, switching; ▧, motor rotation and FliG-FliM interface; ▩, assembly and FliM-FliM interface). In this model, we have not attempted to identify the region of FliM that constitutes its interface with FliN.

In summary, the mutant data indicate that the N-terminal region of FliM is primarily important for switching, the middle region is primarily important for motor rotation and switching, and both the extended N-terminal and the C-terminal regions are primarily important for assembly. We suggest that the N-terminal region of FliM interacts with CheY-P (which induces clockwise rotation), and its removal therefore throws the switch into its default, counterclockwise state. We suggest that the central region of FliM interacts with FliG to induce proper switching and to permit proper motor rotation. Finally, we suggest that the extended N-terminal and C-terminal regions interact with other FliM molecules and that this self-association is necessary for flagellar assembly.

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