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Salmonella typhimurium fliG and *fliN* Mutations Causing Defects in Assembly, Rotation, and Switching of the Flagellar Motor

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FliG, FliM, and FliN are three proteins of *Salmonella typhimurium* that affect the rotation and switching of direction of the flagellar motor. An analysis of mutant alleles of FliM has been described recently (H. Sockett, S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab, J. Bacteriol. 174:793–806, 1992). We have now analyzed a large number of mutations in the *fliG* and *fliN* genes that are responsible for four different types of defects: failure to assembly flagella (nonflagellate phenotype), failure to rotate flagella (paralyzed phenotype), and failure to display normal chemotaxis as a result of an abnormally high bias to clockwise (CW) or counterclockwise (CCW) rotation (CW-bias and CCW-bias phenotypes, respectively). The null phenotype for *fliG*, caused by nonsense or frameshift mutations, was nonflagellate. However, a considerable part of the FliG amino acid sequence was not needed for flagellation, with several substantial in-frame deletions preventing motor rotation but not flagellar assembly. Missense mutations in *fliG* causing paralysis or abnormal switching occurred at a number of positions, almost all within the middle one-third of the gene. CW-bias and CCW-bias mutations tended to segregate into separate subclusters. The null phenotype of *fliN* is uncertain, since frameshift and nonsense mutations gave in some cases the nonflagellate phenotype and in other cases the paralyzed phenotype; in none of these cases was the phenotype a consequence of polar effects on downstream flagellar genes. Few positions in FliN were found to affect switching: only one gave rise to the CW mutant bias and only four gave rise to the CCW mutant bias. The different properties of the FliM, FliG, and FliN proteins with respect to the processes of assembly, rotation, and switching are discussed.

The direction of rotation of the bacterial flagellum of *Salmonella typhimurium* is under the control of the flagellar switch, a structure that on genetic grounds is thought to consist of three proteins, FliG, FliM, and FliN, all of which are needed to permit rotation to occur and to control its direction (27). These proteins also affect the structural integrity of the flagellum, so that some mutations, especially severe ones (19), result in failure to assemble flagella at all.

The default rotational state of the switch is counterclockwise (CCW), with the clockwise (CW) state being stabilized by a component of the sensory transduction system, CheY, following its phosphorylation by another component, CheA; the simplest hypothesis is that phosphorylated CheY acts by binding to the switch. Still another chemotaxis protein, CheZ, catalyzes the dephosphorylation of CheY, rendering it incapable of stabilizing the CW state. Thus, CheZ has the effect of favoring the CCW state of the switch (for a review, see reference 3).

We have recently described an extensive analysis of mutations in *fliM* of *S. typhimurium* (19). The emphasis was on mutations that were isolated as suppressors of defects in *cheY* and *cheZ* and contribute CW and CCW biases, respectively, to the switch (Che^-[CW] and the Che^-[CCW] mutant phenotypes). Mutations responsible for the paralyzed (Mot^-) and nonflagellate (Fla^-) phenotypes were also analyzed. From that study, we concluded that the FliM protein plays a major role in switching and a more limited role in the mechanism of rotation itself.

We now extend the analysis to mutant alleles of the other two switch genes, *fliG* and *fliN*, and compare all three switch proteins in terms of their roles in flagellar structure, rotation, and switching.

MATERIALS AND METHODS

Bacterial strains. The *cheY* and *cheZ* mutant parental strains, the isolation of pseudorevertants from them, the isolation of other switch mutants, and the characterization of flagellation and of motility patterns have been described before (14, 19, 26, 27).

Isolation and amplification of chromosomal DNA. Chromosomal DNA was isolated and then amplified by the polymerase chain reaction method as described before (19). Mutant alleles of the *fliG* gene were amplified as a 1.6-kb fragment with an *a* direction primer in *fliF* and a *b* direction primer in *fliH*. Those for the *fliN* gene were amplified as a 0.5-kb fragment with an *a* direction primer in *fliM* and a *b* direction primer in *fliO*.

Cloning of *fliG* mutant alleles. Mutant alleles of *fliG* were cloned by digesting chromosomal DNA with *Kpn*I, which has sites at bp 16 within *fliG* and at bp 291 in *fliM* (5.6-kb total insert size), ligating it into *Kpn*I-digested pUC18 or pUC19 (IBI), and selecting motile transformants of a *fliI* mutant host, as described before (19).

DNA sequencing. DNA sequencing was done either by direct sequencing of the cloned genes on a plasmid (*fliG* only) or by direct sequencing of amplified linear DNA (*fliG*

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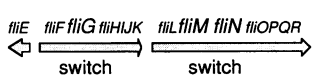

FIG. 1. Flagellar region IIIb of *S. typhimurium*, which is located at 40 min on the genetic map (18). The region contains the three operons shown (not drawn to scale). The three switch genes *fliG*, *fliM*, and *fliN* are emphasized. Immediately upstream of *fliG* is *fliF*, the structural gene for the basal-body MS ring; FliFG protein fusion mutants (5) are discussed in the text.

and *fliN*), as described before (19), with custom-synthesized primers directed at sequences distributed throughout the two genes.

Complementation analysis for polarity effects. Complementation analysis was performed by phage P22-mediated transduction as described before (27).

RESULTS

Isolation and characterization of pseudorevertants of *cheY* and *cheZ* mutants. A set of 10 parental *cheY* mutants (constituting 9 distinct mutations) and 10 parental *cheZ* mutants (10 distinct mutations) were used as the source of a large set of pseudorevertants (14, 19), whose second-site mutations mapped either to flagellar region II (and presumably lay in one of the chemotaxis genes) or to one of the three switch genes, all of which lie in flagellar region IIIb (Fig. 1) (10, 12). Any given *cheY* or *cheZ* mutation was found to be suppressible by any of a large number of mutations that mapped to many locations in all three genes.

For each *cheY* and *cheZ* mutant parent, 50 pseudorevertants with suppressors in the switch genes were chosen at random (i.e., without knowledge of which of the three switch genes was involved) and used for further analysis. As expected from the first-site phenotype, all 500 of the *cheY* suppressors were found to be Che$^-$[CW] alleles, and all 500 of the *cheZ* suppressors were found to be Che$^-$[CCW] alleles. The analysis of the parental *cheY* and *cheZ* mutations and of the suppressors in *fliM* has been described before (19); in a related study with *Escherichia coli*, Roman et al. (17) analyzed *cheY* mutations that had been isolated as suppressors of switch mutations.

The majority of the Che$^-$[CW] alleles were found to lie in *fliM* (Table 1), and most of the remainder lay in *fliG*; only 1% lay in *fliN*. Among the Che$^-$[CCW] alleles, again the majority lay in *fliM*, but in this case the relative contributions of *fliG* and *fliN* were reversed, with *fliG* contributing only 3% of the instances.

TABLE 1. Distribution of suppressor mutations among switch genes

Switch gene	No. (%) of instances[a]	
	cheY suppressors (CW bias)	*cheZ* suppressors (CCW bias)
fliG	116 (23)	16 (3)
fliM	379 (76)	420 (84)
fliN	5 (1)	64 (13)
Total	500 (100)	500 (100)

[a] Suppressor mutations mapping to flagellar region IIIb (which contains the switch genes *fliM*, *fliG*, and *fliN*) were accumulated to a total of 50 per *cheY* or *cheZ* parent and then mapped in detail and sequenced (14, 19; this study). Thus, the numbers in a given column are a direct reflection of the relative probability of finding a mutation in each of the switch genes. Numbers in a given row bear no simple relationship to each other.

By classical deletion mapping methods, each suppressing mutation was localized to a particular segment of a 12-segment map of *fliG*, a 22-segment map of *fliM*, or an 8-segment map of *fliN* (14, 19; this study, data not shown); this mapping greatly simplified the process of identifying these mutations by DNA sequence analysis. The number of mutations found within a given segment is a combination of segment size (which is statistical in nature), the number of positions giving a recognizable mutant phenotype, and the intrinsic mutability of sites within a segment. This situation, which has been described in detail for *fliM* (19), also applies to *fliG* and *fliN* (data not shown).

Amino acid changes in FliG responsible for suppression of *cheY* and *cheZ* mutations. The wild-type sequences of *fliG* and *fliN* have been published (12) (GenBank accession numbers M24462 and M24465). We have sequenced about two-thirds of the *cheY* mutation suppressors in *fliG* (chosen to represent the various segments from the deletion mapping) and all of the *cheZ* mutation suppressors. We have also sequenced a number of other mutations associated with CW and CCW bias in strains that had been isolated in the course of previous studies (26, 27). All of these mutations were found to be missense ones, yielding single amino acid changes.

The *cheY* suppressors and other Che$^-$[CW] mutations together affected 26 amino acid positions in FliG (Fig. 2A); these are therefore positions that, in the wild-type sequence, are important for stabilizing the CCW state of the switch. More than half of them were encountered more than once (not necessarily with the same amino acid replacement), the mean number of instances for a given position being 3.5; this suggests that the FliG map is fairly close to saturated for this phenotype. The N-terminal 30% and (with a single exception at position 313) the C-terminal 30% of the FliG sequence were devoid of instances of CW mutations. The central part of the sequence contained positions scattered throughout in short clusters; the most prominent of these was between positions 165 and 195, where CW mutations were found at 12 of the 31 positions. This cluster included four amino acid changes (G165D, V167L, L172Q, and G185[A/D/S]) which, on the basis of the motility bias rating of cells carrying the *fliG* mutations in a *che* gene deletion background (14), have been identified as conveying a very strong CW bias to the switch.

Far fewer *cheZ* suppressors were generated in *fliG* than *cheY* suppressors (16 versus 116 instances; Table 1). At first this might seem to indicate that *fliG* is much less important for the CW state than for the CCW state of the wild-type switch (CCW versus CW state of the mutant switch). However, recall that suppressors of *cheY* and *cheZ* mutations were isolated without regard to the switch gene involved. The paucity of *cheZ* suppressors in *fliG* is therefore directly related to their abundance in *fliM* and *fliN* but is not directly related to the abundance of *cheY* suppressors in *fliG*. This conclusion is borne out by the fact that we have encountered 10 positions in FliG yielding CCW mutant bias (Fig. 2A); while this is considerably less than the 26 positions yielding CW mutant bias, the difference is not as great as the data in Table 1 superficially seem to indicate. Because of the small numbers of CCW mutants analyzed (25, including 9 first-site and other mutants), the redundancy of identification of a given position is less than for CW mutants, with only 4 of the 10 positions being encountered more than once. Presumably,

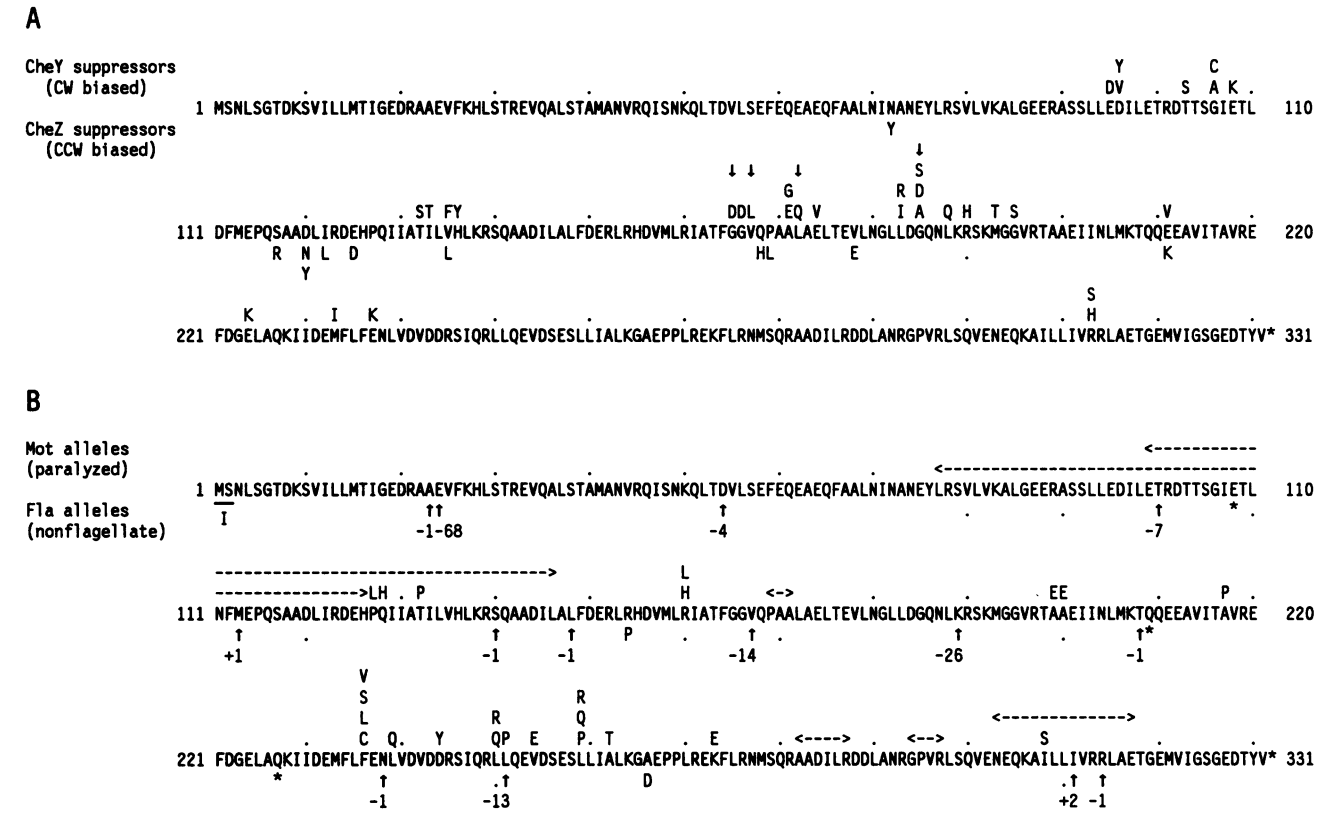

FIG. 2. Wild-type amino acid sequence of the switch protein FliG of *S. typhimurium* (12), together with changes present in mutant alleles of the protein. (A) Mutations responsible for the Che$^-$ phenotype. *cheY* suppressors, which contribute to a CW motor bias (tumbly motility), are shown above the wild-type sequence (mutations causing an especially strong CW bias are indicated by ↓); *cheZ* suppressors, which contribute to a CCW motor bias (smooth-swimming motility), are shown below it. The figure also includes a few instances of mutations in FliG that have been observed in spontaneous *che* mutants but not yet as *cheY* or *cheZ* suppressors. (B) Mutations causing the paralyzed (Mot$^-$) phenotype (above the wild-type sequence) and the nonflagellate (Fla$^-$) phenotype (below). <---->, amino acid deletion of the extent indicated. The start points of frameshift insertions and deletions are indicated by ↑ along with the number of base pairs involved. *, nonsense mutation generating a stop codon.

several other positions causing CCW mutant bias remain to be identified.

Just as with *cheY* suppressors, the N and C termini of the FliG sequence were devoid of instances of *cheZ* suppressors or other Che$^-$[CCW] mutations. The first mutation found was at position 72 of the 331-residue sequence, and the remainder lay between positions 117 and 211. Within the central part of the sequence, *cheZ* suppressors showed a small degree of clustering: a short stretch of nine residues (between positions 117 and 125) contained 4 of the 10 positions identified in this study and was separate from any of the *cheY* suppressor clusters described above. Only two positions were found where the same residue could be changed to give both mutant biases: V135F (CW) versus V135L (CCW), and E211V (CW) versus E211K (CCW).

In the case of FliM, the strength of the CCW bias generated by *cheZ* suppressors was estimated from the average motility bias of a given mutation in all *cheZ* parental backgrounds where it was encountered (19); with FliG, there were too few instances to make this approach feasible.

Amino acid changes in FliG responsible for Mot$^-$ phenotype. From previous studies, we had accumulated a number of switch gene mutants with the Mot$^-$ phenotype (9, 27). These strains, examined by the high-intensity dark-field light microscopy (13), were seen to be well flagellated, but their flagella failed to rotate. We have now sequenced the mutations in many of these strains. For FliG (Fig. 2B), they included a number of instances of single amino acid changes but also several in-frame deletions; there were no instances of nonsense or frameshift mutations. Among the missense mutations, more than one instance was observed at 8 of the 17 positions affected, suggesting that the spectrum of possible mutations was well but not completely represented.

None of the Mot$^-$ mutations lay near the N terminus. The first deletion started at position 77, and the first missense mutation did not occur until position 127. Instances of both types of mutations continued throughout most of the sequence, with the region between positions 236 and 262 containing an especially high density of sites, including three mutations (F236L, D244Y, and L259R) causing a temperature-sensitive Mot$^-$ phenotype (9, 23). Many of the amino acid changes involved the loss or gain of proline residues and therefore presumably caused a fairly major change in structure.

The in-frame deletions were remarkable in their number, extent, and distribution throughout the sequence. The two largest overlapped and together defined a stretch of 70 amino acids from positions 77 to 146 that could be deleted without affecting flagellar assembly. There was a short deletion at positions 169 to 171 and three others closer to the C

TABLE 2. Intergenic suppression of Mot$^-$ alleles of the switch genes[a]

First-site Mot$^-$ mutation	Second-site mutation	Second-site phenotype[b]
FliM (G133D)	FliN (V111L)	CCW
FliM (T147P)	FliG (E211K)	CCW
FliM (L272R)	FliG (N72Y)	CCW
FliN (L78Q)	FliG (N72Y)	CCW
FliN (L78Q)	FliM (R74H)	CCW
FliG (Q128H)	FliN (T82R)	CCW
FliG (Δ169–171, PAA)	MotB (L47R)[c]	[Mot$^-$][d]

[a] The isolation of these mutants is described in reference 26.
[b] Phenotype of the second-site mutation in isolation, i.e., crossed back into the wild-type background at the first site. For phenotypes of the strains containing both mutations, see Table 1 of reference 26.
[c] L47 in the *S. typhimurium* MotB sequence (21) corresponds to L46 in the *E. coli* sequence (20).
[d] Poorly motile (partially paralyzed).

terminus, the last one occurring at positions 303 to 317 of the 331-amino-acid sequence.

Amino acid changes in FliG responsible for Fla$^-$ phenotype. We encountered only two instances of a simple amino acid change giving rise to the Fla$^-$ phenotype, R154P and A266D (Fig. 2B). All others involved major defects, a situation similar to that which we have previously encountered with FliM (19). One of the *fliG* Fla$^-$ mutations was an in-frame deletion of 3 bp at the 5′ end of the gene (ATGAGT→ATT), resulting in loss of the ATG codon and so, presumably, failure of translation initiation. Of the remainder, 14 were frameshift mutations and 3 were nonsense mutations.

Amino acid changes in FliN responsible for suppression of *cheY* and *cheZ* mutations. Only five *cheY* suppressors were found in *fliN* (Table 1). Four of these (from three different parental backgrounds) involve the same amino acid change, Q83K. (For unknown reasons, we were unable to locate the mutation in the fifth strain.)

Because of the relatively large number of *cheZ* suppressors in *fliN*, we anticipated that quite a few amino acid positions would be capable of yielding CCW mutant bias. This proved not to be the case. We sequenced 40 of the 64 strains available and found only five amino acid changes, involving just three positions: G94C (25 instances), G94S (7 instances), E110K (1 instance), V111L (5 instances), and V111M (2 instances). Thus, the large number of mutants is a consequence not of a large number of residues that yield CCW mutant bias, but rather of high intrinsic mutability of just two sites, most notably G94. A similar situation was observed in the analysis of FliM (19), in which several positions were highly mutable; in the most extreme case there, L104, 38 instances of mutation were encountered.

One further FliN position (T82) giving rise to CCW mutant bias was found as a second-site suppressor of a *fliG* Mot$^-$ mutation (Table 2). Surprisingly, it is immediately adjacent to the sole position (Q83) that has given rise to a CW bias, and in both cases the mutation involves a change to a basic amino acid (T82R and Q83K, respectively).

Amino acid changes in FliN responsible for Mot$^-$ phenotype. Seven positions were found where a single amino acid change in FliN resulted in the Mot$^-$ phenotype (Fig. 3). Of these, only two were seen more than once. The region around positions 100 to 105 appeared to be especially important, with four of the six residues giving instances of mutations. There were four instances of frameshift mutations, but no instances of nonsense mutations. All four frameshifts

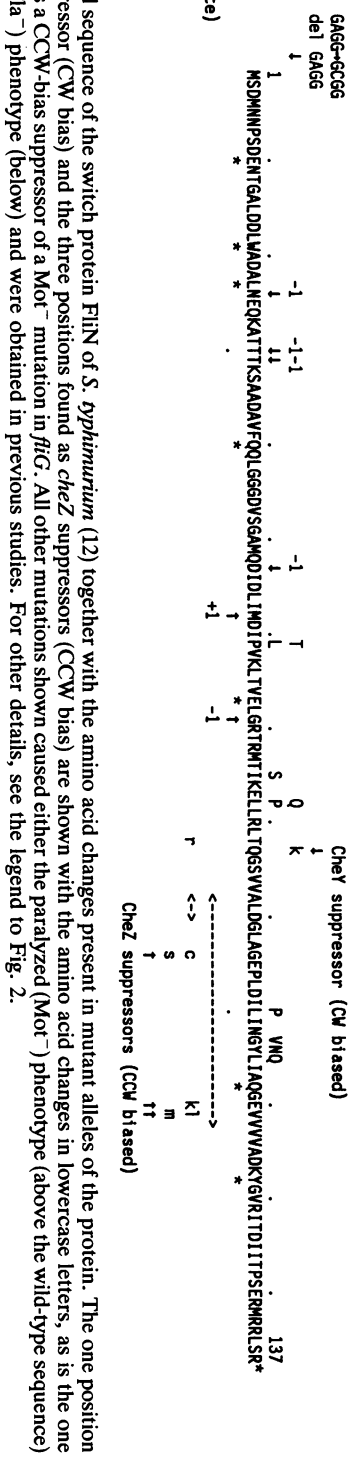

FIG. 3. Amino acid sequence of the switch protein FliN of *S. typhimurium* (12) together with the amino acid changes present in mutant alleles of the protein. The one position found as a *cheY* suppressor (CW bias) and the three positions found as *cheZ* suppressors (CCW bias) are shown with the amino acid changes in lowercase letters, as is the one position (T82) found as a CCW-bias suppressor of a Mot$^-$ mutation in *fliG*. All other mutations shown caused either the paralyzed (Mot$^-$) phenotype (above the wild-type sequence) or the nonflagellate (Fla$^-$) phenotype (below) and were obtained in previous studies. For other details, see the legend to Fig. 2.

were single-base deletions resulting in termination at the same position, yielding polypeptides of 55 residues whose sequences ranged from 23 natural residues plus 32 artificial residues to 52 natural residues plus 3 artificial residues.

Two mutations lay just before the 3′ end of the coding region of *fliM* rather than in *fliN* itself. Both affected the putative *fliN* ribosome-binding site, one by changing it from GAGG to GCGG and the other by deleting the four bases GAGG. These mutations presumably blocked or reduced translation of *fliN*. The changes that these mutations introduced to the C terminus of FliM were minor (in one case, a single amino acid change, and in the other, replacement of the last five amino acids by five artificial ones). It has been demonstrated in previous work that alteration of the FliM sequence near its C terminus has little effect on function (12).

Mutations in *fliN* responsible for Fla$^-$ phenotype. Mutations in *fliN* resulting in the Fla$^-$ phenotype were in-frame deletions or frameshift or nonsense mutations.

Nonpolarity of nonsense and frameshift mutations in *fliN*. As was described above, severe mutations in *fliN* gave in some cases the Mot$^-$ phenotype and in other cases the Fla$^-$ phenotype. The genes downstream of *fliN* within the *fliL* operon (*fliO*, *fliP*, *fliQ*, and *fliR*) all have Fla$^-$ as the only known mutant phenotype, and so if a given mutation in *fliN* was polar, it would be expected to produce that phenotype, regardless of the phenotype associated with the *fliN* mutation itself. Using the general transducing phage P22, we carried out complementation tests with all of the nonsense and frameshift mutants as donors. Transductants in which the recipients were defective in *fliO*, *fliP*, fliQ, or *fliR* (but not in *fliN*) gave trails on semisolid agar plates (data not shown). Such trails are diagnostic of motility achieved as a result of complementation in *trans* (28) and demonstrate that none of the *fliN* defects were polar on the downstream genes. Polarity therefore cannot be the explanation for the ambiguity concerning the null phenotype of *fliN*.

Intergenic and intragenic suppression of *mot* alleles in the switch genes. About half of the 83 Mot$^-$ mutants reported by Yamaguchi et al. (27) gave rise to motile swarms when streaked on semisolid agar. Most of these were the result of intragenic suppression, but in some cases the recovery of motility was a consequence of a second mutation in another switch gene, i.e., of intergenic suppression; these second-site mutations were themselves associated with the Che$^-$ phenotype (26). In the present study, we have obtained the sequences of several of the intergenic suppressor pairs of mutations and many of the first-site mutations that gave rise to intragenic suppressors.

The intergenic suppressor pairs we have analyzed are shown in Table 2. Both the original and the suppressing switch mutations were single amino acid substitutions. The FliN mutation L78Q was suppressed by two mutations, FliG-(N72Y) and FliM-(R74H); FliG-(N72Y) also appeared as a suppressor of another first-site mutation, FliM-(L272R). The Mot$^-$ alleles that could be suppressed intragenically were also simple amino acid changes (not shown in Table 2 but included in Fig. 2), with the exception of a 6-amino-acid deletion (AADILR) at positions 282 to 287. Thus, as expected, both intragenic and intergenic suppression typically involved minor mutations.

One *fliG* Mot$^-$ mutation had been found to be suppressible by a mutation in *motB* (27). This was a small in-frame deletion (PAA) at positions 169 to 171. The suppressing mutation had been located to the first segment of a 15-segment map of *motB* (25). We have now shown that it is the missense mutation L47R (where position L47 in *S. typhimurium* MotB [21] corresponds to L46 in the *E. coli* sequence [20]).

DISCUSSION

Properties of FliG with respect to switching, rotation, and flagellar structure. The central one-third to one-half of the FliG sequence, but not the termini, appears to be important for switching. Of the 331-residue sequence, 26 positions were associated with CW mutant bias and 10 with CCW mutant bias. There is no obvious pattern to the types of amino acid changes that are responsible for altered switch bias. Two of the four positions resulting in a particularly strong CW bias involved loss of a glycine, but with such small numbers, it is difficult to draw any conclusion from this.

FliG is extensively involved in motor rotation. Mutations in a large part of the sequence, beginning about 80 residues in from the N terminus and continuing essentially to the C terminus, cause paralysis. In several cases, the mutations are in-frame deletions; for these, of course, we cannot identify which specific amino acids are critical. However, there are a number of missense mutations, with a highly populated cluster centered about three-quarters of the way through the sequence.

With the exception of two missense mutations, all those conferring the nonflagellate phenotype were ones causing truncation. Two of these were close to the end of the gene and cause loss of about 20 residues of the natural sequence; termination occurs shortly (9 or 10 codons, respectively) before the natural position, making it unlikely that the mutations are polar. The most direct interpretation would be that loss of this sequence prevents FliG from adopting the correct conformation for assembly; another possibility is that loss of this sequence renders FliG susceptible to degradation. Since all of the other frameshift and nonsense mutations would also result in loss of these C-terminal residues, we have no independent evidence (aside from the two missense mutations) that the rest of the FliG sequence is needed in this regard.

A recent study (5) has established that much of the N-terminal sequence of FliG is not necessary for flagellar assembly; a deletion mutation resulting in synthesis of a fusion protein between the basal-body MS-ring protein (FliF) and the switch protein FliG, but with 94 N-terminal amino acids of FliG and also 56 C-terminal amino acids of FliF removed, still permits flagellar assembly and (albeit somewhat impaired) rotation and switching. In light of this result and the fact that no missense mutations with a detectable phenotype, whether Che$^-$, Mot$^-$, or Fla$^-$, have been found, it would appear that the N-terminal portion of FliG is rather unimportant; yet its sequence is highly conserved between *S. typhimurium* and *E. coli* (12). Why it has so little apparent contribution to function is therefore unclear.

The most remarkable result concerning FliG is how much of its sequence can be removed without interfering with flagellar structure. Six different in-frame deletions were encountered, all of which caused the Mot$^-$ rather than the Fla$^-$ phenotype. Between them, they represent loss of 98 residues, or almost one-third of the entire sequence. Combining this result with that for the fusion mutant, one is forced to the conclusion that much of the FliG sequence is not involved in a major way with the integrity of the flagellar structure as a whole (though of course it may be involved with the integrity of the switch).

TABLE 3. Switch protein residues responsible for various mutant phenotypes

Protein	Size (residues)	Null phenotype	Mot⁻		CW bias		CCW bias	
			No. of positions[a]	Fraction (%) of total sequence[b]	No. of positions	Fraction (%) of total sequence	No. of positions	Fraction (%) of total sequence
FliG	331	Fla⁻	17[c] (ns)	5	26 (s)	8	10 (ns)	3
FliM	334	Fla⁻	8 (s)	2	50 (s)	15	39 (s)	12
FliN	137	Fla⁻?[d]	7 (ns)	5	1 (S)	0.7	4 (s)	3

[a] Number of positions in the sequence that gave rise to one or more instances of the given phenotype. The letters in parentheses are qualitative estimates of the extent of saturation of the map for that phenotype: S, probably all positions identified; s, probably most positions identified (more than half of the positions shown were seen more than once); ns, probably several positions remain to be identified (less than half of the positions shown were seen more than once).
[b] Fraction of the positions in the sequence that gave rise to one or more instances of the given phenotype.
[c] Not including deletion mutations.
[d] See text re the ambiguity concerning the null phenotype of *fliN*.

The suppression of a FliG motility defect by a second mutation in MotB is intriguing. The FliG mutation is a small (3-amino-acid) deletion in the middle of the sequence, while the mutation in MotB is an L→R change at position 47. From data for mutants in which FliG is fused to the MS-ring protein FliF, it appears that much if not all of FliG projects from the face of the MS ring into the cytoplasm (5). MotB is a membrane protein (20, 24) with hydrophobic N terminus; data from protease digestion and alkaline phosphatase fusion experiments (4) indicate a single membrane span with a large periplasmic C-terminal domain beginning at about position 50. This would place the suppressing mutation within the membrane-spanning segment, close to the boundary of the membrane and the periplasm. If so, the two mutation sites cannot be in physical proximity to each other, and we therefore suspect that the suppression mechanism is an indirect one. The MotB mutation involves a change from leucine to arginine, which would be expected to perturb the membrane span (probably by displacing it in the direction of the periplasm), thereby affecting the small N-terminal cytoplasmic portion of the molecule, the large periplasmic domain, or both. Changes in the state of the cytoplasmic portion of MotB could affect FliG directly; changes in the periplasmic domain would more likely affect it indirectly via another component, such as MotA.

Properties of FliN with respect to switching, rotation, and flagellar structure. Classical analysis had suggested that *fliN* was principally associated with defects in rotation (27) (indeed, in *E. coli*, the gene was originally called *motD* [15]). Nonetheless, we were surprised to find just how limited its role in switching was. In the case of CCW-bias mutants, although a relatively large number were found, their mutations corresponded to just four positions in the sequence. The few CW-bias mutants found were all defective in the same amino acid, Q83.

Thus, only five positions in FliN have been found to affect the rotational bias. This suggests that FliN may not be directly involved in the switching process. These residues could represent rare cases in which a change in FliN structure is propagated into the structure of either FliG or FliM in such a way that their switching properties are affected. However, it could also be that FliN does participate directly in the switching process but in a way that primarily involves changes in quaternary interactions rather than changes in its own conformation.

There are at least seven FliN residues (and probably several more, since the map was not saturated) that are important for motor rotation. We suspect that this protein may be directly involved in the rotation mechanism. (We cannot exclude the possibility that these residues also play a role in switching. The Mot⁻ mutant phenotype, being epistatic, would prevent the Che⁻ phenotype from being detected.)

It is unclear at this point whether FliN is necessary for flagellar assembly, since some severe mutations gave the nonflagellate phenotype while others gave the paralyzed phenotype. This situation is difficult to understand if the severe consequences predicted from the various mutations are in fact occurring; perhaps in some cases (those giving the Mot⁻ phenotype), the consequences may be less severe than a simple interpretation of the mutation would indicate. For example, when premature termination of translation is predicted, might there be reinitiation so that important C-terminal sequence is in fact synthesized? Further analysis of these mutations is needed before the null phenotype of *fliN* cans be stated with certainty.

Comparison of the properties of the three switch proteins. Although mutations in all three switch proteins can affect assembly, rotation, and switching, the proteins have interesting differences when examined in detail. (Refer to Fig. 2 and 3 for mutations in FliG and FliN; Fig. 3 of reference 19 for mutations in FliM; Table 3 for comparisons of various parameters; and Fig. 4 for schematic diagrams of the primary structures of the three proteins.)

(i) Flagellar assembly. First, consider how important the

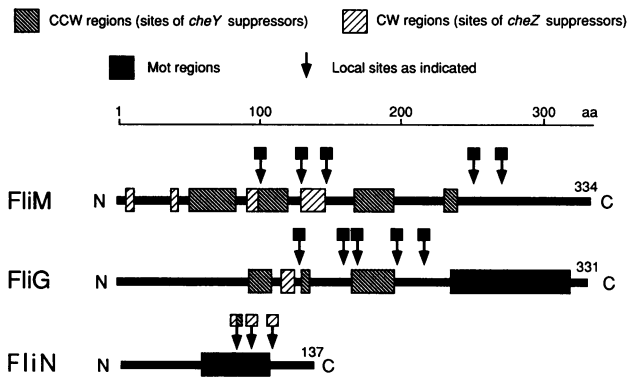

FIG. 4. Schematic illustrations of the primary structures of FliM, FliG, and FliN, indicating regions important for the CCW and CW states of the wild-type flagellar switch. Mutant alleles in these regions result in CW bias or CCW bias and were mostly isolated as suppressors of *cheY* and *cheZ* defects, respectively. Also shown are the regions that gave rise to mutants with the paralyzed (Mot⁻) phenotype. When a region is so local that it consists of only one or two nearby positions, it is indicated by an arrow. The schematic for FliM is modified from reference 19.

three proteins are to the structure of the rest of the flagellum. In other words, what is their propensity to give the Fla$^-$ mutant phenotype? In all three proteins, many of the mutations causing failure of flagellar assembly are highly destructive ones—nonsense or frameshift mutations. Few positions were found where missense mutations gave the Fla$^-$ phenotype; FliM yielded the largest number (six positions). Even with FliM there are strikingly large regions that are devoid of missense mutations: none in the first 104 residues or in the last 73 residues. In the case of FliG, only two positions have been found thus far, one roughly in the middle of the sequence and the other about 20% in from the C terminus. FliG also has the remarkable property that large internal deletions nonetheless permit flagellar assembly. No missense mutations in FliN that prevented flagellar assembly were found.

From these results, we conclude that the three switch proteins are not active participants in the actual mechanism of assembly of the flagellum (or, more precisely, of the filament hook basal-body complex) but, like many other structural components, need to be incorporated in order for assembly to proceed to completion. The most likely process to be affected by their absence is that of flagellar protein export. This process is thought to employ an apparatus at the flagellar base (23), i.e., in the same general location as the switch (5). It would then not be surprising if there was the potential for steric interference between the two structures. In addition, it is likely that the three switch proteins are mutually necessary for their own assembly, and they may be necessary for assembly of the Mot proteins.

(ii) Flagellar rotation. All three proteins are important to motor rotation, judging by the fact that each contains a number of positions where an amino acid change results in paralysis. FliG is involved in the greatest degree, judging by the total number of positions where an amino acid change gives the Mot$^-$ phenotype (Table 3); however, relative to its small size, FliN also contributes in an important way to rotation. The functions of rotation and switching appear to be related to some degree, in that for all three proteins there are examples where Mot$^-$ and Che$^-$ mutations lie close by in the primary structure. In the case of FliM, this is true of most of the positions giving the Mot$^-$ phenotype, although there are two extensive regions, at the N terminus and near the center of the sequence, which are heavily involved in the switching process but not in rotation. In FliG, there is a region with the converse property, in that it contains most of the Mot$^-$ positions but no Che$^-$ mutations. A striking feature of all three proteins is that their N termini do not seem to be important for rotation; the first Mot$^-$ mutations are not encountered until far into the sequence.

(iii) Flagellar switching. This is the function in which the three proteins differ the most (Table 3). FliM is clearly the protein with the most structure implicated in switching, especially for stabilizing the CCW state (judging by the large number of positions at which CW mutant alleles were found). FliG has a substantial number of positions important for the CCW state and fewer for the CW state. FliN has revealed only five positions that affect switching, one for the CCW state and four for the CW state; the very limited degree to which it participates in the switching process is remarkable and makes it at least questionable whether it should be described as part of the switch.

In the case of FliM, approximately the first 70% of the primary structure contains all of the positions found to be important for switching, while in FliG, only the central portion of the sequence is heavily involved. Although the mutations on both proteins responsible for the CCW and CW mutant phenotypes are largely segregated into clusters, in neither case are the clusters large enough to constitute an entire domain of the protein. We have argued elsewhere (19) that they may represent patches of residues that are responsible for the interactions in the two switch states. We noted for FliM that several of the amino acid changes that strongly affected switching were changes in charge and suggested that the switch may utilize electrostatic interactions (19); there are examples of charge changes in the other two proteins, but the pattern is less striking.

Allele specificity of suppression. In our previous analysis of *cheY*, *cheZ*, and *fliM* mutations (19), we considered in detail whether the data indicated allele specificity between the first and second mutations, which could have been used as evidence for physical interactions between CheY or CheZ and FliM. We concluded that suppression was not allele specific but rather was a result of balancing the switch biases introduced individually by the two mutations; this type of suppression could be achieved readily, because any mutation impairing one state (say, CCW) is equivalent to enhancement of the other state (CW). Our results in the present study fully support this conclusion (which does not in any way argue against an interaction between the chemotaxis proteins and the switch).

In the case of intergenic suppression between pairs of switch genes, a different situation was encountered (26). First, examples of suppressors were rare, as would be expected if they derived from compensating physical interactions. Second, suppression was allele specific; when a given Mot$^-$ allele was combined with an arbitrarily chosen Che$^-$ allele in another switch gene, motility was not regained. We interpreted this to mean that suppression was not achieved merely by nonspecifically adjusting the switch bias but rather by some specific structural compensation. We therefore concluded (and still do) that the data provide genetic evidence for a multi-subunit complex consisting of FliG, FliM, and FliN. However, the suppression was not totally specific, because in one case a *fliN* Mot$^-$ allele gave rise to two distinct suppressing alleles, one in *fliG* and the other in *fliM*. This has now been verified by our sequence analysis (Table 2), which has also uncovered an example in which two distinct Mot$^-$ alleles (one in *fliM* and the other in *fliN*) were suppressed by the same allele in *fliG*. This raises the question of whether suppression occurs via direct physical contact between the corresponding amino acids; in the most extreme case indicated by the data, this would require FliM272, FliG72, FliN78, and FliM74 all to be in the same immediate vicinity (Table 2).We suspect that, while some of the examples of suppression may involve direct interactions, others may be occurring less directly—though still specifically—compensating via allosteric changes in the interacting subunits.

Mot$^-$ mutations and the proton conductance pathway. The rotation of the flagellar motor of *S. typhimurium* and *E. coli* is driven by proton potential. All of the five proteins known to be involved in motor rotation (MotA, MotB, FliG, FliM, and FliN) have now been subjected to mutant sequence analysis (1, 2, 19; this study), and in all cases, Mot$^-$ mutations involving acidic or basic residues are rare. (In MotB, although there are a number of mutations involving charged residues, they are in the periplasmic domain, which is not believed to be involved in proton conduction.) In FliM, only 1 of 8 residues affected are charged (replacement of H106); in FliG, 3 of 17 residues affected are charged;

(R160, D244, and K273); and in FliN, 0 residues affected are charged.

If we broaden the criterion to include polar residues that could be involved in hydrogen-bonding relays, a few additional residues meet it: two in FliM (T147 and T149), for a total of three; two in FliG (Q128 and T132), for a total of five; and one in FliN (Y104). However, since in some species the motor is driven by sodium rather than proton potential (7), the most economical hypothesis regarding the mechanism is one involving ion association-disociation events rather than a hydrogen-bonding chain, in which case one would not expect residues like Thr, Gln, or Tyr to participate.

Many of the switch mutations causing the Mot$^-$ phenotype involve loss of hydrophobic residues (Ala, Leu, Ile, Val, Phe, Met, or Pro): 3 of 8 in FliM, 12 of 17 in FliG, and 5 of 7 in FliN. The MotB mutation that suppressed a FliG defect also fell into this category. It seems unlikely that these residues are involved in the actual mechanism by which torque is generated. They may, however, be important structurally and interfere with rotation for this reason.

Thus, there is no clear evidence at this time as to which residues in the motility proteins actually contribute to the proton conductance pathway.

Model for the spatial and functional organization of the switch. Where are the switch proteins located? The only direct evidence available is for FliG (5); mutants synthesizing a fusion protein of FliG and FliF (the protein from which the basal-body MS ring is constructed [6, 8, 22]) were found to be almost normal in function. Electron microscopy showed that the FliG component of the fusion was located at the cytoplasmic face of the MS ring, making it almost certain that this is its natural location.

Since the ring face was largely occluded, we suspect that neither FliM nor FliN is directly associated with the MS ring but may instead be associated indirectly via FliG.

There is good evidence that CheY (a cytoplasmic protein) binds to the flagellar switch (16), but it is not known which switch component(s) contributes to the binding site. Had the pattern of suppressor mutations been allele specific, it might have allowed identification of the site for binding of CheY. Although we were unable to detect any such allele specificity, the analysis does give some clues as to the target for CheY binding. FliN seems the least likely target, given how few of its residues affect switch bias. FliM, on the other hand, has many residues that are important to the switch state (more than twice as many in FliG, which is the same size). The location of FliG, directly at the MS ring and almost occluding it, suggests that FliM may have a location more towards the cytoplasm. Both of these lines of evidence suggest that FliM is the most likely target for binding of CheY and for initiating the subsequent switching process.

If FliG and FliN are the two components most involved in the mechanism of rotation, it seems likely that they will interface with the rest of the motor structure, namely, MotA and MotB; these two proteins are located in the membrane and are thought to surround the basal body. Electron microscopic evidence indicates that FliG extends to the outermost edge of the MS ring. No such information is available for FliN, but in view of its role in motor rotation, we hypothesize that it may exist near the outer radius of the MS ring. A cartoon illustrating this model for the organization of the switch is given in Fig. 5.

We then hypothesize that the following sequence of events takes place during switching. (i) When FliM does not have CheY bound, it is in the CCW state and, as a result of direct interaction with FliG, ensures that it is in the CCW state

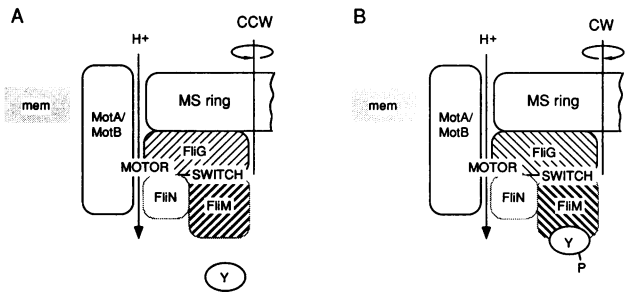

FIG. 5. Model for the structure of the switch and its function. FliG has been shown to be located at the cytoplasmic face of the MS ring (5). MotA and MotB are integral membrane (mem) proteins that are believed to surround the MS ring (11). FliM and FliN are thought on genetic grounds to interact with FliG (26); the locations shown for them are speculative but are consistent with the available evidence (see text). FliM is postulated to be the target for CheY (Y), based on the large number of positions within its sequence that affect the CCW versus CW state of the switch. FliM and FliG are postulated to constitute the switching function, while FliG and FliN (perhaps with MotA and MotB) constitute the motor function. (A) When CheY is not bound to FliM, both FliM and FliG are in their CCW states and the motor rotates CCW. (B) When CheY is phosphorylated (Y-P) and binds to FliM, it places FliM in its CW state; this in turn causes FliG to change to its CW state, and so the motor rotates CW.

also. Proton conductance by FliG and FliN (possibly with the participation of MotA and MotB) therefore results in the generation of CCW torque (Fig. 5A). (ii) CheY, when phosphorylated, binds to FliM and changes its state to CW. This change is propagated to FliG. The altered state of FliG results in an altered path of proton conductance and the generation of CW torque, and so the direction of motor rotation switches (Fig. 5B).

Finally, we consider the current state of knowledge concerning the subdivision of the motor into its rotor and stator elements. The basal-body M and S rings, once thought to be constructed from different proteins and to constitute rotor and stator elements, respectively, are now known to represent domain features of the same protein, FliF (22). It seems likely that the MS ring rotates as part of the passive structure that mechanically communicates torque from the motor to the external filament. The fact that the motor is still able to rotate when FliG is covalently fused to the MS ring then demonstrates that if FliF is part of the rotor, so also is FliG. If we are correct in suggesting that FliM binds CheY and propagates its change in conformation to FliG, it seems likely that the two proteins are in a mutually fixed relationship to each other, in which case FliM would also be part of the rotor. The circumferential location of the MotA and MotB proteins suggests that they are part of the stator.

We are unaware of any evidence that favors a rotor versus a stator role for FliN. Although intergenic suppression evidence (26) has indicated that it interacts with the other switch components, this result does not necessarily imply a static (rotor-rotor or stator-stator) interaction. One possibility is that FliN acts with FliG to develop torque against the Mot proteins, as is depicted in Fig. 5; another is that it is associated with the Mot proteins and acts with them to develop torque against FliG.

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