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

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Defects in the chemotaxis proteins CheY and CheZ of *Salmonella typhimurium* can be suppressed by mutations in the flagellar switch, such that swarming of a pseudorevertant on semisolid plates is significantly better than that of its parent. *cheY* suppressors contribute to a clockwise switch bias, and *cheZ* suppressors contribute to a counterclockwise bias. Among the three known switch genes, *fliM* contributes most examples of such suppressor mutations. We have investigated the changes in FliM that are responsible for suppression, as well as the changes in CheY or CheZ that are being compensated for. Ten independently isolated parental *cheY* mutations represented nine distinct mutations, one an amino acid duplication and the rest missense mutations. Several of the altered amino acids lie on one face of the three-dimensional structure of CheY (A. M. Stock, J. M. Mottonen, J. B. Stock, and C. E. Schutt, Nature (London) 337:745–749, 1989; K. Volz and P. Matsumura, J. Biol. Chem. 266:15511–15519, 1991); this face may constitute the binding site for the switch. All 10 *cheZ* mutations were distinct, with several of them resulting in premature termination. *cheY* and *cheZ* suppressors in FliM occurred in clusters, which in general did not overlap. A few *cheZ* suppressors and one *cheY* suppressor involved changes near the N terminus of FliM, but neither *cheY* nor *cheZ* suppressors involved changes near the C terminus. Among the strongest *cheY* suppressors were changes from Arg to a neutral amino acid or from Val to Glu, suggesting that electrostatic interactions may play an important role in switching. A given *cheY* or *cheZ* mutation could be suppressed by many different *fliM* mutations; conversely, a given *fliM* mutation was often encountered as a suppressor of more than one *cheY* or *cheZ* mutation. The data suggest that an important factor in suppression is a balancing of the shift in switch bias introduced by alteration of CheY or CheZ with an appropriate opposing shift introduced by alteration of FliM. For strains with a severe parental mutation, such as the *cheZ* null mutations, adjustment of switch bias is essentially the only factor in suppression, since the attractant L-aspartate caused at most a slight further enhancement of the swarming rate over that occurring in the absence of a chemotactic stimulus. We discuss a model for switching in which there are distinct interactions for the counterclockwise and clockwise states, with suppression occurring by impairment of one of the states and hence by relative enhancement of the other state. FliM can also undergo amino acid changes that result in a paralyzed (Mot⁻) phenotype; these changes were confined to a very few residues in the protein.

Previous studies have led to the conclusion that in *Salmonella typhimurium* (and also in *Escherichia coli*) there are three flagellar proteins, FliG, FliM, and FliN, which function together in enabling motor rotation and in controlling its direction of rotation (2, 4, 10, 35, 41). The part of the flagellum constructed from these proteins is called the flagellar switch. The state of the switch, counterclockwise (CCW) or clockwise (CW), is influenced by the chemotaxis sensory system, such that CCW rotation is enhanced by favorable stimuli and CW rotation is enhanced by unfavorable stimuli (14). On the basis of intergenic suppression analysis in *E. coli* (24) and the effect of CheY on cell envelopes lacking any of the other chemotaxis proteins (25), CheY is thought to bind to the switch and bias it to the CW state. CheZ has the opposite effect on switch bias (13), and intergenic suppression analysis (23, 24) suggests that it, too, binds to the switch; however, in this case no biochemical evidence is available to support the hypothesis. CheY is activated by CheA (by phosphorylation of aspartate 57) and deactivated by CheZ (by dephosphorylation) (1, 6, 27, 38). The conformation adopted by the phosphorylated form of CheY is believed to be the one that is effective in modulating the switch state, either because it has a higher affinity for the switch or because it has a greater effect on the switch state. Acetyladenylate and fumarate may also affect the state of the switch (3).

Switch gene mutations are often suppressible by *cheY* and *cheZ* mutations and vice versa (16, 23, 24, 40). Parkinson et al. found that the suppression exhibited some degree of allele specificity and used this as evidence for physical interactions between the CheY and CheZ proteins and the switch (23, 24).

In a recent study, Magariyama et al. (16) obtained intergenic suppressors of 10 independently isolated *cheY* mutations in *S. typhimurium*, mapped them locally within the relevant switch gene, and characterized the bias of the pseudorevertant strains carrying the mutations. For each of the 10 parental *cheY* mutations, 50 switch mutations were analyzed. The majority (76%) of these mutations lay within *fliM* and were localized largely to only a few segments of the gene. A similar study has also been carried out for *cheZ* suppressors (see Results); again, the majority lay within *fliM*.

The amino acid sequences of wild-type FliM, CheY, and CheZ are all known (11, 29, 31). We have investigated the

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TABLE 1. *S. typhimurium* strains used in this study

Strain	Genotype/phenotype	Parent	Parental genotype/phenotype	Reference
SJW1103	Wild-type			42
SJW2339[a]	Δ(*fliE-fliN*)/nonflagellate			16
SJW3076[b]	Δ(*cheA-cheZ*)/nonchemotactic			16
SJW3103	*cheB*/CW bias	SJW1103	Wild type	39
SJW2903 (Y_1)[c,d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW2905 (Y_2)[d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW2906 (Y_3)[d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW2908 (Y_4)[d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW3062 (Y_5)[d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW3063 (Y_6)[d]	*cheY*/CCW bias	SJW2323	*fliG*/CW bias	16
SJW3064 (Y_7)[e]	*cheY*/CCW bias	SJW1103	Wild type	16
SJW3066 (Y_8)[d]	*cheY*/CCW bias	SJW3065	*fliM*/CW bias	16
SJW3068 (Y_9)[d]	*cheY*/CCW bias	SJW3067	*fliM*/CW bias	16
SJW3069 (Y_{10})[d]	*cheY*/CCW bias	SJW3067	*fliM*/CW bias	16
SJW3037 (Z_1)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3038 (Z_2)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3039 (Z_3)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3040 (Z_4)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3041 (Z_5)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3042 (Z_6)[e]	*cheZ*/CW bias	SJW1103	Wild type	This study
SJW3044 (Z_7)[d]	*cheZ*/CW bias	SJW3043	*fliM*/CCW bias	This study
SJW3046 (Z_8)[d]	*cheZ*/CW bias	SJW3045	*fliM*/CCW bias	This study
SJW3048 (Z_9)[d]	*cheZ*/CW bias	SJW3047	*fliM*/CCW bias	This study
SJW3049 (Z_{10})[d]	*cheZ*/CW bias	SJW3047	*fliM*/CCW bias	This study

[a] Used as recipient for transfer of *fliM* alleles into the wild-type *che* background.
[b] Used as recipient for transfer of *cheY* or *cheZ* alleles into the wild-type switch background.
[c] Y_1, etc., and Z_1, etc., are used in the text and tables as abbreviated strain names for the *cheY* and *cheZ* parents.
[d] These *cheY* or *cheZ* alleles were obtained as suppressors of switch mutations and then placed in a wild-type switch background for use as parents in reference 16 and the present study.
[e] These *cheY* or *cheZ* alleles are spontaneous first-site mutants used as parents in reference 16 and the present study.

types of changes in FliM that were responsible for changes in switch bias, and we report here the results for a large number of suppressor mutations in *fliM*, as well as their parental *cheY* or *cheZ* mutations.

MATERIALS AND METHODS

Bacterial strains. Strains used for the generation of *cheY* and *cheZ* parents and their pseudorevertants are listed in Table 1.

Isolation of pseudorevertants of *cheZ* mutants, mapping of mutations, introduction of suppressor mutations into different *che* backgrounds, and characterization of motility patterns. Isolation of pseudorevertants of *cheZ* mutants, mapping of mutations, introduction of suppressor mutations into different *che* backgrounds, and characterization of motility patterns were performed essentially as described for the analysis of pseudorevertants of *cheY* mutants (16).

Preparation of chromosomal DNA. A single colony from each mutant was inoculated into 10 ml of LM medium (10 mg of Bacto-Tryptone per ml, 5 mg of Bacto-Yeast Extract per ml, 5 mg of NaCl per ml) supplemented with 40 μg of thymine per ml and 1 μg of thiamin per ml. Following overnight incubation at 37°C, cultures were centrifuged, resuspended in 500 μl of sterile H_2O, frozen at −70°C, thawed rapidly at 95°C, and extracted with phenol. The DNA was precipitated with ethanol, treated with RNase A, reextracted with phenol-chloroform, and reprecipitated. Following resuspension of the DNA in 500 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.9]), the concentration was estimated by reading the optical density at 260 nm.

Cloning and direct plasmid DNA sequencing of mutant alleles. *Eco*RI was a convenient restriction enzyme for cloning *fliM*, since there is an upstream site at bp 658 in *fliG* and no other site for about 5.6 kb, almost at the end of *fliM* (bp 975 of the 1,002-bp amino acid coding sequence). By using a host strain defective in *fliI* (which lies between *fliG* and *fliM*), we were able to use recovery of motility as the basis for selection of the desired clone.

Chromosomal DNA from each *fliM* mutant strain was obtained as described above. Approximately 5 μg was digested with *Eco*RI for 2 h at 37°C, and the digestion mixture was ligated with *Eco*RI-digested pUC18 or pUC19 (U.S. Biochemicals) and incubated overnight at 15°C with T4 DNA ligase (IBI). *E. coli* GMIK123 (a Tn*10*::*fliI* derivative of the restriction-defective strain GM2163 [New England BioLabs]), made competent by $CaCl_2$ treatment, was transformed with the religated mixture and applied as two 100-μl lines on soft tryptone plates (13 mg of Bacto-Tryptone per ml, 7 mg of NaCl per ml, 3.5 mg of Bacto-Agar per ml) containing 50 μg of ampicillin (Sigma) per ml. The plates were dried for 1 h at 30°C and incubated in a humid environment at 30°C. Plates were examined over the next 2 days for flares appearing from the line of the streak. A sample was taken from the periphery of each flare and streaked onto an LM-ampicillin plate. Single colonies from each flare were tested for swarming on soft tryptone plates containing ampicillin, and clones which swarmed were restreaked on LM-ampicillin plates. The plasmid constructions were checked by restriction digestion of minipreparations obtained by the alkali-sodium dodecyl sulfate method (26).

fliM-containing plasmids were transferred to strain DH5α (New England BioLabs), and plasmid DNA was prepared and denatured by the method of Mierendorf and Pfeffer (18) from cells grown from single colonies in 1.5-ml cultures in

LM-ampicillin. Direct plasmid sequencing by the dideoxy-chain termination method (28) was carried out with [^{35}S]dATP and Sequenase version 1.0 DNA polymerase (U.S. Biochemicals).

Polymerase chain reaction amplification and DNA sequencing of mutant alleles. *cheY* and *cheZ* were amplified in a single fragment of 1,237 bp by using primers corresponding to bp 921 to 938 of *cheB* and bp −149 to −166 of *flhB* (32). *fliM* was amplified in a single fragment of 1,506 bp by using primers corresponding to bp 121 to 138 of *fliL* and bp 153 to 136 of *fliN* (11). Reaction buffer comprised 67 mM Tris (pH 8.8), 16 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, and 170 μg of acetylated bovine serum albumin per ml (9) in a final volume of 100 μl. The reaction mixture also included 1 μg of chromosomal DNA, the two primers at a final concentration of 1 μM each, the four deoxynucleotides at a final concentration of 1.5 mM each, and 5 U of AmpliTaq DNA polymerase (Cetus Corp.). Reaction mixtures were overlaid with 100 μl of mineral oil and heated to 94°C for 90 s to denature the DNA. The mixture was then subjected to 30 amplification cycles, each for 1 min at 37°C to anneal the primers, 10 min at 72°C for strand extension, and 1 min at 94°C to denature the DNA. In the final cycle the extension step was continued for 30 min and the denaturation step was omitted.

Sequencing was carried out directly on the amplified DNA after it had been extracted once with chloroform to remove the mineral oil overlay. End-labeled primers were used, eliminating the need to remove excess primers carried over from the polymerase chain reaction.

The sequencing protocol was modified from that described in reference 34. Approximately 0.3 pmol of amplified target DNA was mixed with 2 pmol of end-labeled primer in 15 μl of 40 mM Tris (pH 7.5)–20 mM $MgCl_2$–50 mM NaCl. The mixture was heated at 94°C for 5 min to denature the DNA and then placed on ice for 5 min to allow the primer to anneal.

The mixture was then subdivided into four tubes, containing the four deoxynucleotides at a final concentration of 250 μM each, as well as one of the four dideoxynucleotides at 25 μM, 3.4 mM dithiothreitol, and 3.25 U of the Sequenase version 2.0 DNA polymerase (U.S. Biochemical Corp.). Following a 5-min extension period at 37°C, an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF) was added. Samples were heated to 94°C for 5 min to denature the DNA before loading on 6% acrylamide gels.

Sequencing of *fliM* alleles was carried out with five 18-base primers, two on the *a* (nontemplate) strand at bp 111 to 128 and 602 to 619, and three on the *b* strand at bp 178 to 161, 524 to 507, and 677 to 660 (11). Choice of primer for a given mutation was greatly facilitated by knowledge of the position of the mutation within a given deletion segment; in most cases this resulted in successful detection in the first attempt, especially after previous experience had indicated the approximate extent of each deletion segment.

RESULTS

Strategies for obtaining mutant allele sequences. In this study, we used two different strategies for sequencing mutant alleles. In the first, we digested the chromosomal DNA with an appropriate restriction enzyme, cloned the digestion mixture into a plasmid, selected for the desired insert by using a flanking flagellar gene (*fliI*) as a marker, and then proceeded to direct sequencing of the gene on the plasmid.

TABLE 2. Parental CheY and CheZ mutations[a]

Strain designation[b]	Mutation in CheY	Strain designation[b]	Mutation in CheZ
Y_1	L66Q	Z_1	E103 to stop
Y_2	P110T	Z_2	L110P
Y_3	Duplication of L24	Z_3	Q142 to stop
Y_4	S104R	Z_4	Frameshift, Δ bp 529
Y_5	L9W	Z_5	Q39 to stop
Y_6	L9W	Z_6	T145M
Y_7	A99T	Z_7	D143E
Y_8	T115A	Z_8	Frameshift, Δ bp 271–278
Y_9	K45N	Z_9	F141I
Y_{10}	A113V	Z_{10}	Frameshift, +C at bp 140

[a] The wild-type amino acid sequences of *S. typhimurium* CheY and CheZ are given in references 29 and 31, respectively.
[b] For full strain designations, see Table 1.

However, we eventually found that it was more effective to amplify the desired chromosomal region by the polymerase chain reaction and then sequence the amplified fragment by using end-labeled primers. The majority of the mutant sequences described in this paper were obtained by the latter approach. We checked a few examples by both methods and found the results to be in agreement.

Sequence analysis of parental *cheY* mutant alleles. Among the 10 parental *cheY* mutant alleles (Table 1), which had been isolated independently (16), 9 unique mutations were present (Table 2), with the amino acid change L9W appearing twice. There were no nonsense or frameshift mutations in the set. Given that we encountered only one mutation twice and that the mutations obtained by Matsumura et al. (17) are all different from ours, the map of *cheY* with respect to suppressible mutations is probably not yet close to being saturated.

Sequence analysis of parental *cheZ* mutant alleles. All 10 parental *cheZ* mutant alleles, which had been isolated independently (Table 1), were distinct (Table 2). Only four of them were missense mutations. Of the remainder, three were nonsense mutations, one was a frameshift insertion, and two were frameshift deletions; the earliest was a nonsense mutation at Q39 that generated an N-terminal fragment missing 176 C-terminal residues. Since *cheZ* is the last gene in the *tar* operon, there should be no polarity effects, and so these mutations should reflect the properties of CheZ alone.

***cheZ* mutations conferring CCW phenotype.** In a previous study (16) in which suppressors of mutations in switch genes were analyzed, we were surprised to encounter two suppressors of CW *fliG* mutations mapping to *cheZ*, since *cheZ* mutations themselves normally confer the CW phenotype. When the second-site mutation was isolated, the phenotype was in fact found to be CCW, as would be expected for suppression of a CW switch mutation. Thus the procedure is such as to select for mutant CheZ that is better than the wild type at stabilizing the CCW state. Both mutations were missense, with the amino acid changes responsible being R54C and V166G.

Isolation and mapping of *cheZ* suppressor mutations. We describe here the mapping of *cheZ* suppressors; for the mapping of *cheY* suppressors, see reference 16.

The 10 independently isolated *cheZ* mutants (Tables 1 and 2) were used to generate a total of 940 independent suppressor mutants, which were detected as swarms emerging from a line inoculum of the parent on a semisolid agar plate. Among these, 78% of the suppressor mutations mapped to

TABLE 3. Distribution of *cheZ* suppressor mutations between flagellar region IIIb and the *che* region of flagellar region II

cheZ parent[a]	Total no. of pseudorevertants isolated	% of suppressor mutations mapping to:	
		Switch genes in flagellar region IIIb	*che* genes in flagellar region II
Z_1	118	65	35
Z_2	80	76	24
Z_3	130	61	39
Z_4	100	83	17
Z_5	84	82	18
Z_6	82	95	5
Z_7	82	94	6
Z_8	90	80	20
Z_9	84	76	24
Z_{10}	90	83	17
Total	940	78	22

[a] For full strain designations, see Table 1.

flagellar region IIIb. (Region III has recently been shown to be disrupted by an extensive region of DNA unrelated to flagellar function and has therefore been redefined as two regions, IIIa and IIIb [15].) The remainder mapped close to the original mutation and were probably either intragenic suppressors or compensating mutations in another nearby *che* gene such as *cheY*; they were not examined further. As had been observed with suppression of *cheY* mutations (16), different *cheZ* mutants gave substantially different ratios of suppressors in region IIIb flagellar genes to those in region II chemotaxis genes (Table 3).

Our finding that the majority of *cheZ* suppressor mutations occurred in flagellar rather than chemotaxis genes differs from that of Parkinson et al. (24), who found that only 14% were in flagellar genes, with the remainder occurring closely linked to *cheZ*. There is also a difference for *cheY* suppressors, with Magariyama et al. (16) finding 47% in flagellar genes and Parkinson et al. (24) finding 97% of them there. The reasons for these differences are not clear.

For each of the 10 *cheZ* parents, 50 region IIIb suppressor mutants (pseudorevertants) were chosen at random for further study. The mutations all lay in *fliG* (3%), *fliM* (84%), or *fliN* (13%); the corresponding figures for the *cheY* suppressors described in reference 16 are 23, 76, and 1%, respectively. Similar values for the relative probabilities of suppression by *fliG* and *fliM* have been reported by Parkinson et al. (24).

In this paper, we confine ourselves to analysis of suppressors in *fliM*, which contributed the majority by far. An analysis of those in *fliG* and *fliN* will be presented separately.

Suppressors in *fliM* were fine-mapped to segments of a 22-segment map of the gene that had been defined previously by the recombination properties of deletion mutants (41). Figure 1B shows a histogram of the results; the equivalent data from the previous analysis of suppressors of *cheY* mutations (16) is shown for comparison in Fig. 1A. The *cheZ* suppressors cluster strongly, notably in segments 4, 5, 8, and 19. Segments 1 and 2, 10 to 17, and 21 and 22 are devoid of examples. This is broadly similar to the distribution of *cheY* suppressors (Fig. 1A), for which segments 5, 6, and 18 to 20 predominate.

Switch bias of the *cheZ* mutants and their pseudorevertants. We describe here the switch bias of the 10 parental *cheZ* mutants and their pseudorevertants. For the corresponding

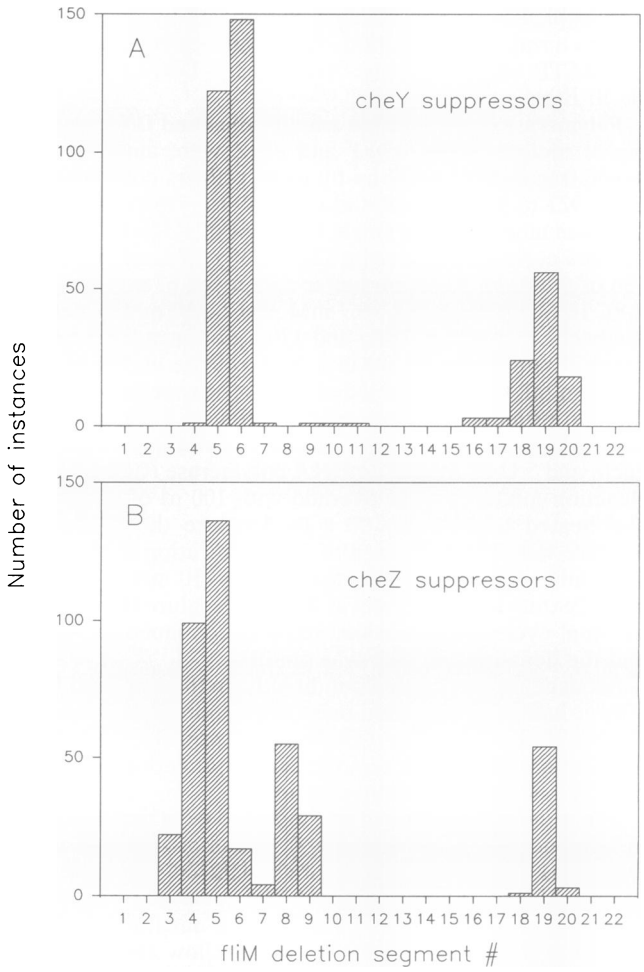

FIG. 1. Suppression of *cheY* mutations (A) and *cheZ* mutations (B) of *S. typhimurium* by mutations in the switch gene *fliM*. Histograms show the numbers of instances of suppressor mutations that were found in the various segments of a 22-segment map of *fliM* that had been constructed by deletion mapping (41). The data for *cheY* suppressors were originally reported in reference 16; those for *cheZ* suppressors are from the present study.

data for the *cheY* mutants, their pseudorevertants, and other derivatives, see reference 16.

All 10 *cheZ* mutants tumbled frequently, being rated 4 on the bias scale from 1 to 5 (extreme CCW to extreme CW) used in reference 16. This agrees with previous characterizations of *cheZ* mutants as having a CW bias (21). Different pseudorevertants ranged from tumbly (rating of 4) to smooth (rating of 1), but almost all were smoother than their *cheZ* parent. Thus *cheZ* suppressors contribute a bias toward the CCW state of the switch, in contrast to *cheY* suppressors, which contribute a CW bias, as has been noted in previous studies. Pseudorevertants from a given parent exhibited a considerable range of bias, indicating various degrees of severity of the compensating switch mutation.

In temporal gradient assays involving a saturating stimulus of L-aspartate (0 to 1 mM), parental *cheZ* strains (even those with nonsense or frameshift mutations), and also the pseudorevertants derived from them, responded with smooth swimming; the duration of the response was typically about 3 min, substantially longer than that of wild-type cells (about 1 min). However, on semisolid agar plates containing mini-

mal medium and glycerol as the carbon and energy source (37), all of the *cheZ* parents swarmed very poorly (typically about 30% of the wild-type rate), and the presence of aspartate (10 μM) increased the swarm rate only slightly (typically by about 10 to 20%, whereas the wild type increased its rate by about 80%). We examined the swarming of at least two examples of pseudorevertants of each *cheZ* parent. In the absence of aspartate, all swarmed significantly better than their parents (50 to 110% of the wild-type rate). The effect of aspartate varied depending on the parental mutation and the suppressing mutation: for pseudorevertants of parents with a severe *cheZ* mutation (Z_1, Z_3, Z_4, Z_5, Z_8, and Z_{10}), aspartate caused little further increase (typically 10 to 20%) in the swarm rate. For those with a missense *cheZ* mutation (Z_2, Z_6, Z_7, and Z_9), aspartate caused increases that depended on the particular suppressing mutation, but frequently were 40% or more. In some of these cases the swarming was at essentially wild-type rates. Thus the enhanced spreading of pseudorevertants compared with their parents is a consequence of achieving a more suitable switch bias, coupled in some cases with the recovery of a true chemotactic capability (see Discussion).

Location of *cheY* and *cheZ* suppressors at the level of amino acid position within FliM. The mapping of suppressors of *cheY* and *cheZ* mutations in *fliM* involved a total of 379 and 420 examples, respectively (16; Fig. 1). We have sequenced more than half of both sets of suppressors, chosen randomly from each segment; when a segment was poorly represented, we sequenced a higher proportion. We also sequenced the mutations of most of the spontaneous Che^- *fliM* mutants that were reported in reference 41.

To facilitate comparison with the genetic mapping analysis, we first present the results simply in terms of the number of observed occurrences of a change at a particular residue, without regard to the specifics of the change. This is shown in Fig. 2A and B for *cheY* and *cheZ* suppressors, respectively. In 34 of the 49 codons at which *cheY*-suppressing missense mutations were detected, more than one example was encountered; for *cheZ* suppressors, the corresponding figures are 24 of 37. This suggests that the majority of the residues that are important to the two switch bias phenotypes have been identified.

The ordering of mutations by deletion segment (Fig. 1) and by amino acid position (Fig. 2) were, for the most part, consistent. Inconsistencies tended to occur at the vicinity of a segment boundary, or where a segment was small, and probably reflect ambiguities in the recombination analysis. Two mutations that were originally mapped to segment 1 of *fliM* (41) actually lie in *fliL*, a gene that was discovered later (7, 12); analysis of the *fliL* gene will be presented separately.

Since the extent of a segment has no functional significance, we did not sequence the deletion mutations to establish segment boundaries precisely. The approximate extents of the most highly populated segments are indicated by bars in Fig. 2.

The degree to which a given segment is represented is a combined result of segment size, mutation probability of codons within the segment, and phenotypic consequences of the mutations. Most of the poorly represented segments were small and contained few positions with phenotypically scorable mutations. Highly represented segments either were fairly large (e.g., segment 5), contained many positions with scorable mutations, or contained mutational hotspots. The most extreme example of the latter was segment 6, which was quite small but generated 38 examples of *cheY* suppressors via the same base change at codon 104 (Fig. 2A). Other strong hotspots occurred as *cheZ* suppressors in segment 4 (26 occurrences at codon 39) and segment 19 (26 occurrences at codon 208) (Fig. 2B). Since mutations at these positions were detected in pseudorevertants ranging widely in their degree of suppression, discrimination in the procedure for isolating the pseudorevertants cannot be the only or even the main reason for these hotspots. A major factor, presumably, is high intrinsic mutability.

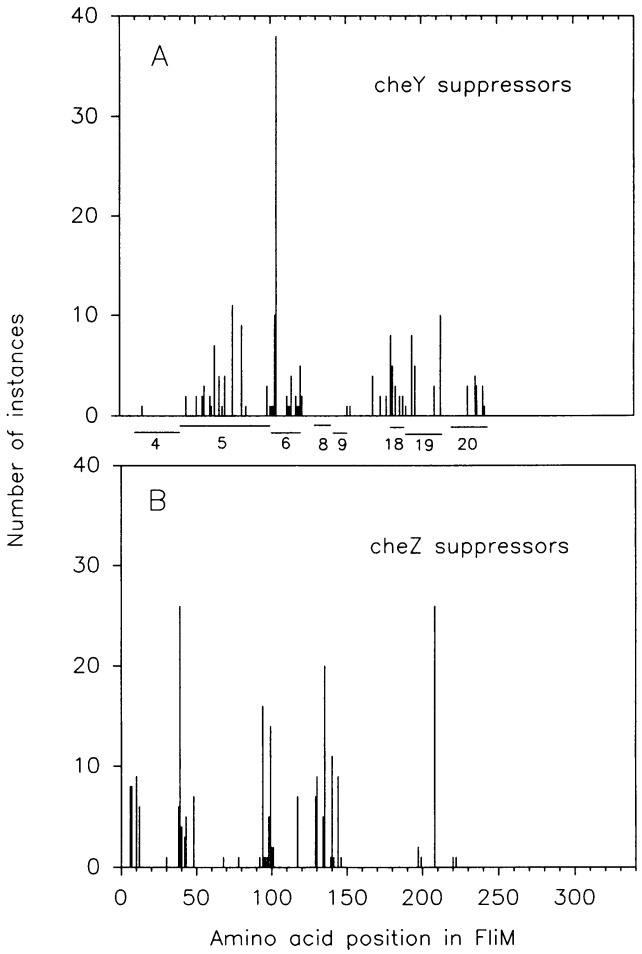

FIG. 2. Suppression of *cheY* mutations (A) and *cheZ* mutations (B) by mutations in the switch gene *fliM*. The data are now at the amino acid level, deduced from sequence analysis of the mutant alleles of the *fliM* gene. More than half of the mutations shown in Fig. 1 were subjected to sequence analysis. The histogram indicates the number of instances of an amino acid change at the corresponding position in the FliM sequence. The approximate extents of the most highly populated deletion segments of Fig. 1 are indicated as bars below Fig. 2A.

Amino acid changes in FliM responsible for suppression of *cheY* and *cheZ* mutations. Figure 3A shows the wild-type amino acid sequence of FliM (11), with *cheY* suppressor (CW) mutations shown above and *cheZ* suppressor (CCW) mutations shown below. Also included are three positions (E59, R74, and G143) that have been observed in spontaneous Che^- mutants, but have not yet been seen as *cheY* or *cheZ* suppressors; there are also a few examples of positions where a particular amino acid change has not yet been observed as a suppressor. Each amino acid change is presented only once, avoiding the distortion shown in Fig. 2, which is caused by multiple occurrences at hotspots.

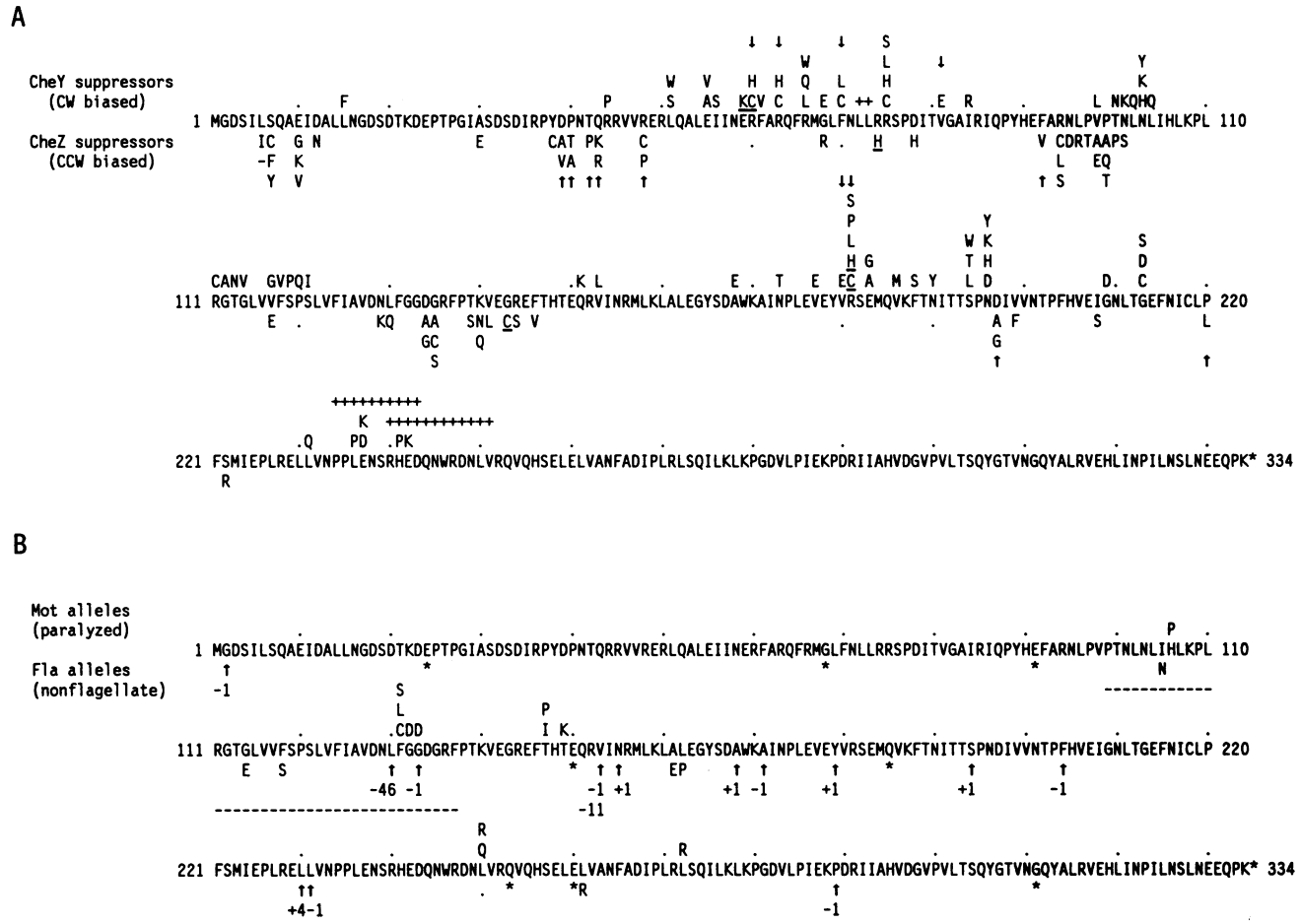

FIG. 3. Amino acid sequence of the switch protein FliM of *S. typhimurium* (11), together with the amino acid changes present in mutant alleles of the protein. A given amino acid change at a given position is indicated only once; i.e., this figure (in contrast to Fig. 2) is not a histogram. (A) Mutations responsible for Che$^-$ phenotype. *cheY* suppressors, which contribute to a CW motor bias (tumbly motility), are shown above the wild-type sequence; *cheZ* suppressors, which contribute to a CCW motor bias (smooth swimming motility), are shown below it. The figure includes a few examples (underlined) of mutations in FliM that have been observed in spontaneous *che* mutants but not yet as *cheY* or *cheZ* suppressors. Mutations causing a strong shift in switch bias are indicated by ↑ or ↓. (B) Mutations causing a paralyzed, or Mot$^-$, phenotype (above the wild-type sequence) and a nonflagellate, or Fla$^-$, phenotype (below) were obtained in previous studies. The start points of frameshift insertions and deletions in the *fla* mutations are indicated by ↑, and their extents are given in numbers of base pairs. Symbols: *, a stop codon; ----, amino acid deletion of the extent indicated; ++++, amino acid duplication of the extent indicated.

The distribution reveals several features that were crudely evident from the deletion mapping. (i) Bias to the CW state is caused by mutations between positions 44 and 242; neither the N nor the C terminus seems to play a role (except for a single example at L15). Within the central portion, regions centered around positions 65, 110, and 180 seem especially important. (ii) Bias to the CCW state is caused by mutations extending from close to the N terminus up to position 222. Particularly prominent are local regions around residues 10, 40, 95, and 135. The C-terminal 90 residues or so apparently play no role in switching (but see the material concerning L272 in the Discussion).

Almost all of the mutations involve simple amino acid substitutions. We encountered one example of a single-amino-acid deletion (L6) and three examples of duplications (L72-L73, P234-D243, and R240-V251). The last two, both *cheY* suppressors, overlap by 4 residues and cover, between them, a stretch of 18 residues. We did not observe any nonsense or frameshift mutations giving rise to the Che$^-$ phenotype.

Among the amino acids, their effect on switching varies widely. Appearing frequently are mutations involving Arg (44% of the Arg residues in the sequence), Phe (43%), Gly (39%), and Val (35%); at the other extreme are Gln (14%), Ala (13%), His (13%), Ile (12%), Lys (10%), and Met (0%). Possibly significant differences between amino acids involved in *cheY* and *cheZ* suppression include the following: (i) five of the six mutations involving a Glu residue are *cheY* suppressors, whereas all five of the mutations involving an Asp residue are *cheZ* suppressors; and (ii) four of the five mutations involving a Pro residue are *cheZ* suppressors.

From previous behavioral analysis (16), we are able to identify FliM mutations that confer a strong CW bias even in a *che* deletion background (which by itself causes a strong CCW bias). Prominent among these are changes from R to X, where X is neutral or weakly basic, and from V to E; therefore, both involve a shift to more negative charge. Mutations at R60, R63, V81, V180, and R181 are examples, the mean bias rating for mutations at these positions (in the *che* deletion background) being in the range of 3 to 4. For the

R-to-X changes, several replacements were effective, even at a given location, suggesting that it is the loss of the Arg that is critical rather than the nature of the replacement. In contrast, all of the instances at V81 and V180 (nine and eight examples, respectively) were V to E; however, part of the reason for this may be the restricted range of other amino acids (Ala, Gly, Met, or Leu) that can result from a single-base change in the GTG codon. A full evaluation of this issue would require generation of all 19 amino acid changes.

cheZ suppressors themselves contribute to CCW phenotype, and so examining them in a *che* deletion background does not yield any new information; we confirmed for a limited set of strains (those from Z_1) that they were all CCW. We also placed the suppressor mutations of Z_1 into a *che*$^+$ background, and, with two exceptions (which gave roughly wild-type bias), the strains were CCW. Thus the best available indication of the strength of a given *cheZ* suppressor is its average effect in different *cheZ* parental backgrounds, based on the behavioral assessments described above. In this way, we have identified (Fig. 3A) several amino acid changes in FliM that contribute strongly to the CCW state, having a mean bias rating of less than 2 among the pseudorevertants where they were encountered. There is no pattern as striking as that seen with *cheY* suppressors, although changes involving charged residues or prolines are common. The region of the sequence around position 40 seems particularly important in generating a strong CCW mutant bias.

Comparison of suppressor mutation distributions for different parents. As described above, the parents of the pseudorevertants represent a set of 9 distinct *cheY* mutations and 10 distinct *cheZ* mutations. We consider next the spectrum of suppressor mutations as a function of the parental mutation.

The spectra for *cheY* and *cheZ* suppressors are very different, as Fig. 2 and 3A clearly indicate. Of the 81 codons at which suppressors of *cheY* or *cheZ* mutations have been observed, only 5 have given rise to suppressors of both: G68E versus G68R, respectively, V98L versus V98(A/E), T100N versus T100P, N101K versus N101S, and V117G versus V117E.

For each of the *cheY* and *cheZ* mutations, there is a wide range of possible compensating mutations. A given *cheY* or *cheZ* mutation can be suppressed by mutations in other chemotaxis genes, and often in all three of the switch genes. Also, within a given switch gene, suppressors of a given parental mutation occur at a number of different positions. For *cheY* suppressors, this has already been illustrated at the level of deletion segments (see Table 3 of reference 16).

We now consider the question at the level of the amino acid position. Tables 4 and 5 are matrices giving, for each of the 10 *cheY* and 10 *cheZ* parents, the number of instances of a suppressing mutation at a given position. The number of positions in FliM that were found to suppress any given *cheY* mutation was large (mean, 11.2; low, 6; high, 19); the wide range is partly a result of variation in the total number of mutations sequenced for each parent. Data for *cheZ* suppressors were similar (mean, 12.8; low, 10; high, 15). We also found that mutation at a given position in FliM could suppress several different parental mutations, although here we were often limited in our interpretation by the small numbers involved: the absence of an instance for a given parent means little if only a few instances were encountered in total. At positions where there were many instances, the number of different parents with instances at that position was large. For example, for the four positions that gave rise to 10 or more instances of *cheY* suppressors, the average

TABLE 4. Distribution of suppressor of different *cheY* parents within FliM

Parental mutant	No. of instances[a] of mutation[b] at FliM amino acid position: 15 44 51 55 56 60 61 63 66 68 70 75 81 84 98 100 101 102 103 104 111 112 113 114 117 118 119 120 121 151 153 168 173 177 180 181 183 186 188 190 194 196 209 213 231 236 237 241 242	Total no. of: Instances	Positions[c]
Y_1	1 –[d] – 1 – – – – – – – – 1 – 2 1 – – – 4 – – – – – 1 1 – – – – – – – – – – – 1 – 2 1 2 – – –	18	12
Y_2	– – – – – – – 1 – – 3 5 – – – – – 1 5 – – – – – – – – – – – – – – – – – 1 – 2 – – –	18	8
Y_3	– 2 – – – 1 – 5 1 – – 1 2 – – – – – 2 3 – – – – – – – – – 4 1 – – – – – 1 – – – 1 –	23	11
Y_4	– – 1 2 1 – 1 – – – – 2 1 – – – 1 – 7 – – – – – 1 1 – – – – 2 1 2 – – – 2 3 – – – 1 – 1	33	19
Y_5	– – – – – – – 1 2 – – 1 2 – – – – 3 3 – – – – – – – – – – 2 2 – – – – 1 – – – – – –	17	9
Y_6	– – – – – 1 – – 1 – – 2 1 – – – – 4 1 – – – – – 1 1 – – – 3 – – 2 – – – 1 – – 2 – –	21	13
Y_7	– – 1 – – – – – – 1 – – – – – – – – 4 – 1 1 – – 1 1 – – – 2 – – – – – 1 3 1 – – –	18	12
Y_8	– – – – – – – – – – – – – – – – – – 5 1 – – – – – – – – – – – – – – – – 2 – 1 1 – –	12	6
Y_9	– – – – 1 – – – – – – 2 – – – 1 – 3 1 – – – – – 1 – – – – 2 – – – – – 1 – 1 – 1 – –	15	11
Y_{10}	– – – – – – – 1 – – – 1 1 – – – – 3 – – – – – 1 – – – – – 2 – – – – – 1 2 – – 1 2 –	18	11

[a] The number of times a change was observed at that amino acid position as a suppressor of the corresponding parental mutation.
[b] Only positions yielding at least one instance of a mutation are shown.
[c] The total number of positions in the amino acid sequence where at least one instance of change was observed.
[d] –, no instances of mutation.

TABLE 5. Distribution of suppressors of different *cheZ* parents within FliM[a]

Parental mutant	No. of instances of mutation at FliM amino acid position:																									Total no. of:	
	6	7	10	12	30	38	39	40	42	43	48	68	78	92	94	95	96	97	98	99	100	101	117	129	130	134	135	139	140	141	144	146	197	199	208	220	222	Instances	Positions
Z_1	1	1	3	2	–	–	1	1	3	–	1	–	–	–	3	–	–	–	–	2	–	–	–	1	2	–	–	–	–	–	1	–	1	1	–	24	15
Z_2	3	–	–	–	–	–	2	1	–	–	2	–	–	–	3	–	–	–	1	–	1	–	–	–	1	–	1	–	3	–	–	1	–	1	–	20	12
Z_3	–	1	–	2	–	–	1	–	–	–	–	–	–	1	2	1	1	–	–	3	–	–	–	2	2	2	3	–	–	–	1	–	2	–	–	24	14
Z_4	1	–	–	–	–	–	5	–	–	–	–	–	–	–	1	–	–	–	–	–	2	1	1	1	1	–	2	–	3	–	–	–	1	–	–	19	11
Z_5	1	1	2	–	–	–	–	1	–	1	–	–	–	–	2	–	–	–	–	1	–	–	2	–	–	1	4	–	1	–	–	–	3	–	–	21	13
Z_6	–	–	–	–	–	1	6	–	–	–	1	–	–	–	2	–	–	1	–	1	–	1	–	2	–	1	1	–	2	–	–	–	4	–	–	23	12
Z_7	–	2	1	–	–	4	–	–	–	1	–	–	–	–	–	–	–	–	1	2	–	–	–	–	2	–	4	–	1	–	1	–	4	–	–	24	12
Z_8	2[b]	2	2	1	–	–	–	–	–	2	–	–	–	–	2	–	–	–	2	2	–	–	2	1	1	–	1	–	1	1	–	–	–	4	–	26	15
Z_9	–	–	–	–	–	1	11	–	–	1	1	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	4	–	3	–	–	1	–	25	10
Z_{10}	2	1	1	1	1	–	–	1	–	–	2	–	–	–	–	–	–	–	1	3	–	–	2	–	–	1	4	–	–	–	–	–	–	5	–	1	26	14

[a] See footnotes to Table 4.
[b] Both of these mutations were deletions of residue 6 rather than amino acid substitutions.

number of parents yielding instances was 6.0. For the seven positions that gave rise to 10 or more instances of *cheZ* suppressors, the average number of parents was 7.3.

However, there are indications that the distribution is not entirely random. For example, where there are only two instances at a given position, they should both arise from the same parent with a probability of 0.1 if the process was random, whereas the observed probability was 0.5. Other examples of apparently nonrandom distribution are evident by inspection of the matrices, such as that for *cheZ* suppressors at position 39 in FliM.

There is also the previous observation (16) of significantly different representation of segments among different parents with respect to *cheY* suppression: mutations in segments 5 and 18 were more prevalent in some parental backgrounds, and mutations in segments 6 and 19 were more prevalent in others. These differences correlated roughly with the degree of CW bias introduced by the switch mutation, with segments 5 and 18 representing a higher CW bias than segments 6 and 19. This is reflected at the amino acid level by the fact that segments 5 and 18 contain the strongly CW-biasing R-to-X and V-to-E mutations described above, whereas segments 6 and 19 do not. These strongly biasing mutations tend to be encountered as suppressors with higher probability in certain parental backgrounds, such as Y_3, Y_5, and Y_6. To understand how this correlation arises, we attempted to estimate the degree of bias associated with a given *cheY* mutation. To do so, we took advantage of the fact that in the preceding study of these mutants and their pseudorevertants (16) the consequence of the switch mutation had been examined not only in the parental *cheY* background, but also in a *che* deletion background. The difference in the bias rating of a given switch mutation in the two backgrounds should then be a rough measure of the severity of the *cheY* mutation, with a small difference implying that the *cheY* mutation confers a strong CCW bias. For each *cheY* parent, we have averaged that difference over the total of 50 suppressor mutations observed in *fliG*, *fliM*, and *fliN*. By this criterion, the mutations in strains Y_5 and Y_6 (both L9W) ranked as the strongest, and that in Y_3 (L24 duplication) ranked next. Thus a balance of the strength of the *cheY* mutation and the strength of the switch mutation seems to contribute to the probability of selecting a particular pseudorevertant, probably because swarming would tend to be detected earlier.

The suppression in pseudorevertants combining a strong *cheY* mutation with a strong *fliM* mutation appears to derive largely from compensation of switch bias rather than chemotaxis, since the swarming rate was not significantly enhanced by aspartate. This is similar to the situation described above for suppression of *cheZ* null mutations.

Introduction of *cheZ* suppressor mutations into a *cheB* background. CheB is a protein that participates in the adaptation phase of bacterial chemotactic behavior (5). The phenotype of *cheB* mutants, like that of *cheZ* mutants, is a marked CW bias (20). CheB acts on the receptors, and there is no evidence to suggest that it physically interacts with the motor. Thus suppression of *fliM* mutations by *cheB* mutations would not be expected to show allele specificity. We transferred the *fliM* allele of all of the pseudorevertants of one of the *cheZ* parents, Z_1, into the same *cheB* mutant background and then examined the switch bias of the *cheB fliM* double mutants. Although the bias ratings of both the *cheY fliM* pseudorevertants and the *cheB fliM* double mutants varied widely (from 1 through 4), the difference between the ratings was quite constant (either 0 or 1). Thus the

differential ability of these two *cheB* and *cheZ* mutations to suppress was largely independent of the particular *fliM* allele being suppressed. Given the presumption of nonspecificity in the case of *cheB*, these data argue against specificity in the CheZ-FliM interaction.

Distribution of *mot* alleles in *fliM*. In a previous study of the switch genes (41), we reported the isolation of Mot$^-$ and Fla$^-$ mutants, as well as Che$^-$ mutants. The *mot* alleles in *fliM* were encountered in four deletion segments: 6, 8, 10, and 20. Sequence analysis (Fig. 3B) showed that the mutations occur at only eight positions (H106, F131, G132, G133, T147, T149, L250, and L272) and, in all cases, produce a simple amino acid change. None of the positions giving rise to *mot* alleles have been observed to give rise to *che* alleles, although some are close or even adjacent to such positions. Mutations at five of the eight positions were encountered at least twice, suggesting that there are not likely to be many other positions that can give rise to the Mot$^-$ phenotype.

Distribution of *fla* alleles in *fliM*. We have sequenced many of the *fla* alleles of *fliM* obtained in the previous study of the switch genes (41). Many are either nonsense or frameshift mutations; one is an in-frame deletion of 39 codons. Of the nonsense mutations, the latest occurs at codon 311, leading to the loss of the C-terminal 24 amino acids. Among the alleles we sequenced, we found six distinct missense mutations (Fig. 3B). Of these, only one appeared more than once, indicating that the map is far from being saturated with respect to *fla* mutants; presumably there are a large number of sites that can give rise to this phenotype.

DISCUSSION

The flagellar switch is one of the most fascinating aspects of the function of the flagellum, yet it is one of the most elusive. Genetic evidence has indicated which proteins are involved—FliG, FliM, and FliN—but little is known about how they are organized in the flagellar structure, what the switching process actually entails at a molecular level, or what the relative roles of the three proteins are.

Intergenic suppression analysis has previously indicated interaction among the three switch proteins and between the switch proteins and the chemotaxis proteins CheY and CheZ (16, 23, 24, 40). In *S. typhimurium*, the mutations responsible were mapped into local regions of the various genes. We have now moved to a molecular level of analysis by identifying the amino acid changes involved in suppression. Because *fliM* yields by far the majority of *cheY* and *cheZ* suppressors, we have focused our attention on it initially.

Location of suppressible CheY mutations in the three-dimensional structure. The three-dimensional structure of the CheY protein (30, 36) consists of a five-strand parallel β sheet surrounded by three α helices on one side and two on the other (Fig. 4). One face of the protein, constructed from the C-terminal portion of the primary structure, consists of (i) helix αD, (ii) the loop connecting αD to strand β5, (iii) β5 itself, (iv) the loop connecting β5 to αE, and (v) the C-terminal helix, αE. Five of the nine mutated residues lie on this face of the protein: A99T at the C-terminal end of αD, S104R at the N-terminal end of β5, P110T and A113V in the β5-αE loop, and T115A near the N-terminal end of αE. Another mutation (duplication of L24) lies at the C-terminal end of αA, which is at one corner of the face described above, close to the C-terminal end of the β5-αE loop. A similar clustering of suppressible mutations (at residues 90, 108, 111, 112, and 117) on the three-dimensional structure of *E. coli* CheY has been noted by Matsumura et al. (17). We agree with their

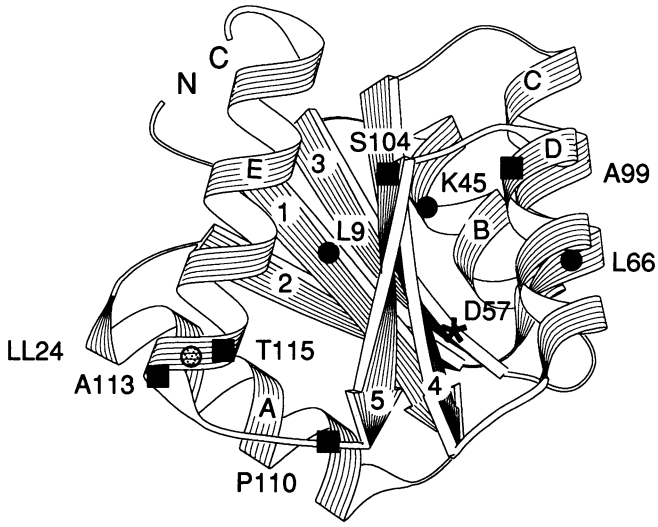

FIG. 4. Sites of mutations in *S. typhimurium* CheY for the parental *cheY* strains used in this study, placed on a ribbon diagram showing the three-dimensional structure of the protein. CheY consists of a five-strand parallel β sheet flanked by three α helices on one side and two on the other (30, 36). In the figure, the molecule is oriented so that a face consisting of αD, β5, and αE is at the front. Several of the mutation sites (A99, S104, P110, A113, and T115; shown as black squares) lie on this face. Other mutation sites are shown as black circles, except for mutation LL24 (a duplication of L24), which lies on αA and is obscured by αE in this view (stippled circle). Aspartate 57 (asterisk) is the site of phosphorylation of CheY, an event which is believed to affect its ability to interact with the switch. The figure was constructed by using coordinates from a 0.19-nm (1.9-Å) map kindly provided by Ann Stock.

suggestion that this face may constitute a major part of the binding site of CheY for the switch.

The mutation L9W is on β1 and lies fairly close to the phosphorylated residue of CheY, D57, which is on the adjacent strand in space, β3. The mutation L66Q lies at the N-terminal end of αC and also may be close enough to D57 to perturb it. Matsumura et al. (17) noted suppressible mutations at residues 11 and 56, also in the proximity of D57.

The mutation L9W was the most severe among the set and, in the original isolation of pseudorevertants (16), gave an extremely low yield of suppressor mutations in the *che* region versus the switch genes. This suggests that severe interference with phosphorylation may be less readily compensated for by changes in the chemotaxis proteins than by alteration of intrinsic switch bias.

The mutation K45N lies on the opposite side of the protein from the putative switch-binding face and also far from D57. It is not clear why this mutation affects CheY function.

Both CheY and CheB are substrates for phosphorylation by CheA and for dephosphorylation by CheZ (6, 19) and show some sequence similarity to each other (29). Of the nine independent mutation positions in CheY obtained in this study, four (L9, K45, L66, and P110) are conserved in CheB. One of these, P110, lies on the face of the protein that is the putative binding site for the switch (a site which is not expected to be present on CheB). P110 is at the start of the β5-αE loop and is part of a short stretch of similarity between CheY and CheB that runs from residues 102 to 110 and includes all of β5. K109 is absolutely conserved over a large family of phosphorylated proteins involved in regulation of a variety of cellular responses (33). Volz and Matsu-

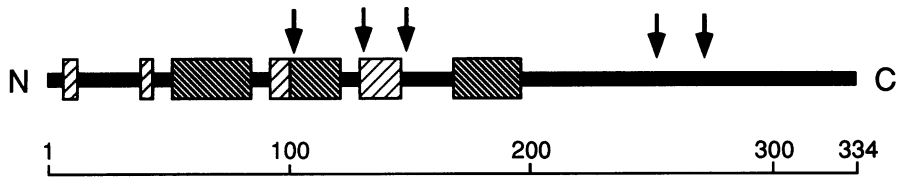

FIG. 5. Schematic illustration of the sequence of FliM, indicating local regions important for the CCW bias and CW bias states of the wild-type flagellar switch. Mutant alleles in these regions result in CW bias and CCW bias and were isolated as suppressors of CheY and CheZ defects, respectively. Also shown are the highly localized regions that gave rise to mutants with the paralyzed (Mot$^-$) phenotype. Symbols: ▨, CCW regions in FliM (CW mutant phenotype, sites of *cheY* suppressors); ▨, CW regions in FliM (CCW mutant phenotype, sites of *cheZ* suppressors); ↓, motility regions in FliM (sites of Mot mutations).

mura (36) describe how the *cis* turn between K109 and P110 positions the ε-amino group of K109 adjacent to the β-carboxyl of D57; it would not be surprising, therefore, if mutation of P110 affected the phosphorylation properties of CheY. However, the presence of proline at position 110 does not appear to be mandatory, since the mutation P110T in strain Y_2 could be quite effectively suppressed by certain mutations in FliM (see Fig. 4 of reference 16). Also, P110 is not absolutely conserved in the response regulator family; interestingly, one member of the family (ComA) has, like the P110T mutant CheY, a threonine residue at the corresponding position (33).

Suppressible *cheZ* mutations and the phenomenon of pseudotaxis. The *cheZ* mutations we encountered among the 10 parents fell into two classes, missense mutations and the more severe class of nonsense or frameshift mutations.

In the absence of a three-dimensional structure of CheZ, it is not possible to comment in detail about the missense mutations in the manner that was done above for CheY. They are clustered toward the center of the primary structure, but this may not be significant in view of the small numbers involved. Two of the mutations which gave an extremely low probability of suppressors in the *che* region (Table 3) are at almost the same position (D143E and T145M) in CheZ; by analogy with the L9W mutation in CheY, this could mean that this region of CheZ is important to its enzymatic activity as a CheY/CheB phosphatase.

We were surprised at first to find that nonsense and frameshift mutations in *cheZ* could be suppressed. Since the most extreme example involved truncation of about 80% of the protein sequence, there could be little doubt that we were dealing with the null phenotype, in which rates of dephosphorylation of CheY and CheB would be severely reduced. We found that both the parents and the pseudorevertants could respond in temporal gradient experiments, and so in that context CheZ was not essential; they did, however, show defective adaptation. In the more realistic context of swarming in semisolid agar, the presence of attractant caused little or no enhancement of the swarm rate. We conclude that CheZ is necessary for an appreciable chemotactic response in spatial gradients, where a proper balance of the excitation and adaptation processes is important.

Wolfe and Berg (37) have concluded from microscopic observations that cells with either an extreme CW bias or an extreme CCW bias both fail to swarm well, but for quite different reasons: in the former case the mean run length between directional changes is too short, whereas in the latter case the cells get trapped in local dead ends in the agar and cannot make the directional changes that are necessary to escape. Cells with wild-type or slightly tumbly unstimulated motility have reasonably long runs, can avoid trapping, and hence swarm more rapidly. Parkinson (22) has coined the term "pseudotaxis" for this phenomenon of enhanced swarming in the absence of a true chemotactic response. Pseudotaxis appears to contribute to enhanced swarming in all of the pseudorevertants we isolated, and in cases in which the parental mutation is sufficiently severe, it is virtually the only contribution. When the parental mutation is less severe, pseudotaxis contributes by enabling more effective expression of a true chemotactic response.

Nature and location of switch bias (*che*) mutations in FliM. The overall view that emerges from the analysis of *cheY* and *cheZ* suppressor mutations is that the N-terminal 60% of FliM contains essentially all of its information that is important to switching (Fig. 5). Within that portion the information important for the CCW state (therefore contributing a CW bias when defective) and the CW state (CCW bias when defective) occurs in mostly nonoverlapping stretches of sequence. These stretches are relatively short, arguing against a model in which there are separate protein domains, one active in the CCW state and the other in the CW state. It seems likely, instead, that all of the sequence important for switching in FliM occurs within a single domain, with the C-terminal 40% of the protein perhaps forming a second domain and fulfilling a static structural role such as binding to the M ring or other switch subunits. Since the residues in the putative switching domain that are important to the two states are for the most part segregated in the primary structure, we suggest that in three-dimensional space they may generate two separate surface sites on the protein, with one site acting to generate the CCW state and the other the CW state. This point is discussed further below.

We encountered two quite extensive duplications (of 10 and 12 residues), which nonetheless had relatively minor consequences, namely, alteration of switch bias rather than paralysis or loss of flagellation. Both occur at essentially the same position in the protein, defining between them an 18-residue stretch that is tolerant of duplication. This suggests that this region of the protein must be at the surface and must be quite mobile, so that the duplication does not severely disrupt the structure. However, since this 18-residue stretch is, like most of FliM (11, 12), highly conserved between *S. typhimurium* and *E. coli* (the only nonconserved position within the 18 residues being H241), the sequence is likely to be functionally important rather than just being a floppy connecting loop. A prediction from this would be that deletion of the region would be a serious defect, probably resulting in lack of flagellation.

Allele specificity. In principle, a defect in CheY might be compensated for by increasing the affinity of the switch for the mutant version of CheY, or it might be compensated for (at least to some degree) by adjusting the intrinsic switch

bias. The former mechanism would be expected to exhibit considerable allele specificity, whereas the latter would not.

We already had strong indications from classical mapping that a given *cheY* or *cheZ* mutation could be suppressed by mutations in multiple locations in each of the three switch genes. We have now refined the information by determining the actual mutations involved and find that a given amino acid change in CheY or CheZ can be suppressed by many different amino acid changes in FliM (and in the other switch proteins [8]). Conversely, different changes in CheY (or CheZ) may be suppressed by the same amino acid change in FliM. Therefore, our data do not provide evidence for allele specificity, at least in the narrow sense of a given mutation in CheY being suppressible only by a very limited set of mutations in FliM, with another mutation in CheY being suppressible by a different limited set. We do not believe, therefore, that our data can be used as evidence for (or against) a direct interaction between CheY and FliM.

We also note that if a mutation in the switch were to be specifically effective in binding a mutant version of CheY, it should in general be less effective in binding wild-type CheY (Fig. 6), and as a consequence the bias phenotype associated with the switch mutation in a wild-type *cheY* background should be toward CCW compared with the bias in the mutant *cheY* background. Our data indicate just the opposite: the phenotype associated with a given switch mutation in a wild-type *cheY* background is at least as strongly (and usually more strongly) CW biased as in the pseudorevertant background. Thus, if there is an effect of enhanced CheY binding, it appears to be a rather general one, not one specific to a particular mutant form of CheY. A similar result was obtained regarding CheZ, in the one case where we examined this issue, i.e., Z_1. The various switch mutations in combination with wild-type *cheZ* produced at least as strong a CCW bias (and usually a stronger one) as was produced when they were in combination with the parental *cheZ* mutation.

There is also likely to be a steric problem concerning binding sites at the switch (16). This device is thought to be (i) a multisubunit complex consisting of subunits of FliM, FliG, and FliN (40), (ii) mounted onto the M ring, (iii) capable of binding CheY and possibly CheZ, and perhaps (iv) interacting with the MotA and MotB proteins to cause motor rotation. It seems unlikely that any one switch protein could make contacts with all of these other components.

In our view there is no clear evidence yet about which among FliM, FliG, and FliN contribute(s) directly to the binding site for CheY, or whether any of them contribute to a binding site for CheZ.

It is true that we encountered some suppressor mutations more commonly in some parental backgrounds than in others. A factor contributing to this result seems to be that if the CheY mutation results in a given severity of CCW bias, it will be most effectively suppressed by a switch mutation with a corresponding severity of CW bias. Perhaps the clearest indication of this involves the strong CW bias mutations in FliM, such as R to X and V to E, which were seldom encountered as suppressors of mild CheY defects but were encountered often as suppressors of the most severe CheY defects. A similar, although less striking, argument can be made for weaker CW bias switch mutations being encountered most commonly in mildly defective *cheY* backgrounds. However, by itself, we do not view this type of compensation as evidence for physical interaction.

It should be emphasized that we do not discount the possibility that some combinations of amino acid changes

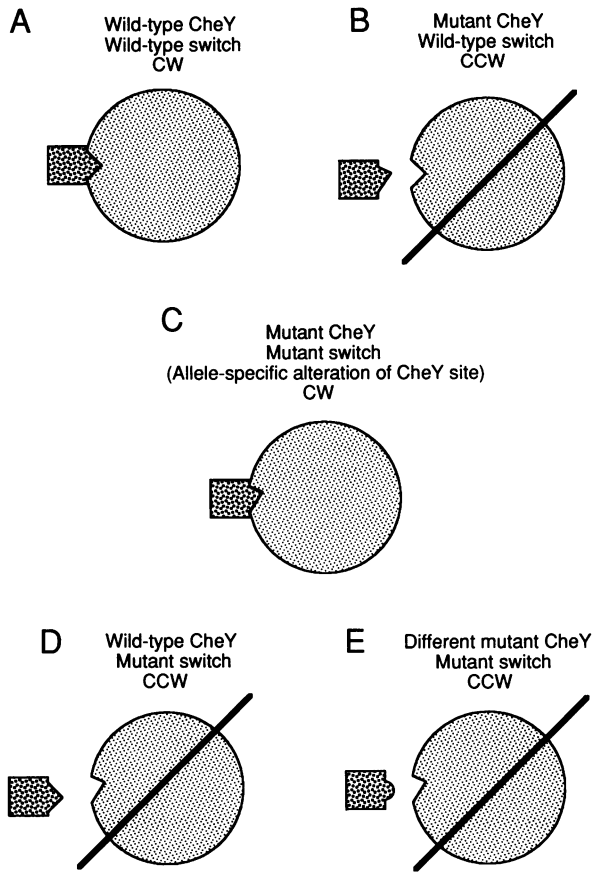

FIG. 6. Model of allele-specific modification of the switch-binding site for CheY. (A) Wild-type CheY binds to the wild-type switch, biasing it toward the CW state. (B) Mutant CheY binds poorly to the wild-type switch, failing to bias it to the CW state. (C) After the switch has undergone an allele-specific change in its binding site, mutant CheY binds to the switch and biases it to the CW state. (D and E) Wild-type CheY (panel D) or other arbitrarily chosen mutant versions of CheY (panel E) do not bind as well to the mutant switch and so are less effective in biasing it to the CW state. The results of the present study are not consistent with this model, since wild-type CheY was usually more effective than mutant CheY in reversing the bias introduced by the suppressing *fliM* mutation.

are especially effective because of a more detailed specificity in maintaining a suitable Che-switch interface. Indeed, we suspect that such examples must exist, but we have not been able to identify them against the more general phenomenon of balancing of bias contributions.

Parkinson et al. (23, 24), in their pioneering genetic analyses of CheY, CheZ, and the switch, also found that any given parental mutation could be suppressed to various degrees by a number of different second-site mutations. However, a suppressor that was effective in one background was not necessarily effective in another. From a detailed analysis of this type of evidence they concluded that there was at least some allele specificity, although they noted that the achievement of a suitable balance between CCW and CW rotation also played an important role and was likely to be rather nonspecific. At the time, it was not fully appreciated that the latter effect is significant even in the absence of any chemotactic capability, giving rise to the phenomenon of pseudotaxis on swarm plates. The issue of allele specificity of *cheY* and *cheZ* suppression, and its interpretation in terms

of binding interactions at the switch, is a difficult one given the number of different proteins involved. Ultimately the question of which proteins interact, and where their sites of interaction are, will be most convincingly answered by biochemical rather than genetic approaches.

A model for switching. We present here a speculative model for switching that attempts to bring together two observations: (i) the clustering of switch mutations on the primary structure of FliM, and (ii) the ease with which suppressor mutations were obtained, in a wide range of locations in FliM as well as in the other two switch proteins, FliG and FliN (8; see above).

Random mutagenesis will normally produce far more examples of impaired rather than enhanced function or activity of the gene product: put simply, it is easier to destroy than to improve, and only if there is a strong selection pressure does one normally encounter an up mutation, i.e., one that results in improved function. For a heterodimeric protein, conformational suppression of a mutation in the first subunit by a mutation in the second will generally be possible only for a limited subset of first-site mutations (those that affect the state of the interface) and then will arise from only a very limited subset of second-site mutations that are specifically capable of compensating for the first one. Thus conformational suppression should be a rather rare and specific event.

We did, however, encounter suppression with all of the *cheY* and *cheZ* mutants we used (including ones with spontaneous first-site mutations and ones with isolated second-site mutations), and we encountered it in many different places in several different genes. What is special about this situation? We suggest that it is intrinsic to the binary character of the switch, which we postulate derives from alternative sets of interactions that exist at different locations within the switch structure (Fig. 7) and are generated by clusters of primary structure as revealed in the mutant sequence analysis.

Thus the CCW state of the switch is stabilized by one set of interactions among the switch proteins (Fig. 7A), and the CW state is stabilized by another set among the switch proteins and also by CheY (Fig. 7B). Then any change that impairs one set of interactions (provided that it is not so severe as to make a major change in overall structure) will be equivalent to an up mutation as far as the other set of interactions is concerned.

Take the specific case of suppression of a CheY mutation that results in a reduced affinity for the switch (Fig. 7C), for any of a variety of reasons: because the binding site for the switch has been altered directly, because the ability to be phosphorylated has been impaired, or because the communication between the phosphorylated site and the switch-binding site has been disrupted. Suppression of the defect could then arise by improving the binding site for the mutant CheY (Fig. 7D) or by improving the intrinsic (CheY-independent) interactions responsible for the CW state of the switch (Fig. 7E). Both these classes of events are intrinsically less likely to occur than the class of events that impair the intrinsic interactions responsible for the CCW state of the switch (Fig. 7F), which will have the indirect effect of enhancing the CW state of the switch and hence enhancing the possibility of CheY binding to the switch.

We therefore favor the model shown in Fig. 7F. Since it involves adjustment of intrinsic switch bias rather than direct improvement of the binding site for CheY, suppression is not automatically guaranteed to result in true chemotaxis beyond the enhanced swarming generated by pseudo-

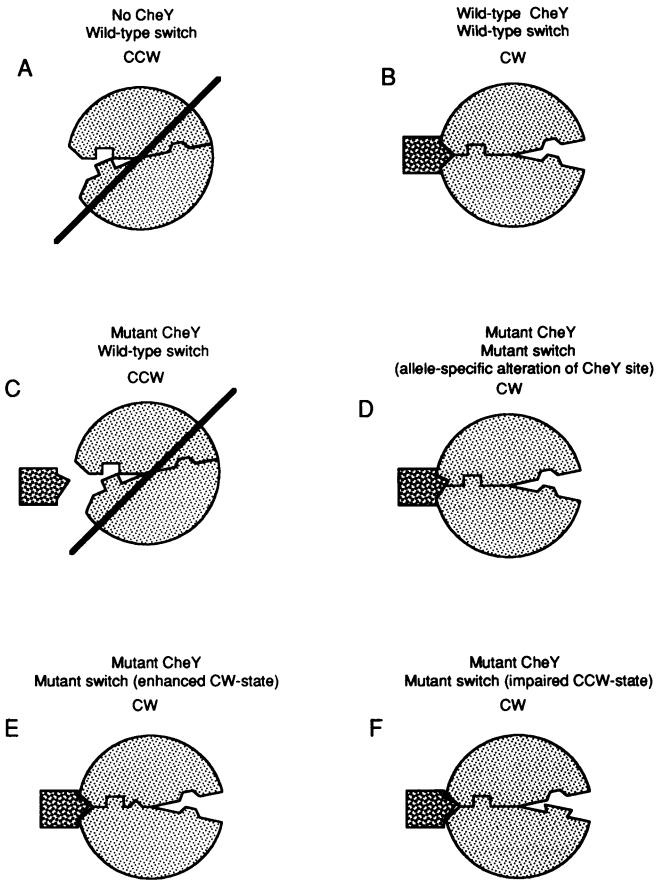

FIG. 7. Model for switching and for intergenic suppression between CheY and the switch. The switch is postulated to utilize different surfaces for the interactions that are important to the CCW and CW states. (A) In the absence of CheY, the interactions responsible for the CCW state completely dominate; i.e., the CW state cannot be accessed and the cell is nonchemotactic. (B) Wild-type activated (phosphorylated) CheY, by binding to the switch, favors the interactions responsible for the CW state (and modulation of the level of activated CheY by the sensory system results in chemotaxis). (C) Mutant CheY fails to bind to the switch, and the interactions responsible for the CCW state dominate. Compensation for the mutant CheY can be accomplished in different ways (panels D, E, and F). (D) Allele-specific alteration of the CheY-binding site restores the high affinity of binding and access to the CW state (cf. Fig. 6C). (E) Strengthening of the interactions responsible for the CW state occurs at the switch and so indirectly enhances binding of the mutant CheY. (F) Weakening of the interactions responsible for the CCW state makes the CW state more favorable and again indirectly enhances binding of the mutant CheY. Mutations of types D and E can be thought of as up mutations; they have to be highly specific and are therefore relatively rare. Mutations of type F are down mutations, can be accomplished in many more ways, and so are more common. We suspect that most of the suppressor mutations we isolated and sequenced fall into the latter category.

taxis. Whether it does so depends on whether the mutant version of CheY has retained sufficient ability to be phosphorylated, to bind to the switch, and thereby to modulate switch state.

In terms of this model, we suggest that the prevalence of R-to-X and V-to-E changes among those conferring the strongest CW bias as suppressors of *cheY* mutations may mean that electrostatic forces are important for interactions

responsible for the CCW state of the switch and that impairment of these interactions results in the CW state's becoming more dominant.

How suppression of *cheZ* mutations should be viewed depends on whether CheZ does in fact bind to the switch. If it does, it presumably is antagonistic to CheY binding either because of competitive binding or because binding to the switch enhances its effectiveness as a phosphatase. In either case a CheZ mutation could be compensated for in an equivalent manner to that just discussed for *cheY* suppressors, by improving the CheZ-binding site, by improving the interactions responsible for the CCW state of the switch (both relatively improbable events), or by impairing any of the interactions responsible for the CW state (a much more probable event). If CheZ does not bind to the switch but exerts its influence indirectly via the phosphorylation state of CheY, a *cheZ* mutation is equivalent to an up mutation of *cheY*. It can then be compensated for by impairing the CheY binding site or the interactions responsible for the CW state of the switch (both probable events) or by improving the interactions responsible for the CCW state of the switch (an improbable event).

Nature and location of *mot* and *fla* mutations in FliM. We found very few positions within the FliM sequence that could be altered in such a way as to inhibit motor rotation. However, we cannot rule out the possibility of other residues that are important for motor rotation, but are also important for flagellar structure, in which case the mutant phenotype would be nonflagellate. Of the five positions in the sequence giving rise to *mot* mutations, three are at boundaries of *che* regions, in one case at a CCW-CW boundary and in the other two cases flanking a CCW region (Fig. 5). This suggests that the mechanisms of rotation and switching are in some way related, with some local regions of the protein being involved in both functions. It is interesting that we never encountered any examples of *mot* and *che* mutations arising at the same residue. Whether this means that there is strict segregation of function at the level of individual amino acid position is not certain. A residue might be involved in switching as well as rotation, but defects in switching would be masked by the fact that the flagellum was paralyzed. Evidence for this hypothesis was obtained in a suppression analysis of *mot* alleles of the switch genes (40), where it was possible in some cases, by comparing the behavior of the pseudorevertant and the isolated second-site mutant, to ascribe a switch bias phenotype to the first-site *mot* mutation. The examples included one in *fliM*, namely, a *mot* allele causing the amino acid change L272R, which contributed a CW bias to the pseudorevertant phenotype.

With the exception of H106, none of the wild-type amino acids at positions that can give a Mot^- phenotype have readily dissociable protons. This may mean that the role of FliM in motor rotation does not involve the actual proton conductance pathway.

Concerning the role of FliM in flagellation, we have confirmed what was already known from classical genetics, namely that FliM is essential for flagellar structure, as well as for rotation and switching. Nonsense and frameshift mutations occurring throughout the gene resulted in a nonflagellate phenotype; although some of these may have had polar effects on downstream flagellar genes within the *fliL* operon, it seems unlikely that they all would. We did not encounter any missense or in-frame deletions in the first 100 residues, suggesting that it may not be important for flagellar structure. Most of the remainder is likely to be important in this regard, since there are missense mutations scattered throughout much of the central part of the sequence and a nonsense mutation fairly close to the C terminus.

High prevalence of *cheY* and *cheZ* suppressors in FliM. In the isolation of the pseudorevertants of both *cheY* and *cheZ* mutants, suppressors were encountered much more frequently in *fliM* than in the other two switch genes, *fliG* and *fliN*. What is the significance of this finding? *fliG* is essentially the same size as *fliM*, and so that cannot be a contributing factor (although it could be for *fliN*, which is much smaller). A major factor is that there are more positions in the *fliM* sequence that give rise to suppression (49 and 37 independent amino acid positions for *cheY* and *cheZ* suppressors, respectively) than there are in the *fliG* or *fliN* sequences (8). This implies that a larger proportion of the FliM protein is critical for the switch states, both CCW and CW. This could mean that FliM contributes extensively to the binding site for CheY. It could also mean that it contributes extensively to the interactions responsible for intrinsic switch bias.

An analysis of the other two switch proteins, FliG and FliN, comparable to that described here for FliM, is in progress and should further elucidate the roles of the three proteins in the processes of switching, rotation, and flagellar structure.

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