Genetic Evidence for a Switching and Energy-Transducing Complex in the Flagellar Motor of *Salmonella typhimurium*

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The *flaAII.2*, *flaQ*, and *flaN* genes of *Salmonella typhimurium* are important for assembly, rotation, and counterclockwise-clockwise switching of the flagellar motor. Paralyzed and nonchemotactic mutants were subjected to selection pressure for partial acquisition of motility and chemotaxis, and the suppressor mutations of the resulting pseudorevertants were mapped and isolated. Many of the intergenic suppressor mutations were in one of the other two genes. Others were in genes for cytoplasmic components of the chemotaxis system, notably *cheY* and *cheZ*; one of the mutations was found in the *cheA* gene and one in a motility gene, *motB*. Suppression among the three *fla* genes was allele specific, and many of the pseudorevertants were either cold sensitive or heat sensitive. We conclude that the FlaAII.2, FlaQ, and FlaN proteins form a complex which determines the rotational sense, either counterclockwise or clockwise, of the motor and also participates in the conversion of proton energy into mechanical work of rotation. This switch complex is probably mounted to the base of the flagellar basal body and, via binding of the CheY and CheZ proteins, receives sensory information and uses it to control flagellar operation.

Bacteria swim using rotary flagella (2, 27) driven by the transmembrane proton motive force (18). Each motor can rotate in both the clockwise (CW) and counterclockwise (CCW) sense (14), i.e., it contains a binary switch. The switch operates even in unstimulated cells; stimuli bias the switching events in a way that results in migration toward more favorable environments (3, 17).

The motors are located at the cell surface. After digestion of the peptidoglycan layer and dissolution of the outer and cytoplasmic membranes, a structure can be isolated which is termed the flagellar basal body and consists of four rings and a rod (7, 8). A variety of evidence, including a recent study of *Salmonella typhimurium* (1), indicates that the basal body is not the entire motor. It does not contain any of the proteins known to be involved in the two central functions of the motor, conversion of proton motive force into rotational work and switching between the two rotational senses.

Five proteins are known to play important roles in rotation and switching. They are MotA and MotB (6, 9, 25, 28, 29, 33) and FlaAII.2, FlaQ, and FlaN (4, 5, 9, 11, 12, 19, 22, 23, 26, 31-33). The last three are the subject of this paper; they are essential for flagellar assembly but also participate in energy transduction and switching, as judged by the fact that some missense mutations cause paralysis or an abnormal switch bias. They are referred to as switch proteins (16, 21). In a recent study (32) we established that within each switch-gene sequence, subregions responsible for flagellar assembly, energy transduction, and switching are substantially segregated.

If the switch proteins are not part of the basal body, where are they located, and with what other components do they interact? In an attempt to answer these questions, we selected for phenotypic suppression of defects in the switch proteins and then identified the genes carrying the suppres-

sor mutations. The pattern of intergenic suppression that emerged suggests that the three proteins form a complex that functions in energy transduction and switching and interacts with certain cytoplasmic components of the sensory transduction apparatus.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium flaAII.2*, *flaQ*, and *flaN* strains used to generate pseudorevertants were spontaneous paralyzed or nonchemotactic derivatives of wild-type strain SJW1103 or SJW806 (32) and are listed in Table 1.

Isolation of pseudorevertants. A single colony of a given paralyzed or switch-biased mutant was grown in liquid culture, and a 10- μ l portion was applied as a line onto a soft nutrient gelatin agar (NGA) plate (32), incubated open at 30°C for 1.5 h (to dry the surface), and then incubated closed at 37°C. For up to 5 days, the plates were inspected for spontaneous swarms emerging from the growth line. Swarms that spread less rapidly than the wild-type swarms were presumed to be the result of pseudoreversion. Cells from these swarms were picked for further study.

Mapping of mutations. Mutations were mapped by using the general transducing bacteriophage P22 (wild type); the recipients were a series of deletion mutants in the three main flagellar/motility regions of the genome (13, 32). Recombinant wild-type swarms indicated that the donor did not have a mutation within the range of the deletion of the recipient.

Isolation of second-site mutants. Cells (10 μ l at 5×10^8 cells ml^{-1}) of a nonflagellate recipient, with a deletion covering the site of the second mutation, and P22 (*int*) phage (10 μ l at 10^9 to 10^{10} PFU ml^{-1}) grown on the pseudorevertant donor strain were applied as a line onto soft NGA and incubated as described above for ca. 18 h. Where a colony with a morphology differing from that of the donor was noted, the overlying carpet of recipient cells was swabbed off and the colony was picked and streaked onto soft NGA; from these plates, single colonies with partial swarm morphology were

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TABLE 1. First-site mutants, pseudorevertants, and second-site mutants used in this study^a

First-site mutant			Pseudorevertant		Second-site mutant		
Strain	Genotype and gene segment	Phenotype	Strain	Phenotype	Strain	Genotype and gene segment	Phenotype
SJW1759	<i>flaAII.2-6</i>	Mot ⁻	MY110 ^b	CW [Mot ⁻]	MY119	<i>flaN-3</i>	CCW
SJW2811	<i>flaAII.2-7</i>	Mot ⁻	MY112 ^c	CW [Mot ⁻]	SJW2879	<i>motB-1</i>	[Mot ⁻]
SJW1783	<i>flaQ-8</i>	Mot ⁻	MY104	CCW [Mot ⁻]	SJW2866	<i>flaN-7</i>	CCW
SJW1808	<i>flaQ-10</i>	Mot ⁻	MY106	CCW [Mot ⁻]	SJW2864	<i>flaAII.2-4</i>	CCW
SJW1808	<i>flaQ-10</i>	Mot ⁻	MY105 ^b	CW [Mot ⁻]	SJW2863	<i>cheZ-2</i>	CW
SJW1763	<i>flaQ-20</i>	Mot ⁻	MY103	CW [Mot ⁻]	MY107	<i>flaAII.2-5</i>	CCW
SJW1809	<i>flaN-3</i>	Mot ⁻	MY304	CCW [Mot ⁻]	SJW2286	<i>flaQ-5</i>	CCW
SJW1809	<i>flaN-3</i>	Mot ⁻	MY502	CCW [Mot ⁻]	SJW2842	<i>flaAII.2-4</i>	CCW
MY107	<i>flaAII.2-5</i>	CCW	MY108	CCW	MY115	<i>flaQ-6</i>	CW
MY107	<i>flaAII.2-5</i>	CCW	MY109	CCW	MY123	<i>cheZ-1</i>	CW
SJW2286	<i>flaQ-5</i>	CCW	MY344 ^c	CW	SJW2288	<i>flaAII.2-5</i>	CW
SJW2286	<i>flaQ-5</i>	CCW	MY349	CW	SJW2291	<i>flaAII.2-9</i>	CW
SJW2286	<i>flaQ-5</i>	CCW	MY370 ^c	CCW	SJW2841	<i>flaN-3</i>	CW
SJW2287	<i>flaN-6</i>	CCW	MY388 ^c	CCW	SJW2835	<i>flaAII.2-9</i>	CW
SJW2287	<i>flaN-6</i>	CCW	MY391 ^c	CCW	SJW2836	<i>cheZ-2</i>	CW
SJW2287	<i>flaN-6</i>	CCW	MY394 ^c	CCW	SJW2838	<i>cheZ-1</i>	CW
SJW2323	<i>flaAII.2-5</i>	CW	SJW2896 ^b	CW	SJW2907	<i>cheA-7</i>	CCW
SJW2323	<i>flaAII.2-5</i>	CW	SJW2892	CW	SJW2903	<i>cheY-1</i>	CCW
SJW2323	<i>flaAII.2-5</i>	CW	SJW2890	CW	SJW2901	<i>cheZ-1</i>	CCW
SJW2330	<i>flaAII.2-9</i>	CW	SJW2914	CW	SJW2923	<i>cheY-1</i>	CCW
SJW2330	<i>flaAII.2-9</i>	CW	SJW2910 ^c	[WT]	SJW2919	<i>cheZ-1</i>	CCW

^a The location of each mutation is indicated by gene and (following the hyphen) gene segment (32). Pseudorevertants contain both first-site and second-site mutations. Phenotypes are indicated as follows: Mot⁻, paralyzed; [Mot⁻], partially paralyzed; CW and CCW, nonchemotactic, with the switch bias indicated; [WT], close to normal switch bias.

^b Strain showed pronounced heat sensitivity (cf. Fig. 6).

^c Strain showed pronounced cold sensitivity (cf. Fig. 6).

picked and streaked on nutrient agar. Single colonies from these plates were grown and tested for P22 sensitivity by smearing 10 µl of cell suspension onto nutrient agar, allowing it to dry, spotting 2 µl of phage [clear-plaque isolate from P22 (*int*)] in the center, incubating overnight at 37°C, and inspecting for a cleared central area. P22-sensitive colonies were tested by transduction for the presence of the second-site mutation and the absence of the first-site mutation.

When no colonies with partial swarm morphology were detected, the transduction mixture was first enriched for flagellated cells. Equal volumes (0.1 ml) of recipient cells and P22 (*int*) phage grown on the donor strain were mixed, diluted in 1 ml of nutrient broth, and grown overnight at 37°C. A drop of a suspension of Formalin-killed wild-type cells (SJW1103) was added as carrier, followed by a drop of anti-flagellin antibody. The sample was allowed to stand for ca. 30 min at 37°C and then for 1 h at 4°C. A portion of the precipitate that formed was transferred by Pasteur pipette into 1 ml of broth and incubated overnight at 37°C. This precipitation procedure was repeated four times, and the final culture was applied to soft NGA, grown, and inspected as described above.

Introduction of deletion mutations into missense mutants.

For testing the allele specificity of suppression (see Results), it was necessary to introduce deletions that would ensure that motility could only be achieved by recombination of the first and second mutations that were being tested.

P22 (*int*) was grown on the appropriate deletion mutant, mixed with the missense mutant, and incubated in nutrient broth overnight at 37°C. Flagellated cells were agglutinated with anti-flagellin antibody and centrifuged, and 50 µl of the supernatant was transferred into 1 ml of broth and incubated overnight at 37°C. This procedure was repeated three times, and the final supernatant was spread on soft NGA. Nonflagellate, phage-sensitive colonies were tested by P22

transduction for the combined presence of the missense and deletion mutations.

Physiological characterization of pseudorevertants and second-site mutants. Cells were examined by high-intensity dark-field light microscopy (15) for extent of flagellation, motility, and switching bias of the motors.

Temperature sensitivity. Cells from an overnight culture in nutrient broth were spotted onto soft tryptone agar (0.38% [wt/vol]; 5) and incubated at either 20 or 37°C for up to 2 days. Once a swarm was established, its diameter increased linearly with time at a rate that was independent of inoculum size. The swarm rate was calculated from the diameter at an early time (typically 8 h after inoculation) and at the last usable time (at the end of the experiment, when the swarm was about to reach the edge of the plate, or when revertant flares were beginning to obscure the swarm). The relative swarm rate of a strain was defined as the ratio of its rate at 20°C to its rate at 37°C. The normalized relative swarm rate of a mutant was defined as its relative swarm rate divided by the relative swarm rate of wild-type strain SJW1103.

RESULTS

Strategy. A common strategy (e.g., see reference 23) for mapping intergenic suppressor mutations uses functionally unrelated markers in the vicinity of the genes of interest. It requires that the site of the second mutation be fairly distant from the site of the first (to distinguish from intragenic suppression) and close enough to the marker to be cotransducible with it.

In the present case, we suspected that intergenic suppressor mutations might occur in genes closely linked to the first (Fig. 1). We therefore decided to rely, not on markers, but on the ability to recognize phenotypically a pseudorevertant with partially restored function; pseudorevertants with wild-

FIG. 1. Flagellar regions II and III of *S. typhimurium*; both are at 40 min on the genetic map. Mutants in the switch genes, *flaAII.2*, *flaQ*, and *flaN* (large boldface), were subjected to pseudoreversion analysis. Intergenic suppressor mutations mapped either to another of the switch genes or to *cheY*, *cheZ*, *motB*, or *cheA* (boldface).

type motility and taxis, as well as true revertants, would be ignored. Intragenic and intergenic suppressors would then be distinguished by detailed mapping, as is indicated schematically in Fig. 2a and b.

Pseudorevertants from paralyzed (*Mot*⁻) mutants. Of the 83 paralyzed switch-gene mutants from our previous study (32), roughly one-half failed to give any swarms. The remainder gave swarms of various sizes. Those that spread more rapidly than the parent but less rapidly than the wild type were judged to be pseudorevertant swarms. Single-colony isolates were obtained from these swarms.

None of the suppressor mutations in the pseudorevertants mapped to region I. Two mapped to region II (Fig. 1), which contains some flagellar genes and the genes involved in the central processes of sensory transduction; since the first mutation was in region III, the suppression was clearly intergenic. All others mapped to region III (Fig. 1), which

contains the switch genes; many of the pseudorevertants with such suppressor mutations fully complemented deletions in all genes except the one carrying the original mutation and were presumed to be intragenic (Fig. 2a); others failed to complement deletions in another region III gene and were therefore intergenic (Fig. 2b).

From the original pool of 83 first-site mutants, only 6 gave rise to intergenic pseudorevertants. The intergenic suppressor mutations were next mapped in detail to a single segment of the deletion map of the relevant gene; a description of the fine-mapping protocol was given previously (32). Among the intergenic pseudorevertants, at least eight demonstrably distinct classes were obtained, each with a unique combination of first-site and second-site mutations. Different pseudorevertants within a class, although isolated independently, could in principle have been genetically identical. For this reason, the remaining description is confined to one example from each distinct class.

The genetic analysis of the intergenic pseudorevertants, along with their phenotypic description, is given in Table 1 and Fig. 3a. Of the two region II mutations, one (suppressing a *flaQ* defect) lay in the *cheZ* gene and the other (suppressing a *flaAII.2* defect) lay in the *motB* gene. All of the six region III intergenic suppressor mutations were found to reside in another switch gene. We found at least one example of each of the six possible pairwise combinations except *flaAII.2* → *flaQ*. Given the small numbers of isolates, this exception is probably not significant.

Pseudorevertants from switch-biased (*Che*⁻) mutants. Provided the swarming rate of a switch-biased mutant was fairly low, we were able to select for suppression of its defect. We did this with several isolated second-site mutants (see below) and also several nonchemotactic mutants that had been isolated previously (32).

Five mutants gave rise to at least 13 distinct intergenic pseudorevertant classes (Table 1 and Fig. 3b). The suppressor mutations mapped either to another switch gene (five classes) or to *cheY* (two classes), *cheZ* (five classes), or *cheA* (one class). The last class was quite rare; of 200 pseudorevertants from the same parent, only one suppressor mutation mapped to the *cheA* gene.

Phenotypes of pseudorevertants. Pseudorevertants were in most instances either more smooth swimming (CCW biased) or more tumbling (CW biased) than the wild type (Table 1). Those deriving from paralyzed parents were also less vigorous than the wild type. Some pseudorevertants of nonchemotactic parents retained the switch bias of their parents, whereas others had the opposite switch bias. In all instances, pseudorevertants swarmed less well than the wild type, as would be expected from the isolation protocol. The swarming behavior of the wild type, a paralyzed mutant, a pseudorevertant of that mutant, and the isolated second-site mutant (see below) is illustrated in Fig. 4.

Isolation and characterization of second-site mutants.

FIG. 2. Analysis of pseudorevertants of switch-gene mutants by P22-mediated transduction and recombination. Since first-site, pseudorevertant, and second-site swarming phenotypes were all distinguishable from the wild type, a recipient with a deletion covering either mutation (or both) could not acquire wild-type function, and so both mutations in a pseudorevertant could be mapped. For simplicity, only large intragenic deletions are shown, but smaller deletions were used for mapping to local regions within a gene. Only the minimum recombinational replacement of a given deletion is shown, but mutations not covered by the deletion might also recombine. (a) Intragenic suppression within gene A. (b) Intergenic suppression between genes A and B and isolation of second-site mutants (*). (c) Test of allele specificity. The two alleles that were being tested for mutual suppression were first combined with deletions covering the site of the other allele by using the paralyzed or switch-biased (but flagellate) mutant as the recipient and selecting for the nonflagellate phenotype. They were then tested for mutual suppression, i.e., generation of pseudorevertant swarms. Symbols: ●, first-site mutation; ○, second-site mutation; □, deletion. PR, Pseudorevertant; WT, wild type.

FIG. 3. Pseudoreversion analysis of the switch genes of *S. typhimurium*, showing intergenic suppression of mutations conferring the paralyzed (Mot^-) phenotype (a) and the CW-biased or CCW-biased phenotype (b). The sides of the triangles represent the *flaAII.2*, *flaQ*, and *flaN* genes, divided into numbered segments defined by deletion mapping (32). Each line connects the site of a mutation (thick initial arrow) and the site of a second mutation (thin final arrow) that suppressed it phenotypically. The phenotypes of the first-site mutant and the isolated second-site mutant are indicated at the initial and final arrows, respectively; phenotypes associated with the pseudorevertants (thin middle arrow) were omitted for clarity but are given in Table 1. Second sites in other genes are indicated by the gene symbol and segment number; *cheY* and *cheZ* are divided into two segments, *motB* is divided into 15 segments, and *cheA* is divided into 12 segments (S. Yamaguchi, unpublished data). When the first mutation was itself isolated as a suppressor, this is indicated by an incoming arrow from the appropriate site. See Table 1 for identification of strain numbers.

Strains carrying only the suppressor mutation of each pseudorevertant were isolated (Fig. 2b and Table 1). In all instances, mapping of the isolated second mutation confirmed its mapping in the corresponding pseudorevertant.

The flagella on the second-site *motB* mutant rotated more slowly than those on wild-type cells but had a normal switch bias. All other second-site mutants, which had defects in switch or chemotaxis genes, were highly motile (unlike many of the pseudorevertants from which they were derived); they also had a pronounced switch bias, which resulted in poor

swarming (Fig. 4). The six classes of switch-gene second-site mutants that had arisen from suppression of paralysis defects were all CCW biased (Table 1 and Fig. 3a), including examples in which the pseudorevertant was CW biased; we do not know whether this was a statistical accident or whether it will prove to be generally true. It was not true of the *cheZ* mutant, which was CW biased. Second-site mutants that had arisen from suppression of switch defects invariably had a switch bias opposite to that associated with the first site (Table 1 and Fig. 3b). One outcome of this was the isolation of *cheZ* mutants with a CCW bias; those described previously had a CW bias (20).

Allele specificity of suppressor mutations. Allele specificity of intergenic suppression usually indicates direct physical interaction between the gene products. Using the approach indicated in Fig. 2c, we tested the ability of various switch-bias mutations to suppress the paralyzed phenotype associated with each of two first-site mutations, one in *flaN* the other in *flaQ* (Fig. 5). As expected, the alleles naturally selected to suppress the first mutation were able to do so, generating small pseudorevertant swarms in transductional crosses. No swarming whatever was observed in crosses with any of the unselected alleles, indicating that they were totally unable to suppress the first mutation.

Cold and heat sensitivity. Other studies (e.g., reference 10) have indicated that intergenic suppression deriving from physical interactions of the products commonly results in unusual temperature sensitivity. We examined the temperature dependence of swarming of (i) wild-type strain SJW1103, (ii) the pseudorevertants isolated in the present study (Table 1), and (iii) a variety of single-site CW- and CCW-biased mutants, including some first-site and second-site mutants from the present study and some other spontaneous mutants (32). The relative swarm rate (at 20°C versus 37°C) of wild-type cells on soft tryptone agar was 0.29. Of the 21 pseudorevertants, 4 swarmed too poorly for a reliable estimate to be made. The remainder showed a broad range of

FIG. 4. Swarming ability of an *S. typhimurium* wild-type (WT) strain (SJW1103), a paralyzed (Mot^-) *flaQ* mutant (SJW1763), a pseudorevertant (PR) (MY103) of SJW1763 with a suppressor mutation in *flaAII.2*, and a nonchemotactic (Che^-) CCW-biased mutant (MY107) carrying the isolated *flaAII.2* mutation of MY103. Cells were spotted onto soft tryptone agar and incubated at 37°C for 10 h. The wild-type swarm was 45 mm in diameter.

relative swarm rates (Fig. 6). Seven pseudorevertants were markedly cold sensitive, with relative rates less than one-half the wild-type value, whereas three were markedly heat sensitive, with relative swarm rates greater than twice the wild-type value (one strain, MY110, was actually more motile at 20°C than at 37°C); these mutants are identified in Table 1. In contrast, single-site mutants had a rather narrow range of relative swarm rates; none of the rates was less than one-half or greater than twice the wild-type value.

DISCUSSION

Three proteins, FlaAII.2, FlaQ, and FlaN, have been shown to be central to the mechanisms of rotation and switching of the flagellar motor of *S. typhimurium*, as well as being essential for its assembly (32). The location of these switch proteins with respect to each other and to other components of the sensory and motility apparatus has not been established. We used intergenic suppression analysis to address this question.

A small fraction of mutations in the three switch genes could be suppressed intergenically. From these, we were able to identify 21 distinct classes of such suppressor mutations (meaning that no two classes map to the same location and derive from the same parent). Eleven of the classes of suppressor mutations lay within another of the three switch genes, eight lay within two genes (*cheY* and *cheZ*) that code for cytoplasmic components of the sensory transduction apparatus and two were within other chemotaxis and motility genes (*cheA* and *motB*).

The mutual suppression of defects in the switch genes is the first evidence that their products interact and will be discussed below. The suppression of switch defects by mutations in the *cheY* and *cheZ* genes reinforces a previous finding (23) that certain *cheY* and *cheZ* mutations in *Esche-*

FIG. 5. Allele specificity of suppression of paralyzed phenotype. A mutation causing paralysis (*Mot⁻*) was combined (cf. Fig. 2c) with various mutations causing abnormal switch bias (CCW or CW), which either had been originally selected for the ability to suppress the first mutation (thick connecting arrow) or had not (thin connecting arrow). Success (recombinant pseudorevertant swarms) or failure (no swarms) in suppression is indicated by ⊕ or ⊖, respectively. SJW strain numbers of the mutants (32; this study) that contributed the first-site and second-site mutations are indicated at the appropriate positions.

FIG. 6. Temperature sensitivity of pseudorevertants and single-site mutants. Normalized relative swarm rates of less than 1 indicate cold sensitivity compared with the wild type; rates greater than 1 indicate heat sensitivity. See the text for further explanation. Note that the rates are displayed on a logarithmic scale.

richia coli could be suppressed by mutations in the genes homologous to *flaAII.2* and *flaQ*. We extended this relationship to include a third switch gene, *flaN*, and showed that *cheY* and *cheZ* are by far the most prominent chemotaxis genes with respect to suppression of motor switch defects; only a single pseudorevertant was obtained with suppression by another chemotaxis gene (*cheA*).

Assessment of evidence for a switch complex. Intergenic suppression need not arise from physical contact between the gene products; the effect could be indirect, involving, for example, compensating effects on the pool size of another molecule that determines the phenotype under consideration. However, for the following reasons we conclude that the suppression data we obtained reflects physical contact. (i) Suppression was allele specific. The defects of paralyzed *flaN* and *flaQ* mutants were suppressible only by second mutations that had been selected for in this regard; unselected alleles, including ones conferring the same switch bias, totally failed to suppress (Fig. 5). Indirect effects are not predicted to be allele specific. (ii) Most of the pseudorevertants showed either pronounced cold sensitivity or heat sensitivity relative to the wild type (Fig. 6). Unusual temperature characteristics are a common feature of intergenic pseudorevertants in which the defective proteins are in physical contact (10). (iii) Most paralyzed mutants failed to give rise to intergenic pseudorevertants. This is to be expected if the mechanism of suppression involves direct physical interaction, since many defects in the first protein may be insensitive to quaternary interactions with the other protein, whereas many others may be too severe to be suppressible. (iv) Mutant phenotypes were not necessarily equivalent. First-site mutations conferring a paralyzed phenotype were suppressed by mutations conferring a switch-bias phenotype. Compensation for such qualitatively different defects can be readily understood if the gene products are in physical contact but not if the mechanism of suppression is indirect. (v) Suppressor mutations occurred in a limited set of genes. We found a tight pattern of interactions,

FIG. 7. (a) Model of FlaAII.2-FlaQ-FlaN switch-complex of flagellar motor of *S. typhimurium*, with protein-protein interactions (arrows) inferred from intergenic suppression data. The FlaAII.2, FlaQ, and FlaN proteins form a complex of unspecified stoichiometry, with three-way physical interactions among its components. The complex is presumed to be attached (dotted lines) to the basal body, probably at the M ring. The other face of the complex is exposed to the cytoplasm (cyto) and binds two sensory proteins, CheY and CheZ. The complex may also interact with the MotB and MotA proteins in the cytoplasmic membrane (CM) to create the mechanism for converting proton motive force to rotational work. (b) FlaAII.2-FlaQ-FlaN complex is bistable (right and left diagonal patterns), intermittently switching between conformational states that result in CW and CCW motor rotation. The states are stabilized by binding of CheY and CheZ, respectively.

involving almost exclusively the three switch genes plus *cheY* and *cheZ*. No suppressor mutations were found in a chemotaxis gene such as *cheR*, *cheB*, or *cheW*, even though their products are important in determining switch bias. This suggests that suppression by indirect mechanisms is not readily achieved, perhaps because there are additional consequences of such suppressor mutations (e.g., adaptation failure) that interfere with the chemotactic response. Based on the above reasoning, we conclude that the suppression data reflected direct physical interactions among the switch-gene products.

Cellular location of switch complex. Where in the cell do these interactions occur? Since the *flaAII.2*, *flaQ*, and *flaN* genes are necessary for flagellar assembly, the simplest hypothesis is that their products are part of the flagellar apparatus (FlaAII.2, for example, is known to be necessary for flagellar function as well as assembly; [5]). The genes are necessary for the earliest detectable stages of flagellar assembly (30), suggesting that their products are cytoplasmic proximal, a conclusion that is reinforced by the finding (23; this study) that switch-gene mutations can be compensated for by mutations in the *cheY* and *cheZ* genes, which code for cytoplasmic sensory components. It is known that the CheY and CheZ proteins can in the absence of other sensory components influence motor switching (4, 24a), further indicating that the interaction is direct. Finally, the FlaAII.2 and FlaQ proteins have been shown to be associated with the cell envelope (24).

We therefore suggest that the FlaAII.2, FlaQ, and FlaN proteins form a switch complex at the cytoplasmic face of the basal body (Fig. 7a) and that the cytoplasmic sensory proteins CheY and CheZ physically interact, probably in a reversible fashion, with the complex, with their relative extent of binding determining the conformational state of the switch and hence the sense of rotation of the motor (Fig. 7b). The switch complex may also interact with the MotB or MotA proteins to jointly form the energy transduction machinery for converting the proton motive force into rota-

tional work. The suppression of a switch-gene mutation by one in the *cheA* gene might indicate that CheA also interacts with the switch, but because such suppression was rare, this interaction must be regarded as tentative.

A number of questions arise in connection with this model. (i) If the switch complex is part of the flagellar motor, why are its components not found in basal body preparations (1, 4)? We view the basal body not as the entire flagellar motor but as a substructure. The procedure for isolating basal bodies is a fairly harsh one; peripherally associated structures such as the switch complex could easily be lost in the process. (ii) If the switch complex is mounted to the basal body, why were no suppressor mutations found in basal body genes such as *flaAII.1*, which is thought to code for the M ring (Fig. 7a; 1)? Because we were interested in interactions among energy-transducing and switching components of the motor, we chose paralyzed or switch-biased mutants as our starting point. The regions of the switch proteins critical for rotation and switching are likely to be distinct from those that interface with the basal body (cf. reference 32). We suspect that if we had chosen to analyze assembly-defective (nonflagellate) mutants in the switch genes, we would have encountered suppressor mutations in basal body genes. (iii) If the switch complex is part of the energy transduction machinery (as the MotA and MotB proteins are believed to be), why was only a single example found of suppression by *motB* and none by *motA*? The switch complex and the MotA and MotB proteins may well constitute the rotor and stator elements of the motor, respectively (cf. R. M. Macnab, in J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, H. E. Umbarger, and F. C. Neidhardt, ed., *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, in press), in which case their surfaces would need to continuously move past each other. The implications of this for intergenic suppression analysis are difficult to predict because it is a situation for which there is no precedent. It may be that the surfaces interact closely in only a few places, with the

interacting residues so critically important for the proton translocation process that defects are not readily suppressible.

It is interesting that a *cheZ* mutation was able to suppress a paralysis defect in the FlaQ protein (Table 1 and Fig. 3a), suggesting that CheZ binding to the motor can affect not only its switching properties but also its energy-transducing ability. Since no paralyzed *cheZ* mutants have ever been found, it seems unlikely that CheZ plays any direct role in motor rotation. We interpret the result to mean that some conformational defects in the switch, which block the process of energy transduction, are correctable by binding of a mutant CheZ protein but that the conformation of a wild-type switch with respect to energy transduction is too stable to be affected by binding of CheZ, mutant or otherwise.

Phenotypes of pseudorevertants and second-site mutants. Pseudorevertants of paralyzed mutants usually had abnormal switching, with a bias that was not necessarily the same as that of the second-site mutant; an extreme example of this was the *flaAII.2 motB* mutant, which had a strong CW bias, whereas the *motB* mutation alone conferred a normal switch bias. Such results suggest that paralyzed mutants in the switch genes may have a cryptic switch-bias phenotype that is only revealed when the paralysis defect is suppressed. Thus, the observed segregation of switching phenotypes within the primary structures of the FlaAII.2, FlaQ, and FlaN proteins (32) may have revealed only the regions that are important for switching alone and concealed those that are important for both switching and energy transduction. These two functions of the motor, although to some extent separable, are not entirely so.

Interestingly, the second-site *motB* mutant was partially paralyzed, indicating that the protein was only marginally functional. Probably a more serious defect in MotB function would have been incapable of providing motility in the pseudorevertant.

The phenotypes of pseudorevertants of switch-bias mutants represented a balance between the opposite phenotypes associated with the two alleles taken separately. Thus, the switch bias associated with the second site was forced by the selection protocol.

A novel phenotype for *cheZ* mutants. The selection pressure mentioned above resulted in the isolation of CCW-biased *cheZ* mutants. All those isolated previously were CW biased (20), a result that is interpreted to mean that binding of the CheZ protein, which places the motor in CCW rotation, was weakened. The simplest interpretation of the CCW-biased mutants would then be that the mutant CheZ protein actually binds to the switch more strongly than does the wild-type protein.

Significance of detailed location of suppressor mutations. In a previous study (32), we found that switch-gene mutations were highly clustered. The mutations generated in this study confirm that trend, all but three of them lying in deletion segments that had already yielded that phenotype in single-site mutants. Of a total of 42 segments defined for the three genes, only 16 have given rise to paralyzed mutants, only 6 have given rise to CW-biased mutants, and only 9 have given rise to CCW-biased mutants.

Conclusions. In this study, we used intergenic suppression analysis primarily to make inferences regarding protein-protein interactions. However, the results (as well as those from intragenic suppression, which was not analyzed here, and from our previous detailed mapping of the switch genes [32]) potentially contain much more information. The detailed locations of the mutations and the amino acid changes

they represent must reflect the higher-order structure of the components and the mechanisms of motor rotation and switching. Sequencing of the mutant alleles should give further insights into this remarkable biological device.

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