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Genetic and Behavioral Analysis of Flagellar Switch Mutants of *Salmonella typhimurium*

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At the interface between the sensory transduction system and the flagellar motor system of *Salmonella typhimurium*, the switch complex plays an important role in both sensory transduction and energy transduction. To examine the function of the switch complex, we isolated from 10 *cheY* mutants 500 pseudorevertants with a suppressor mutation in one of the three genes (*fliG*, *fliM*, and *fliN*) encoding the switch complex. Detailed mapping revealed that these suppressor mutations were localized to several segments of each switch gene, suggesting localization of functional sites on the switch complex. These switch mutations were introduced into the wild-type background and into a chemotaxis deletion background. Behavior of the pseudorevertants and their derivatives (1,500 strains in all) was observed by light microscopy. In the chemotaxis deletion background, about 70% of the switch mutants showed smooth swimming and the rest showed more or less tumbly swimming. There was some correlation between the mutational sites and the swimming patterns in the chemotaxis deletion background, suggesting that there is segregation of functional sites on the switch complex. The interaction of the switch complex with the chemotaxis protein, CheY, and the stochastic nature of switching in the absence of CheY are discussed.

Chemotactic bacteria such as *Salmonella typhimurium* swim with alternating intervals of swimming and tumbling (reviewed by Macnab [14]). Both modes have been shown to be essential to taxis: mutants that exclusively use one or the other mode fail to respond to environmental stimuli. Bacteria swim by rotating flagellar filaments driven by rotary motors (flagellar motors) embedded in the cell surface. A cell swims smoothly when all flagella are rotating counterclockwise (CCW), resulting in the formation of a flagellar bundle. Tumbling is a consequence of the disruption of the flagellar bundle caused by reversal of the rotation of flagella. The duration of the reversal, i.e., the period of clockwise (CW) rotation of a flagellum, varies according to the strength of the stimuli experienced by the cell. Unstimulated wild-type cells randomly switch the direction of flagellar rotation.

The flagellar basal body, a multisubunit complex at the base of the flagellum, is a major part of the flagellar motor (1, 15). However, it is not the entire motor because it lacks many proteins, including FliG, FliM, and FliN, which are indispensable for the generation of torque and for reversal of the direction of rotation; defects can result in abnormal switching (Che^-), paralysis (Mot^-), or lack of flagella (Fla^-) (29). (The nomenclature for the flagellar genes of *Escherichia coli* and *S. typhimurium* has been recently unified [8]; the *S. typhimurium* switch genes *fliG*, *fliM*, and *fliN* were formerly called *flaAII.2* [or *cheV*], *flaQ* [or *cheC*], and *flaN*, respectively.)

As one missing part of the motor, Yamaguchi et al. (29) have proposed a structure composed of the FliG, FliM, and FliN proteins, which works as a switching and energy-transducing complex (switch complex). Ravid and Eisenbach (22) found that the cell envelopes, which retained intact flagella but were devoid of cytoplasm, prepared from a *fliM* mutant and a *fliG* mutant still retained the tumbly nature of the parent cells, indicating that those gene products were associated with the cytoplasmic membrane. The idea that the switch complex might be located at the cytoplasmic face of the cell membrane was supported by the analysis of the deduced amino acid sequences of the switch genes (11). However, the physical existence of the switch complex has not been demonstrated either morphologically or biochemically (1, 15).

The flagellar genes of *S. typhimurium* are clustered in four regions of the chromosome; the switch genes (*fliG*, *fliM*, and *fliN*) are in region III, and the chemotaxis (*che*) genes are in region II (8, 14). The *che* genes encode most of the components of the sensory transduction system, and the roles of several Che proteins in the flow of information from chemoreceptors to the flagellar motor have been revealed (reviewed by Stewart and Dahlquist [25], Koshland [12], and Stock et al. [26]). In the last step of sensory transduction, a signal is transmitted to the flagellar motor, causing the motor to rotate in the CW direction and cells to tumble. Several lines of evidence indicate that CheY is this tumble signal. If CheY is absent from an otherwise wild-type cell, the motor rotates CCW exclusively (27). Parkinson et al. (20, 21) showed that in *E. coli*, most intergenic *cheY* and *cheZ* pseudorevertants contained compensatory mutations in the *fliG* or *fliM* locus and that the suppression between the *che* genes and the *fla* genes was at least partially allele specific, suggesting physical interactions between the CheY and CheZ proteins and the motor components. Yamaguchi et al. (28) expanded on their work using *S. typhimurium* and also came to the conclusion that the CheY and CheZ proteins probably bind directly to the switch complex, which they further showed was composed of the *fliG*, *fliM*, and *fliN* gene products. Interaction between CheY and two of the switch components was also demonstrated by analysis of the behavior of various *fliG* and *fliM* mutants in various genetic backgrounds by Wolfe et al. (27). Furthermore, they showed that CheZ counteracted the effects of CheY at the level of the motor. This relationship among CheY, CheZ, and the switch complex has been described at the molecular level by Simon and colleagues (2, 6, 24), who found that CheY could

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be phosphorylated and that CheZ specifically dephosphorylated CheY, suggesting that the phosphorylated form of CheY might be the tumble signal.

To elucidate the mechanism of the switch complex in detail, it is desirable to examine the behavior of various mutants of the switch genes. Koshland and colleagues (3, 10, 23) isolated several *fliM* mutants that showed exclusive CW rotation and, despite having abnormal switching probabilities, could swim smoothly; this so-called inverse-smooth swimming is the result of conformational change in the shape of flagellar filaments. Chemotactic mutants defective in the *fliG*, *fliM*, and *fliN* genes isolated by Yamaguchi et al. (29) displayed either smooth or tumbly swimming, indicating that an aberration in one of these gene products enhanced the stochastic nature of switching. In extreme cases, switch mutants can tumble in a *che* deletion background, indicating that mutations in the switch complex can reverse rotation even without CheY or the other chemotaxis proteins.

In this study, in order to obtain many switch mutants, we used the same strategy as did Parkinson et al. (21) by isolating spontaneous pseudorevertants of *cheY* mutants in *S. typhimurium*. The locations of 500 compensatory switch mutations obtained were determined by deletion mapping. The influence of these mutations on cellular motility in the presence or absence of the sensory transduction system was examined by light microscopy. From the results, we could specify the regions within the switch genes which we presume to be responsible for the interaction with CheY and for determination of the direction of flagellar rotation.

MATERIALS AND METHODS

Strains. All bacterial strains used in this study are derivatives of *S. typhimurium* SJW1103 (30); some of them, including *cheY* strains used as parents for the isolation of pseudorevertants, are listed in Table 1. Pseudorevertants of *cheY* mutants and their derivatives are not included in the table. Deletion mutants used for the mapping of suppressor mutations have been previously described (29).

Chemicals. Agar powder was obtained from Shouei Agar Co., Inc., gelatin powder was obtained from Nitta Gelatin, Ltd., yeast extract was obtained from Oxoid Ltd., and tryptone was obtained from Difco Laboratories. The other reagents (special grade) were from Wako Pure Chemical Industries, Ltd.

Media. Nutrient broth, nutrient agar, and soft nutrient agar were prepared as described by Yamaguchi et al. (29). Motility medium contained 10 mM potassium phosphate buffer and 20 mM KCl (pH 7.0). Attractant and repellent media consisted of 0.1 mM serine (4) and 10% glycerol (19), respectively, in motility medium.

Isolation of pseudorevertants and mapping of their suppressor mutations. A single colony of a given *cheY* mutant was grown in nutrient broth, and a 10-μl portion was applied as a line onto a soft nutrient agar plate. After 1 to 3 days of incubation at 37°C, spontaneous swarms emerging from the growth line were isolated. Pseudorevertants derived from different single colonies of *cheY* parents were considered to be independent from each other.

Mapping of *cheY* suppressor mutations. Second-site mutations (that is, *cheY* suppressor mutations) of pseudorevertants were mapped by transduction, using the general transducing bacteriophage P22 as mediator and mutants having a deletion in flagellar region I, II, or III as recipients, as described by Yamaguchi et al. (29). The mixture of equal volumes of the donor phage suspension (ca. 10^{10} PFU/ml) and the recipient culture (ca. 10^9 cells per ml) was streaked as several lines (ca. 15 μl per line) on a soft nutrient agar plate. Not only transductants introduced with the wild-type allele but also those with a suppressor mutation in place of the deletion formed swarms. However, they were easily distinguished from each other by their swarm size; the latter always formed smaller swarms than did the former.

Mapping was carried out in two steps. First, to determine in which region their suppressor mutations were, pseudorevertants were subjected to transductional crosses with long-deletion mutants whose deletions together cover flagellar regions I, II, and III. Then, to determine the precise location of suppressor mutations, pseudorevertants shown to have suppressor mutations in region III were subjected to crosses with a series of deletion mutants having deletion ends in region III (for the detailed deletion map, see reference 29).

Introduction of *cheY* suppressor mutations into a strain with wild-type *che* genes and into a strain with no *che* genes. The *cheY* suppressor mutation from each pseudorevertant was transduced into nonflagellate strains SJW2339 Δ(*fliE-fliN*) and SJW3077 Δ(*cheA-cheZ*) Δ(*fliE-fliN*); SJW3077 was constructed by introducing the deletion from SJW2339 Δ(*fliE-fliN*) into SJW3076 Δ(*cheA-cheZ*) (Table 1). The procedure is summarized in Fig. 1.

Transduction and isolation of transductants were performed essentially by the method of Yamaguchi et al. (29).

TABLE 1. *S. typhimurium* strains used

Strain	Relevant genotype	Source (reference)	Parent and parental phenotype
SJW1103	Wild type	S. Yamaguchi (30)	
SJW2323	*fliG*-5	S. Yamaguchi (28)	
SJW2339	Δ(*fliE-fliN*)	S. Yamaguchi (29)	
SJW2903[a]	*cheY*-4[b]	S. Yamaguchi (28)	SJW2323; not smooth
SJW2905[a]	*cheY*-5	This study[c]	SJW2323; not smooth
SJW2906[a]	*cheY*-3	This study[c]	SJW2323; not smooth
SJW2908[a]	*cheY*-5	This study[c]	SJW2323; not smooth
SJW3062[a]	*cheY*-2	This study[c]	SJW2323; not smooth
SJW3063[a]	*cheY*-2	This study[c]	SJW2323; not smooth
SJW3064[d]	*cheY*-2	This study[c]	SJW1103; wild type
SJW3065	*fliM*-19	This study[c]	SJW3064; smooth
SJW3066[a]	*cheY*-5	This study[c]	SJW3065; not smooth
SJW3067	*fliM*-5	This study[c]	SJW3064; smooth
SJW3068[a]	*cheY*-1	This study[c]	SJW3067; not smooth
SJW3069[a]	*cheY*-5	This study[c]	SJW3067; not smooth
SJW3076	Δ(*cheA-cheZ*)	This study	
SJW3077[e]	Δ(*fliE-fliN*) Δ(*cheA-cheZ*)	This study	

[a] *cheY* mutant obtained from pseudoreversion analysis of the switch mutants shown.
[b] The *cheY* gene has been divided into six segments. No mutant has yet been found in segment 6.
[c] Isolated as described by Yamaguchi et al. (28).
[d] Spontaneous *cheY* mutant.
[e] Constructed by introducing the *fla* deletion of SJW2339 into SJW3076 (see Materials and Methods).

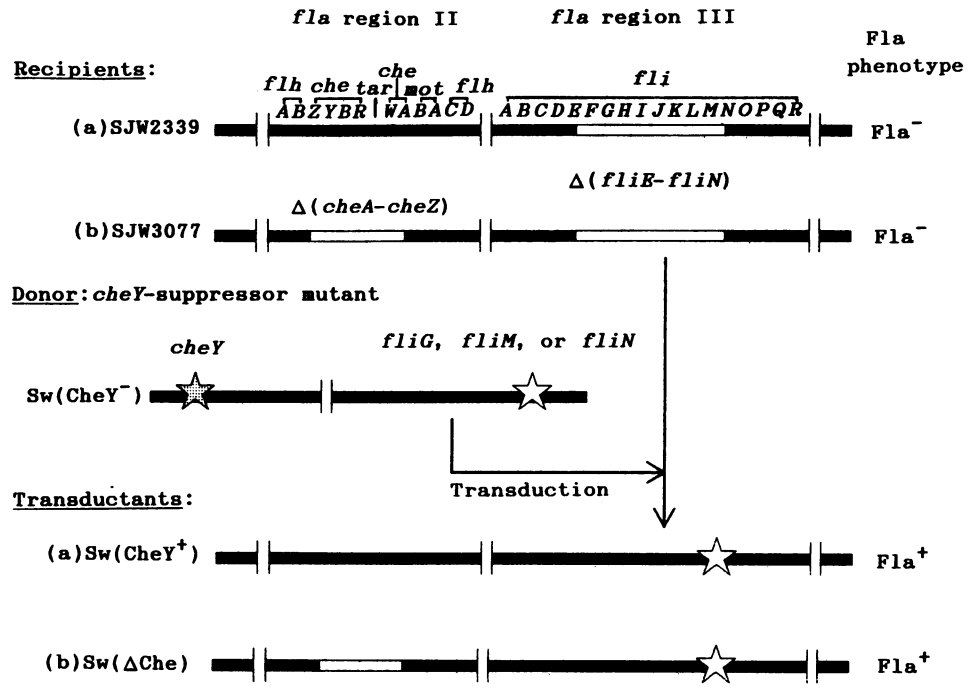

FIG. 1. Introduction of region III *cheY* suppressor mutations into a strain with (a) or without (b) all of the *che* genes of flagellar region II. Cells of the recipient strain, which was Fla^- as a result of a large deletion in region III, and P22 phage grown on a *cheY* suppressor mutant were applied as a line onto a soft nutrient agar plate and incubated overnight. Fla^+ transductional recombinants, which appeared as swarms, were isolated. Symbols: ■, bacterial chromosome; □, deleted region; ★, site of an original mutation; ★, site of a suppressor mutation.

Equal volumes of a culture of SJW2339 or SJW3077 (ca. 10^{10} cells per ml) and of a P22 *int* suspension (ca. 10^{10} PFU/ml) that had been grown on a pseudorevertant were mixed and applied as a line (15 μl per line) onto a soft nutrient agar plate and incubated overnight at 37°C. Fla^+ transductants that appeared as swarms (sizes varied with the donor pseudorevertant) were isolated and tested for P22 sensitivity. P22-sensitive clones were then examined for the presence of the donor suppressor mutation by transductional crosses with deletion mutants used for mapping of the suppressor mutations.

Characterization of motility patterns of pseudorevertants and constructed strains. Free-swimming cells were observed by phase-contrast microscopy and high-intensity dark-field light microscopy (7, 13). Tethered cells were prepared and examined by the method of Manson et al. (17). Motility tracks (1-s exposure) of free-swimming cells were recorded by using a phase-contrast microscope (Olympus BH-2) and a Polaroid camera (type 665 film) or an SIT video camera (Hamamatsu Photonics C1000), an image processor (Avionics Image Sigma IS-100), and a monitor TV (Hamamatsu Photonics C2130).

RESULTS

***cheY* suppressor mutations outside of the *che* gene cluster are localized to the switch genes.** We isolated 10 *cheY* mutants that had mutations in various segments of the gene (Table 1); of the *cheY* mutations, one (that of SJW3064) was spontaneous; the remainder had been obtained as second-site suppressors of *fliG* or *fliM* switch mutations obtained either in this study or in a previous one (28). All of the *cheY* mutants swarmed poorly but gave rise to revertants of various swarm sizes. Those that spread more rapidly than the parent but less rapidly than the wild-type strain were presumed to be pseudorevertants. From each *cheY* mutant, 72 to 184 pseudorevertants were isolated, to yield a total of 1,225. Genetic analysis showed that about half of the second-site *cheY* suppressor mutations mapped to the *che* gene cluster and that most of these lay in *cheY*, i.e., were intragenic suppressors; the remainder were in other *che* genes (possibly *cheZ* or *cheA*) but were not investigated further.

All of the other suppressor mutations (578 in all) mapped to flagellar region III (Table 2) and were located in one of the switch genes, *fliG*, *fliM*, or *fliN*. (Interestingly, the ratio of suppressor mutations in flagellar region III versus the *che* region varied considerably among the *cheY* mutants; thus, strains SJW3062 and SJW3063 yielded about 94% of suppressor mutations in flagellar region III, whereas other

TABLE 2. Crude mapping of *cheY* suppressor mutations in pseudorevertants derived from *cheY* mutants

cheY parent strain	No. of pseudorevertants	No. of *cheY* suppressor mutations mapped to:	
		fla region III	*che* genes
SJW2903	133	50	83
SJW2905	89	61	28
SJW2906	84	63	21
SJW2908	91	53	38
SJW3062	74	69	5
SJW3063	72	68	4
SJW3064	184	52	132
SJW3066	162	56	106
SJW3068	178	56	122
SJW3069	158	50	108

strains yielded much lower values [e.g., SJW3064, 28%].) Fifty such pseudorevertants were chosen randomly from each *cheY* mutant, and their mutations were mapped in detail. Of the 500 pseudorevertants, 379 (76%) were in *fliM*, 116 (23%) were in *fliG*, and only 5 (1%) were in *fliN*.

***cheY* suppressor switch mutations are localized to several hot spots within the switch genes.** The sites of *cheY* suppressor mutations were not evenly distributed within the switch genes but were localized in a few segments (defined by deletion endpoints; 29) (Fig. 2). Segments 5 and 6 of *fliM* (*fliM*-5 and *fliM*-6) contained more than half of them (125 and 144, respectively). *fliG*-9 and *fliM*-19 also contained many mutations (56 each).

Different *cheY* parents tended to give different distributions of suppressor mutations. For example, SJW2903 (Table 3) and SJW3064, SJW3066, SJW3068, and SJW3069 (not shown) gave rise to many suppressor mutations in *fliM*-6 and *fliM*-19 but few in *fliM*-5 and *fliM*-18. At the other extreme, SJW3062 (Table 3) and SJW2906 and SJW3063 (not shown) gave rise to few in *fliM*-6 and *fliM*-19 and many in *fliM*-5 and *fliM*-18. SJW2908 (Table 3) and SJW2905 (not shown) gave an intermediate pattern, with comparable numbers of mutations in *fliM*-5, *fliM*-6 and *fliM*-19 and mutations also in *fliG*-9.

Transfer of switch mutations into other genetic backgrounds. We transferred the *cheY* suppressor mutations into two other genetic backgrounds: (i) a wild-type strain, in order to characterize the interaction of switch defects with wild-type CheY protein, and (ii) a strain deleted for all chemotaxis genes, in order to determine the intrinsic properties of the switch defects in the absence of interaction with the chemotaxis system.

Hereafter we focus on the switching function of the gene products and therefore describe the *cheY* suppressor mutations as switch mutations. To simplify the presentation, we adopt the following shorthand nomenclature: strains carrying the switch mutation in the original mutant *cheY* background (i.e., pseudorevertants) are called Sw(CheY$^-$) mutants; those in a wild-type *cheY* background are called Sw(CheY$^+$) mutants; and those in the *che* deletion background are called Sw(ΔChe) mutants.

Classification of swimming patterns of switch mutants. Because of the large numbers of switch mutants to be tested, we observed the swimming patterns of free cells rather than the switching probabilities of tethered cells. Before describing the results in detail, it is necessary to define the swimming patterns clearly.

By convention, there are four classes of swimming patterns: smooth, normal (wild type), tumbly, and inverse smooth, corresponding to progressively increased CW bias (9, 10). In the course of examining many mutants, we noticed that among tumbly mutants, there were really two distinguishable patterns: cells of one type jumped rapidly from one spot to another with little long-term displacement (perhaps the "twidding" described by Manson et al. [18]), whereas those of the other type appeared to tumble vigorously in one spot. Analysis of tethered cells of 10 tumbly mutants chosen randomly showed that the latter type of cells were more CW biased than the former. Instead of introducing additional terms, we use a scale from I to V to describe these motility patterns according to the degree of tumbliness (CW bias): I, cells that swam smoothly and never tumbled; II, cells with wild-type behavior, i.e., swimming punctuated by tumbling; III, cells that jumped about from one spot to another; IV, cells that tumbled in one spot; and V, cells that swam slowly but tumbled with about the same frequency as wild-type cells; these cells were using inverse swimming as a result of a high CW bias (10).

The differences between adjacent swimming patterns was not always easily distinguished. Therefore, to be as consistent as possible, only one of us (Y.M.) judged these patterns.

Figure 3 shows motility tracks of cells exhibiting these five classes of swimming patterns. Although the patterns do not represent the exact CCW/CW ratio of the motor, they do reflect it semiquantitatively. (Incidentally, differences between mutants that could be detected by inspecting cells in the light microscope were not necessarily detected as differences in swarming rates on soft nutrient agar plates.)

Comparison of swimming patterns of switch mutants. All of the parental *cheY* strains swam smoothly without tumbling (class I), whereas the switch mutants showed a variety of swimming patterns, ranging from smooth swimming (class I) through inverse swimming (class V), as shown in histogram form for all mutants in Fig. 2 and in more detailed form for three representative mutants, SJW2903, SJW2908, and SJW3062, in Table 3.

Generally, Sw(CheY$^+$) mutants were tumbly, Sw(ΔChe) mutants were more smooth swimming, and Sw(CheY$^-$) mutants were somewhere in between. Typically, the degree of tumbliness of a Sw(CheY$^+$) mutant was about one step greater than that of the corresponding Sw(CheY$^-$) mutant, which in turn was about one step greater than that of the corresponding Sw(ΔChe) mutant; e.g., if the Sw(CheY$^+$) mutant fell in class III, often the corresponding Sw(CheY$^-$) mutant fell in class II and the corresponding Sw(ΔChe) mutant fell in class I. In some cases, the differences were as large as two steps, whereas in others the differences were too small to be scored. In a few cases (e.g., *fliM*-18 mutants derived from SJW3062; Table 3), the differences were so small that even the Sw(CheY$^+$) and Sw(ΔChe) mutants fell in the same class; this was especially true when the motility was very tumbly (e.g., class IV).

Some switch mutations introduced a sufficiently strong CW bias that Sw(CheY$^+$) and Sw(CheY$^-$) mutants exhibited inverse motility, but in no case did this extend to a Sw(ΔChe) mutant.

Although most of the mutant switch complexes seemed to be quite stable as judged by swimming patterns, they did give some evidence of defective rotation, with cells pausing or slowing down more frequently than did the wild type (S. Kudo, personal communication). This may be a consequence of their higher reversal frequency (cf. Eisenbach et al. [5]).

Relationship between parent, switch mutational site, and swimming pattern. Different *cheY* mutants tended to give rise to different spectra of phenotypes. A good example of this was the occurrence of inverse motility, which was not seen at all with suppressor mutations derived from most *cheY* parents but was seen with high frequency with two parents, SJW3064 and SJW2906, which yielded 19 and 10 examples, respectively.

The different spectra were also strikingly seen by comparing the phenotypes of Sw(ΔChe) mutants from various backgrounds. Of the 500 mutants, 70% were smooth swimming and the remainder were more or less tumbly (Fig. 2C). Most of the former derived from *cheY* strains SJW2903, SJW2908, SJW3064, SJW3066, SJW3068, and SJW3069, whereas most of the latter derived from strains SJW2905, SJW2906, SJW3062, and SJW3063.

This classification of phenotype by *cheY* parental origin agrees fairly well with that noted above for the different distributions of suppressor mutations in the switch genes as

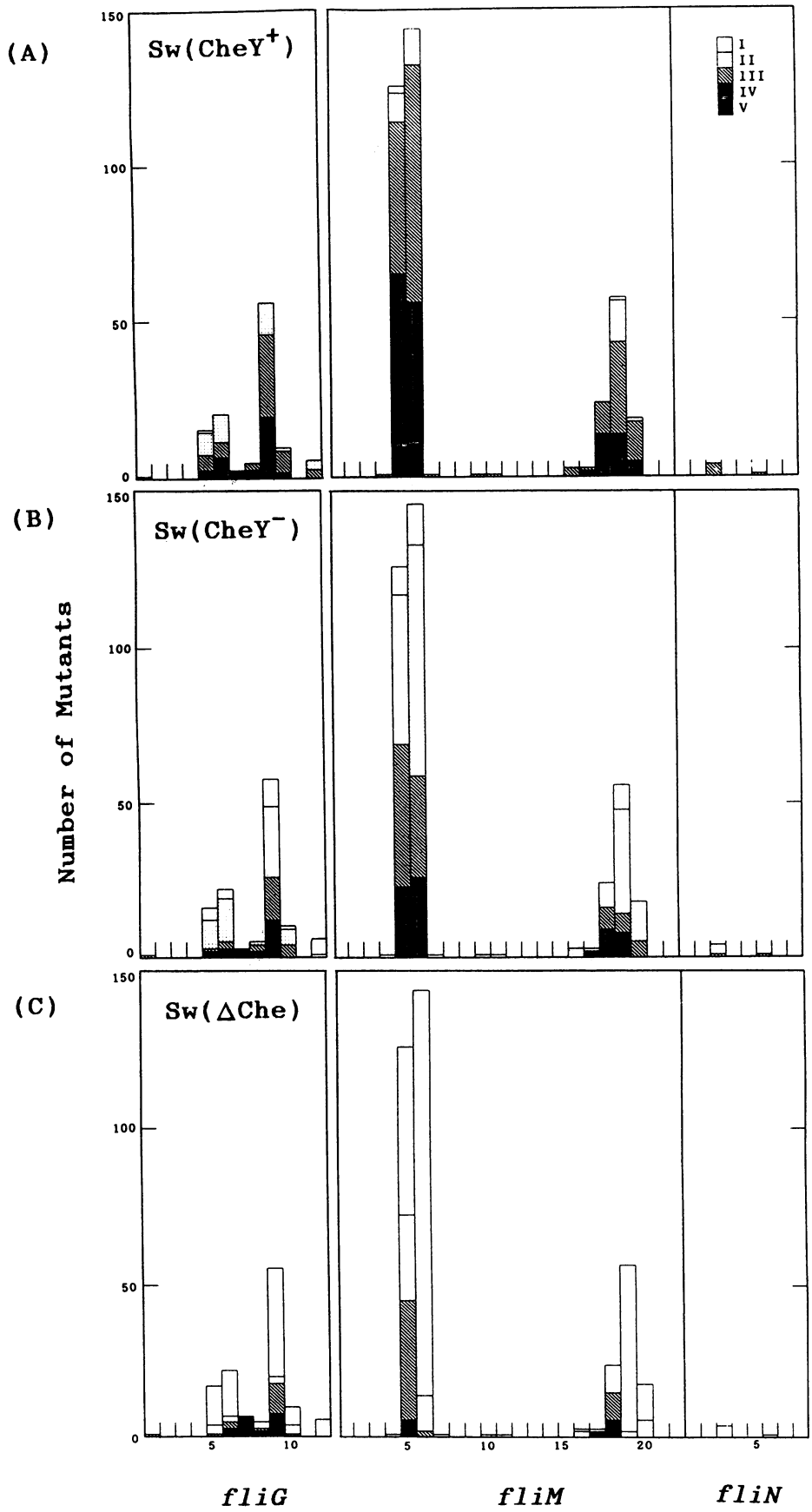

FIG. 2. Distribution of various swimming patterns of switch mutants in three genetic backgrounds. (A) Mutant *cheY* Sw(CheY$^+$) mutants; (B) Sw(CheY$^-$) mutants; (C) Sw(ΔChe) mutants. The mutational site of each switch mutant was mapped to deletion segments of each gene. The swimming pattern of each mutant (cf. Fig. 3) is indicated by different patterns (see key) such that those at the bottom of bars are more tumbly than those at the top.

TABLE 3. Locations of switch mutations and swimming patterns of switch mutants derived from SJW2903, SJW2908, and SJW3062

Source of mutation	Mutation no.	Location of switch mutation	Swimming pattern		
			Sw(CheY$^+$)	Sw(CheY$^-$)	Sw(ΔChe)
SJW2903	1	*fliG*-5	II	I	I
	2	*fliG*-6	II	I	I
	3	*fliG*-6	II	II	I
	4	*fliG*-6	III	II	I
	5	*fliG*-9	II	I	I
	6	*fliG*-9	II	I	I
	7	*fliG*-9	III	II	I
	8	*fliG*-10	III	III	I
	9	*fliM*-4	III	II	I
	10	*fliM*-5	III	III	II
	11	*fliM*-5	IV	III	III
	12	*fliM*-6	II	I	I
	13	*fliM*-6	III	I	I
	14	*fliM*-6	III	II	I
	15	*fliM*-6	III	II	I
	16	*fliM*-6	III	II	I
	17	*fliM*-6	III	II	I
	18	*fliM*-6	III	II	I
	19	*fliM*-6	III	II	I
	20	*fliM*-6	III	II	I
	21	*fliM*-6	III	III	I
	22	*fliM*-6	III	III	I
	23	*fliM*-6	III	III	I
	24	*fliM*-6	III	III	I
	25	*fliM*-6	III	III	I
	26	*fliM*-6	III	III	I
	27	*fliM*-6	III	III	I
	28	*fliM*-6	IV	II	I
	29	*fliM*-6	IV	III	I
	30	*fliM*-6	IV	III	I
	31	*fliM*-6	IV	III	I
	32	*fliM*-7	III	II	I
	33	*fliM*-18	III	II	I
	34	*fliM*-18	III	II	I
	35	*fliM*-18	III	II	I
	36	*fliM*-19	III	I	I
	37	*fliM*-19	III	I	I
	38	*fliM*-19	III	II	I
	39	*fliM*-19	III	II	I
	40	*fliM*-19	III	II	I
	41	*fliM*-19	III	II	I
	42	*fliM*-19	III	II	I
	43	*fliM*-19	III	II	I
	44	*fliM*-19	IV	II	I
	45	*fliM*-19	IV	II	I
	46	*fliM*-19	IV	III	I
	47	*fliM*-20	III	II	I
	48	*fliM*-20	III	II	I
	49	*fliM*-20	IV	III	II
	50	*fliM*-20	IV	III	II
SJW2908	1	*fliG*-8	IV	III	III
	2	*fliG*-9	III	I	I
	3	*fliG*-9	III	I	I
	4	*fliG*-9	III	II	I
	5	*fliG*-9	III	II	I
	6	*fliG*-9	III	II	I
	7	*fliG*-9	III	II	I
	8	*fliG*-9	IV	III	III
	9	*fliG*-9	IV	III	III
	10	*fliG*-9	IV	IV	IV
	11	*fliG*-10	III	I	I
	12	*fliG*-10	III	II	II
	13	*fliG*-12	II	II	I
	14	*fliM*-5	I	I	I
	15	*fliM*-5	II	I	I
	16	*fliM*-5	II	II	I
	17	*fliM*-5	III	I	I
	18	*fliM*-5	III	II	I
	19	*fliM*-5	III	II	I
	20	*fliM*-5	III	II	I
	21	*fliM*-5	IV	III	II
	22	*fliM*-5	IV	III	III
	23	*fliM*-5	IV	IV	IV
	24	*fliM*-6	II	I	I
	25	*fliM*-6	II	I	I
	26	*fliM*-6	II	I	I
	27	*fliM*-6	III	II	I
	28	*fliM*-6	III	II	I
	29	*fliM*-6	III	II	I
	30	*fliM*-6	III	II	I
	31	*fliM*-6	III	II	I
	32	*fliM*-6	III	II	I
	33	*fliM*-6	III	II	II
	34	*fliM*-6	IV	III	I
	35	*fliM*-16	III	II	II
	36	*fliM*-17	III	II	I
	37	*fliM*-18	III	II	I
	38	*fliM*-18	IV	IV	IV
	39	*fliM*-18	IV	IV	IV
	40	*fliM*-19	II	I	I
	41	*fliM*-19	II	II	I
	42	*fliM*-19	II	II	I
	43	*fliM*-19	II	II	I
	44	*fliM*-19	III	I	I
	45	*fliM*-19	III	II	I
	46	*fliM*-19	III	II	I
	47	*fliM*-19	III	II	I
	48	*fliM*-19	III	II	I
	49	*fliM*-19	III	II	I
	50	*fliM*-20	IV	III	II
SJW3062	1	*fliG*-5	I	II	I
	2	*fliG*-6	II	I	I
	3	*fliG*-6	II	II	II
	4	*fliG*-6	III	I	I
	5	*fliG*-6	III	II	II
	6	*fliG*-6	IV	III	III
	7	*fliG*-6	V	IV	IV
	8	*fliG*-9	III	II	II
	9	*fliG*-9	IV	III	III
	10	*fliG*-9	IV	III	III
	11	*fliM*-5	I	I	I
	12	*fliM*-5	III	I	I
	13	*fliM*-5	III	II	I
	14	*fliM*-5	III	II	I
	15	*fliM*-5	III	I	II
	16	*fliM*-5	III	II	II
	17	*fliM*-5	III	II	II
	18	*fliM*-5	III	II	II
	19	*fliM*-5	III	II	II
	20	*fliM*-5	III	II	II
	21	*fliM*-5	III	III	II
	22	*fliM*-5	III	III	III
	23	*fliM*-5	III	III	III
	24	*fliM*-5	IV	III	II
	25	*fliM*-5	IV	III	III
	26	*fliM*-5	IV	III	III
	27	*fliM*-5	IV	III	III
	28	*fliM*-5	IV	III	III
	29	*fliM*-5	IV	III	III
	30	*fliM*-5	IV	III	III
	31	*fliM*-5	IV	III	III

Continued

TABLE 3—*Continued*

Continued on following page

TABLE 3—*Continued*

Source of mutation	Mutation no.	Location of switch mutation	Swimming pattern		
			Sw(CheY$^+$)	Sw(CheY$^-$)	Sw(ΔChe)
	32	*fliM*-5	IV	III	III
	33	*fliM*-5	IV	III	III
	34	*fliM*-6	III	I	I
	35	*fliM*-6	III	I	I
	36	*fliM*-6	III	I	I
	37	*fliM*-6	III	I	I
	38	*fliM*-6	III	II	I
	39	*fliM*-6	III	II	I
	40	*fliM*-6	III	II	I
	41	*fliM*-18	III	III	III
	42	*fliM*-18	IV	III	III
	43	*fliM*-18	IV	III	III
	44	*fliM*-18	IV	IV	IV
	45	*fliM*-18	IV	IV	IV
	46	*fliM*-18	IV	IV	IV
	47	*fliM*-19	III	I	I
	48	*fliM*-19	III	I	I
	49	*fliM*-19	III	II	II
	50	*fliM*-20	III	II	I

a function of parental origin. Although no precise correlation exists between the site of a switch mutation and the phenotype associated with it, we were able to make some generalizations, especially in the context of the Sw(ΔChe) mutants. Most notably, segments *fliM*-6 and *fliM*-19 tended to yield smooth-swimming mutants, whereas segments *fliM*-5 and *fliM*-18 tended to yield tumbly mutants.

Switch mutants respond to temporal gradients of chemoeffectors. Sw(ΔChe) mutants showed negligible swarming on soft nutrient agar plates. Sw(CheY$^-$) mutants, of course, swarmed moderately well (that being the basis of their original selection). Most Sw(CheY$^+$) mutants, however, also made small swarms, suggesting they were chemotactic. All 50 Sw(CheY$^+$) mutants derived from the *cheY* mutant SJW2908 were subjected to temporal gradients of the attractant serine (4) or the repellent glycerol (19) to see whether this was the case. Most became less tumbly upon serine addition and more tumbly upon glycerol addition (representative examples are shown in Table 4). However, a few (e.g., strain 39) failed to respond to either stimulus, suggesting that the switch complex was so altered that it was unaffected by wild-type CheY.

Allele specificity of the CheY-switch interaction. To examine the allele specificity between mutations in the *cheY* and switch genes, 18 different switch mutations were introduced into a variety of *cheY* mutant backgrounds, and the swarm sizes of the resulting double mutants on soft nutrient agar plates were measured (Fig. 4). Sizes ranged from those of the *cheY* parents (ca. 4 mm) up to about 30 mm, roughly half that of wild-type cells. For any given switch mutation, the swarm size also varied according to the *cheY* allele. For example, switch mutation 38 originating from *cheY* parent SJW2903 was effective in combination with some *cheY* alleles (e.g., those of SJW2903, SJW2905, and SJW2908) but not with others (e.g., those of SJW2906, SJW3062, and SJW3063). Interestingly, although the parental allele (e.g., the one giving rise to the switch mutation in the original pseudorevertant) tended to be fairly effective, it was not necessarily the most effective; for example, switch allele 47 (in *fliM*-19) originating as a suppressor of SJW3062 yielded better swarming in combination with the *cheY* alleles of SJW2903, SJW2905, or SJW2908.

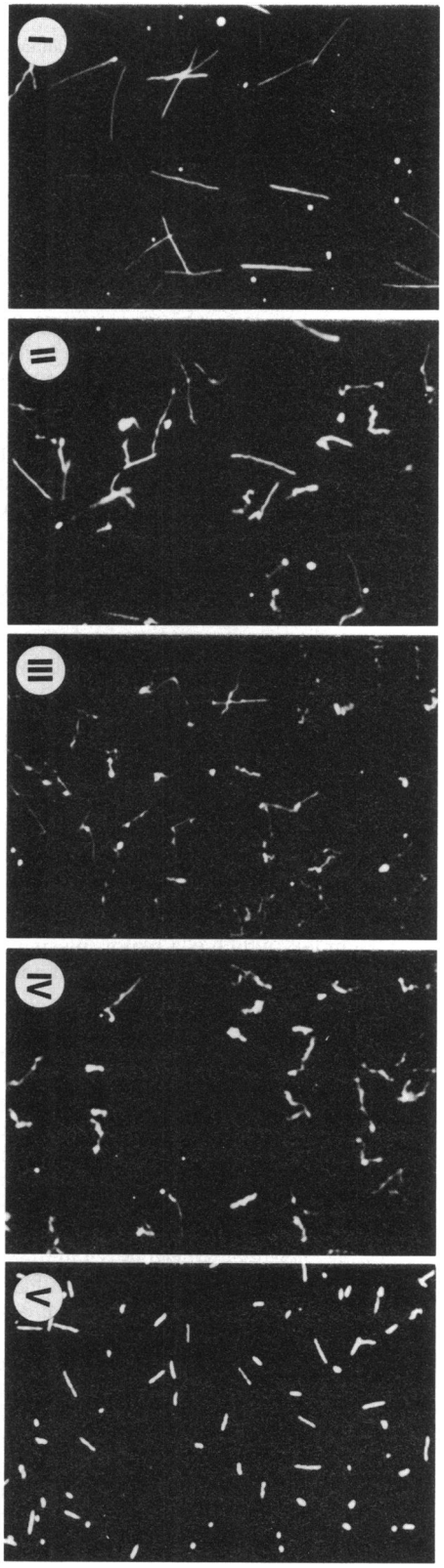

FIG. 3. Five categories (I to V; see text for definitions) of trajectories of free-swimming cells.

TABLE 4. Chemotactic response of Sw(CheY$^+$) mutants from SJW2908

Strain	Location of switch mutation	Phenotype of Sw(ΔChe)	+Attractant	No addition	+Repellent
SJW1103	Wild type	I	I	II	III
3	*fliG*-9	I	II	III	IV
22	*fliM*-5	III	III	IV	IV
27	*fliM*-6	I	II	III	IV
39	*fliM*-18	IV	IV	IV	IV
42	*fliM*-19	I	I	II	III

The mutant *cheY* alleles from strains SJW2903, SJW2905, and SJW2908 generally combined most effectively with those switch mutations which (in the *che* deletion background) were associated with the smooth-swimming phenotype. Conversely, mutant *cheY* alleles from strains SJW2906, SJW3062, and SJW3063 combined most effectively with switch mutations associated with the tumbly phenotype. This subclassification of the *cheY* strains closely resembles the one described above, which was based on the spectra of switch mutant phenotype generated in the original pseudorevertant isolation.

Overall, these results imply that there is some allele specificity between *cheY* and switch mutations but that it is not so narrow as to correspond to a unique specificity between one allele and another, but rather between groups of *cheY* alleles and groups of switch alleles, the latter being distributed among all three switch genes. Similar results have been reported for *E. coli* by Parkinson et al. (21).

The weak allele specificity between CheY and the switch contrasts with the strong specificity found among the switch proteins themselves (28), which has been used as evidence for the existence of a switch complex.

DISCUSSION

The switch complex, which is a major missing component of the flagellar motor as it is currently isolated, has been studied by analyzing the swimming patterns of switch mutants in a variety of backgrounds. Since the yield of spontaneous *che* switch mutants by conventional methods (29) is low, we adopted the strategy used by Parkinson et al. (21) and isolated spontaneous pseudorevertants from different *cheY* mutants. Of the suppressor mutations, about half lay in *cheY* or adjacent chemotaxis genes and the remainder lay within the three known switch genes, *fliG*, *fliM*, and *fliN*. We have analyzed 500 such mutations, 50 each derived from each of 10 *cheY* parents.

These mutations clearly clustered into a limited number of hot spots within the switch genes; more than half lay within just two segments (*fliM*-5 and *fliM*-6) out of a total of 42 segments defined by deletion mapping of the three genes. Although the parental *cheY* mutations were (with one exception) themselves isolated as suppressors of switch mutations, they were fairly uniformly distributed throughout the *cheY* gene. This, plus the fact that the distribution of switch alleles closely resembles that obtained in a previous study with spontaneous switch mutants (29), suggests that the hot spots are not a simple consequence of the isolation procedure but rather reflect the intrinsic character of the switch genes.

To examine the properties of the switch proteins in the presence and absence of the sensory transduction, each switch mutation was transferred into a wild-type and a *che* deletion background. We can summarize the main features of the analysis of the 1,500 mutants [500 each of Sw(CheY$^+$), Sw(CheY$^-$), and Sw(ΔChe)] as follows. (i) About 1% of Sw(CheY$^+$) mutants were smooth swimming, about 13% had essentially wild-type motility, and the remaining 86% showed tumbly swimming. For Sw(CheY$^-$) mutants, the corresponding figures were 10, 48, and 42%; for Sw(ΔChe) mutants, they were 70, 12, and 18%. Thus, as a group, Sw(CheY$^+$) mutants were more tumbly than Sw(CheY$^-$) mutants, which in turn were more tumbly than Sw(ΔChe) mutants. (ii) For any given switch mutation, the Sw(CheY$^+$) mutant was almost always more tumbly than the corresponding Sw(CheY$^-$) mutant, which in turn was almost always more tumbly than the corresponding Sw(ΔChe) mutant.

The simplest interpretation of these results, in the context of accumulating evidence that the critical event in modulating the rotational state of the motor is CheY binding to the switch (21, 28), is as follows. (i) The contribution of the mutant *cheY* allele is to enhance the probability of CCW rotation relative to that with the wild-type allele, as a result of weaker binding to the switch caused either by impaired phosphorylation (2, 24) or an intrinsically weaker affinity (the latter perhaps being more likely in the case of the majority of the *cheY* mutations used in this study, which were isolated as suppressors of switch mutations). (ii) The contribution of the *che* deletion is to further enhance the probability of CCW rotation, since CheY is now totally absent. (iii) The contribution of the mutant switch allele is to enhance the probability of CW rotation relative to that with the wild-type switch allele; this is to be expected in view of their origin as suppressors of the CCW *cheY* mutations just described. The results suggest that there is a considerable range in the degree of CCW bias introduced by the various *cheY* alleles and in the degree of CW bias introduced by the various switch alleles, since the swarming ability of mutants carrying a given *cheY* allele and various switch alleles varied widely, as did that of mutants carrying various *cheY* alleles and a given switch allele (Fig. 4).

The fact that about 30% of the Sw(ΔChe) mutants exhibited some degree of tumbling underscores the fact that the flagellar switch has an intrinsic capacity to rotate in the CW as well as the CCW direction, perhaps switching between the two as a result of thermal fluctuations, as was suggested by Macnab and co-workers (9, 16). The intrinsic ability of the switch to rotate in both senses has been demonstrated previously by Ravid and Eisenbach (22), who observed CW rotation of cell envelopes of certain switch mutants, and by Wolfe et al. (27), who observed CW rotation of *E. coli* Sw(ΔChe) strains similar to the ones used in this study.

Thus, while CheY greatly enhances CW rotation, it is not an absolute requirement for it. However, the wild-type switch complex is intrinsically far more stable in the conformation for CCW rotation. Under ordinary conditions, only CheY binding to the switch can generate an appreciable probability of CW rotation and so allow the sensory transduction system to effectively modulate motility.

What is the mechanism whereby a given switch mutation results in an enhancement of CW bias? In principle, it could be a consequence of enhanced CheY binding, altered intrinsic CW bias, or both. In the case of those mutations which result in detectable CW rotation even in the *che* deletion background, altered intrinsic bias is clearly a major contributing factor. Where the switch mutation in such a *che* deletion background results in exclusively CCW rotation (as is also the case with the wild-type switch alleles), it could be that the intrinsic bias is truly unaltered compared with wild

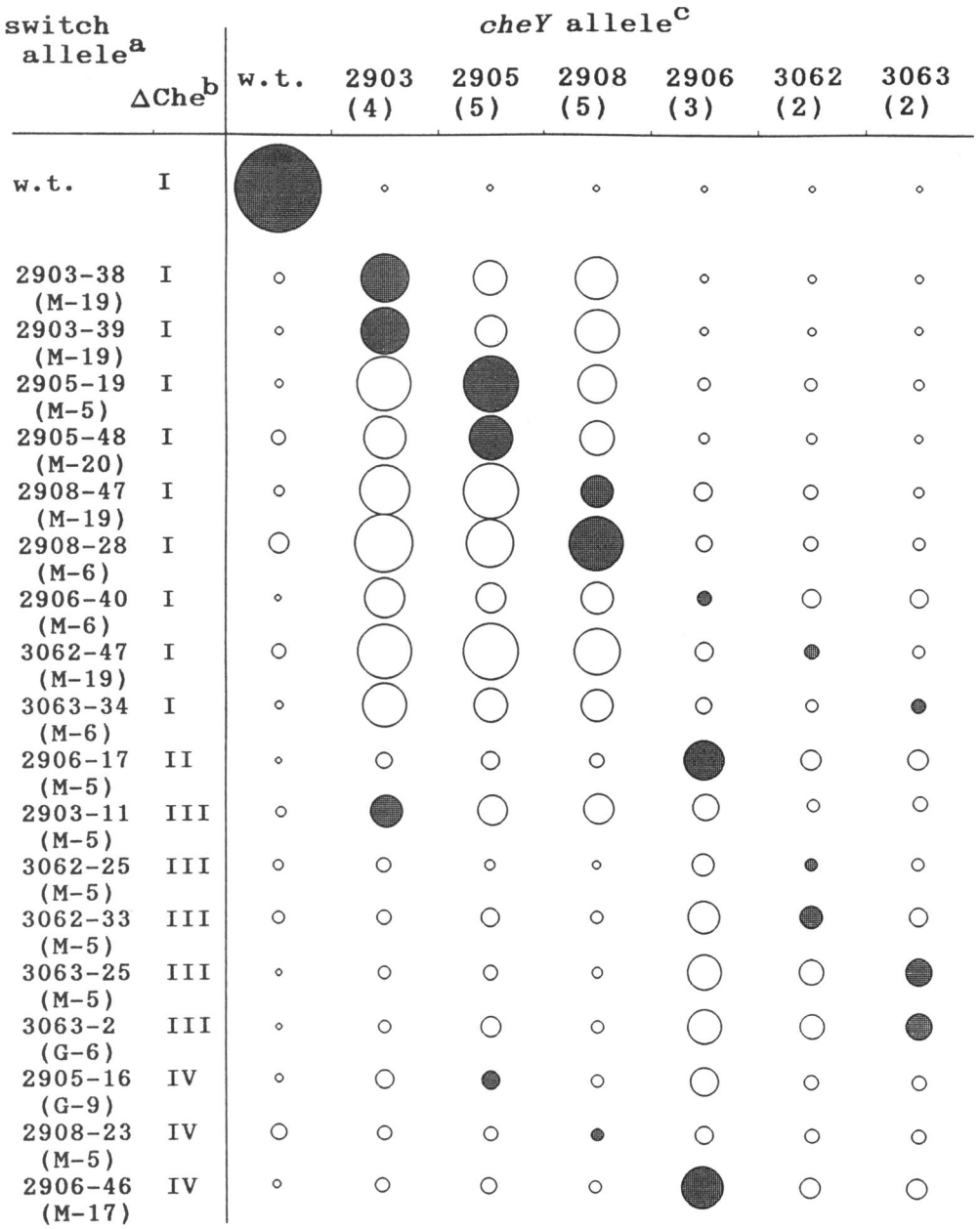

FIG. 4. Allele specificity of the interaction between *cheY* and the switch genes. The chemotactic abilities of double mutants defective in *cheY* and one of the switch genes were assessed by their swarm sizes on soft nutrient agar plates. The sizes of circles represent the swarm sizes; shading indicates original (pseudorevertant) combinations of *cheY* and switch mutations. [a]w.t. represents the wild-type switch genes in the wild-type strain, SJW1103. The others are mutant switch genes. The numbers before and after the hyphen show the parent and mutation number, respectively; e.g., 2903-38 is the 38th switch mutation derived from SJW2903. The location of the switch mutation is shown in parentheses; e.g., M-19 is the gene segment *fliM*-19. [b]The swimming pattern of switch mutants in a *che* deletion background. [c]w.t. represents the wild-type *cheY* in SJW1103. The others are mutant *cheY*; e.g., 2908 is a mutant *cheY* in SJW2903. Location of the *cheY* mutation is shown in parentheses; e.g., 4 is the *cheY*-4.

type, in which case one must assume that the altered bias in the presence of CheY is because of an enhanced affinity for its binding. However, the bias might have been altered but by too small an amount to be detected in the absence of CheY; for example, if the intrinsic CW probability of the wild-type switch was 0.1% whereas that of the mutant switch was 1%, this difference would probably not be detectable but might result in an easily detectable difference with CheY present. In this case, there would be no need to assume that the affinity for CheY had been affected by the switch mutation.

In this regard, it is interesting to consider the results of the allele specificity analysis (Fig. 4). These findings can generally be explained by the postulate that if the original *cheY* mutation requires a given degree of enhanced CW bias in the switch mutation to give a strongly swarming pseudorevertant, then other switch mutations with a similar degree of enhanced CW bias will also tend to give strong swarming; in

those cases where the original pseudorevertant swarmed poorly, another switch mutation often gave better swarming, presumably because the mutation conveyed a bias that matched that of the original *cheY* mutation more effectively. The fact that different *cheY* mutants gave rise to different distributions of the locations of suppressor mutations might at first seem to reflect allele specificity, but closer inspection of the data indicates that they are more simply explained on the basis of the strengths of CW bias associated with a particular location. Thus, there is little if any evidence for a degree of specificity that would indicate pairwise matching between a given altered CheY and a given altered switch protein. Indeed, the fact that the CW bias of most Sw(CheY$^+$) mutants was greater than that of the corresponding pseudorevertants argues against such a pairwise matching and suggests instead that if there is an enhanced affinity for CheY, it is general and not specific for any given CheY allele; this was true of those switch mutations in which the intrinsic bias clearly had been altered as well as those in which this effect was uncertain.

It seems questionable on steric grounds whether all three switch proteins could be contributing directly to the CheY binding site; this is especially true in light of the fact that a single *cheY* mutation could give rise to suppressor mutations in a variety of locations in all three genes. Also, given the fact that mutations generally impair rather than enhance function, it would perhaps be surprising if our study were to produce many examples of stronger CheY binding sites. (In this regard, studies of pseudorevertants of mutants in which CheY was more highly phosphorylated, for example as a result of a *cheZ* mutation, might be more likely to yield examples of altered [but in this case lowered rather than enhanced] affinity of the switch binding site for CheY.)

The most economical hypothesis therefore seems to be that most of the switch mutations result in an altered intrinsic CW bias that compensates for weaker binding of the mutant CheY proteins. This does not preclude the possibility that there could also be some examples of binding-site mutations in our study, since we did not analyze the allele specificity of all of the switch mutations.

It should also be noted that if CheY binding favors the CW state of the switch, then by a reciprocal argument we can expect that the CW state of the switch will favor CheY binding. Thus, a mutation that alters the intrinsic bias of the switch toward the CW state is also likely to result in a greater mean degree of CheY binding, even though the alteration in the switch protein may not occur in the CheY binding site.

Yamaguchi et al. (29) have demonstrated that the different aspects of switch protein function (flagellar assembly, torque generation, CW rotation, and CCW rotation) segregate into different segments of the protein sequence. The present study emphasizes this point even more strongly. Because of the manner in which the switch mutations were selected (as suppressors of *cheY* mutations), they were all associated with a CW-biased rather than CCW-biased phenotype. However, they varied greatly in the degree of that bias. Segments 6 and 9 of *fliG* tended to produce a strong CW bias. So too did segments 5 and 18 of *fliM*; the adjacent segments, 6 and 19, produced many examples of switch mutations, but with a weaker CW bias. *fliN* produced only 1% of the switch mutations in this study, emphasizing the conclusion reached in a previous study (29) that *fliN* is only weakly associated with switching phenotype but is strongly associated with rotation (Mot) phenotype. Perhaps it does not play a direct role in switching but interfaces with the FliG and FliM proteins that do.

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