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Subdivision of Flagellar Genes of *Salmonella typhimurium* into Regions Responsible for Assembly, Rotation, and Switching

SHIGERU YAMAGUCHI,[1] HIROSHI FUJITA,[1] AKIRA ISHIHARA,[2]† SHIN-ICHI AIZAWA,[3]‡ AND ROBERT M. MACNAB[3]*

Department of Biology, School of Education, Waseda University, Nishi-waseda, Shinjuku-ku, Tokyo 160,[1] and Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606,[2] Japan; and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511-8112[3]

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Three flagellar genes of *Salmonella typhimurium* (*flaAII.2*, *flaQ*, and *flaN*) were found to be multifunctional, each being associated with four distinct mutant phenotypes: nonflagellate (Fla$^-$), paralyzed (Mot$^-$), nonchemotactic (Che$^-$) with clockwise motor bias, and nonchemotactic (Che$^-$) with counterclockwise motor bias. The distribution of Fla, Mot, and Che mutational sites within each gene was examined. Fla sites were fairly broadly distributed, whereas Mot and Che sites were more narrowly defined. Local subregions rich in sites of one type were not generally rich in sites of another type. Among Che sites, there was little overlap between those corresponding to a clockwise bias and those corresponding to a counterclockwise bias. Our results suggest that within the corresponding gene products there are specialized subregions for flagellar structure, motor rotation, and control of the sense of rotation.

The bacterial flagellum is a reversible rotary apparatus driven by proton motive force. Counterclockwise (CCW) rotation causes smooth swimming of the bacterium, whereas an abrupt switch to clockwise (CW) rotation causes tumbling. Bacteria migrate toward beneficial environments by modulating the frequency of switching between CCW and CW senses (see references 9 and 16 for reviews).

In *Salmonella typhimurium*, nearly 50 genes are involved in the formation and function of the flagella (12, 14, 24). They have been placed into three categories: those necessary for flagellar formation (*fla*), for flagellar rotation (*mot*), and for modulation of switching (*che*). The corresponding mutant phenotypes are nonflagellate, paralyzed, and nonchemotactic, respectively.

Two of the *fla* genes, *flaAII.2* and *flaQ*, are particularly interesting because they give rise to several types of mutants, not only nonflagellate (the null phenotype), but also paralyzed (*flaAII.2*) and nonchemotactic (both *flaAII.2* and *flaQ*) (5, 7, 11, 12, 21, 25). The gene products are therefore needed for rotation and for modulation of switching as well as for flagellar structure, i.e., they can be regarded as multifunctional. Elucidation of their properties and those of any other similar gene products is obviously important for the understanding of bacterial behavior.

Within each gene, the locations of mutational sites associated with the various phenotypes should reflect structural and functional features of the gene product. In the present study, a large number of spontaneous Fla$^-$, Mot$^-$, and Che$^-$ mutants of *S. typhimurium* were isolated. Based on deletion mutants from that collection, genes were subdivided into units we term "segments." The mutational sites—those conferring the Fla$^-$, Mot$^-$, and Che$^-$ phenotypes—of all of the remaining mutants were then localized to single segments.

This work demonstrates that there is a considerable degree of segregation of function within *flaAII.2* and *flaQ*, and also within *flaN*, which we have established is another multifunctional gene.

Note regarding nomenclature. In the literature, Mot$^-$ alleles of *flaAII.2* have been referred to as *motC*, Che$^-$ alleles of *flaAII.2* have been referred to as *cheV*, and Che$^-$ alleles of *flaQ* have been referred to as *cheC* or *cheU* (5–7, 11, 21). Here, we use a single symbol for each gene and refer in parentheses to the phenotype of a particular allele; thus, instead of *cheC*, we use *flaQ*(Che). We recommend that this convention be adopted by others and that use of secondary gene symbols such as *motC*, *cheV*, and *cheC* be discontinued.

MATERIALS AND METHODS

Bacterial and phage strains. The *S. typhimurium* strains used were as follows. (i) SJW1103 (*H1-i*$^+$ *H2-enx*$^{-,off}$ *vh2*$^-$) is a phase-1-stable strain (23) used as the parent for all of the Fla$^-$ and Mot$^-$ and some of the Che$^-$ mutants in this study. (ii) SJW806 (*H1-gt*$^-$ *H2-enx*^{on} *vh2*$^-$) is a phase-2-stable strain (24) used as the parent for some of the Che$^-$ mutants in this study. (iii) SJW1556 (Δ*fla-2195*), SJW1572 (Δ*fla-2211*), and SJW1411 (Δ*fla-2050*) (24) are known large deletion mutants used for detection of region III mutants; see Fig. 2a for the ranges of the deletions. (iv) SJW1448 (*flaL2087*), SJW1567 (*H1-1004*), SJW2149 (*flaV2380*) (24), SJW1371 (*flaAI2021*) (12), SJW1684 (*flaAII.1-2323*), SJW1365 (*flaAII.2-2015*), SJW1680 (*flaAIII2319*), SJW124 (*flaS1124*), SJW123 (*flaR1123*), SJW1559 (*flaQ2198*), SJW1549 (*flaN2188*), SJW1357 (*flaP2008*), SJW1401 (*flaB2041*), SJW1535 (*flaD2174*), and SJW161 (*flaX1161*) (24) are representatives of region III *fla* and *H1* complementation groups, used for complementation studies.

The flagellotropic bacteriophage χ (15) was used to select

* Corresponding author.
† Present address: Department of Anatomy, The School of Medicine, University of North Carolina, Chapel Hill, NC 27514.
‡ Present address: Research Development Corp. of Japan, Tokodai 5-9-5, Toyosato, Tsukuba, Japan.

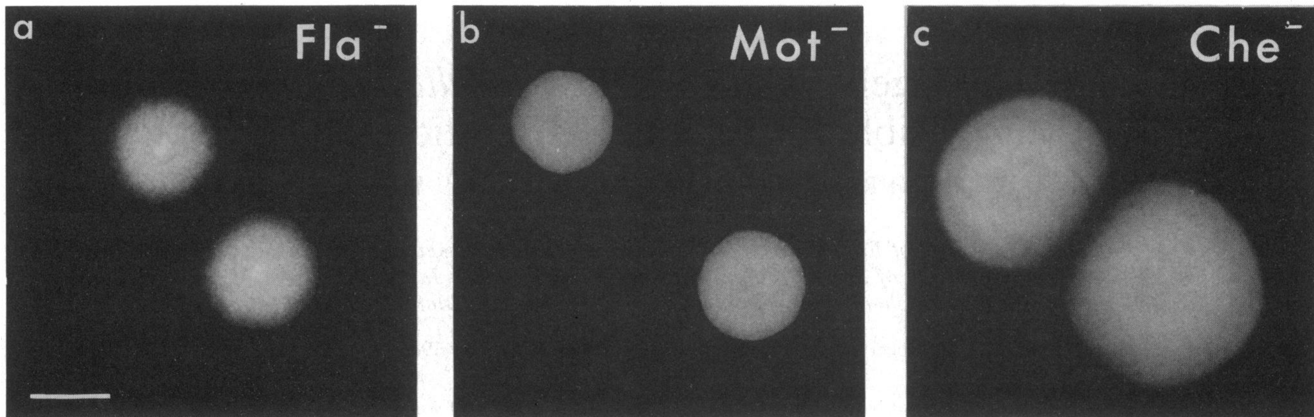

FIG. 1. Colony morphology of motility mutants on soft NGA plates (see Results). The Fla$^-$ mutant phenotype is exemplified by SJW1551 (Δ*flaQ*), the Mot$^-$ phenotype is exemplified by SJW1809 [*flaN*(Mot)], and the Che$^-$ phenotype is exemplified by SJW2287 [*flaN*(Che)]. Bar, 1 mm.

Mot$^-$ and Fla$^-$ mutants. Bacteriophage P22 (wild type) was used as the mediator in transduction experiments.

Media. Nutrient broth (NB) contained 1.0% polypeptone (Daigo-Eiyo, Osaka) and 1.0% meat extract (Mikuni Chemical Industry, Tokyo), adjusted to pH 7.0; alternatively, NB was supplied by Difco Laboratories, Detroit, Mich. Bacto-Agar (1.5%; Difco) was added to NB to give nutrient agar (NA). Soft nutrient gelatin agar (NGA) contained NB plus 8.0% gelatin (Nitta Gelatin, Osaka; or Knox Gelatin, Sioux City, Iowa) and 0.3% agar. Hard NGA contained NB plus 4.0% gelatin and 0.4% agar.

Isolation and characterization of Fla$^-$ and Mot$^-$ mutants. Phage χ, which attacks only motile cells, was used as the selecting agent. A single colony of the parent strain was inoculated from an NA plate into 0.5 ml of NB, and a drop of χ phage suspension (ca. 10^{10} PFU ml^{-1}) was added. After overnight incubation at 37°C, a small drop of the culture was spread on a hard NGA plate. Spontaneous Fla$^-$ and Mot$^-$ mutants from the liquid culture formed compact colonies; the swarms of motile survivors of the χ infection were sufficiently small that mutant colonies could be picked without contamination by motile cells. Furthermore, Fla$^-$ and Mot$^-$ mutants could be distinguished from each other by the detailed appearance of their colonies on NGA plates (see Results; Fig. 1). Confirmation of the distinction between the two categories of mutants was obtained by microscopic inspection for agglutination by antifilament antiserum (25). Mutants derived from different single colonies on the original NA plates (before χ phage selection) were considered to be independent from each other. The revertibility of each mutant was checked by streaking a 0.1-ml sample of an overnight broth culture (ca. 10^9 cells ml^{-1}) in five lines on a soft NGA plate; where no swarm was observed after 48 h of incubation at 37°C, the mutant was regarded as stable.

Isolation of Che$^-$ mutants. A single colony of the parent strain was inoculated from an NA plate into 1.0 ml of NB in a test tube and incubated for 3 or 4 days at 37°C without shaking. A drop of 10^6-fold-diluted culture was then spread on a hard NGA plate. After overnight incubation at 37°C, well-isolated compact colonies with Che$^-$ appearance were transferred to a soft NGA plate for confirmation of phenotype (see Results; Fig. 1).

Test for complementation and recombination. Complementation and recombination between pairs of mutants were examined by P22-mediated transduction. The donor phage suspension (ca. 10^{10} PFU ml^{-1}) was mixed with an equal volume of the recipient bacterial culture (ca. 10^9 cells ml^{-1}). The transduction mixture was streaked as several lines (ca. 20 μl per line) on a soft NGA plate. Production of trails (abortive transductants) or swarms (complete transductants) was used as the criterion for complementation or recombination, respectively (25). When 0.5 ml of the transduction mixture did not produce any swarms, the mutational sites of the donor and the recipient were regarded as overlapping each other, with at least one of the mutations presumed to be a deletion.

High-intensity dark-field microscopy. Flagella were visualized in the light microscope by the high-intensity dark-field technique of Macnab (13).

Observation of passive rotation of flagella on Mot$^-$ mutants. Observation of passive rotation was performed by the method of Ishihara et al. (10).

RESULTS

Distinction between Fla$^-$, Mot$^-$, and Che$^-$ mutants by colony morphology. An important factor in the success of this study was the ability to rapidly and reliably distinguish wild-type and Fla$^-$, Mot$^-$, and Che$^-$ mutant colonies. This distinction was difficult to make on conventional semisolid agar plates, but on semisolid agar plates containing gelatin (NGA plates) the four types displayed recognizably different morphologies. Fla$^-$ and Mot$^-$ mutants both formed compact colonies with sharp boundaries and no evidence of chemotactic banding; however, Fla$^-$ colonies had a dense central spot (Fig. 1a), whereas Mot$^-$ colonies had an ivory color and were of uniform density (Fig. 1b). On hard NGA plates, Che$^-$ colonies were almost as compact as Fla$^-$ or Mot$^-$ colonies, but on soft NGA plates they were larger (the actual size varied from mutant to mutant) and had a soft appearance at their outer edge (Fig. 1c); there was often evidence of chemotactic banding. Overall, Che$^-$ colonies resembled a feeble example of wild-type swarming.

Isolation of Fla$^-$ mutants of region III. A collection of 1,984 spontaneous nonflagellate mutants had previously been isolated from strain SJW1103 (24). From these, 897 clones (220 stable, 677 revertible) were chosen for further testing in the present study. All of the expressed flagellar genes in SJW1103 are contained in three regions of the genome, regions I, II, and III (25), with *flaAII.2*, *flaQ*, and *flaN* all falling in region III (25). As a result of recombination tests

with three mutants whose deletions together cover region III (SJW1411, SJW1572, and SJW1556; Fig. 2a), 452 clones were identified as Fla$^-$ mutants from that region. (Of the remainder, 325 belonged to region I and 120 belonged to region II. The relative numbers of mutants found among regions I, II, and III correspond closely to the numbers of *fla* genes they contain—12, 5, and 17, respectively.)

Deletion mapping of the *flaAII.2* gene and its vicinity. Of the 452 Fla$^-$ mutants from region III, 203 (55 stable, 148 revertible) did not yield Fla$^+$ recombinants with SJW1572 (Fig. 2a) and were used for the analysis of *flaAII.2* and neighboring genes. The following series of transductional crosses was performed. Complementation tests with standard strains representing the *flaAI*, *flaAII.1*, *flaAII.2*, *flaAIII*, *flaS*, and *flaR* complementation groups; recombination tests between all pairwise combinations of stable mutants; and recombination tests between revertible and stable mutants.

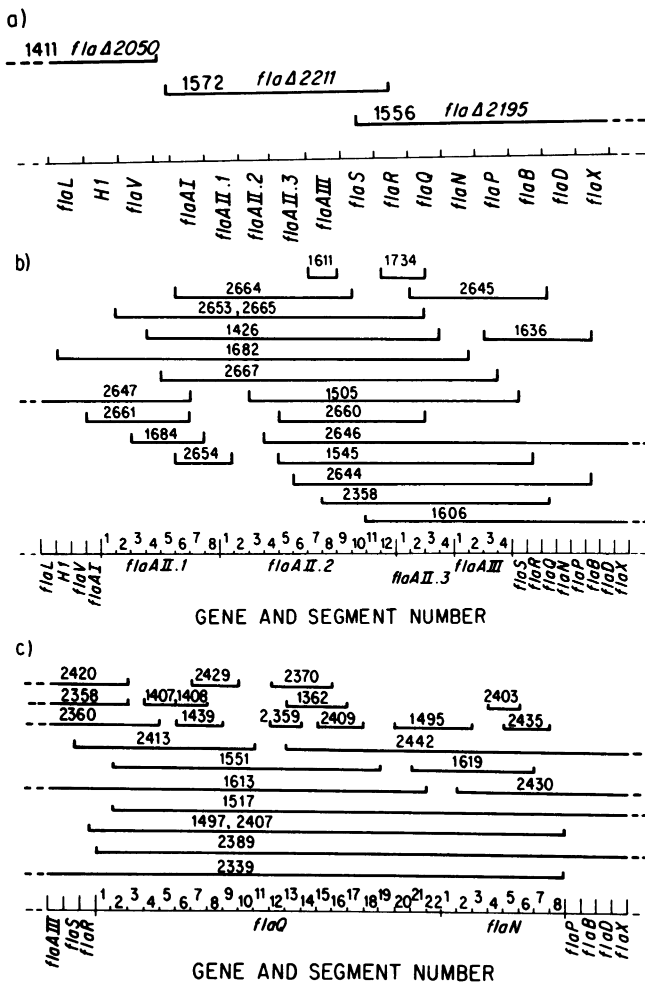

FIG. 2. Flagellar region III of *S. typhimurium* and mutants with deletions in that region. *flaAII.3* was defined by the present study; all other genes were known previously. Segments defined by the deletion mutants were used for allocating mutational sites in Fig. 3 and 4. Strain numbers all carry the prefix SJW (e.g., 1661 refers to strain SJW1611). Bar lines indicate the extents of the deletions. (a) Large-deletion mutants used for identifying region III mutants. The deletions of SJW1411 and SJW1572 do not overlap; SJW1411 complements *flaAI* mutants and SJW1572 complements *flaV* mutants, suggesting there may be an appreciable intergenic region. (b) Deletion map of *flaAII.2* and its vicinity. (c) Deletion map of *flaQ* and *flaN*.

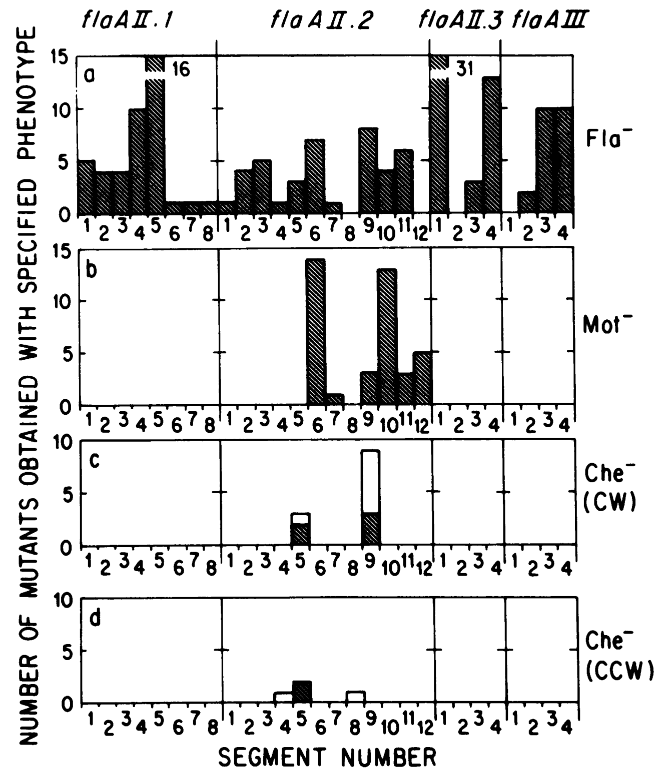

FIG. 3. Fine mapping of mutational sites within *flaAII.2* and adjacent genes. Sites were allocated to gene segments defined by the deletion map shown in Fig. 2b. Each single-site mutant was separately categorized as being (a) nonflagellate (Fla$^-$), (b) flagellate but paralyzed (Mot$^-$), or (c, d) motile but nonchemotactic (Che$^-$). Che$^-$ mutants were further subdivided into (c) CW biased and (d) CCW biased, based on observations by high-intensity dark-field light microscopy of flagellar function of free-swimming cells. In addition to the Che$^-$ mutants isolated in the present study, a number of mutants obtained elsewhere are included in the histograms (as unfilled bars). The sources of these mutants and the segment locations of the mutations within *flaAII.2* are as follows: MY402 (segment 4), SJW2288 (segment 5), and SJW2289, SJW2290, SJW2291, and SJW2835 (segment 9), all isolated in the course of a pseudoreversion analysis that will be published elsewhere; SL2516 (segment 8) (21); ST134 and ST155 (segment 9) (19); in that study, ST134 and ST155 were erroneously classified as *flaQ* mutants). Strain numbers of all strains contributing to this figure and Fig. 4 are available on request from the authors.

By such tests, 25 of 56 stable mutants (including one previous mutant, SJW1684) were shown to carry deletions and were used to generate the deletion map shown in Fig. 2b, in which each gene was subdivided into units defined by adjacent deletion endpoints; we term these units segments. *flaAII.2* was subdivided in this way into 12 segments.

The other stable mutants, together with revertible ones, were regarded as having single-site mutations. By a single-site (or point) mutation we mean any one that falls entirely within one segment; it could be a missense mutation, an insertion, or a small deletion. By complementation tests with standard strains, the single-site mutants (148 revertible, 31 stable) were classified as follows: 12 *flaAI*, 42 *flaAII.1*, 43 *flaAII.2*, 22 *flaAIII*, 5 *flaS*, 8 *flaR*, and 47 unclassifiable. All of these last 47 clones were found to belong to a single new complementation group; the corresponding gene, which

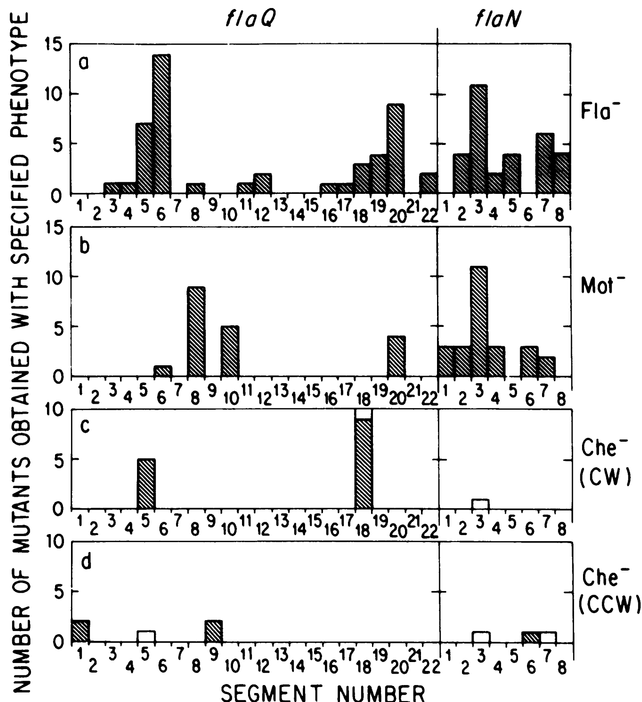

FIG. 4. Fine mapping of mutational sites within *flaQ* and *flaN* (see legend to Fig. 3). Sites were allocated to gene segments defined by the deletion map shown in Fig. 2c. Che⁻ mutants in addition to those isolated in the present study are as follows: SJW2286 (*flaQ*-5) and SJW2841 (*flaN*-3), isolated in the course of a pseudoreversion analysis that will be published elsewhere; ST120 (*flaQ*-18), ST203 (*flaN*-3), and ST221 (*flaN*-7) (19, 22; in those studies, ST203 and ST221 were erroneously classified as *flaQ* mutants).

maps between *flaAII.2* and *flaAIII*, we have named *flaAII.3*. All of the *flaAII.3* mutants, even stable ones, were phenotypically leaky (see Discussion).

The sites of the single-site mutations in Fla⁻ mutants were then localized to individual gene segments (Fig. 3a). Within *flaAII.2*, Fla sites were broadly distributed, possibly into two regions peaking at segments 6 and 9.

Deletion mapping of the *flaQ* and *flaN* genes. Of the region III Fla⁻ mutants, 168 (48 stable, 120 revertible) failed to yield Fla⁺ recombinants with SJW1556 (Fig. 2a). They were subjected to complementation tests with standard strains for the *flaQ* and *flaN* groups. As a result, 72 revertible mutants and 35 stable mutants were shown to be *flaQ*, *flaN*, or *flaQ flaN*.

Twenty-nine of the stable mutants were shown to contain deletions and were used to generate the map shown in Fig. 2c, in which *flaQ* and *flaN* were divided into 22 and 8 segments, respectively.

Of the single-site mutants (72 revertible, 6 stable), 47 were defective in *flaQ* and 31 were defective in *flaN*. Their mutational sites were then mapped to individual segments. The distribution (Fig. 4a) appeared nonrandom, especially in the case of *flaQ*, where sites were highly clustered; nearly half were located at segments 5 and 6, and most of the remainder were located between segments 18 and 20. In *flaN*, segment 3 was especially rich in *fla* sites.

Isolation of region III Mot⁻ mutants and mapping of their mutational sites. By the use of phage χ and inspection of colony morphology (see above), 840 Mot⁻ mutants were isolated from SJW1103. Of these, 83 were found to belong to region III and could be assigned to one of three groups: 39 *flaAII.2*(Mot), 19 *flaQ*(Mot), and 25 *flaN*(Mot). (Those Mot⁻ mutants not belonging to region III all belonged to region II and were defective in *motA* or *motB*.)

Mot sites were then located to particular segments of *flaAII.2*, *flaQ*, and *flaN* (Fig. 3b and 4b). The distribution, particularly in *flaAII.2* and *flaQ*, appeared nonrandom. In *flaAII.2*, most sites were located in segments 6 and 10. In *flaQ*, most were in segments 8, 10, and 20. In *flaN*, segment 3 contained almost half of the sites, but the remainder were distributed rather broadly.

Isolation of region III Che⁻ mutants and mapping of their mutational sites. In a previous paper (8) we reported that during prolonged static cultivation, cultures became enriched with spontaneous flagellar-shape mutants and Mot⁻ mutants. Subsequently, we noticed that Che⁻ mutants also arose under those conditions. Taking advantage of this, we isolated putative Che⁻ mutants from SJW1103 and SJW806 (186 and 120 mutants, respectively). Of these, 31 proved to have mutations in region III. Dark-field light microscopy revealed that 26 were Che⁻ and the remainder were leaky Fla⁻. Among the Che⁻ mutants, 7 were *flaAII.2*(Che), 18 were *flaQ*(Che), and 1 was *flaN*(Che). (Of the Che⁻ mutants with mutations outside region III, more than 80% were found to be defective in region II, which contains all of the known chemotaxis genes, except those for receptors and certain transducers.)

To determine which alleles were responsible for CCW and CW bias, the swimming patterns of the mutants were studied by high-intensity dark-field light microscopy (13), with careful attention paid to flagellar function to avoid confusion between, for example, tumbling deriving from CW flagellar rotation and erratic motility deriving from few or short flagella. The collections of *flaAII.2*(Che) and *flaQ*(Che) mutants both contained examples of CCW bias and of CW bias; the one *flaN*(Che) mutant was CCW biased.

The mutational sites were then assigned to individual segments [Fig. 3c, 3d, 4c, and 4d; also shown is information on several *fla*(Che) mutants obtained from other sources]. A high degree of localization of Che sites was seen. In *flaAII.2*, Che sites were found in only four segments—4, 5, 8, and 9; in *flaQ*, Che sites were also found in four segments—1, 5, 9, and 18. In both genes, segments rich in Che sites either were poor in Mot sites or lacked them entirely. Based on the one *flaN*(Che) mutant obtained in the present study and three we identified in other studies, there may be two Che subregions (segment 3 and segments 6 and 7) in that gene.

The rotational biases (CW or CCW) of the mutants showed a strong correlation with the location of their mutational sites. Within the present collection of mutants, the correlation for *flaQ* is complete except for one mutant; of a total of 20 mutants, 15 of 16 defective in segments 5 and 18 showed CW-biased phenotype (tumbling), whereas the 4 defective in segments 1 and 9 all showed CCW-biased phenotype (smooth swimming). In *flaAII.2*, all nine strains defective in segment 9 showed CW-biased phenotype; only one segment, segment 5, has so far yielded examples of both CW and CCW phenotypes. The data for *flaN* are too limited to permit any definitive statement regarding segregation of CW and CCW phenotypes.

Passive rotation of flagella on Mot⁻ mutants by external driving force. Ishihara et al. (10) found that the flagella of *motA*, *motB*, and *flaAII.2*(Mot) mutants rotated passively when external torque was applied. We wished to examine *flaN*(Mot) and *flaQ*(Mot) mutants in this respect. Representative strains [SJW1767 and SJW1785, both *flaN*(Mot);

SJW1760, SJW1762, and SJW1763, all *flaQ*(Mot)] with mutations in different segments were examined, along with two *flaAII.2*(Mot) mutants, SJW1759 and SJW1766.

When subjected to viscous flow (see Materials and Methods), the flagella of all of these mutants pivoted to point in the direction of the flow and began to rotate, suggesting that the paralyzed phenotype was not caused by a physical obstruction to rotation.

DISCUSSION

There are at least three multifunctional flagellar genes. Among all of the genes involved in the motility of *S. typhimurium*, three—*flaAII.2*, *flaQ*, and *flaN*—seem to be of special importance. The present study, together with previous work, reveals that these genes can each give rise to four mutant phenotypes, Fla$^-$, Mot$^-$, Che$^-$ (CW), and Che$^-$ (CCW). Thus, they are essential for flagellar structure or assembly, energy transduction, and both senses of switching. For these reasons, we term them multifunctional genes. In contrast, other *fla* genes give rise to Fla$^-$ phenotype only, *motA* and *motB* give rise to Mot$^-$ phenotype only, and the *che* genes (*cheA*, *cheB*, etc.) give rise to either Che$^-$ (CCW) only or Che$^-$ (CW) phenotype only.

Although the three multifunctional flagellar genes identified here are likely to be the ones of primary importance, it is possible that other examples may occur less commonly. Specifically, regions I and II were not examined for multifunctional flagellar genes in the present study; however, they have not yielded examples in previous work.

In this study, we obtained more Che$^-$ (CW) than Che$^-$ (CCW) mutants (19 versus 7), whereas some previous studies (2, 22) overwhelmingly generated Che$^-$ (CCW) mutants. The selection technique used in the latter case (migration in a preformed liquid gradient) is known to favor CCW bias; we do not know whether ours favors CW bias. The use of additional selection techniques might extend the spectrum of Che sites we have obtained in the present study.

Statistical significance of the variability in allele incidence. The genes were divided into segments defined by deletion endpoints. For simplicity, these segments are represented in Fig. 2 through 4 as being of equal length, although the actual physical lengths undoubtedly vary. For several reasons, however, we do not believe that the wide variation in the observed incidence of mutant alleles is simply a reflection of segment length variation.

The strongest argument comes from a comparison of the incidence of different allele types for a given segment. For example, *flaQ* segment 6 (herein designated *flaQ*-6) contains 30% of the observed Fla alleles, but only 5% of the Mot alleles and none of the Che alleles; *flaQ*-8 contains 47% of the Mot alleles, but only 2% of the Fla alleles and none of the Che alleles; *flaQ*-18 contains 64% of the Che (CW) alleles, but only 6% of the Fla alleles and none of the Mot or Che (CCW) alleles. Thus the relative incidences reflect phenotypic specificity, not just segment size or, for that matter, hypermutability within certain segments.

Other arguments support this conclusion. If deletions occur more or less randomly, the segment lengths will conform closely to a Poisson distribution, provided the segments are short compared with the gene; we have also carried out Monte Carlo simulations (data not shown) of the statistics that apply without this restriction. For example, Fig. 4a shows that *flaQ*-6 generated 6.6 times the mean number of Fla alleles per segment for that gene; the probability of finding a segment as large as this is only 0.01. For Mot and Che alleles, the argument against segment size being the cause of the pronounced localization becomes overwhelming.

An independent argument concerns clustering of maxima of allele incidences. Fla alleles, and to some extent Mot alleles, tend to be in roughly graded clusters spanning several segments. Since there should be no correlation between the lengths of adjacent segments, the clustering suggests a high probability of producing that type of allele in that region.

Small segments are not particularly improbable. There is about a 10% chance that a given segment will be less than 0.1 the mean size, and so the absence, for example, of any Mot alleles in *flaQ*-9 (when flanking segments have considerable numbers) could simply reflect small segment size. However, clusters of empty segments (e.g., the absence of any Fla alleles in segments *flaQ*-13 to *flaQ*-15) are probably phenotypically significant.

One caveat applies to the frequency data for Fla alleles. Since Fla$^-$ is the null phenotype, some of the single-site clones may carry mutations (either single-base or frameshift) resulting in chain termination, in which case the position of the mutation is irrelevant.

The mean physical size of the segments is not the same for each gene. The *Escherichia coli* genes corresponding to *flaAII.2*, *flaQ*, and *flaN* contain 987, 1002, and 405 bases, respectively (P. Matsumura and D. E. Koshland, Jr., personal communications). Assuming the *S. typhimurium* genes are of comparable size, the mean segment length in *flaAII.2*, *flaQ*, and *flaN* is 82, 46, and 51 bases, respectively. Thus (fortuitously) the mean segment lengths of *flaQ* and *flaN* are likely to be similar, but that of *flaAII.2* is likely to be about 70% larger.

Relative importance of the multifunctional genes for a given phenotype. For a given phenotype such as Fla$^-$, the present study was impartial among genes; a large number of mutants were isolated and then mapped. It was not, however, impartial regarding the different phenotypes for a given gene, since the searches for Fla$^-$, Mot$^-$, and Che$^-$ mutants were conducted separately. We may therefore make useful comparisons concerning the relative numbers of mutants of a given phenotype found in the various genes, but not concerning the relative numbers of mutants of different phenotypes found within the same gene. [An exception to the latter statement concerns Che$^-$ (CW) versus Che$^-$ (CCW) mutants, since they were both obtained in the same search.]

A major conclusion is that, of the 15 *fla* genes in region III, Mot$^-$ and Che$^-$ phenotypes were only found in *flaAII.2*, *flaQ*, and *flaN*. However, although these genes are all multifunctional, there are differences in the extent to which they contribute to each function. Of Fla$^-$ alleles in the three genes, 36% were in *flaAII.2*, 39% were in *flaQ*, and 25% were in *flaN*; this is approximately what would be expected just on the basis of gene size. However, with regard to Mot$^-$ mutants (and especially if the small size of *flaN* is taken into account), *flaQ* appears to be less important than the other two (47% *flaAII.2*, 23% *flaQ*, 30% *flaN*); with regard to Che$^-$ mutants, *flaQ* appears to be the most important and *flaN* the least (27% *flaAII.2*, 69% *flaQ*, 4% *flaN*).

The information to date is insufficient to support the idea that any of the three genes are much more important for one rotational state (CW or CCW) than for the other; each of these genes clearly plays a role in enabling both rotational states.

It is interesting that the great majority (ca. 90%) of the Mot$^-$ and Che$^-$ mutants isolated in this study were defective in genes outside region III. All of the Mot$^-$ mutants and most

of the Che$^-$ mutants in this category were defective in region II, which contains only two *mot* genes and six *che* genes. Thus the yield of Mot$^-$ and Che$^-$ mutants from region III is disproportionately low. We suspect this may be because, for *flaAII.2*, *flaQ*, and *flaN*, the Fla$^-$ phenotype is epistatic and so prevents many potential Mot and Che defects from being detected.

Molecular significance of observed phenotypic distributions. Given the segregation of phenotypic alleles (Fig. 3 and 4), it is reasonable to think in terms of segregation of function within the primary sequence of the gene product. The segregation is less pronounced in the case of Fla alleles, which is perhaps to be expected, since any mutation resulting in substantial change in protein conformation would be likely to block flagellar assembly.

How are the segregation patterns to be understood in terms of protein structure and function? Where—as is the case with Fla alleles—several adjacent segments constitute a region of the gene that tends to yield a given mutant phenotype, the corresponding region of the protein may constitute an actual tertiary structural domain. Where, as with Mot alleles and even more with Che alleles, the phenotype is more narrowly focused, this may reflect a more limited portion of the primary sequence that is especially important for the function in question (cf., the active site of an enzyme); several such local regions might, of course, combine into a single tertiary structural domain.

Although there is considerable segregation at the segment level, alleles of all types tend to be found in the same overall regions within the gene (Fig. 3 and 4). We tentatively suggest that each of the genes has two such regions, which may correspond to structural domains: for *flaAII.2*, near the center and the 3′ end of the gene; for *flaQ*, in the 5′ and 3′ thirds; for *flaN*, in the 5′ third and (less marked) the 3′ end.

It should be realized that the multiple functions we have been distinguishing may not be completely separable. In one trivial sense this is obviously true—there can be no rotation or switching unless there is a flagellar structure, but it may also be true that the mechanisms for enabling the CCW and CW states are related, and that both mechanisms may be related to the mechanism of rotation itself.

Other knowledge concerning the multifunctional genes. To place the present study in context, we summarize briefly other information concerning these genes and their products. The *flaAII.2*, *flaQ*, and *flaN* gene products are not part of the basal body complex (1, 4), yet they are essential for the early stage of flagellar assembly (9a, 20). The Che$^-$ mutant phenotype is retained in cytoplasm-free cell envelopes (18). Mutations in multifunctional genes can suppress defects in CheY and CheZ, which are cytoplasmic proteins (17; results obtained in *E. coli*). Intergenic suppression occurs among the three multifunctional genes (manuscript in preparation).

From these various lines of evidence, Macnab and Aizawa (14) have postulated the existence of a "switch complex" constructed from the FlaAII.2, FlaQ, and FlaN proteins, which is at the cytoplasmic-proximal face of the basal body and is a prerequisite for its assembly, is exposed to the cytoplasm and available for interaction with cytoplasmic components of the sensory transduction apparatus such as CheY and CheZ and is part (perhaps in conjunction with the MotA and MotB proteins) of the mechanism for converting proton motive force into rotational energy.

Possible causes of phenotypic defects. Given the available evidence concerning the multifunctional genes, can we make any predictions regarding the causes of the observed phenotypic defects—Fla$^-$, Mot$^-$, Che$^-$ (CW), and Che$^-$ (CCW)?

Fla$^-$ defects are likely to fall into two general categories, those that prevent the gene product from assembling and those that prevent subsequent flagellar components from assembling. Examples of the latter may emerge if intergenic suppression of Fla$^-$ mutants can be found.

The observed Mot$^-$ defects presumably involve the actual process of energy transduction rather than a simple jamming of the machinery—otherwise the observed passive rotation of flagella (see Results) should not have been possible. These defects might occur in the interior of the protein but more likely are at a surface that interacts with other components of the energy-transducing machinery.

Che$^-$ defects might result from incorrect interactions with other components of the sensory transduction chain. It seems likely (17) that at least FlaAII.2 and FlaQ have binding sites for CheY and CheZ. If these sites were distinct, the substantial segregation of CW and CCW alleles would be readily explained, since mutants defective in CheY and CheZ binding are predicted to be of CCW- and CW-biased phenotype, respectively.

However, the FlaAII.2, FlaQ, and FlaN proteins may well possess intrinsic properties with regard to the CW and CCW states. Thus a mutant might have an abnormal bias even though its ability to bind cytoplasmic effectors is normal. The observed allele segregation suggests that, if there are regions of the primary structure that are important to the intrinsic stability of the CW and CCW states of the switch, they are in general distinct.

In summary, the multifunctional flagellar proteins are likely to require for their proper functioning not only a stable internal structure, but also proper interactions with a variety of other proteins, both within the flagellar motor and in the cytoplasm. The study of intragenic and intergenic suppression of mutant defects should be a useful approach toward understanding these interactions; in such a study, the phenotypic probability distributions in Fig. 3 and 4 will be of great value.

Definition of an additional *fla* gene. In the course of this work, an additional gene, which we have named *flaAII.3*, was found between *flaAII.2* and *flaAIII*. The *E. coli* gene *flaB* was originally presumed to be the homologue of *S. typhimurium flaAII.1* (12). Recently, *flaB* has been subdivided into three genes, *flaBI*, *flaBII*, and *flaBIII*, with *flaBII* being capable of yielding the Che$^-$ phenotype (3). Based on gene order and phenotype, *flaBI* corresponds to *flaAII.1*, *flaBII* corresponds to *flaAII.2*, and *flaBIII* corresponds to the newly described *flaAII.3*.

All strains with mutations in *flaAII.3*, even stable ones, were phenotypically leaky. A number of other genes have this characteristic: *flaFIII*, *flaFIV*, *flaFVIII*, *flaFX*, *flaW*, and *flaU* in region I and *flaP*, *flaR*, *flaS*, and *flaV* in region III (S. Yamaguchi, unpublished observations). The frequent occurrence of leaky phenotype suggests that the product of the gene in question, although important for reliable assembly and function or for regulation, is not absolutely required; several of the genes mentioned above have roles that can be understood in this way. The product of the newly described gene, *flaAII.3*, apparently falls into this category, although nothing is yet known regarding its function.

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