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GENETICS OF STRUCTURE AND FUNCTION OF BACTERIAL FLAGELLA

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INTRODUCTION

Eight years ago I wrote a review article on the genetics of bacterial flagella (1). Since then, the knowledge of the structure and function of bacterial flagella has advanced considerably.

Owing to the success of the isolation of an "intact flagellum" (a flagellar filament joined with a hook and a basal body) from osmotically lysed bacterial cells, basal body structures, which had been ambiguous for a long time, were clarified (2–6).

In gram-negative bacteria, such as *Escherichia, Salmonella,* and *Pseudomonas,* a basal body is composed of four rings, a rod passing through the centers of the four rings, and a cylinder filled in the space between two outer rings (Figure 1). The four rings are termed L, P, S, and M; each is connected by its periphery with a lipopolysaccharide outer layer, a peptidoglycan layer, a periplasmic space, and a cytoplasmic membrane respectively. Thus the basal bodies are entirely mounted in the cell envelope. In gram-positive bacteria, only two rings are present in a basal body (4). At an end adjacent to the L ring, the rod connects longitudinally to the hook, which extends out of the cell body and is joined to the filament at the other end of its tubular structure.

These flagellar structures are composed principally of protein. Purification and amino acid analysis of hook protein (7–9) as well as filament protein, i.e. flagellin (10–19), has been established. The basal body of *Escherichia coli* was fractionated into at least seven component proteins with differing molecular weights (20, 21). The helical arrangement of flagellin molecules along the longitudinal rows in a filament was visualized on some bacterial strains, mainly with optical diffraction and filtering methods (22–30).

Flagella function as locomotive organelles of bacteria by propagating helical waves and by pushing bacterial bodies forward. Recent investigations have provided sound experimental evidence that the flagellar filaments rotate relative to the cell

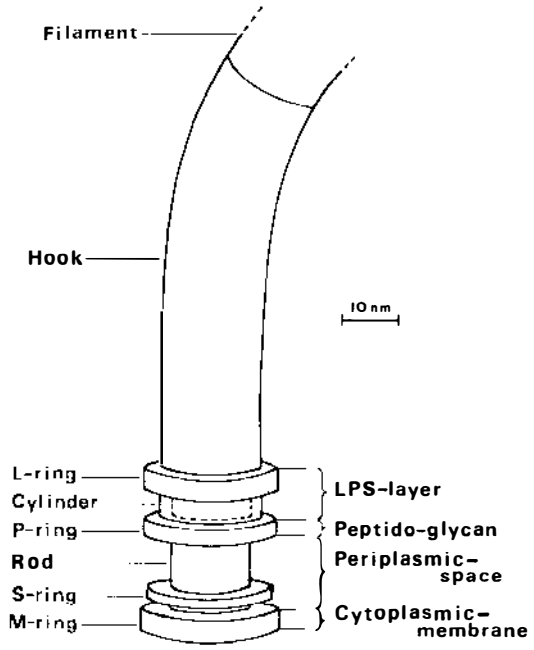

Figure 1 Model of the basal structure of a flagellum of *Salmonella typhimurium.*

body and that their motor machineries reside at their bases (31–36). The role of the basal structures in the locomotive function of flagella is thus gaining attention.

Genetic studies of bacterial flagella in recent years have complemented these researches on their structure and function. This review covers the progress in the knowledge of the structure and function of bacterial flagella since publication of my earlier review article (1). Taxis, a pronounced response of flagella, is to be covered in the review by Parkinson in this volume (37).

GENETIC ANALYSIS OF FLAGELLAR CHARACTERS

Flagella Genes and Selection of Their Mutants

The flagella genes whose functions were most clearly identified are the structural genes for flagellin, namely *H1* and *H2* in *Salmonella* (1), *H* in *Bacillus subtilis* (38) and *Pseudomonas aeruginosa* (39), and *hag* in *E. coli* (40). Their mutation results in the change of any one of the following phenotypes: efficiency of filament formation, shape of filaments, sensitivity to flagellotropic phage, and specificity of flagellar antigen (41–43).

Other flagella genes involved in flagellar formation are commonly given the gene symbol *fla* followed by a capital letter and/or a Roman numeral designating a cistron (1, 38–40, 44). The mutant phenotype generally used for genetic analysis of the *fla* genes is nonflagellate.

The flagella genes of the third category are termed *mot* (45–48). They control the locomotive function of flagella without affecting their overall structure. A mutation from mot^+ to mot^- results in flagellar paralysis. The genes for chemotaxis are termed *che* (37).

In addition to these genes essential for the structure and function of flagella, a modifier gene, *nml,* is present in some serotypes of *Salmonella* (49, 50). In the presence of nml^+, about half of the lysine residues of each flagellin molecule are methylated at the ϵ-position. This chemical modification sometimes is associated with the change of antigenicity of flagella (49).

Neither nonflagellate nor paralyzed mutants can spread and form swarms on semisolid medium (51), and both are resistant to flagellotropic phage (52–56). Therefore, by the combined use of semisolid medium and flagellotropic phage for selection, both types of nonmotile mutants are very efficiently isolated from a culture of motile bacterial strains.

Change of flagellar shape by mutation often associates with the change of motility of the bacteria without alteration in the sensitivity to flagellotropic phage (57). Consequently, flagellar shape mutants are detected among nonswarmers, e.g. straight mutants, or slow-swarmers, e.g. curly mutants, on semisolid medium, and they are differentiated from fla^- or mot^- by their sensitivity to flagellotropic phage (51).

An appropriate titer of antiserum prepared against a specific type of flagellar antigen inhibits motility of the bacteria of the same antigenic type but allows multiplication of their cells. The mutants of flagellar antigenic type are isolated as motile swarmers that escape from the inhibition by the antibody supplemented in semisolid medium (41).

Overall Maps of Flagella Genes

Genetic analyses of flagella genes were extensively carried out on *Salmonella,* mainly *S. typhimurium* and *S. abortusequi,* and on *E. coli.* The analyses on *Salmonella* were performed with P22-mediated transduction (41, 43, 58), and Hfr X F^- conjugation (46). On *E. coli,* F' X F^- conjugation (44, 59) and special transduction with λ-*fla* (60–63) as well as Pl-mediated transduction (47) were applied. Bacteriophage Mu-induced mutations have been known to prevent the expression of the genes that are transcribed later than the mutant genes in the same operon (64). Mu-induced flagellar mutations were successfully used for the disclosure of the organization of flagella genes into operons (65).

Distributions of the flagella genes on the chromosomes of *Salmonella* and *E. coli* are summarized in Figure 2. In both of these bacteria, the largest cluster of flagella genes resides at about the same map position, i.e. near the *his* operon (58, 59, 62, 66, 67). At the middle of the cluster, a flagellin gene, *H1* in *Salmonella* or *hag* in *E. coli*, is located. Besides, *fla, mot,* and *che* have been found in this cluster. Sixteen *fla* genes have been detected in *Salmonella* and 14 in *E. coli.* Two *mot* genes are present adjacent to each other in both bacteria. Near *mot,* a group of *che* genes was identified (37).

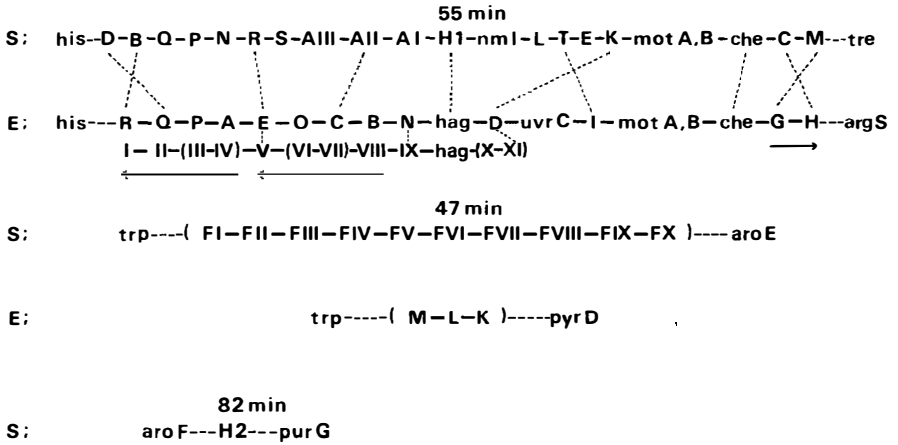

Figure 2 Linkage map of the flagella genes of *Salmonella* (S) and *Escherichia coli* (E). his: histidine operon including A-I cistrons; trp: tryptophan operon including A-E cistrons; tre: trehalose; arg: arginine; aro: aromatic amino acids; pyr: pyrimidine; pur: purine; uvr: ultraviolet light sensitivity; nml: N-methyl lysine in flagellin; hag, H1, H2: flagellin; mot: motility; che: chemotaxis. Other symbols on which only cistron designations are given are *fla* (flagellation). An *arrow* indicates the direction of transcription of an operon. A *dotted line* indicates a pair of homologous cistrons. The chromosomal position of each linkage group is shown as "min" in the 138 min linkage map of *Salmonella typhimurium* (66). The arrangement of the genes in parentheses to their outside genes is not known. For the composition of cistrons in *che,* refer to Parkinson (37).

Functional homologies between the *fla* genes of *Salmonella* and *E. coli* in this cluster were examined by complementation tests between *fla*⁻ mutants in intergeneric transductional heterogenotes and conjugal hemizygotes of these bacteria (68; B. Stocker, personal communication). The relative positions of the functionally homologous genes on their chromosomes is not exactly the same in these two genera (Figure 2). This suggests the occurrence of inversion or translocation in the evolutionary history of these genera. In addition to these genes, *nml* is present in the same cluster of *Salmonella* (49, 50). Flagella genes of this cluster are separated into several operons (Figure 2) (59, 61, 67).

The second cluster of flagella genes in *Salmonella* and *E. coli* is located near the *trp* operon (44, 66, 69). In *Salmonella,* ten *fla* genes were identified in this cluster, while in *E. coli* three *fla* genes were found. This difference in number may simply reflect the extensiveness of the examined *fla* mutants of this cluster. The complementation test on transductional heterogenotes between *S. typhimurium* and *Shigella dysenteriae* indicated that in the latter bacterium at least a part of this *fla* cluster is present, but that the largest cluster near *his* is deleted (68). In diphasic *Salmonella,* an additional flagellin gene, *H2,* was mapped apart from the above-mentioned two clusters. Its location is near *purG* (66, 70). *E. coli* entirely misses the *H2* locus. When *H2* is transduced from *Salmonella* to *E. coli*, it is translocated to the *hag* locus or more occasionally to a locus unrelated to flagella genes (71).

No advance has been made on mapping of the flagella genes in *B. subtilis* since Joys & Frankel (72) reported three nonallelic genes, *H, fla,* and *mot,* with transformation experiments. On *Proteus mirabilis,* eight *fla* and one *mot* were disclosed (73). Except for one *fla,* they were located in a cluster transduced by phage 34.13 simultaneously. Flagella genes of *Pseudomonas aeruginosa* were also mapped in a cluster, in which at least one *hag,* ten *fla,* and one *mot* were detected by phage F116-mediated transduction (T. Iino, unpublished information).

REGULATION OF FLAGELLAR FORMATION

Cell Cycle and Flagellation

The distribution of the number of flagella per cell is characteristic for each bacterial strain under a given cultural condition. During a cell cycle of peritrichously flagellate bacteria, the number of flagella doubles and upon cell division they distribute to daughter cells approximately evenly. This indicates that the formation of flagella is genetically regulated so as to be coupled with the cell cycle.

When light-density spores of *B. subtilis* are germinated at 46°C and grown at 37°C, they are well synchronized in both cell division and chromosome replication. In such bacteria, the doubling of the rate of flagellar formation was found to correspond to the time of replication of the *hisA1* gene (74). The synchronization of flagellar formation in a synchronized culture of *E. coli* was also reported (75). Several temperature-sensitive mutants of *E. coli* defective in DNA-replication, e.g. *fts* and *dna,* are retarded in flagellar formation at a nonpermissive temperature (76). Although these observations have not demonstrated that the responsible reaction step is at the initiation of flagellar formation, they strongly suggest that the initiation

of flagellar formation is coupled with DNA replication. Whether the coupling is at the transcriptional level or, more indirectly, whether it involves a sequential induction system proceeding through a cell cycle or is associated with a specific state of the cell surface appearing at a stage in a cell cycle is left unanswered.

A regulatory factor presumably involved in the initiation of flagellation is adenosine 3',5'-cyclic phosphate (cAMP) and the protein specifically bound to it (CRP). Either cAMP-deficient mutants (*cya*$^-$) or CRP-deficient mutants (*crp*$^-$) of both *E. coli* and *S. typhimurium* are defective not only in sugar fermentation but also in flagellar formation (77–79). Thus the complex of cAMP and CRP has a pleiotropic regulatory function for both sugar fermentation and flagellation. Their function, however, is not invariably common to these phenomena, because suppressor mutants [constitutive flagella synthesis (*cfs*)] which restore flagella-forming ability in *cya*$^-$ and *crp*$^-$ were detected (78, 79). Sugar fermentation of these mutants remained defective in the presence of the suppressor. *cfs* is dominant over its wild allele. The mutant site of *cfs* was mapped in *flaI* in *E. coli* and *flaT* in *S. typhimurium* (78, 79). The step of flagellar formation sensitive to cAMP may be at a very early stage of flagellar formation because none of the precursor structures of flagellar bases were detected on *cya*$^-$ cells. The most plausible explanation regarding the role of cAMP in flagellation is as follows: cAMP receptor protein together with cAMP modulates the wild allele of the gene in which the *cfs* mutation resides, and the gene in turn acts as a positive effector on the initial step of flagellar formation. Then, the *cfs* mutant gene may be regarded as the constitutive effector.

Sequence of Flagellar Morphogenesis

The intensive electron microscopical observation of the cell envelope fractions of various nonflagellate mutants enabled us to detect a series of precursor structures of flagella (69). The simplest precursor structure so far detected among the nonflagellate mutants of *Salmonella* is the complex of two inner rings and a rod (RIV particle). The next simplest is the complex of a RIV particle and a P ring (CAS particle). Then comes the complex of a CAS particle, a cylinder, and an L ring (BAB particle). A BAB particle is morphologically indistinguishable from a basal body of an intact flagellum. The precursor between BAB and the intact flagellum is the complex of a BAB particle and a hook (HOB particle). These precursor structures were cistron-specific. Summing up these results, the sequential process of flagellar morphogenesis is constructed as shown in Figure 3, and several *fla* cistrons were assigned to the resolved steps. Whether a *fla* cistron at a step is the structural gene of the forthcoming component or the positive regulator gene of the morphogenetic step is still not clear.

Among the *fla*$^-$ mutants belonging to 13 cistrons listed at the left side of Figure 3, none of the precursor structures were detected electron microscopically. Some of these cistrons may be responsible for the synthesis of the components of RIV particles and the others for the regulation of the initiation of flagellar formation.

The existence of the complex of a rod and two inner rings as the first detectable structure suggests that the flagellar morphogenesis starts from the assembly of inner rings associated with the cell surface layers. Even if the genes specifically responsible

for the formation of flagella are functional, flagellar formation is retarded by a mutation that causes a defect in the surface layers. Deep-rough mutants in *S. typhimurium* have no flagella (80). Flagellar formation in uridine diphosphoglucose pyrophosphorylase–deficient *galU*$^-$ mutants of *E. coli* is remarkably depressed (81). The lipopolysaccharide layer is incomplete and some kinds of membrane-bound proteins are released in these mutants (80, 82). In a mutant of *B. subtilis,* production of exoenzymes and formation of flagella are pleiotropically affected presumably through certain alterations of the surface layers (83). Although the presence or absence of basal bodies has not been examined in these mutants, it is highly plausible that some components or structures of the surface layers are essential for the early stages of flagellar morphogenesis.

Because flagellar morphogenesis proceeds from the inner portion to the outer, the components of the latter structures constructed must be transported from the site of their synthesis in the cytoplasm to the outer surface layer. The intralayer protein transport systems may be responsible for this phenomenon, although they have not yet been identified.

In the sequential pathway of flagellar morphogenesis, three branches were found (Figure 3). Each of these branches resulted from a block of the main pathway by a *fla*$^-$ mutation. The first and the second branches, as shown in Figure 3, indicate that (*a*) the formation of hooks can proceed to some extent even if outer rings are absent and (*b*) the L rings are formed inefficiently when cylinders are missing. The third branch consists of polyhooks or, in other words, superhooks. A polyhook is structurally homologous with a normal hook but exceedingly longer than that (8, 80, 82). Polyhooks have been detected in *flaE* mutants of *E. coli* (84) and *flaR* mutants of *S. typhimurium* (85). The mutant allele is recessive to its wild-type allele. In these mutants, few polyhooks bear flagellar filaments. The product of the

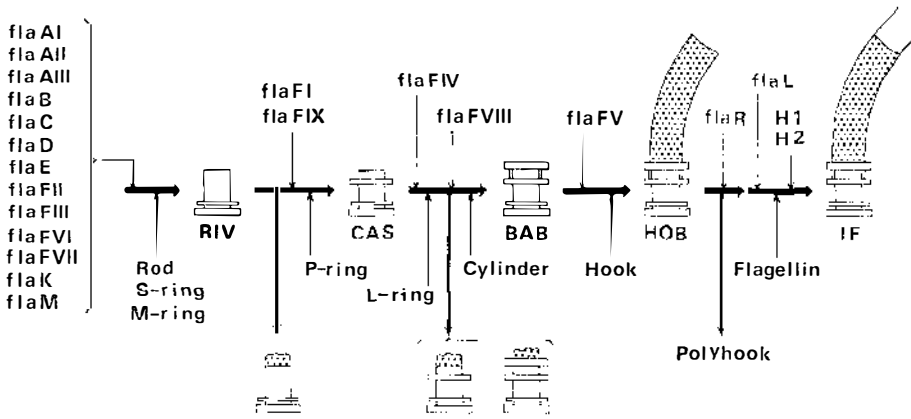

Figure 3 The process of flagellar morphogenesis in *Salmonella.* RIV: rod-inner ring complex; CAS: RIV-P ring complex; BAB: basal body; HOB: hook-basal body complex; IF: intact flagellum.

wild-type allele may control the length of the hooks and the initiation of filament growth, presumably acting as "stopper" for hook protein assembly and "initiator" for flagellin assembly at the tip of each hook.

Regulation of Flagellin Synthesis

The synthesis of flagellin, whose assembly occurs at the final step of flagellar morphogenesis, seems to be under the control of a complex regulatory system. So far, none of the proteins immunologically cross-reacting with flagellin have been detected in any *fla*$^-$ mutants of *Salmonella* (1). A mutant assigned as *flaG* in *Salmonella* has been reported as the exception to this rule. However, it was later found to carry paralyzed flagellar filaments in less than 1% of the cells and its mutant site was mapped in *flaAII* (86).

In vitro synthesis of flagellin was carried out with a cell-free extract of *E. coli* directed by mRNA of *Salmonella* (86, 87). When RNA extracted from the flagellate strains was used, flagellin synthesis was demonstrated together with the synthesis of other proteins. When RNA of the nonflagellate mutants of various *fla* cistrons was used, no detectable flagellin synthesis occurred even though the overall synthesis of protein proceeded as efficiently as in the experiments with RNA of the flagellate strains. The same result was observed when a *cya* mutant, which has a primary defect in cAMP synthesis and consequently fails to produce flagella, was used as a source of mRNA (79). Extracts of *fla*$^-$ mutant cells have no inhibitory effect on the synthesis of flagellin directed by mRNA of *fla*$^+$ strains. Further, RNA-free extracts of *fla*$^+$ strains do not promote the synthesis of flagellin by mRNA of *fla*$^-$ strains (86).

These results mean that not only flagellin but also mRNA for flagellin are not synthesized in any *fla*$^-$ mutant. In other words, when any one step of flagellar formation is genetically blocked, flagellin synthesis stops at the transcriptional level. The structural gene for flagellin constitutes an operon independent of any other known *fla* cistrons, and the *fla* cistrons are distributed among several discrete operons (65). Therefore, an interoperonic regulation system must be operating in the synthesis of flagellin mRNA. It is plausible that *flaL* of *Salmonella* functions as a "linker" between the induction system and the operons for flagellin synthesis (88). The *flaL* mutants can produce flagellar structures other than filaments, and synthesis of mRNA specific for both *H1* and *H2* is blocked simultaneously in the mutants. This is the case even in the deletion mutants of *flaL*. Further, some revertants of *flaL* partially recover the ability to synthesize flagellin, and the resulting partial revertant cells produce flagellar filaments shorter than those of the normal *fla*$^+$ cells. It is possible that (*a*) flagellin monomers in an amount not detectable by the techniques applied so far work as an antirepressor of flagellin synthesis when they fail to polymerize in the cells of *fla*$^-$ mutants, or (*b*) the product of *flaL* works as the antirepressor.

Growth of Flagellar Filaments

The process of assembly of flagellin to flagellar filaments has been clarified in detail at the molecular level by the in vitro polymerization experiments with *Salmonella*

flagellin (89). A flagellin monomer binds to an end of an existing filament. The end where the monomer binds corresponds to the distal end of a flagellar filament on living bacteria. Next, the monomer is firmly incorporated into the filament accompanied by its conformational change. The incorporated monomer then acts as a part of the nucleus for polymerization of the next monomer to begin. Thus, a conformational change of flagellin molecules upon their assembly confers structural polarity to the flagellar filaments and also confines the assembling site to the distal end of each filament. These processes are regarded as self-assembly and proceed in the presence of appropriate concentrations of flagellin monomers and flagellar filaments under proper temperature, pH, and ionic strength (89). The growth of flagellar filaments in vivo may be essentially homologous as is that in vitro. The in vivo growth of a flagellar filament occurs by the polymerization of flagellin at the distal end of the filament (90–92) , and under optimal conditions the maximum speed at the initial stage of the filament growth in vivo was the same as that in vitro (93).

Although polymerization of flagellin monomers in vitro is initiated without the presence of the filaments under conditions of high ionic strength (94), the filament must be present for polymerization to occur in the ordinary physiological environment. This suggests that the initiation of filament growth in vivo under ordinary physiological conditions occurs through a somewhat different process from that in vitro. In living bacteria, the proximal end of a filament is connected to the distal end of a hook, and by the binding of flagellin at this end, the filament begins to grow. Thus the distal end of each hook serves as a nucleus for the polymerization of flagellin. In fact, isolated hooks were successfully used as a heteronucleus for the polymerization of flagellin in vitro, although the efficiency of the process was low compared with the polymerization in which fragments of flagellar filaments were used as the homonucleus (93). The requirement of a heteronucleus for the initiation of assembly of certain structural components seems to be an elaborate mechanism to confine the site of structure formation to a proper site in living organisms.

The rate of in vitro polymerization of flagellin monomers to flagellar filaments depends on the species and the amount of flagellin, and on the physicochemical condition of the environment (89). However, when these are fixed, the average rate is maintained at a constant, and filament growth proceeds constantly except for an occasional abrupt termination by an error occurring at the growing end of the filament (95). Consequently, it is experimentally possible to obtain filaments longer than 50 μm by in vitro polymerization. On the other hand, the average rate of in vivo elongation was found to decrease exponentially with the increase of filament length according to the following equation:

$$V = V_0 e^{-KL}$$

where V denotes the rate of filament growth at length L, V_0 the initial rate at $L = 0$, and K the constant characterizing the degree of decrease in rate per unit of length (93). This relationship is observed even among the filaments of various lengths growing on a single cell or a filament mechanically shortened by breakage. Therefore, the contribution of aging of a cell or of a flagellum-forming apparatus to the decrease in the growth rate of filaments is implausible. For the growth of a

flagellar filament, flagellin monomers must be transported, probably by a sort of diffusion, from the cell body to the tip of the filament through its central canal (90–93). Consequently, the decrease in growth rate must be caused by the decrease in the efficiency of transportation with the increase in length of the filament. This means that the filament limits its own growth rate. The observed maximal length of flagellar filaments on living bacteria is explained by such autoregulation of the growth rate, although the possibility of a termination factor for filament elongation is not entirely excluded (93).

POLYMORPHISM OF FLAGELLAR FILAMENTS

Fine Structure and Polymorphism of Flagellin

Although flagellin molecules of various bacteria have common biochemical features as the component protein of flagellar filaments, they also exist as extensively polymorphic molecules in nature. Molecular weights of flagellin so far reported ranges from 33,000 to 60,000 among different bacterial species (14–17, 96).

Various mutants that differ in the spiral shape of flagellar filaments have been reported, and their mutant sites were all assigned to the structural genes for flagellin (1, 28, 42). In a straight flagellar mutant of *B. subtilis* an alanine residue in the polypeptide of normal flagellin is replaced with valine (97).

In some bacterial genera, such as *Salmonella,* many varieties of flagellar (H) antigen have been observed, and the difference of amino acid composition among different *Salmonella* serotypes has been demonstrated (1). The antigenic mutants of flagellin-i of *S. typhimurium* were attributed to either substitution of an amino acid or a small deletion in the flagellin (98). Even in flagellar filaments of the same shape and antigen type, their component flagellin molecules differ in primary structure between different strains of the same species (99).

Polymorphism of flagellin appears even in a clone of some bacteria. Flagellar phase variation in *Salmonella* is a remarkable example which will be described later. A single cell of *Vibrio parahaemolyticus* produces both polar and peritrichous flagella. Interestingly enough, flagellar filaments of these two types differ from each other in both shape and antigenicity, suggesting that they are composed of different types of flagellin (100, 101). Although mutants that produce only one type of flagella have been isolated, their genetic analysis has not yet been carried out.

This pronounced polymorphism of flagellin among bacteria suggests the existence of evolutionarily variable and conservative regions in the molecule. Genetic fine structure analysis of the *H1*-g cistron of *Salmonella* revealed that a quarter of the cistron from the terminus adjacent to *ah1* predominantly produces nonflagellate phenotype ("essential region" in Figure 4) (43). This region may correspond to a section of polypeptide chain essential for the specific conformation of flagellin. Evolutionally, this region must have been conservative. Antigenic mutations occur in the remaining region ("antigenic region" in Figure 4). In the region primarily responsible for antigen-type determination, the sites of specific antigen-type determinants are arranged linearly. The antigenic region may correspond to an exposed part of the flagellin polypeptide. The relatively rare detection of nonflagellate mutations

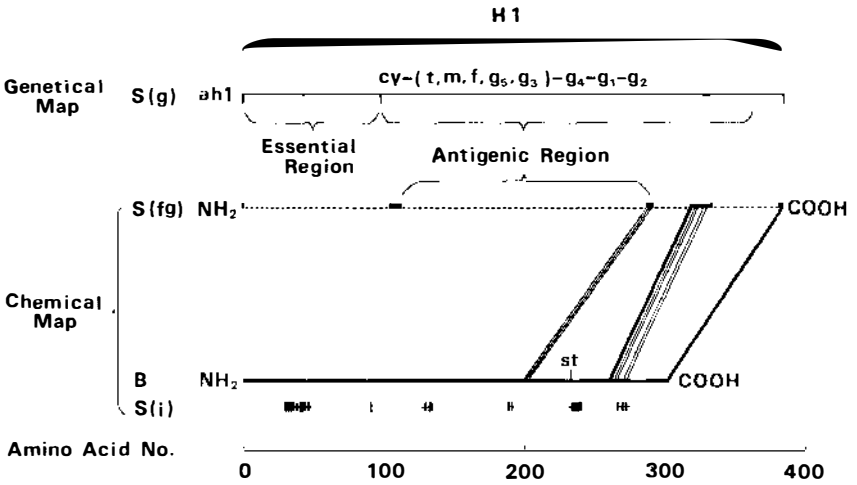

Figure 4 Comparison of the map of flagellin gene *H1-g,* shown as S(g), of *Salmonella* and the chemical maps of flagellin of *Salmonella adelaide,* S(fg), and *Bacillus subtilis* (B). Amino acid sequences of the regions indicated by dotted lines are not determined. The lines between S(fg) and B connect the identical amino acids of the homologous peptide segments. S(i) shows peptide segments of flagellin-i of *Salmonella typhimurium* whose homologous regions were identified in B. The identical amino acids between them are shown by vertical bars. st indicates straight mutant site; cy indicates curly mutant site.

in this region may mean that conformational alteration of flagellin caused by a mutational change in its surface is usually not so severe as to result in the loss of ability to polymerize. This region may be evolutionarily variable. These genetic results conform with the conclusion drawn from immunochemical studies on fg-type flagellin that all the antigenic specificities reside in the central region of flagellin polypeptide (Figure 4) (11, 12). The sites of amino acid substitution responsible for antigen-type mutations in the whole flagellin polypeptide are left for future investigations.

In *Salmonella,* partial amino acid sequences were determined on flagellin-i of *S. typhimurium,* and flagellin-fg of *S. adelaide* (12, 14). Flagellin-i and flagellin-fg are composed of 470 and 386 amino acids respectively. In the former, amino acid sequences were determined on 29 tryptic peptides of in total 217 amino acids. The order of these peptides in a flagellin polypeptide has not been determined yet. On flagellin-fg, the N-terminal amino acid, sequences of 37 amino acids of 4 tryptic peptides and the positions of these peptides in the polypeptide were determined.

It is premature to correlate this chemical information with the fine structure of the corresponding flagellin gene. However, the complete amino acid sequence of a flagellin molecule of *B. subtilis* strain 168, designated as flagellin-B for further discussion, was recently clarified (19), and the comparison of the sequence with the known partial amino acid sequences of *Salmonella* flagellin provides some interest-

ing information. A flagellin-B molecule is composed of 304 amino acid residues. Out of the known 29 tryptic peptides of flagellin-i, 7 peptides correspond to homologous regions of flagellin-B (Figure 4). The largest homologous region, which corresponds to two consecutive peptides of flagellin-i, is assigned between the 30th and the 51st amino acids from the N terminus of flagellin-B. These two flagellin molecules are identical in 16 of the 22 amino acids in the region. The amino acids between the 233rd and the 241st of flagellin-B are identical in 7 of 9 amino acids with peptide-10 of flagellin-i. These two regions correspond to antigenically inert sections of flagellin-fg, and the former resides in a region corresponding to the "essential region" in the genetic map. In the homologous peptides assigned in the "antigenic region," flagellin-B and flagellin-i are identical in only 7 out of 16 amino acids. Thus as far as the identified homologous regions are concerned, the "essential region" seems to be more conservative than the antigenic region. Remarkable variation in molecular size among these three different flagellins indicates that numerous deletions or additions of genetic codes have occurred in the flagellin genes during the course of evolution. Distribution of the homologous peptide regions among flagellin-B, -i, and -fg suggests that the region responsible for such chromosomal variation is mainly in the antigenic region in the middle of the flagellin polypeptide (Figure 4).

As regards the sites of flagellar shape mutations, curly mutant sites in *Salmonella* were genetically mapped in a region proximal to the N terminus, and a straight mutant site of *B. subtilis* was chemically identified to be close to the C terminus. The antigenic region was located between them. These two separate regions on a flagellin polypeptide may jointly play an essential role for the determination of the mode of molecular assembly.

Polymorphism in Helical Shape of Flagellar Filaments

As described in the foregoing section, the genetic change of helical shape in flagellar filaments is attributed to a mutation at a specific site in the flagellin gene. The primacy of the type of flagellin for the determination of helical shape was also shown by in vitro assembly of flagellin into flagellar filaments (89). However, the helical shape manifested by a flagellar filament is not always restricted to only one form; transformation from one form to another occurs often. For example, the normal shaped filaments of *Salmonella* strain SJ25 undergo sequential transformation to coiled, semicoiled, and curly when the pH of the environmental solution is lowered from neutral to acidic (102). The transformation is also affected by ionic strength of the environment. The transformation from one form to another is discontinuous and the intermediate filaments appear as segmental chimera of two discrete forms. The comparable polymorphism is observed when the ratio of two different types of flagellin are changed in in vitro copolymerization of flagellin (103).

In order to explain the polymerization of a single kind of flagellin into a helical tubular structure and their discontinuous transformation, a hypothesis was presented that a single kind of flagellin can take two different conformations, namely R and T, as metastable states and that the molecules of each state lie along the longitudinal strands of a filament (89). Then, the various helical shapes appearing by the transformation are explained by the difference in the number of R and T

strands in a filament. Another explanation assumes the presence of alternative bonding sites in a flagellin molecule (104). Whether it is attributed to the conformation or the bonding, the presence of at least two different states in a single kind of flagellin and their reversible transition from one to the other seem to be essential for the discontinuous transformation of the helix of flagellar filaments. If this is the case, the different flagellar shape mutants may produce flagellin differing not only in detailed conformation of its molecule but also in the stability of these states under ordinary physiological conditions. The polymorphous mutants detected on *Salmonella* may produce "flexible" flagellin which undergoes frequent transition between two states (42). Even though more than one form appears among a population of the polymorphous flagella, each individual flagellum comprises a single form except on rare occasions. For a flagellar filament to manifest a regular helical shape, flagellin molecules of the same state must be regularly arrayed along each of the longitudinal strands. This means that the terminus of an existing flagellar filament plays a decisive role in the choice of the state of polymerizing flagellin and successively determines the helical shape of the growing portion of the filament.

Flagellar Phase Variation

Flagellar phase variation in *Salmonella* is a dimorphic expression of flagellar characters in a clone. Many *Salmonella* species are characterized by their possession of a pair of nonallelic structural genes for flagellin designated *H1* and *H2*, whose expression is alternative in a clone. For example, in *S. typhimurium* the alternative expression of *H1-i* and *H2–1.2* occurs with the probability of about 10^{-3} per bacterial division. This phenomenon was first detected as the alternative expression of two discrete flagellar antigen types, and later observed on the shape of flagellar filaments (1). A bacterial strain that undergoes phase variation is termed a *diphasic strain* and a cell expressing *H1* or *H2* is said to be in *phase 1* or *phase 2* respectively. Early genetic studies on phase variation interpreted the process as follows: *H2* can exist in two different states, active and inactive; when *H2* is in the active state, the production of the phase-1 flagellin by *H1* is repressed, while *H2* carries out the production of phase-2 flagellin, and when *H2* changes to the inactive state the production of phase-1 flagellin, specified by *H1*, proceeds (1). As for the expression of each *H* gene, a closely linked factor *ah* was found to be responsible for activation of the adjoining *H* gene. Mutation of $ah1^+$ to $ah1^-$ or $ah2^+$ to $ah2^-$ results in the failure of production of phase-1 or phase-2 flagellin respectively (Table 1).

The process of phase variation has raised two questions associated with fundamental problems of genetics. The first is how the production of phase-1 flagellin is repressed in the *H2*-active state and the second is by what mechanism *H2* changes its state. Recent efforts to answer these questions have met with considerable success. Concerning the first question, a repressor gene, *rh1*, was found to exist and constitute an operon with *H2* and *ah2* (105). The genotype of diphasic cells is $ah2^+$-$H2^+$-$rh1^+$ (Table 1). *Ah2* controls the activity of both *H2* and *rh1*, and is regarded as the operator region. In the $ah2^-$ mutants the abilities of both phase-2 flagellin synthesis and inhibition of phase-1 flagellin synthesis are lost. Consequently, the phenotype of such mutants becomes stable phase 1. On the contrary, *rh1* remains

Table 1 Mutants of H1 or H2 operon and their phenotypes in *Salmonella typhimurium*

H1 operon		H2 operon				Phase	Phenotype in	
ah1	H1	vh2	ah2	H2	rh1	variation	phase 1	phase 2
+	i	+	+	1.2	+	+	i	1.2
–	i	+	+	1.2	+	+	nf[a]	1.2
+	–	+	+	1.2	+	+	nf	1.2
+	i	–	+	1.2	+	s[b]	i	1.2
+	i	+	–	1.2	+	–	i	—
+	i	+	+	–	+	+	i	nf
+	i	+	+	1.2	–	+	i	i and 1.2

[a] nf = nonflagellate.
[b] s = stable in existing phase.

active in phase 2 of the *H2*⁻ mutants, and neither phase 1 nor phase 2 flagellins are synthesized in this phase. Thus, the mutant clones reveal H-O variation. In the *rh1*⁻ mutants, synthesis of both phase-1 and phase-2 flagellin proceeds in phase 2, and flagellar filaments are composed of both types of flagellin. In phase 1, they produce only phase-1 flagellin.

Studies on a cell-free system for in vitro protein synthesis directed by RNA of *Salmonella* were extended to the elucidation of the step blocked by the phase-1 repressor in phase-2 (106). Chromatographic analysis of the in vitro products showed the presence of synthesized flagellin corresponding to the phenotype of the cells from which the RNA was derived. That is, when RNA was extracted from the cells of the diphasic strain propagated from a single colony, expressing either phase 1 or phase 2, the in vitro synthesized flagellin was predominantly the same as that produced by the original colony. Translation of mRNA specific for phase-1 flagellin was not inhibited by the presence of mRNA specific for phase 2. This experimental evidence indicates that phase variation is due to the alternative synthesis of phase-specific mRNA and that the phase-1 repressor, i.e. the product of *rh1,* blocks the transcription of the *ah1-H1* operon (Figure 5).

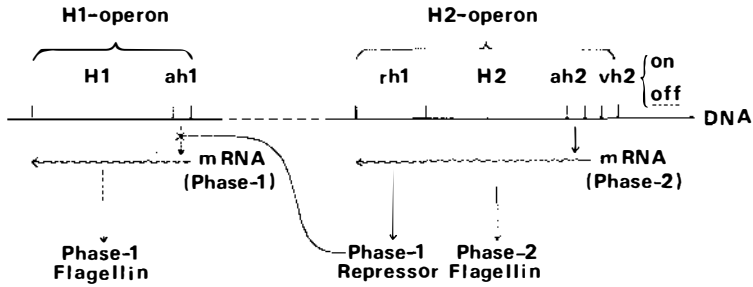

Figure 5 The regulation system operating on phase variation of *Salmonella.*

The repression of the *ah1-H1* operon by *rh1* is not complete throughout the growth phases of diphasic bacteria. Temporary derepression of phase-1 flagellar formation occurs in a fraction of the phase-2 cell population at late exponential and early stationary phase when a cell generation time exceeded 80 min (107). The resulting flagellar filaments carry a segment region composed of both phase-1 and phase-2 flagellin molecules. The copolymer segments are formed almost simultaneously in every growing flagellar filament of the derepressed cells for an average of about 8 min until the supply of phase-1 flagellin is exhausted after reestablishment of repression. The duration of detectable copolymer formation in a cell is of the same order as the half-life of flagellin mRNA (108). These phenomena show that a flagellum-forming apparatus can accept newly synthesized flagellin of phase 1 even after it has already started filament formation in phase 2. Thus, the flagellum-forming apparatus of each flagellum was found to be phase nonspecific. Formation of a homogeneous copolymer filament of two different kinds of flagellin was demonstrated also on the merodiploids of *E. coli* carrying two distinguishable *hag* loci (109).

As for the mechanism of the alternative activation and inactivation of the *ah2-H2-rh1* operon, presence of a chromosomal factor, *vh2,* which is closely linked to the operon was reported (1). Replacement of $vh2^+$ in a diphasic clone by $vh2^-$ of a monophasic clone reduced the rate of shifting from the existing state to the alternative one below 10^{-7} per bacterial division. Recently, molecular cloning techniques were applied to isolate the segment of *Salmonella* DNA which contain the *H2* region attached to λ (M. Simon, personal communication). When the cloned DNA molecules were denatured and then renatured, presumed heteroduplexes having a region similar to an "inversion bubble" were detected adjacent to *H2* on the molecules. The frequency of such molecules correlated with the ratio of phase-1 and phase-2 cells in a bacterial culture from which DNA was derived. Thus, the explanation was proposed that an inversion of DNA adjacent to *H2* is responsible for the flagellar phase variation in *Salmonella.* The correspondence of this inversion region with the genetically assigned stability controller, *vh2,* remains to be investigated. Destabilization of the *H2* state when the *H2* region of the phase-2 stable *Salmonella* is incorporated in the *E. coli* chromosome may be also associated with some sort of structural anomaly of the chromosomal region (71).

GENETIC CONTROL OF MOTILITY

Function of the mot Genes

Recently it has been established that the bacterial flagellum provides the thrust for cell movement by means of a rotary device at its base (31–36). This finding has led the studies of bacterial motility to focus on the rotary mechanism at the base of a flagellum. Two cistrons *motA* and *motB,* primarily responsible for motility of flagella, have been disclosed in both *Salmonella* (45, 46) and *E. coli* (48, 61). In *E. coli, motA* and *motB,* together with *cheA,* were found to be organized into an operon termed *Mocha* (61).

Neither electron microscopy of the flagellar basal structures nor SDS-acrylamide gel electrophoresis of their component proteins could detect differences between *mot*$^+$, *motA*$^-$, and *motB*$^-$ cells (21). However, the efforts to construct the λ-*mot* plasmids by molecular cloning techniques and to let them express *mot* functions in *mot*$^-$ host bacteria helped identify the specific proteins corresponding to these two *mot* genes (48, 61). The molecular weights for *motA* and *motB* are 31,000 and 39,000 respectively. These proteins may be either the components of flagellar basal bodies contained in an amount too small to be detected in ordinary analytical procedures or regulatory factors not firmly bound to basal bodies. As intercistronic weak-complementation occurs in many combinations of *motA*$^-$ and *motB*$^-$ mutants (45–47), it is possible that the product proteins of these *mot* cistrons function as a structural complex. Although the actual function of these proteins in motility is left for further investigation, it is quite plausible that they participate in the link of the rotary apparatus of a basal body with the connecting membrane structure or with the machinery supplying energy for the rotation. It was shown in *E. coli* that chemoattractants or repellents induce a change of the membrane potential of motile *mot*$^+$ cells but not of a *mot*$^-$ mutant cells of *E. coli* (110).

The third *mot* cistron, *motC,* reported in *Salmonella* (45), was later found to be identical with *flaAII* (58). Interestingly enough, a mutation in this cistron results in a defect of flagellar formation or paralysis of flagella depending on the site of mutation. Furthermore, the site of a never-tumbling *che*$^-$ mutant was mapped in *flaAII* (111). Thus the product of *flaAII* is responsible for formation, rotation, and reversal of rotation. In nonflagellate mutants of *flaAII,* none of the flagellar structures were found to be associated with the membrane fraction (T. Suzuki and T. Iino, unpublished information). As mentioned in a foregoing section, the basal structure detectable in the earliest stage of flagellar morphogenesis is the rod–inner ring complex. Therefore, it may be assumed that this structure is the major portion of the rotary apparatus of a flagellum and that the product of *flaAII* either is a component of this complex structure or is associated with it.

The source of energy for the rotation of flagellar bases was inferred to be an intermediate in oxidative phosphorylation and not ATP directly (112), because, unlike their parents, mutants of *E. coli* and *S. typhimurium* that are blocked in the conversion of ATP to the intermediate of oxidative phsophorylation fail to swim anaerobically, even when they produce ATP. Further, carbonylcyanide *m*-chlorophenylhydrazone, which uncouples oxidative phosphorylation, completely inhibits motility even though ATP remains present. Similarly, anaerobic infection with flagellotropic phage χ is blocked in energy-transducing ATPase-defective (*uncA*$^-$) mutants and is restored by the addition of NO_3^-, which functions as a terminal electron acceptor for anaerobic respiration (113).

Role of Flagellar Filaments on Bacterial Motility

Rotation of the base of a flagellum lets the connecting filament rotate jointly and exerts the driving force for bacterial movement. When multiflagellate eubacteria swim translationally, a number of flagella on a cell form a bundle and rotate in unison (114, 115). For translational movement of such bacteria, the direction of rotation

of the base must be the same as the handedness of the helix of the filament. The demonstration of basal rotation and identification of its direction were accomplished with an elaborate use of two kinds of flagella mutants, i.e. polyhook mutants and straight mutants of *E. coli* (32). Neither of these mutant cells can swim in liquid medium because they are missing normal filaments. However, when a cell of these mutants was tethered to a glass slide with antihook or antifilament antibodies, the cell body rotated alternatively clockwise (CW) and counterclockwise (CCW). This phenomenon indicates that, although the cell is nonmotile, its flagellar bases are actively rotating relative to the cell body. This experiment was further extended to cells with only one normal flagellar filament, and the frequency of rotation to each direction was measured (33). The direction of flagellar rotation was frequently CCW when it was seen from the side where the flagellum is attached, but abrupt stops of the rotation and successive CW rotation were also observed intermittently. For bacteria whose flagella rotate CCW, translational movements must be made when the helix is left-handed. Recent improvement of dark-field microscopy helped confirm the expectation (116) that the helix of normal-type flagellar filaments is left-handed. It was shown by dark-field microscopy that abrupt stop and tumbling of a normal flagellate cell during its translational movement corresponds to abrupt reversal of rotation, and flagellar filaments disperse around the cell at this stage (114, 115).

Information on the coordination between direction of basal rotation and handedness of the filament helix in translational movement explained a curious movement of the curly mutant cells, which continue to tumble in liquid medium and intermittently swim straight ahead. In contrast to normal flagellar filaments, curly filaments were found to be right-handed (116). Therefore, when flagellar bases of a mutant cell rotate CCW, its curly filaments cannot form a bundle and exert the necessary driving force for translational movement of the cell.

Reversal of the rotation of flagellar bases is an important event for the manifestation of tactic behavior, whose detail is described in the review article of Parkinson in this volume (37).

SUMMARY

Early genetic studies of bacterial flagella were focused mainly on their filament portion. It is now possible to describe the process of filament formation at the molecular level as the "self-assembly" of flagellin into filaments. The shape and length of the growing portion of a flagellar filament are determined by its preexisting portion. This may be taken as a typical example of the autoregulatory systems operating in molecular assembly in living organisms.

Fine structure analysis of flagellin is in progress both genetically and chemically. Structural and functional differentiation in the molecule is being investigated in relation to its assembly and antigenicity. In connection with the extensive polymorphism of flagellin, the completion of these investigations is expected to contribute to studies on molecular evolution. Investigation of the mechanism of phase variation disclosed a regulatory system operating at the transcriptional level between the

operons in which the flagellin genes reside and demonstrated a possible contribution of a chromosomal inversion for the phase shift.

In addition to the above-mentioned studies on flagellar filaments, the studies on basal structures of flagella are progressing remarkably well. Through the elaborate use of nonmotile mutant cells, it was shown that the motility of a bacterium is activated by rotation of its flagellar bases. Two proteins specifically responsible for the rotation were identified by comparative chemical analysis of a motile bacterial strain and its paralyzed mutants. Studies on the energy transfer system for the basal rotation is also proceeding with appropriate mutants.

The structure of a flagellar basal body was clarified in detail on a number of bacterial species. An outline of the morphogenesis of flagellar basal structures was disclosed, in combination with the identification of the flagella genes responsible for the disclosed steps in the process. Detailed biochemical studies on the product of each step are in progress with the use of the cloned flagella genes.

Studies on the relation of flagellation to the cell cycle and cell envelope have been undertaken. They are expected to provide an excellent model for genetic regulation of cellular differentiation.

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