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Incomplete Flagellar Structures in *Escherichia coli* Mutants

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Escherichia coli mutants with defects in 29 flagellar genes identified so far were examined by electron microscopy for possession of incomplete flagellar structures in membrane-associated fractions. The results are discussed in consideration of the known transcriptional interaction of flagellar genes. Hook-basal body structures were detected in *flaD, flaS, flaT, flbC,* and *hag* mutants. The *flaE* mutant had a polyhook-basal body structure. An intact basal body appeared in *flaK* mutants. Putative precursors of the basal body were detected in mutants with defects in *flaM, flaU, flaV,* and *flaY*. No structures homologous to the flagellar basal body or its parts were detected in mutants with defects in *flaA, flaB, flaC, flaG, flaH, flaI, flaL, flaN, flaO, flaP, flaQ, flaR, flaW, flaX, flbA, flbB,* and *flbD*. One *flaZ* mutant had an incomplete flagellar basal body structure and another formed no significant structure, suggesting that *flaZ* is responsible for both basal body assembly and the transcription of the *hag* gene.

Bacterial flagella are responsible for motility and chemotaxis. Electron microscopic observations of isolated flagella have revealed that they consist of three parts: filament, hook, and basal structure (2). The filament, which extends into the extracellular space, is connected by the hook to the basal structure embedded in the cell membrane. Bacterial flagellar formation is one of the interesting problems in the study of morphogenesis and its genetic control (3, 11). Genetic analyses revealed that 29 *fla, flb,* and *hag* genes are involved in the formation of a morphologically complete flagellum in *Escherichia coli* K-12 (5), and 28 *fla* and *H* genes are involved in that in *Salmonella* (3, 8). Some genes have been shown to code for the proteins of flagellar structure in *E coli* (11). The *hag* and *flaK* genes were identified as the structural genes for flagellin (the protein component of flagellar filaments) and the hook protein, respectively. (7, 9, 10). The following genes are required for the constituent proteins of the basal structure: *flaL, flaM, flaV, flaS,* and *flaT* (6). The remaining genes are considered to be either those coding for the constituents or those regulating the formation of flagella (3, 11).

An in vivo gene fusion technique has revealed a transcriptional interaction of these flagellar genes in *E. coli* K-12 (4; Y. Komeda, manuscript in preparation). The *hag* gene is transcribed in the presence of most of the other flagellar gene products (4). The *hag* mutants have the most complex precursor structure, carrying a hook-basal body structure. Accordingly, the structure appeared to exist in a sequential transcriptional pathway beginning with the basal body constituents, followed by the hook protein and, finally, the flagellin. Identification of the precursor structure of each mutant will reveal the significance of the transcriptional interaction for flagellar assembly. Therefore, precursor structures of each mutant were examined by the method of Suzuki et al., who succeeded in identifying these precursor structures of flagella in *Salmonella typhimurium* with the aid of electron microscopy (13). So far, electron microscopy seems to give us the most reliable data for analysis of flagellar precursors. *E. coli fla* mutants used in this study included more flagellar genes than those identified in *S. typhimurium*. A cooperative approach with electron microscopic observations and an in vivo gene fusion study enabled us to understand the functions of flagellar genes for morphogenesis of *E. coli* flagella.

MATERIALS AND METHODS

Bacterial strains. All of the nonmotile mutants used, *fla, flb, hag,* or *mot* of *E. coli* K-12, were derivatives of strain YK410, described previously (5), and are shown in Table 1.

Media. L broth contained, per liter of distilled water: tryptone (Difco Laboratories, Detroit, Mich.), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; thymine, 0.2 g. The pH was adjusted to 7.2 with 5 N NaOH. L-agar plates contained 1.2% (wt/vol) agar in L broth. Semisolid agar plates containing 2.5 g of agar, 0.2 g of thymine, and 0.1 g of uracil per liter of tryptone broth (1% tryptone, 0.5% NaCl) were used to test for motility.

Cultivations. A single colony grown on L-agar plates was inoculated in 1 ml of L broth. The culture was incubated at 30°C overnight. Fresh L broth (100 ml for fractionations of flagella) was inoculated with 0.002 volume of the overnight culture and incubated

at 30°C with gentle shaking (90 strokes per min). When the bacterial concentration reached about 10^9 cells per ml, the culture was divided into three portions. Two 1-ml portions were fixed with 1 ml of 10% (wt/vol) formaldehyde solution. One of them was used for total cell counting, and the other was used for observing intact cells by negative staining, using the method of Suzuki et al. (13). The remaining portion was used for fractionation of flagella.

Fractionation of flagellar basal structures. Fractionation of flagellar basal structures was performed according to the method of Suzuki et al. (13). The method consisted of forming spheroplasts in the presence of EDTA and lysozyme, followed by detergent treatments and differential centrifugations (13).

Fraction BMII (a membrane fraction that was treated with Triton X-100 and Nonidet P-40; 13) contained flagellar basal structures of nonflagellate mutants, and it was possible to study them by electron microscopy.

Electron microscopy. Negative staining was carried out with 1% sodium phosphotungstate (pH 7.0) according to the method of Brenner and Horne (1).

The specimens were observed in a JEM 100C electron microscope. Observations were performed at an accelerating voltage of 100 kV.

RESULTS

Fractionated materials in the flagellate parent. Observations were made on the fractions prepared from the flagellate parental strain YK410. The results obtained with this *E. coli* strain conformed to the previous data for *S. typhimurium* (13). Fraction BMI (crude membrane fraction; 13) contained fragments of outer and inner membranes, as well as intact flagella (IF) bound to either one or both of these membranes. The strain formed a few pili, which accumulated in this fraction. Triton X-100 treatment of fraction BMI resulted in the solubilization of the membranes and the release of IF from them. Nonidet P-40 treatment further clarified the BMI fraction, resulting in a fraction termed BMII. BMII contained IF (Fig. 1). The nomenclature (Fig. 2) of the structures and their components was that of Suzuki et al. (13) and DePamphilis and Adler (2). HOB and BAB particles are homologous to the hook-basal body complex and basal body of IF, respectively. An RIV particle, whose overall shape resembled a rivet, corresponded to the M ring-S ring-rod complex of IF. A CAS particle, which looked like a candlestick, was the M ring-S ring-rod-P ring complex of IF. Figure 2 gives the dimensional data for these structures taken from electron micrographs. Fractions BSI and BSII, supernatant fractions of BMI and BMII, respectively (13), did not contain a significant amount of IF or fragmented flagellar filaments, as described previously for *S. typhimurium*.

TABLE 1. *Bacterial strains examined*

Mutant	Strain (mutant no.)
Parent (flagellate)	YK410
hag	YK4130, YK4146
flaD	YK4104(867), YK4181
flaS	YK4140, YK4429
flaT	YK3475, YK4176
flbC	YK3415, YK1101
flaE	YK4105(694)
flaK	YK2008, YK4151
flaY	YK4164
flaM	YK2017, YK4148
flaU	YK3440, YK4122
flaV	YK4451(2022), YK4452(2023)
flaA	YK4160, YK4166
flaB	YK4126, YK4141
flaC	YK4127, YK4147
flaG	YK4195
flaH	YK4171, YK4172
flaI	YK4136, YK4155
flaL	YK4405(721), YK4118
flaN	YK4145, YK4193
flaO	YK4112(1862)
flaP	YK4144, YK4154
flaQ	YK4035, YK4120
flaR	YK4150, YK4170
flaW	YK4410(971), YK4455(2841)
flaX	YK4453(2033), YK4454(2058)
flaZ	YK4402(51), YK3449
flbA	YK4457(2861)
flbB	YK4116, YK4131
flbD	YK4458(*am*52)
motA	YK4119, YK4179
motB	YK4183
motD	YK4117

Fractionated materials in the nonflagellate mutants. The procedure for the fractionation of IF was applied to various flagellar mutants. Since structural entities homologous to the flagellar basal structures or their parts were detected previously predominantly in fraction BMII in *E. coli* K-12 and *S. typhimurium*, we examined this fraction extensively by electron microscopy.

Mutants defective in *flaD, flaS, flaT, flbC,* and *hag* genes were found to contain HOB particles (Fig. 3a). The frequencies of the appearance of the structure were comparable to those

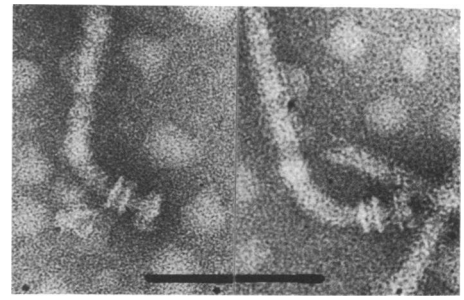

FIG. 1. *IF isolated from parental strain YK410. Negatively stained with sodium phosphotungstate. Bar, 100 nm.*

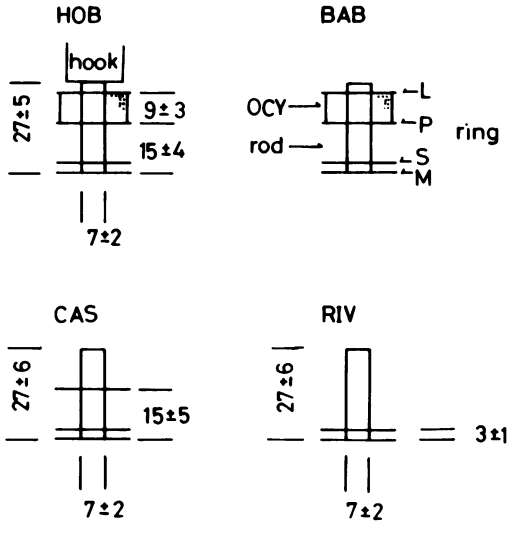

FIG. 2. *Dimensional data for the structures. The data were taken from electron micrographs and values are expressed in nanometers ± standard deviation (95% confidence). BAB had the same value as HOB. Abbreviations: HOB, hook-basal body; BAB, basal body; CAS, candle stick (M ring-S ring-rod-P ring complex); RIV, rivet structure (M ring-S ring-rod complex); OCY, a cylinder filling-up structure between the L and P rings. Nomenclature for the component structures is as described previously (2, 13).*

of IF in the flagellate parent. These HOB particles were not different from the basal structures of IF in gross morphology and dimension (see Fig. 2).

The *flaE* mutant had polyhooks. They had the same morphology as reported previously (12). The mutant also had normal hooks and IF as a minor fraction (about 1% of polyhooks). We also noted some flagellar filaments attached to the tips of polyhooks.

Specimens from a mutant with a *flaK* defect, strain YK4151, contained BAB particles (Fig. 3b). This result conformed to the previous report that *flaK* is the structural gene for the hook subunit protein. We also detected the existence of degraded structures of BAB, CAS and RIV. CAS particles comprised the major fraction (about 60% of the particles) and BAB accounted for relatively minor constituents (about 10%). This suggests the instability of the BAB structure. The other mutant defective in *flaK* showed the same phenotype as strain YK4151.

CAS particles were detected in the fraction of *flaV* mutants (Fig. 3c). The number of CAS particles was almost equal to the number of IF in strain YK410 (parent). RIV particles were also detected as minor constituents. CAS particles in these mutants tended to adhere to one another (top over tail) on the specimen grids (Fig. 3c), as described previously (13). The dimensional data clearly showed that the CAS structure consisted of the rod-S ring-M ring complex of the HOB structure.

A fraction of a *flaY* mutant, strain YK4164, contained particles similar to CAS (Fig. 3d). The dimensional data for CAS were the same as those shown in Fig. 2. However, about 10% had about 4-nm-longer rods. The structure was thought to be CAS with the amorphous hook that was revealed in the *Salmonella* study. The difference was not as clear as shown for *S. typhimurium* (13).

The *flaU* and *flaM* mutants contained RIV (Fig. 3e). The RIV particles were not different from those of *flaV* mutants in gross morphology. However, half of these particles had about 4-nm-longer rods than the hook-basal body (HOB). The distal tips of their RIV particles (Fig. 3e) were split, as seen in the distal hook end of HOB (as Fig. 3a). Accordingly, it was suggested to correspond to the RIV particles with the amorphous hook in *Salmonella*. RIV particles were present in equal frequency with the IF in strain YK410 (parent).

Upon observation of BMII fractions from two *flaZ* mutants, we encountered a discrepancy between these mutants. BMII preparations from strain YK4402 (*flaZ51*) possessed CAS particles, as shown in Fig. 3f. The structure was homologous to that of the *flaY* mutants. However, the specimens isolated from strain YK3449 [*flaZ3449*::Mud (Ap^r *lac*)] carried no significant structure. The former strain is very likely to swarm and is revertible. The latter mutant was generated by Mud phage insertion and was thought to have a null type of mutation. Thus, it was plausible to conclude that the null phenotype of the *flaZ* defect corresponded to the absence of any significant structure. The missense phenotype of strain YK4402 should be taken into account in the function of *flaZ*.

The mutants defective in the cistron, *flaA, flaB, flaC, flaG, flaH, flaI, flaL, flaN, flaO, flaP, flaQ, flaR, flaW, flaX, flbA, flbB,* or *flbD* contained none of the putative precursors of the basal structure in any fraction, as seen by electron microscopy. However, they might have aberrant incomplete basal structures that were difficult to identify.

The paralyzed mutants with defects in *motA, motB,* or *motD* showed no significant difference in gross morphology of the basal structure compared with that of the IF from strain YK410 (parent).

DISCUSSION

Incomplete flagellar structures specific for each mutant with a defect in either the *fla* or

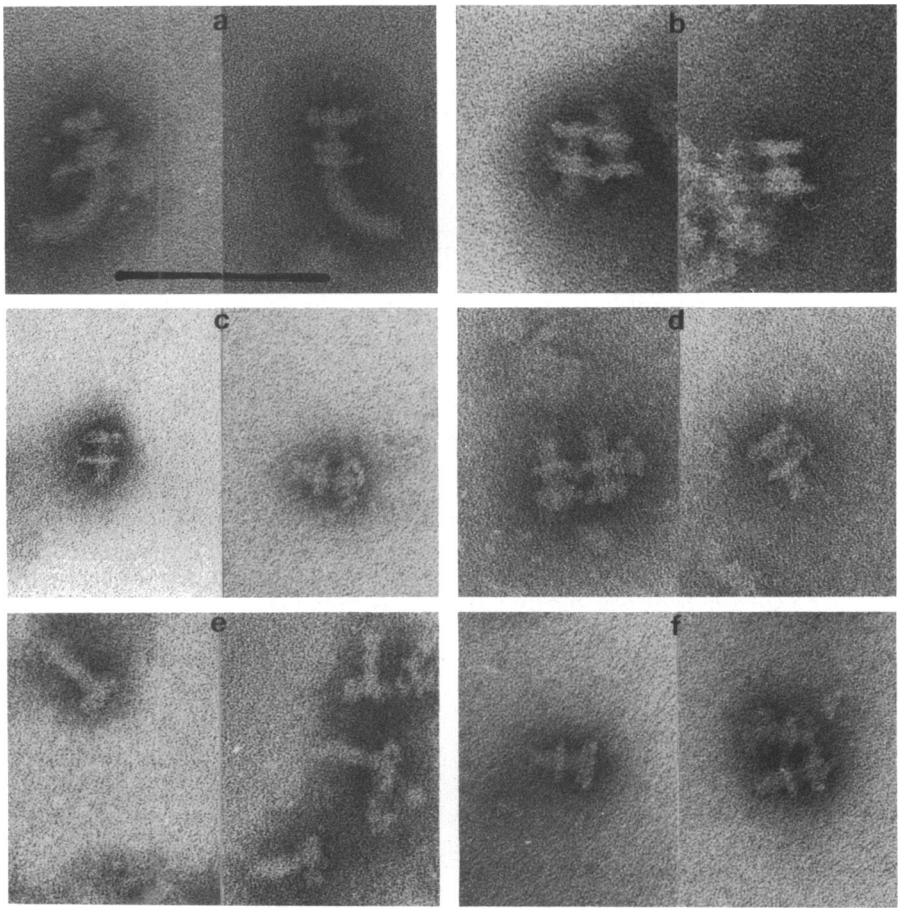

FIG. 3. *Incomplete flagellar structures detected in the membrane fraction. Negatively stained with sodium phosphotungstate. Bar, 100 nm. (a) HOB (hook-basal body) particles detected in the fraction of a flaS mutant, YK4140. Homologous particles were also observed in the flagellar mutants with defects in flaD, flaT, flbC, and hag. (b) BAB (basal body) particles in a flaK mutant, YK4151. (c) CAS (candlestick) particles in a flaV mutant, YK4451. (d) Particles detected in a flaY mutant, YK4164. CAS particles were identified. (e) Particles appearing in a flaM mutant, YK4148. RIV particles were detected in this fraction. Homologous structures were identified in flaU mutants. (f) Particles detected in a flaZ mutant, YK4402. CAS particles were detected.*

the *flb* gene of *E. coli* K-12 were detected by electron microscopy. The present study contributed to the confirmation of the existence of incomplete flagellar structures, which were first discovered in *Salmonella* flagellar mutants (13). Essentially the same precursors of the flagella were detected in *E. coli* mutants as in the homologous *Salmonella* mutants (8). Moreover, we detected hook-basal body structures (HOB particles) in mutants with defects in *flaS*, *flaT*, and *flbC*, which have no counterparts in *S. typhimurium*. The *flaD* and *hag* mutants had HOB particles also. The *flaE* mutant formed a polyhook-basal body structure. BAB particles (basal body) were detected in the preparation of the *flaK* mutants. A presumptive precursor of a basal body was found in the preparations of *flaM*, *flaU*, *flaV*, and *flaY* mutants. One *flaZ* mutant also formed a precursor of the basal body. RIV particles (M ring-S ring-rod complex) were detected in mutants of *flaU* and *flaM*. CAS particles (M ring-S ring-rod-P ring complex) appeared in *flaV*, *flaY*, and one *flaZ* mutant. The RIV structure was the simplest structural entity observed. Thus, up to the formation of RIV, the following genes are required: *flaA*, *flaB*, *flaC*, *flaG*, *flaH*, *flaI*, *flaL*, *flaN*, *flaO*, *flaP*, *flaQ*, *flaR*, *flaW*, *flaX*, *flbA*, *flbB*, and *flbD*. Each *E. coli* flagellar mutant had the same incomplete structures as the homologous *Salmonella* mutant. However, *Salmonella flaFVIII*, *flaFI*, and *flaFIX* mutants (homologous to *E. coli flaY*, *flaU*, and *flaM*, respectively) had distinct amorphous hooks at the tips of their CAS or RIV

structure. We could not detect distinct amorphous hooks; the length of RIV or CAS was a little longer than that of the rod-S ring-M ring part of the hook-basal body (HOB) in *flaU*, *flaM*, and *flaY*. Unlike *Salmonella*, this kind of structure may be unstable, or the hook assembly may be completed after construction of the complete BAB structure in *E. coli*. A definite conclusion will have to await more extensive observations. With the same argument as described in the *Salmonella* study (13), we can rewrite a rather simple pathway of flagellar morphogenesis in *E. coli* (see Fig. 4).

An in vivo *fla-lac* fusion study has revealed a transcriptional interaction (4; Komeda, in preparation). This interaction will be discussed below in consideration of the results of the present study. It was noted that the mutants expressing the *hag* gene (4) had the hook-basal body structure (HOB). They had defects in the *flaS*, *flaT*, *flbC*, or *hag* gene. The *flaU* mutants were an exception. Therefore, it was likely that the existence of the hook-basal body was tightly coupled to ability to transcribe the *hag* gene. In other words, the *hag* gene coding for flagellin is transcribed after the synthesis of the next less complex structural entity, the hook-basal body structure. Since mutants with defects in *flbC* could not form the flagellar filament, the gene product may be necessary for transport of flagellin to outside of the cell or for assembly of flagellin into a filament. Komeda et al. previously identified the products of the flagellar genes in region I (at 23.7 min on the *E. coli* map, near gene *pyrC*) (6). Although six more cistrons have been identified in that region (5) after more study and the assignment should be reconsidered in considerable detail, it is clear that the 42,000-, 60,000-, and 35,000-dalton polypeptides are assigned to *flaK*, *flaS*, and *flaT*, respectively. The *flaS* product was one of the main spots on the polyhook-basal body after two-dimensional gel electrophoresis (6). The quantity of *flaT* product was less than that of other spots and varied from one preparation to another. The present study identified the hook-basal body structure (HOB) in the mutants of these genes. Accordingly, the products of *flaS* and *flaT* may not contribute to the gross morphology of HOB or may be loosely associated with HOB. Also, they may contribute to the assembly of flagellins at the tip of the hook-basal body structure. Although the mutants with defects in *flaU* could express all of the other flagellar genes, they had incomplete basal body structures. Thus, the *flaU* product is thought to be responsible for the assembly process after the synthesis of constituent proteins. The *flaD* gene defect resulted in the existence of a hook-basal body structure. It is suggested that the hook subunit protein is synthesized without the next less complex structure, the basal body structure (BAB), because *flaK* (the structural gene of hook protein) was transcribed only if *flaI* and *flbB* products existed (Komeda, in preparation). The *flaD* gene and the genes for basal body proteins were also transcribed by the same mechanism as the *flaK* gene. Accordingly, the construction of the hook structure appeared to be accomplished after synthesis of its whole components (of the hook-basal body) together. The mechanism contrasts with that of flagellar filament assembly (see above). The *flaU* gene product is expected to be a regulatory or catalytic protein for this assembly process. The self-assembly mechanism may also be used in this step, as shown in the in vitro study of flagellin assembly. The results obtained in the *flaZ* gene study were suggestive from the point of view of flagellar assembly. In the *Salmonella* study (13), the *flaFIX* gene that is homologous to the *E. coli flaZ* gene was included in a group functioning to form the simplest structure, RIV particles. However, from the in vivo *fla-lac* fusion study, the *flaZ* gene was shown to be expressed through the existence of flagellar genes (*flaA* through *flbD*, see above paragraph) whose functions are supposed to be required to form RIV particles. One mutant (strain YK4402) formed the CAS structure; the other (strain YK3449) was shown to have no significant structure. Since the latter was supposed to be of the null phenotype, the representative phenotype of the *flaZ* defect resulted in the absence of any significant structure. Taken together with the facts that one *flaZ* mutant has a presumptive precursor and the *flaZ* gene is not required for transcription of constituent proteins other than flagellin, it is plausible that the *flaZ* gene has a dual role: (i) a regulatory (or cata-

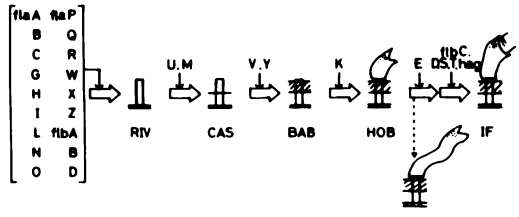

FIG. 4. *Schematic assembly pathway of a flagellum. The scheme is constructed under the following assumptions. (i) The simpler the structure, the earlier in the sequence it appears. (ii) If a mutant with a certain gene defect has a major fraction of the incomplete flagellum, the gene is responsible for the next more complex structure.*

lytic) role for basal body assembly, and (ii) that as a positive effector for *hag* gene expression. Identification of the product will elucidate these functions in molecular terms.

Since the precise function of the flagellar motor is still obscure, the critical question of whether or not an essential component of the flagellum was lost during preparation cannot be satisfactorily answered. It was also noted that some limitations or difficulties existed with the use of electron microscopy. No structure could be detected until the formation of the simplest detected structure, RIV. The genes *flaL*, *flaX*, *flaW*, and *flbA* are thought to be responsible for the formation of inner rings (S and M rings) and rod. The structural entity carrying only rod or outer rings (L and P rings) is degraded quickly or is hard to find in the specimen due to interference of background particles. A difference among the HOB particles of *flaD*, *flaS*, *flaT*, *flbC*, and *hag* mutants might exist. However, the structural difference might be below the resolution power of an electron microscope and less significant.

The combination of the identification of *fla* gene products, the chemical identification of the structural entities of a basal body, and the electron microscopic studies used here promises clarification of the refined process of flagellar morphogenesis.

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