JOURNAL OF BACTERIOLOGY, Feb. 1978, p. 904-915
0021-9193/78/0133-0904$02.00/0

Vol. 33, No. 2
Printed in U.S.A.

Incomplete Flagellar Structures in Nonflagellate Mutants of *Salmonella typhimurium*

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Received for publication 21 October 1977

Incomplete flagellar structures were detected in osmotically shocked cells or membrane-associated fraction of many nonflagellate mutants of *Salmonella typhimurium* by electron microscopy. The predominant types of these structures in the mutants were cistron specific. The incomplete basal bodies were detected in *flaFI, flaFIV, flaFVIII,* and *flaFIX* mutants, the structure homologous to a basal body in *flaFV* mutants, the polyhook-basal body complex in *flaR* mutants, and the hook-basal body complex in *flaL* and *flaU* mutants. No structures homologous to flagellar bases or their parts were detected in the early-*fla* group nonflagellate mutants of *flaAI, flaAII, flaAIII, flaB, flaC, flaD, flaE, flaFII, flaFIII, flaFVI, flaFVII, flaFX, flaK,* and *flaM*. From these observations, a process of flagellar morphogenesis was postulated. The functions of the early-*fla* group are essential to the formation of S ring-M ring-rod complexes bound to the membrane. The completion of basal bodies requires succeeding functions of *flaFI, flaFIV, flaFVIII,* and *flaFIX*. Next, the formation of hooks attached to basal bodies proceeds by the function of *flaFV* and by *flaR*, which controls the hook length. Flagellar filaments appear at the tips of hooks because of the functions of *flaL, flaU,* and flagellin genes.

Genetic studies have shown the presence of more than 20 cistrons indispensable for the formation of bacterial flagella (18, 29, 40, 47, 50). Among them, *H1* and *H2* in *Salmonella* and *hag* in *Escherichia coli* were identified to be the structural genes for flagellin, the component protein of flagellar filaments (16, 40, 44). The remaining genes are termed *fla*, which have been supposed to be either positively regulating the formation of flagella or producing constituents of the flagellar basal structures (10, 14, 18, 20, 45). However, the actual role of each *fla* cistron has remained largely unknown, except that of *flaT*, which is involved in the step of flagellar formation sensitive to cyclic AMP (28) or *flaR*, which controls the length of hooks (35) in *Salmonella*. The homologous cistrons have been reported to be in *E. coli* (39, 41). The proteins synthesized directed by some cloned *fla* cistrons were identified in *E. coli* (27, 31, 38). Among them, only the hook protein product of *flaK* was, so far, assigned as a defined constituent of flagellar basal structures.

The success in isolating a bacterial intact flagellum, i.e., a flagellar filament joined to a hook and a basal body, led to the clarification of the fine structure of the flagellar base, which had been ambiguous for a long time (9, 10, 12, 43). In the present work, by applying the procedures for isolating intact flagella, incomplete flagellar structures were detected by electron microscopy on the membrane-associated fractions and osmotically shocked cells of nonflagellate mutants in *S. typhimurium*. These results led to the assignment of a function for each *fla* cistron in terms of the morphogenesis of flagella.

MATERIALS AND METHODS

Bacterial strains. All the nonmotile mutants, either *fla* or *mot*, of *S. typhimurium* used were derivatives of either LT2 or SJW797 (Table 1). Their complementation tests had been carried out by P22 phage-mediated transduction. In this study, the stable mutants were chosen from each of the known cistrons. The mutants assigned in *flaT* (28) were excluded because they were highly unstable.

Media. L broth contained per liter of distilled water: tryptone (Difco Laboratories, Detroit, Mich.), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; glucose 1 g. The pH was adjusted to 7.0 by 1 N NaOH. L agar plates (LA) contained 1.5% (wt/vol) agar in L broth. Semisolid agar plates containing 3 g of agar and 80 g of gelatin per liter of L broth were used to test for motility.

Cultivations. A single colony grown on LA was inoculated in 1 ml of L broth. The culture was incubated at 37°C overnight. Fresh L broth (250 ml for

TABLE 1. *Bacterial strains examined*

Determination	Strain (mutant no.)	Reference[a]
Flagellate	LT2	19
Parent	SJW797	UD[b]
H1⁻, H2⁻	SJW900 (*H1*-2187)	UD
flaL	SJW800 (1650), SJW801 (1651)	UD
flaU	SL4060 (655)	47, UD
flaR	SL4055 (598)	35
	SJW880 (1656)	49
flaFV	SL4047 (530)	47, UD
	SJW1124 (1668), SJW1160 (Δ-1704)	UD
flaFVIII	SL4053 (577)	47, UD
	SJ376 (43), SJ408 (45)	19, UD
	SJW1155 (1699), SJW1200 (1744)	UD
flaFI	SJW1135 (1679), SJW1136 (1680)	UD
flaFIX	SJW1144 (1688), SJW1182 (1726), SJW1339 (1764), SJW1154 (Δ-1698)	UD
flaFIV	SJ34 (33)	19, UD
	SJW1178 (1722), SJW1169 (Δ-1713)	UD
flaAI	SJ80 (37)	19, 50
flaAII	SJ35 (34), SJ482 (23), SL829 (57)	19, 50
(*motC*)	SJ600 (272), SJ608 (279)	13, 50
flaAIII	SL481 (22)	19, 50
flaB	SL821 (50), SL822 (59)	22
flaC	SJ31 (31), SJ81 (38)	19
	SL4119 (546), SL4122 (569)	47
flaD	SJ374 (42)	19
	SL831 (58)	22
flaE	SL825 (60)	22
	SW1154 (28)	19, 50
flaFII	SJW1117 (1661), SJW1162 (1706), SJW1165 (Δ-1709)	UD
flaFIII	SJW1325 (1750), SJW1179 (Δ-1723)	UD
flaFVI	SL4054 (586)	47, UD
	SJW1177 (1721), SJW1346 (1771)	UD
flaFVII	SL4124 (711)	47, UD
	SJW1121 (1665), SJW1148 (1692)	UD
flaFX	SJW1125 (1669), SJW1139 (1683)	UD
flaK	SJ400 (44)	19
	SL4044 (513), SL4118 (511)	47
flaM	SL4062 (682), SL4063 (699)	47

[a] The *flaF* cistron in reference 19 was further divided into at least 10 complementation groups, whose details will be published elsewhere.

[b] UD, Unpublished data (T. Horiguchi, S. Yamaguchi, T. Suzuki, and T. Iino).

fractionations of flagella or 20 ml for observations of osmotically shocked cells) was inoculated with 1/500 volume of the overnight culture and incubated at 37°C with gentle shaking (90 strokes per min). When the bacterial concentration reached about 10^8 cells per ml in exponential growth phase, 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 by the method of Suzuki and Iino (46). The remaining portion was used for fractionation of flagella or preparation of osmotically shocked cells.

Preparation of osmotically shocked cells. The method of Nossal and Heppel (33) was modified as follows. Bacteria harvested by centrifugation (8,000 × *g* for 7 min) were suspended in 10 ml of 0.03 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 7.4) containing sucrose (0.6 M). A 10-ml amount of the same buffer containing sucrose (0.6 M) and disodium ethylenediaminetetraacetate (EDTA, 2 mM) was added to the suspension. After incubation at 30°C with shaking (180 strokes per min) for 20 min, the suspension was centrifuged (8,000 × *g* for 7 min), and the pellet was rapidly dispersed in 10 ml of cold, distilled water. The resulting osmotically shocked cells were washed twice with cold distilled water by centrifugation (8,000 × *g* for 7 min) and finally suspended in 0.1 ml of distilled water.

Fractionation of flagellar basal structures. The method for fractionizing flagellar basal structures consisted of forming spheroplasts in the presence of EDTA and lysozyme, followed by detergent treatments and differential centrifugations. For preparing spheroplasts, the method of Osborn et al. (34) was modified as follows. Bacteria harvested by centrifugation (6,000 × *g* for 7 min) at 0°C were suspended rapidly in 10 ml of cold 0.1 M Tris-hydrochloride buffer (pH 8.0 at 0°C) containing sucrose (0.75 M) and lysozyme (150 μg/ml). The suspension was slowly

diluted with 18 ml of the cold 0.1 M Tris-hydrochloride buffer containing sucrose (0.5 M) and EDTA (4 mM) over a period of 8 to 10 min. This modification was necessary to protect intact flagella from falling out of the membranes. The suspension was then warmed to 30°C, kept for a few minutes at this temperature, and again chilled to 0°C.

To the suspension, 3.2 ml of 10% (wt/vol) aqueous solution of Brij-58 was added. The mixture became clear within a few minutes due to lysis of the spheroplasts. To decrease the viscosity of the lysate, 3.5 ml of 0.1 M $MgCl_2$ containing bovine (deoxyribonuclease) (0.2 mg/ml) was added, and the mixture was maintained at 0°C for 5 min, followed by incubation at 30°C for 3 min and chilling to 0°C. To the mixture, 3.5 ml of the 0.1 M Tris-hydrochloride buffer containing EDTA (0.1 M) was added to chelate Mg^{2+} ions. The resulting Brij-58 lysate was centrifuged at 30,000 × *g* for 120 min. The pellet thus obtained was termed fraction BMI. The pellet was then suspended in 15 ml of 0.1 M Tris-hydrochloride buffer (pH 7.8 at 25°C) containing EDTA (2 mM) and NaCl (0.2 M). The suspension was slightly turbid, but upon addition of 1.7 ml of 20% (wt/vol) aqueous solution of Triton X-100 (9, 36), it became clear immediately. This lysate was centrifuged at 18,000 × *g* for 40 min at 2°C to remove cell debris and degraded membraneous materials. The supernatant fraction was further centrifuged at 78,000 × *g* for 90 min. To the pellet, 2 ml of 10 mM Tris-hydrochloride buffer (pH 7.8 at 2°C) containing EDTA (2 mM) and 0.2 ml of Nonidet P-40 (5%, wt/vol) (17) was added. The centrifuge tube was left overnight at 2°C until the pellet was dispersed. The suspension was then diluted with 10 mM Tris-hydrochloride buffer containing EDTA (2 mM) and centrifuged at 78,000 × *g* for 120 min. The precipitated fraction at this stage was termed fraction BMII.

The supernatant solution obtained after the removal of fraction BMI from Brij-58 lysate was further sedimented at 100,000 × *g* for 120 min. The pellet thus obtained was termed fraction BSI. It was treated by essentially the same procedure used for the preparation of fraction BMII from fraction BMI, and the resulting pellet was termed fraction BSII.

Fraction BMII contained flagellar basal structures of nonflagellate mutants, and, although it was possible to study them by electron microscopy, further fractionations were necessary for their examination by antibody staining. Therefore, the following procedures were performed. Fraction BMI was suspended in 3 ml of the 0.1 M Tris-hydrochloride buffer containing EDTA (4 mM) and NaCl (0.2 M), and 2 ml of Triton X-100 (20%, wt/vol) was added. This lysate was centrifuged at 18,000 × *g* for 40 min to remove cell debris. In a tube for the Spinco SW27 rotor, 3 ml of 55% and 4 ml each of 50, 45, 40, and 35% (wt/vol) sucrose in 0.1 M Tris-hydrochloride buffer (pH 8.0) containing EDTA (2 mM) and Triton X-100 (0.5%, wt/vol) were overlaid in this order to form a density gradient, and the supernatant fluid, obtained as described above, was placed on it. Centrifugation was performed at 80,000 × *g* for 14 to 16 h at 5°C. After perforation of the bottom of the tube, 1-ml fractions were collected. Flagellar structures were detected in tubes 5 to 9. These fractions were pooled and dialyzed against 5 liters of 10 mM Tris buffer (pH 7.8) containing EDTA (2 mM) and NaCl (0.1 M) for 4 to 5 h at 2°C. The material in a dialysis bag was diluted about 15 ml of the same buffer and sedimented at 78,000 × *g* for 120 min, and the precipitating material was dispersed into 0.1 ml of 0.1 M NaCl to serve for staining with a specific antiserum.

Electron microscopy. Negative staining was carried out with 1% sodium phosphotungstate (NaPT, pH 7.0) according to the method of Brenner and Horne (5). The surface of carbon film on the mesh-grid was washed beforehand with 0.005% Diamiete 315 solution (polyoxyethylene stearylpropylenediamine in distilled water). By this procedure, the surface of carbon film was discharged. An antiserum against hook protein (23) was kindly given to us by H. Kagawa at the Institute of Molecular Biology, Noagoya University, Nagoya, Japan. The preparation of an antiserum against flagellin and the method of antibody staining were according to Asakura et al. (3).

Proportional counts were made to estimate the relative number of various structures in the fraction. 50 μl of styrene-butadiene latex spheres (87 ± 4.6 nm in diameter, prepared to 2.5×10^{11} spheres per ml in distilled water, Dow Chemical Co., Midland, Mich.) was added to a sample suspension in a centrifuge tube. The mixture was diluted with the addition of about 0.5 ml of 0.1% (wt/vol) NaPT and sprayed on the mesh grids.

The specimens were observed in a JEM 7A electron microscope, or in a JEM100C electron microscope equipped with a side-entry goniometer. Observations were performed with an accelerating voltage at 100 kV. A liquid nitrogen decontamination device was routinely used. Magnifications were calibrated using the 2,195-lines per mm spacing of a carbon replica grating. Micrographs were taken of osmotically shocked cells at magnifications between ×5,000 and ×20,000, and of fractionated materials between ×26,000 and ×100,000.

For quantitative analysis of fractionated materials, 5 to 30 micrographs were taken on droplets of the sample, and several mesh-grids were examined. From the ratio of the number of a specific type of particles to the number of latex spheres in the micrographs, the total number of the type of particles recovered in a fraction was calculated. The coefficient of variation in the determination of the number (25) was around 30%. The ratio of the number of a specific type of particles in a nonflagellate mutant to the number of intact flagella recovered in its flagellate parent was then calculated.

RESULTS

Detection of hooks in some nonflagellate mutants by examination of osmotically shocked cells. The difficulty of observing flagellar hooks in the intact cells of flagellate strains was overcome by the observation of osmotically shocked cells that had been negatively stained with NaPT (Fig. 1). Basal parts of flagella were observable in the cell surfaces both close to and away from the supporting film. Therefore, the

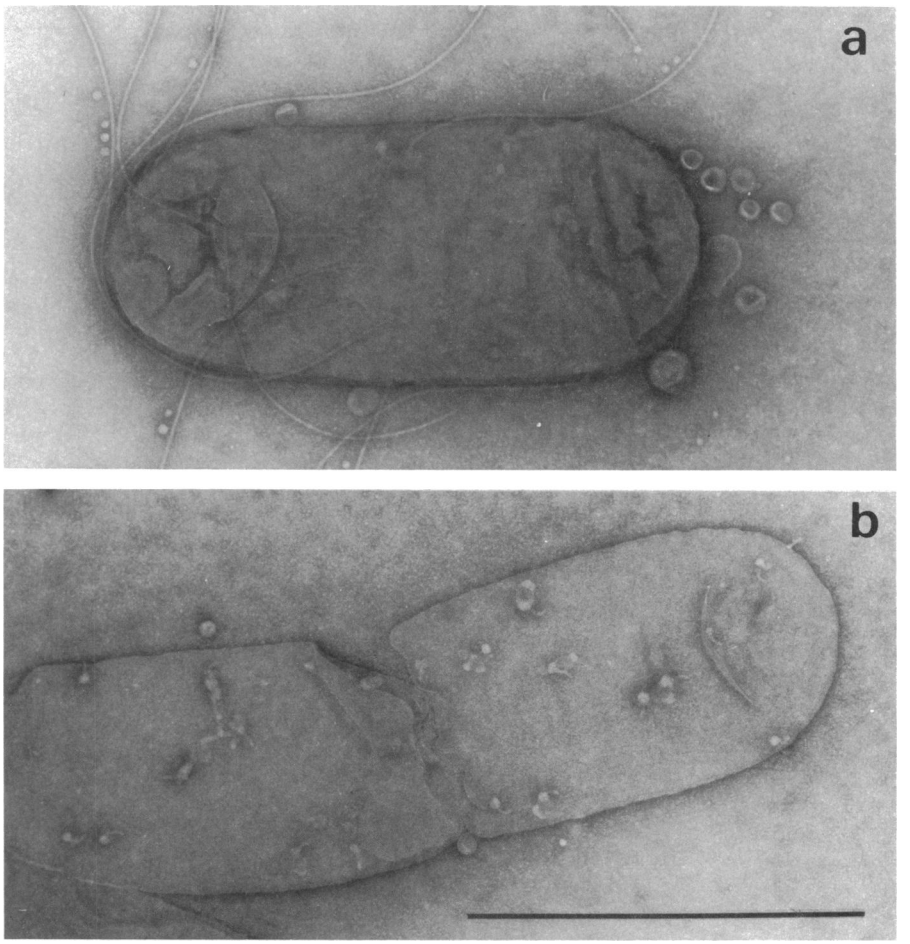


FIG. 1. *Osmotically shocked cells of a flagellate strain, LT2 (a), and a flaL mutant, SJW801 (b), S. typhimurium. Hooks are observable on their cell surface. Negatively stained with NaPT. Bar, 1 μm.*

number of hooks per cell could be counted.

By examination of osmotically shocked cells, the nonflagellate mutant defective in the flagellin genes or the *flaR* mutants were found to carry hooks or polyhooks, respectively. These results conform with previous reports (35, 51). Furthermore, hooks were observed on all of the *flaL* mutants. They had not previously been detected by the observation of intact cells or of the materials detached from these bacteria by mechanical shaking (51). A nonsense motility mutant, SL4060, which was newly assigned as a *flaU* mutant, was also found to carry hooks. The distribution of the number of hooks or polyhooks per cell in these mutants was indistinguishable from that of flagella in their flagellate parents, LT2 or SJW797 (Table 2).

In the remaining nonflagellate mutants, no hooks were detected.

Observations of fractionated materials in the flagellate parents. Observations were made on the fractions prepared from the flagellate parent strains, LT2 and SJW797. Fraction BMI contained fragments of outer and inner membranes, as well as intact flagella (IF) bound to both or either one of these membranes. These two strains were found to carry few pili, which were collected in this fraction. The treatment of fraction BMI with Triton X-100 resulted in the solubilization of membranes and the release of IF from them (9, 36). About 80 to 90% of the flagella counted in the intact cells were found to be recovered as IF in fraction BMII (Fig. 2). As minor constituents, there were several types of particles homologous to the basal parts of IF. The nomenclature of the component structures of IF was that of DePamphilis and Adler (11).

We have used three-letter designations for structures observed electron microscopically. HOB and BAB particles were homologous to

TABLE 2. *Number of hooks in osmotically shocked cells of flagellate parents and nonflagellate mutants*

Strain	Total no. of cells examined	Avg	Counts per cell		
			Maximum	Minimum	Mode
LT2 (parent)[a]	53	10	19	4	9
SJW797 (parent)[a]	19	9	19	2	7
SJW900 (*H1*$^-$, *H2*$^-$)	20	9	18	4	8
SJW801 (*flaL*)	35	10	19	2	8
SL4060 (*flaU*)	25	8	15	3	10
SJW880 (*flaR*)[b]	24	10	18	4	8
SL4055 (*flaR*)[b]	52	8	12	1	7

[a] Both hooks and filament hooks were counted.
[b] Both polyhooks and apparently normal-length hooks were counted.

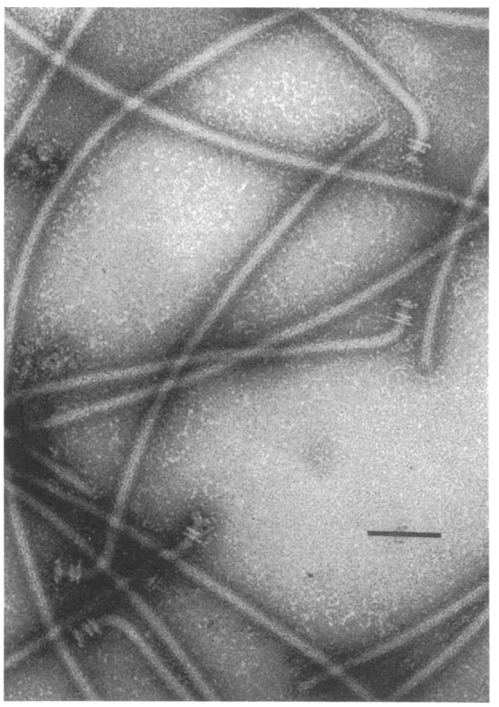


FIG. 2. *IF isolated from a flagellate parent strain, SJW797, of S. typhimurium. Negatively stained with NaPT. Bar, 100 nm.*

the hook-basal body complex and the basal body of IF, respectively. An RIV particle, whose overall shape resembled a rivet, was homologous to the M ring-S ring-rod complex of IF. A CAS particle, which appeared like a candlestick, was detected less frequently. It was homologous to the M ring-S ring-rod-P ring complex of IF.

Fraction BSI contained fragmented outer and inner membrane components. IF were present infrequently. When present, they always attached to small fragments of membranes.

Fraction BSII contained vesicles of outer and inner membranes and a minor fraction of IF or fragmented flagellar filaments.

Examination of the fractionated materials in the nonflagellate mutants. The procedure for the fractionation of IF was applied to various nonflagellate mutants, and the fractions obtained were examined by electron microscopy. Structural entities homologous to the flagellar basal structures or their parts were detected in a number of nonflagellate mutants. They were predominantly found in fraction BMII and scarcely detectable in fractions BSI and BSII. When a structure detected in the fraction of a nonflagellate mutant appeared to be homologous to a particle in the fraction of flagellate parent strains, it was given the same designation as the homologous particle of the latter. The frequency of occurrence of these structural entities was examined in fraction BMII of various nonflagellate mutants and compared with the frequency of IF in flagellate parents (Table 3).

The mutants defective in the flagellin genes, *flaL* or *flaU*, which were shown to contain hooks by the observations of osmotically shocked cells, were found to contain HOB particles (Fig. 3). Their frequencies were comparable with those of IF in the flagellate parents. These HOB particles were not different from the basal structures of IF and the HOB particles in their flagellate parents in gross morphology and dimension.

Specimens from a deletion mutant defective in the *flaFV* cistron, SJW1160, contained RIV and rectangular structures (RCT particles). Structures homologous to RCT have been detected by DePamphilis and Adler (10, 11), when IF of *E. coli* were degraded by mechanical pressure or urea treatment. They suggested that these rectangular structures may be identical with L ring-P ring complexes. The RCT particle was frequently attached to or near the tips of rods of the RIV particle (Fig. 4). These RCT and RIV particles were presumed to be produced by artifical breakages of BAB during sample preparations, because the number of RIV was almost equal to the number of RCT in a fraction

TABLE 3. *Frequencies of flagellar structures detected in nonflagellate mutants*[a]

Determination	Structural entity								
	IF	HOB	BAB	CAS	RIV	RCT	HCS	HRV	HOC
Flagellate parent	++[b]	+[c]	+	+ or −[d]	+	+ or −	−	−	−
H1⁻, H2⁻	−	++	+	+ or −	+	−	−	−	−
flaL	−	++	+	+ or −	+	−	−	−	−
flaU	−	++	+	+ or −	+	−	−	−	−
flaR	−	++[e]	+	+ or −	+	−	−	−	−
flaFV	−	−	+	+	++	++	−	−	−
flaFVIII	−	−	−	+	+	−	++	+ or −	+
flaFI	−	−	−	−	++	−	−	++	−
flaFIX	−	−	−	−	++	−	−	++	−
flaFIV	−	−	−	++	+	−	−	−	−
flaAI	−	−	−	−	−	−	−	−	−
flaAII	−	−	−	−	−	−	−	−	−
(*motC*)	++[f]	+	+	+ or −	+	−	−	−	−
flaAIII	−	−	−	−	−	−	−	−	−
flaB	−	−	−	−	−	−	−	−	−
flaC	−	−	−	−	−	−	−	−	−
flaD	−	−	−	−	−	−	−	−	−
flaE	−	−	−	−	−	−	−	−	−
flaFII	−	−	−	−	−	−	−	−	−
flaFIII	−	−	−	−	−	−	−	−	−
flaFVI	−	−	−	−	−	−	−	−	−
flaFVII	−	−	−	−	−	−	−	−	−
flaFX	−	−	−	−	−	−	−	−	−
flaK	−	−	−	−	−	−	−	−	−
flaM	−	−	−	−	−	−	−	−	−

[a] The frequencies of IF and flagellar basal structures detected in fraction BMII of flagellate parents and nonflagellate mutants are shown.

[b] ++, The count of each structural entity from ⅕ to 5× the count of IF in its flagellate parent.

[c] +, The count of each structural entity from 1/250 to ⅕ the count of IF in its flagellate parent.

[d] −, The count of each structural entity less than 1/250 the count of IF in its flagellate parent.

[e] Polyhook basal body complexes were detected.

[f] Paralyzed flagella, which were not discriminated morphologically from the IF of their flagellate parent.

and the frequency of occurrence of BAB particles varied among the different preparations. In addition, the information obtained from micrographs made by tilting specimen grids with the goniometer supported these presumptions; the inclined image of an RCT appeared to be a cylinder with a hole in the center, and the tilted image of a cylinder consisting of two rings revealed an RCT particle (Fig. 5). The sum of the numbers of BAB and either RIV or RCT was comparable to the number of IF in the parent, SJW797. As a minor constituent, CAS particles were also detected. These four types of particles were not labeled with the antiserum prepared against hook protein. The other mutants defective in *falFV* showed the same phenotype with that of SJW1160.

In the fraction of *flaFIV* mutants, including a deletion mutant, SJW1169, CAS particles were detected. The number of CAS was almost equal to that of IF in their flagellate parents. RIV were present as a minor constituent, but RCT were not detected. These CAS and RIV particles did not react with the antiserum against hook protein. CAS particles in these mutants tended to adhere top over tail to one another on the specimen grids (Fig. 6). The mutual adhesion of CAS was decreased by the addition of bovine serum albumin (0.001%, wt/vol) to the suspension.

The fraction of *flaFVIII* mutants contained the particles similar to CAS. However, these particles, designated as HCS, carried hazy or cloudy structures at the tips of rods (Fig. 7). Furthermore, other types of particles existed. One of them was similar to BAB but lacked the structure homologous to a cylinder filling up the space between L ring and P ring (OCY). The particles of this type also carried cloudy structures at the tips of their rods and were named HOC. Particles that were similar to RIV but carried hazy structures at their tips of rods were detected less frequently and were designated as HRV. Hazy structures in HCS, HOC, and HRV were stained by the antiserum against hook protein, but not stained by the antiserum against flagellin (Fig. 7d). RIV were detected as minor constituents, whereas CAS existed almost at the

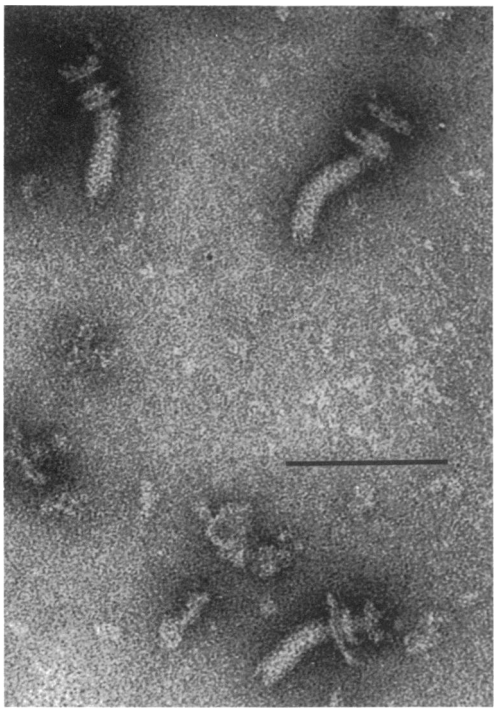


FIG. 3. *HOB particles detected in the fraction of a flaL mutant, SJW801. Homologous particles were also observable in the nonflagellate mutants defective in flagellin genes or flaU. Negatively stained with NaPT. Bar, 100 nm.*

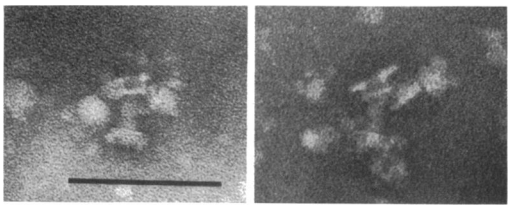


FIG. 4. *BAB particles in a flaFV mutant, SJW1160, of S. typhimurium. P ring-OCY-L ring complexes are often observed to be detached from rods. These complexes have been described by DePamphilis and Adler (10) and are designated as RCT. Bar, 100 nm.*

same frequency as HCS. The structures similar to IF but lacking L ring-OCY portions were also present (Fig. 7c). Their frequency of occurrence was always less than 1/500 the frequency of HCS particles. HCS and CAS particles were the predominant entities in *flaFVIII* mutants, and the sum of the numbers of HCS, HOC, and CAS was comparable to the number of IF in the flagellate parents.

FlaFI or *flaFIX* mutants contained two types of particles, RIV and HRV (Fig. 8). HRV and RIV were present at equal frequencies, and the sum of these two was comparable with the number of IF in their flagellate parent. Structures similar to IF but lacking L ring-OCY-P ring portions were also detected at a frequency of occurrence less than 1/500 the frequency of RIV and HRV.

The mutants defective in the cistron *flaAI, flaAIII, flaB, flaC, flaD, flaE, flaFII, flaFIII, flaFVI, flaFVII, flaFX, flaK,* or *flaM* contained none of the electron microscopically detectable basal structures of flagella in any fraction.

Among *flaAII* mutants, some were flagellate but paralyzed (13, 50). In these mutants, no significant difference was detected in gross morphology of the basal structure, compared with that of the IF of their motile parent. Similar results have been reported by DePamphilis and Adler (10) or Hilmen et al. (14) on the paralyzed or nonchemotactic mutants of *E. coli*. Although present nonflagellate *flaAII* mutants, none of the particles homologous to flagellar basal structures or their parts were detected.

DISCUSSION

In the present study, incomplete flagellar structures specific for each mutant cistron were detected by electron microscopy in the nonflagellate mutants of *S. typhimurium*. Under the following assumptions, it is possible to infer the process of flagellar morphogenesis and the cistron responsible for each step (Fig. 9): (i) the more complex basal structure is a structural entity appearing later in the process of the morphogenesis than simpler ones; (ii) when a precursor structure is present in a mutant at almost the same frequency as that of IF in its flagellate parent, the function of the cistron, to which the mutant site belongs, is indispensable for the step proceeding to the next more complex structure.

The step blocked by the mutation of *flaFI* or *flaFIX* apparently appears to lie between RIV and CAS, and that of *flaFIV* and *flaFVIII* lies between CAS and BAB. However, amorphous hooks were detected in the mutants of *flaFI, flaFIX,* and *flaFVIII,* but not of *flaFIV*. The absence of amorphous hooks in the *flaFIV* mutants suggests that the function of *flaFIV* is necessary for the attachment of hook protein at the tips of rods as well as the formation of L ring-OCY portions, and that the step of the function is before the steps blocked by the mutations of *flaFI, flaFIX,* or *flaFVIII*. A plausible hypothesis to explain such multiple functions of *flaFIV* is that the cistron modifies the rods of RIV particles so as to permit the joining of L rings, OCY, and hooks to them. If this is the case, RIV particles in the *flaFIV* mutants must

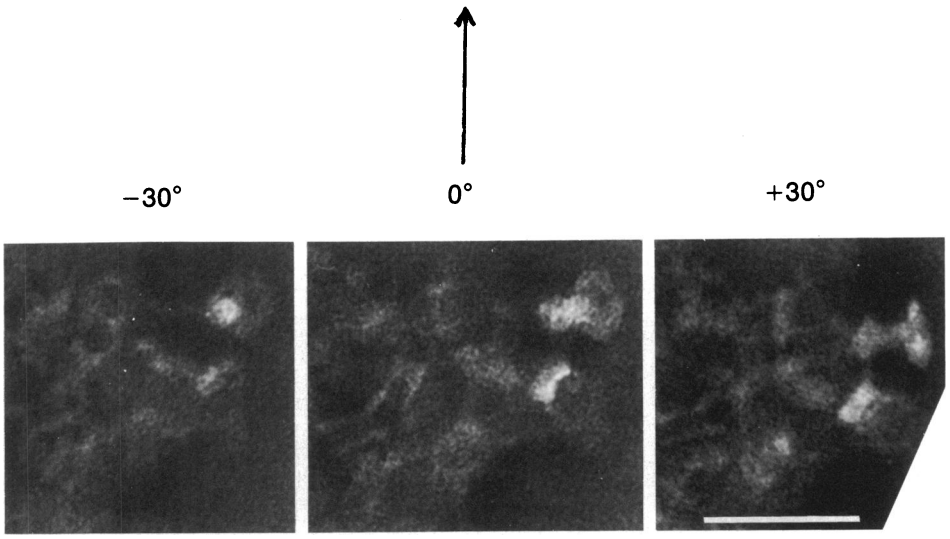


FIG. 5. *Particle images showing the transition from left-featured frontal to right lateral images of RCT and RIV particles detected in a falFV mutant, SJW1160, of S. typhimurium. Images at a negative angle (−30°) are left-featured frontal and those at a positive angle (+30°) resemble right lateral images. Negatively stained with NaPT. Bar, 50 nm.*

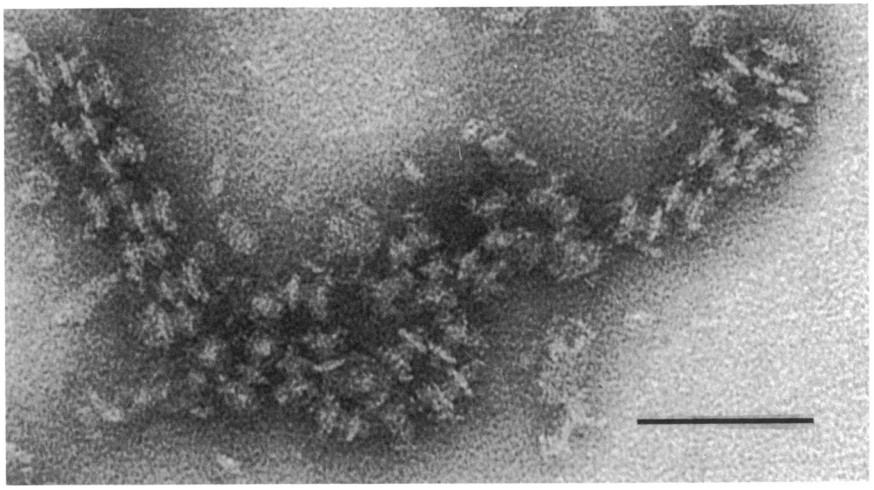


FIG. 6. *CAS particles in a flaFIV mutant, SJW1169, of S. typhimurium. Negatively stained with NaPT. Bar, 100 nm.*

be different from those in the *flaFI, flaFIX,* and *flaFVIII* mutants, although they are morphologically indistinguishable by electron microscopy. These two types of RIV were discriminated in Fig. 9 as RIVo for nonmodified and as RIVm for modified. Similarly, the CAS-type particles in the *flaFIV* mutants are named CASo, indicating the absence of the modification of their rods, and those already having been modified at their rods in the *flaFVIII* mutants are named CASm. CASo are inferred to result from bypassing the pathway to RIVm (Fig. 9). For the present, an alternative explanation that can not be excluded is that *flaFIV* simultaneously modifies the components of hooks and OCY and probably L rings to permit their association with rods. If this is the case, the discrimination of the two types of RIV or CAS is not necessary.

The step blocked by the mutation of *flaFI* or *flaFIX* seems to occur between RIVm and CASm (Fig. 9), suggesting that these cistrons

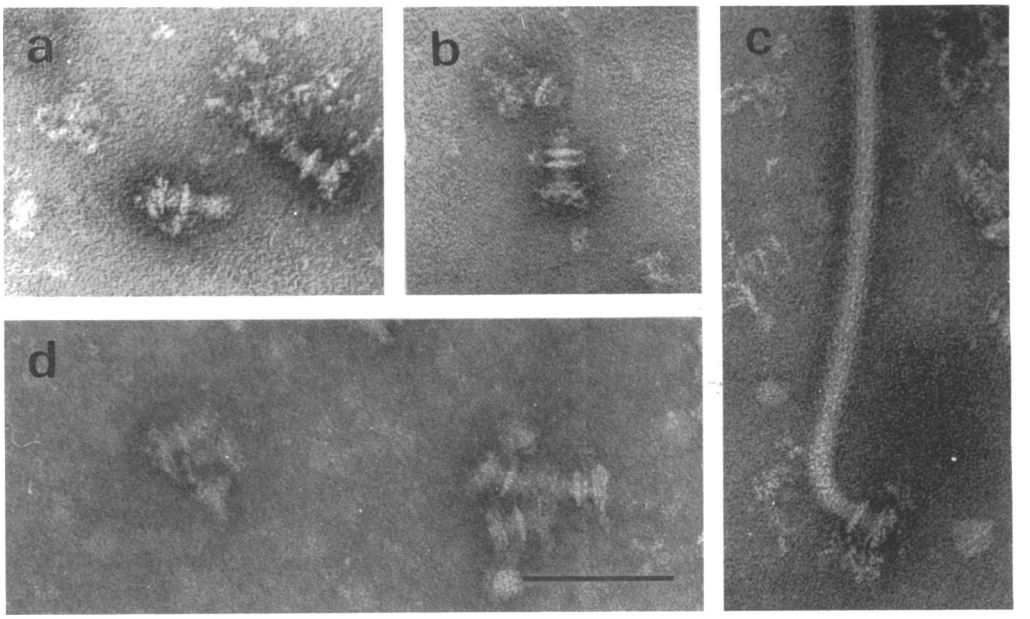
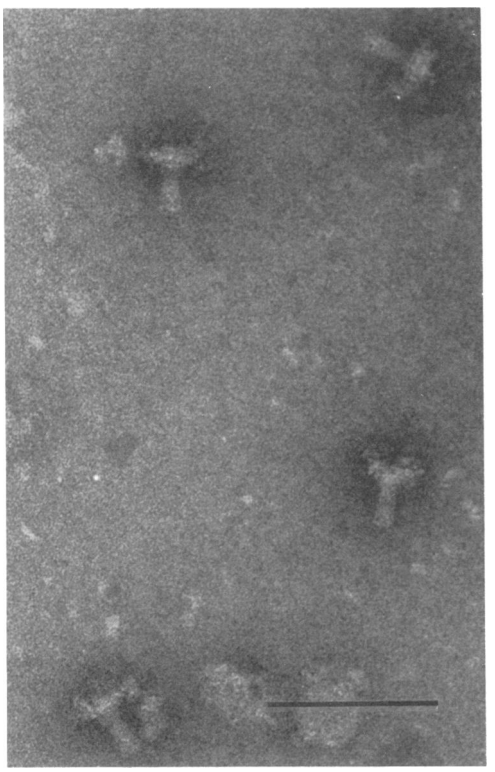


FIG. 7. *Particles detected in a flaFVIII mutant, SJW1155, of S. typhimurium. HCS particles (a) and HOC (b) are characterized by the presence of hazy structures at the tips of their rods. These puffed, hazy structures were stained by a specific antiserum against hook protein (d). Flagella lacking L ring-OCY portions, whose frequency of occurrence is less than 1/500 the frequency of HCS, were also detected (c). Negatively stained with NaPT. Bar, 100 nm.*



participate in the formation of P rings. The P ring of a flagellar basal body has been suggested to be associated with the peptidoglycan layer of bacterial cells (8, 11). These two cistrons may be responsible for either the synthesis of P ring components or the interaction of the components with the peptidoglycan layer.

The step blocked by the mutation of *flaFVIII* is located between CASm and BAB (Fig. 9). This cistron may be primarily responsible for the formation of OCY, L rings, or both. The low frequency of occurrence of HOC structures may be due to the leakiness of the mutant phenotype.

In *flaFV* mutants, the step between BAB and HOB is blocked. This cistron is, therefore, inferred to be necessary for the formation of hooks. The efficient and complete formation of hooks may require the completion of P ring-OCY-L ring portions, as suggested by the existence of amorphous hooks in *flaFI, flaFVIII,* or *flaFIX*

FIG. 8. *Particles detected in a flaFIX mutant, SJW1154, of S. typhimurium. RIV and HRV were detected. Flagella without P ring-OCY-L ring complexes less than 1/500 the frequency of occurrence of RIV and HRV were also detected. Homologous structures were also detected in flaFI mutants. Negatively stained with NaPT. Bar, 100 nm.*

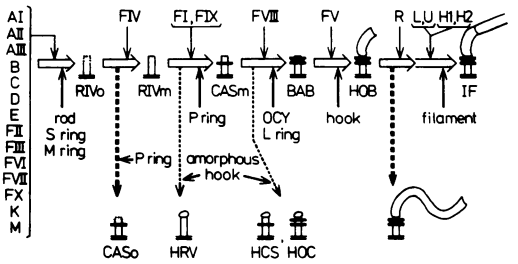


FIG. 9. *Hypothetical scheme of the pathway of flagellar morphogenesis. The step blocked by a mutation in each cistron (represented by letters) is shown by an arrow. The structures to the left of each blocked step represent the complexes formed in that mutant with almost the same frequency as the IF in their parent, when the corresponding fla cistron becomes defective due to mutation.*

mutants. *Salmonella* hooks have been reported to be composed of a single kind of protein (23). In some temperature-sensitive nonflagellate mutants in the *flaFV* cistron, abnormally shaped hooks have been detected (K. Kutsukake, T. Suzuki, H. Kagawa, and T. Iino, unpublished data). These facts strongly suggest that *flaFV* is the structural gene for the hook protein in *S. typhimurium*.

It has been suggested that *flaR* cistron controls the length of hooks (14, 35). There has been no report on the chemical difference of the components between the polyhooks and the normal hooks of flagellate parents. However, a possibility exists that minor components or tenuously bound ones are present in the hook structure and the lack of these components brings about the uncontrolled growth of hooks, as has been demonstrated for the polytube and the polytail in the assembly of the tails of coliphage lambda (24).

Both *flaL* and *flaU* are indispensable for the formation of flagellar filaments at the tips of hook-basal body complexes. Flagellin is supplied by the structural gene *H1* or *H2*. Because mRNA specific for flagellin is absent in *flaL* mutants, this cistron may participate in the transcription of the flagellin genes (45). Whether *flaU* regulates the synthesis of flagellin or the formation of flagellar filaments is not known.

An interesting phenomenon is the rare presence of flagellar filaments in *flaFI, flaFVIII,* and *flaFIX* mutants. In spite of the absence of P ring-OCY-L ring portions in *flaFI* or *flaFIX* mutants and the absence of OCY-L ring portions in *flaFVIII* mutants, the filaments appear when normally shaped hooks are formed at the tips of rods. This indicates that the formation of flagellar filaments proceeds whenever structurally normal hooks are formed even if some portions of basal bodies are missing.

Up to the formation of the simplest detected structure, RIV, functions of *flaAI, flaAIII, flaB, flaC, flaD, flaE, flaFII, flaFIII, flaFVI, flaFVII, flaFX, flaK,* and *flaM* are inferred to be essential: the mutants in this early-*fla* group do not posses any flagellar structures detectable by electron microscopy. Some of these cistrons may be required for the synthesis of the components of RIV particles or the catalytic functions in their formation. Some mutants defective in cell surface formation, namely, deep rough mutants of *S. typhimurium* (2, 21) and *galU* mutants of *E. coli* (26), were found to lose, simultaneously, the ability of flagellar formation. These observations suggest that flagellar basal bodies are formed in association with cell envelopes. Therefore, some cistrons of this early-*fla* group may function in the interactions between the components of basal bodies and cell envelopes. The coordination of the formation of bacterial flagella with cell cycle has been reported in *Caulobacter crescentus* (37) and DNA replication and division mutants of *E. coli* (A. Nishimura, H. Suzuki, and Y. Hirota, unpublished data). Therefore, it is also possible that some of these cistrons participate in the initiation of flagellar formation through the synthesis of the regulatory factors coupled with a step of the cell cycle.

It must be stressed that the phenotype of *flaAII* is not cistron specific, but is mutant specific: some mutants are unable to form flagellar structures at all, while others form paralyzed or motile but nonchemotactic flagella (7, 13, 48, 50). Bacteria swim by rotating their flagellar filaments (4, 42). Chemotaxis is brought about by the change in direction of flagellar rotation (1, 30). The components of the basal structure are inferred to be involved in these phenomena (1, 4, 7). The *flaAII* cistron is suggested to be necessary for both rotary function and morphogenesis of flagella associated with inner rings and rods.

There are several limitations to the use of electron microscopy for inferring the process of flagellar morphogenesis as described in this paper. For example, the step in which a minor structural component is produced is liable to be overlooked, or that in which structural components other than IF are produced is not definitely discriminated. In addition, the regulator gene and the structure gene of a step are not always identified with each other. The identification of the *fla* gene products has been performed, using lambda or colE1 carrying *fla* genes in *E. coli* (31, 38). The combination of the identification of *fla* gene products, chemical identi-

fication of the structural entities of a flagellar base, and the electron microscopical studies used in this work promise clarification of the refined process of flagellar morphogenesis.

The present data indicate that the flagellar morphogenesis proceeds at the bacterial cell envelope from the inner to the outer portion. This means that flagellar components are transported from the sites of their synthesis to the specific sites of the cell surface layers, in some cases passing through the cell envelope. This feature is distinct from that which occurs in the formation of bacteriophages or bacterial ribosomes in cytoplasm (6, 32). The studies of bacterial flagella may be expected to yield unique insights in the investigation of the intracellular regulatory system of protein transportation.

ACKNOWLEDGMENTS

This work was supported by research grants 144003, 111403, and 744001 from the Ministry of Education, Science and Culture, Japan. T.S. is indebted to the Japan Society for the Promotion of Science for a postdoctoral fellowship during the present work.

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