JOURNAL OF BACTERIOLOGY, Jan. 1984, p. 100–108
0021-9193/84/010100-09$02.00/0

Vol. 157, No. 1

Hook-Associated Proteins Essential for Flagellar Filament Formation in *Salmonella typhimurium*

MICHIO HOMMA,[1]* KAZUHIRO KUTSUKAKE,[1] TETSUO IINO,[1] AND SHIGERU YAMAGUCHI[2]

Laboratory of Genetics, Faculty of Science, the University of Tokyo, Hongo, Bunkyoku, Tokyo 113,[1] and Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku-ku, Tokyo 160,[2] Japan

Received 26 May 1983/Accepted 30 September 1983

The hooks of the flagella of *Salmonella typhimurium* were purified by a newly developed method, using a *flaL* mutant without a filament, and the hook components were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As a result, we detected three protein species in addition to hook protein. We call these three proteins hook-associated proteins (HAPs). Their molecular weights were 59,000 for HAP1, 53,000 for HAP2, and 31,000 for HAP3. The HAP1/hook protein/HAP3/HAP2 molar ratio, calculated from their relative amounts and their molecular weights, was 1:10:1.1:0.53. The compositions of HAPs were analyzed in the hooks from the other filamentless mutants which were defective in *Hl H2*, *flaV*, *flaU*, or *flaW*. Hooks from the *Hl H2* mutant had the same HAP composition as hooks from the *flaL* mutant. Hooks from the *flaV* mutants contained HAP1 and HAP3. Hooks from the *flaU* mutants contained HAP1. Hooks from the *flaW* mutants contained a very small amount of HAP3. From these results, the process of hook morphogenesis and the genes responsible for each step were postulated. Electron micrographs of hooks from the filamentless mutants showed that hooks which contained all three HAPs had a sharp clawlike tip, whereas hooks lacking any HAP had a flat tip. Electron micrographs of hooks treated with antibody against the hook protein showed that each claw-shaped end was not covered with antibody. These results strongly suggest that all three HAPs or at least some of them are located at the claw-shaped end and play an essential role in filament formation.

A bacterial flagellum consists of three distinct parts: a basal body, a hook, and a filament (1, 3–6, 15, 16). The filament, which extends from the cell body, is connected by the hook to the basal body, which is embedded in the cell surface layers (6, 43). The formation of bacterial flagella has been studied as a model system for elucidating genetic regulatory mechanisms in relation to the molecular basis of cellular morphogenesis (9, 20, 37).

The assembly pathway of a flagellum and the genes responsible for each step in assembly have been postulated from observations of incomplete flagellar structures which were detected on osmotically shocked cells or membrane-associated fractions of various nonflagellate mutants by electron microscopy of *Salmonella* species (41) and *Escherichia coli* (42). At the final step of the pathway, flagellin molecules, i.e., the component protein of the flagellar filament, polymerize one by one at the tip of the hook, and the filament is constructed (11, 18, 19, 40). The mutants, in which this final step is blocked, produce a hook-basal body structure missing the filament (41, 42). The genes responsible for this step are *flaL*, *flaU*, *flaW*, *flaV*, and *Hl H2* in *Salmonella* (41; Yamaguchi, unpublished data). It is thought that the functions of these genes belong to one of the following categories: (i) synthesis of flagellin, (ii) transport of flagellin to the tip of the hook, or (iii) polymerization of flagellin at the tip of the hook. Because *Hl* and *H2* are the structural genes for flagellin (17) and flagellin mRNA is not detected in a *flaL* mutant (39), *Hl* and *H2* belong to category (i). The functions of other genes, namely, *flaW*, *flaV*, and *flaU*, have not been elucidated. We considered the possibility that these genes were responsible for the production or regulation or both of some hook-associated materials essential for the filament formation. Although it was reported that the hook was composed of a single kind of protein (8, 23, 34, 36), we reexamined the composition of the hook, developing a new method for the purification. As a result, we detected hook-associated proteins (HAPs) in the hook structure, and the functions of *flaW*, *flaV*, and *flaU* were correlated to HAPs.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and phage. All of the nonflagellate mutants of *Salmonella typhimurium* used in the present study are listed in Table 1. Bacteriophage used for transduction was P22L4 (38).

Media. L broth contained (per liter of distilled water): tryptone (Difco Laboratories), 10 g; yeast extract (Difco), 5 g; and NaCl, 5 g. L agar plates contained 1.2% agar in L broth. Semisolid agar plates contained 0.25% agar in L broth.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (29).

Proteins in the gel were stained with 0.1% Coomassie brilliant blue R250 in methanol-acetic acid-water (5:1:4) and destained in methanol-acetic acid-water (0.5:0.7:8.8). The destained gel was scanned at 575 nm by a densitometer (Joyce-Loebl Chromoscan200/201).

Isolation of hooks from a filamentless mutant. A culture of bacteria was grown to late-log phase in 30 liters of L broth. The cells were collected by centrifugation and suspended in 150 ml of TN-buffer (50 mM Tris-hydrochloride, pH 7.8, 0.5% NaCl). Hooks were removed from cells by blending ca. 25 ml of suspension in the homogenizer (Polytoron, type PT10/35; Kinematica) under conditions where the rotation speed dial was set at 8 for 5 min. The cells were sedimented by centrifugation at 10,000 × *g* for 20 min, and the superna-

TABLE 1. *Salmonella* strains used

Strain	Relevant genotype	Reference
LT2	*H1*-i *H2*-1,2 *vh2*$^+$	21
SJW797	*H1*-gt *H2*-enx *vh2*-(*H2*-off)	12
SJW1103	*H1*-i Δ*H2*	12
SJW900	*H1*-gt2187 Δ*H2*	41
SJW800	*flaL1650 H1*-gt *H2*-enx *vh2*$^-$ (*H2*-off)	41
SJW1411	Δ(*flaV-H1-flaL*) of SJW1103	S. Yamaguchi, unpublished
SJW2149	*flaV2380* of SJW1103	S. Yamaguchi, unpublished
SJW2150	*flaV2381* of SJW1103	S. Yamaguchi, unpublished
MH101	*flaU655*(Am) of SJW797	This study
KK2078	*flaU*::Tn*10* of LT2	K. Kutsukake, unpublished
SJW2160	*flaW2391* of SJW1103	S. Yamaguchi, unpublished
SJW2177	*flaW2408* of SJW1103	S. Yamaguchi, unpublished
SL4049	*H1*-i *H2*-enx *vh2*-(*H2*-off)	44; S. Yamaguchi, unpublished
SL4060	*flaU655*(Am) of SL4049	44; S. Yamaguchi, unpublished
SL4055	*flaR598*(Am) of SL4049	33
SJW880	*flaR1656 H1*-gt *H2*-enx *vh2*-(*H2*-on)	45

tant fluids were pooled. The sedimented cells were resuspended in 100 ml of TN-buffer. Hooks still attached to the cell bodies were removed by blending the suspension under the conditions described above. The resulting suspension was centrifuged again and the supernatants were pooled. These procedures were repeated twice. The pooled supernatants were centrifuged at 78,000 × *g* for 90 min. The pellet was suspended in 90 ml of TNE-buffer (50 mM Tris-hydrochloride, pH 7.8, 0.6 M NaCl, 2.5 mM EDTA) and then 10 ml of 20% Triton X-100 was added to the suspension. The mixture was incubated at 4°C for 30 min with shaking and then centrifuged at 15,000 × *g* for 15 min. The supernatant was centrifuged again at 78,000 × *g* for 90 min. The pellet was suspended in 1.5 ml of 10 mM Tris-hydrochloride (pH 7.8), and the suspension was centrifuged at 15,000 × *g* for 15 min. The supernatant was called the crude hook fraction.

Purification of hooks. The crude hook fraction was loaded onto a DEAE-cellulose (Whatman DE52) column (1.3 by 4.5 cm) which was equilibrated with 10TT-buffer (10 mM Tris-hydrochloride, pH 7.8, 0.2% Triton X-100) containing 0.04 M NaCl. The column was washed with 10TT-buffer containing 0.04 M NaCl at a flow rate of 12 ml/h and eluted at 12 ml/h with an 80-ml linear gradient of 0.04 to 0.3 M NaCl in 10TT-buffer. Each 2-ml fraction was collected. These fractions were observed by electron microscopy and analyzed by SDS-PAGE to determine which fractions contained hooks. The fractions containing hooks were pooled and then centrifuged at 78,000 × *g* for 90 min. The pellet was suspended in 0.5 ml of TN-buffer. The suspension was layered on 15 ml of 5 to 30% linear sucrose gradient in 50 mM Tris-hydrochloride (pH 7.8) containing 0.3 M NaCl, 2.5 mM EDTA, and 0.2% Triton X-100 in a nitrocellulose tube for the Beckman SW27 rotor. Centrifugation was carried out for 7 h at 25,000 rpm. The gradient was collected in about 30 fractions from the top. Portions of the fractions were analyzed by SDS-PAGE. The fractions containing hooks were collected and diluted 1/10 with TN-buffer. Hooks were sedimented by centrifugation at 78,000 × *g* for 7 h, and the pellet was suspended in 0.5 ml of TN-buffer.

Preparation of the antihook antibody. Polyhooks were prepared from strain SJW880 by the method of Kutsukake et al. (28). The purified polyhooks were dissociated by acid treatment, and the resulting monomer protein was purified by DEAE-cellulose chromatography by the method of Kagawa et al. (23) and used as the antigen.

A 0.1-mg sample solution of the purified hook protein in Freund complete adjuvant was injected into about 10 sites in the footpads of a rabbit and injected again into the back of the rabbit after 2 weeks. The rabbit was bled 2 weeks after the second injection. After clotting of the blood, serum was collected by centrifugation. Saturated $(NH_4)_2SO_4$ solution was added to the serum to 40% saturation. After 30 min at 0°C with stirring, the resulting precipitate was collected by centrifugation at 10,000 × *g* for 15 min. The pellet was suspended in a two-thirds volume of distilled water against the original volume of the serum, and saturated $(NH_4)_2SO_4$ solution was added to the suspension to 33% saturation. After 30 min at 0°C with stirring, the resulting precipitate was collected by centrifugation at 10,000 × *g* for 15 min. The pellet was suspended in one-third volume of distilled water against the original volume of the serum. The suspension was dialyzed against 10 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl.

Preparation of hook-basal body structure. Hook-basal body structure was prepared by the method of Suzuki et al. (41) with the following modifications. A 1-liter culture of bacteria was grown to exponential phase in L broth. The cells were sedimented by centrifugation and suspended in 120 ml of 0.1 M Tris-hydrochloride (pH 7.8) containing 0.75 M sucrose. Then, 2 ml of a 20 mg/ml solution of lysozyme was added into the suspension, and the mixture was incubated for 60 min at 30°C without shaking. Spheroplasts thus formed were lysed by adding 20 ml of a 10% aqueous solution of Brij-58, and then 0.8 ml of a 10 mg/ml solution of DNaseI and 0.8 ml of 1 M $MgSO_4$ were added. The mixture was incubated at 30°C for 5 min, and 4 ml of 2.5 mM EDTA was added. The resulting Brij-58 lysate was centrifuged at 30,000 × *g* for 90 min. The pellet was suspended in 80 ml of TNE-buffer, and 16 ml of 20% Triton X-100 was added. After 30 min at 4°C with shaking, the suspension was centrifuged at 18,000 × *g* for 30 min. The supernatant was centrifuged at 78,000 × *g* for 90 min. The pellet was suspended in 2 ml of 10 mM Tris-hydrochloride (pH 7.8) containing 1% Triton X-100 and 2.5 mM EDTA, and then 18 ml of distilled water was added into the suspension. The mixture was centrifuged at 18,000 × *g* for 30 min, and the supernatant was centrifuged at 78,000 × *g* for 90 min. The pellet was suspended in 0.1 ml of distilled water and observed by electron microscopy.

Electron microscopy. Negative staining was carried out as follows. A 5-μl sample was put on a carbon-coated 400 mesh copper grid and then 5 μl of 1% potassium phosphotungstate (pH 7.2) or 1% uranyl acetate (pH 4.2) was added. Excess fluid was removed after 2 min.

When the hook structure was fixed by glutaraldehyde before the negative staining, a 5-μl sample was put on a carbon-coated grid. After 5 min, 5 μl of 6% glutaraldehyde was added, and the grid was washed with distilled water.

Antibody labeling of hooks was carried out as follows. A 5-μl sample was put on the carbon-coated grid and then 5 μl of the antihook antibody was added. After 5 to 10 min, the grid was washed with TNE-buffer and then with distilled water. The sample was then negatively stained.

The specimens were observed in a JEM100C electron microscope (JEOL).

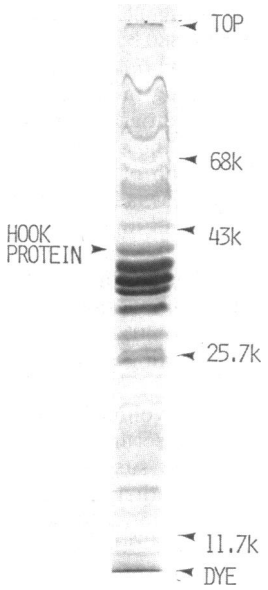

FIG. 1. SDS-PAGE of the crude hook fraction from a *flaL* mutant (SJW800). The crude hook fraction was prepared as described in the text. A 50-μl portion was analyzed by electrophoresis in 12% polyacrylamide gel. The arrows on the right side show the positions of molecular weight markers: bovine serum albumin (68,000 [68K]), ovalbumin (43K), chymotrypsinogen (25.7K), and cytochrome *c* (11.7K).

RESULTS

Isolation and purification of the hooks from a *flaL* mutant. The SDS-PAGE profile of the curde hook fraction showed that it contained many species of proteins from low to high molecular weight (Fig. 1). Most of these proteins were not thought to be part of the hook structure. For example, the proteins detected in the largest quantity had molecular weights of around 40,000, and they were regarded to correspond to the major outer membrane proteins (32). We performed DEAE-cellulose chromatography to separate the hook structure from the contaminating proteins.

The crude hook fraction was loaded onto a DEAE-cellulose column. The column was washed with a sufficient volume of 10 mM Tris-hydrochloride (pH 7.8) containing 0.04 M NaCl and then eluted with a linear gradient of NaCl. Absorbance of the fractions eluted from the column was monitored at 235 nm for detection of proteins. The wavelength of 235 nm was chosen to eliminate the disturbance caused by the absorbance of Triton X-100 contained in the elution buffer. Three peaks were identified by this procedure (Fig. 2). The fractions of these three peaks were observed by electron microscopy, and hooks were found to exist in the peak II fraction. The concentration of NaCl in the peak II fraction was 0.18 M. Proteins contained in the peak II fraction and in fractions around it were analyzed by SDS-PAGE (Fig. 3). The peak II fraction contained more hook protein than the other peaks on SDS-PAGE, and three additional proteins were copurified with the hook protein. The molecular weights of these three proteins estimated by SDS-PAGE were 59,000, 53,000, and 31,000.

Fractions around peak II (see Fig. 4) were pooled together and purified further by sucrose density gradient centrifugation. Portions of these fractions were analyzed by SDS-PAGE (Fig. 4). The SDS-PAGE profiles showed that these three proteins copurified with the hook protein. This result strongly suggests that the hook structure contains three species of protein in addition to the hook protein. We called these three proteins HAP1 (59,000), HAP2 (53,000), and HAP3 (31,000).

Composition of proteins in hooks isolated from filamentless mutants. The hooks were isolated as described above from the filamentless mutants (Table 1), each with a defect in a *fla* gene other than the *flaL* gene. The purification step of the sucrose density gradient centrifugation was omitted. Compositions of proteins in those hooks were determined by SDS-PAGE (Fig. 5). All three HAPs were detected in hooks from the *H1 H2* mutant, which had defects in the structural genes of the flagellin. This profile was the same as that of hooks from the *flaL* mutant. However, in hooks from a mutant with a defect on *flaR*, *flaU*, *flaV*, or *flaW*, all or some HAPs were not detected. In hooks from the *flaV* mutant, HAP1 and HAP3 were detected but HAP2 was not. The hooks from the mutant with the defect covering the *H1*, *flaV*, and *flaL* genes and the defect of the *H2* gene gave the same SDS-PAGE profile as hooks from the *flaV* mutant. In hooks from the *flaU* mutants, HAP1 was detected but not HAP2 or HAP3. In hooks from the *flaW* mutants, hook protein and a very small amount of HAP3 were detected. None of the HAPs was detected in polyhooks from the *flaR* mutant.

Relative amounts of hook protein and HAPs in hooks from the filamentless mutants. Relative amounts of HAPs were estimated by measuring the absorbance of stained bands on the SDS-PAGE profiles. The amount of HAP was much smaller than that of hook protein. If the proper amount for measuring the peak area of hook protein was analyzed by SDS-PAGE (Fig. 6), peak areas of HAP were too small to measure correctly. Therefore, various amounts of hooks were analyzed by SDS-PAGE, and their peak areas were coordinated with each other. The results gave the relative amounts of HAP3, HAP2, and hook protein against HAP1 in the hooks from the filamentless mutants (Table 2). The relative amount of HAP1/hook protein/HAP3/HAP2 was regarded as the same between the hooks from the *H1 H2* mutant and from the *flaL* mutant. The relative amounts of

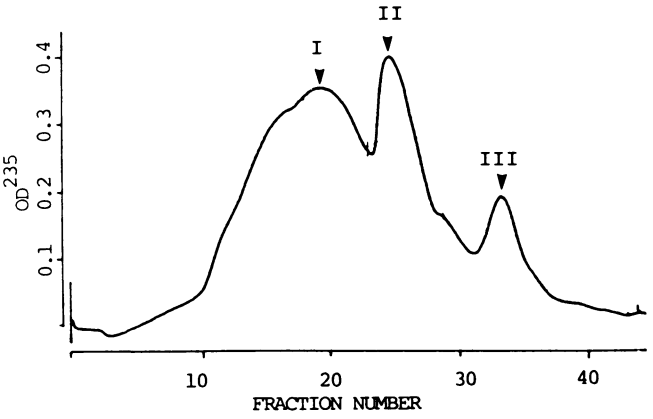

FIG. 2. Chromatography on DEAE-cellulose of the crude hook fraction from the *flaL* mutant. The crude hook fraction was applied to the DEAE-cellulose column, and the column was eluted with a linear gradient of 0.04 to 0.3 M NaCl. Fractions of 2 ml were collected. Hooks were observed in the fraction of peak II by electron microscopy.

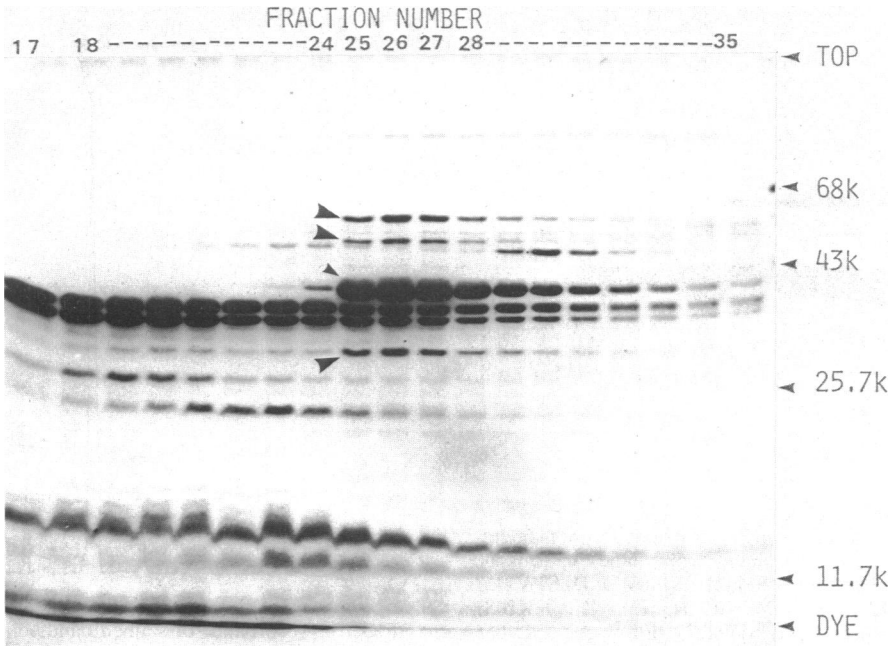

FIG. 3. SDS-PAGE of DEAE-cellulose fractions of the crude hook fraction from the *flaL* mutant. Samples (50 μl) from DEAE-cellulose fractions (see Fig. 2) were analyzed by electrophoresis in a 12% polyacrylamide gel. The small arrow indicates hook protein and large arrows indicate HAPs. The arrows on the right side show the positions of the moleculer weight markers (see legend to Fig. 1).

HAP1 and HAP3 in hooks from the *flaV* mutant and the *H1 flaV flaL* mutant were regarded as the same. A mutation on the *flaV* gene gave not only a defect of HAP2 but also an ca. 40% decrease of HAP3 as compared with HAPs of the *flaL* mutant or the *H1 H2* mutant. Because the hooks from the *flaW* mutants did not have HAP1, the amount of hook protein instead of the amount of HAP1 was used as the standard for the comparison. The relative amount of HAP3 versus hook protein in hooks from the *flaW* mutant was about 1/10 as compared with HAP3 of the *flaL* or *H1 H2* mutant.

The molar ratio of HAP1/hook protein/HAP3/HAP2 in the

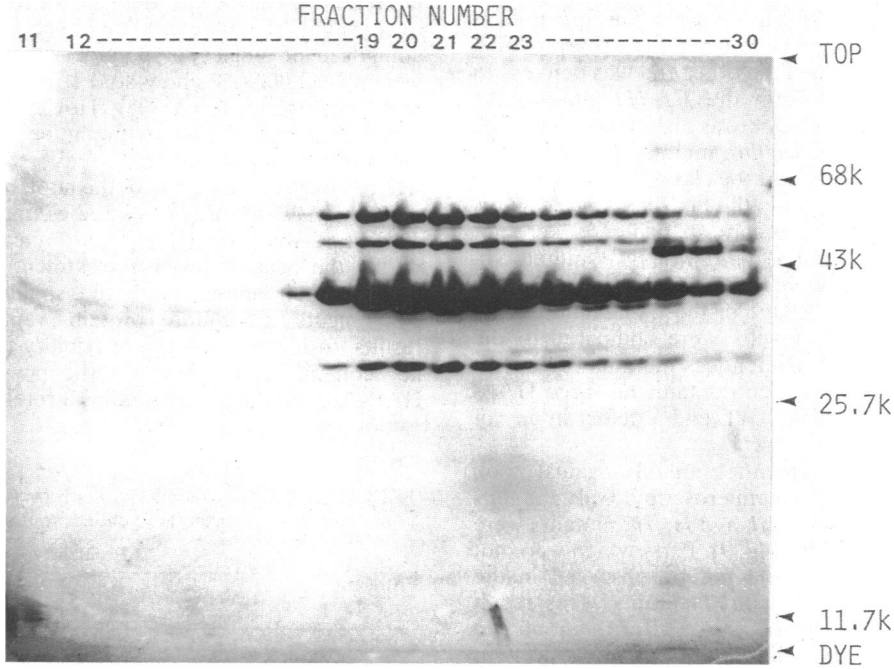

FIG. 4. SDS-PAGE of fractions of a sucrose density gradient in the hooks from the *flaL* mutant. The DEAE-cellulose fraction 25 to 29 (Fig. 3) were pooled and the centrifuged at 78,000 × *g* for 90 min. The pellet was suspended in 0.5 ml of TN-buffer. The suspension was layered on a 5 to 30% linear sucrose gradient. After centrifugation, the gradient was displaced at 0.5-ml fractions from the top. Portions (50 μl) of the fractions were anlayzed by electrophoresis in 12% polyacrylamide gel. The arrows on the right side show the positions of molecular weight markers (see legend to Fig. 1).

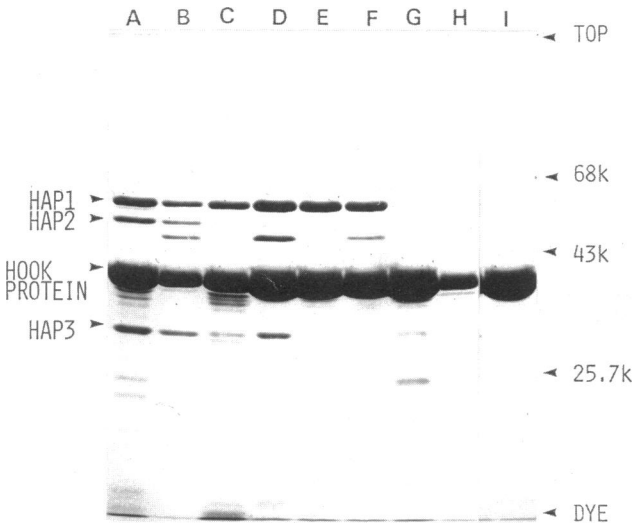

FIG. 5. Composition of proteins in hooks isolated from filamentless mutants. The hooks were isolated from: lane A, SJW800 (FlaL$^-$); B, SJW900 (H1$^-$ H2$^-$); C, SJW2150 (FlaV$^-$); D, SJW1411 (H1$^-$ H2$^-$, FlaL$^-$, FlaV$^-$); E, MH101 (FlaU$^-$); F, KK2087 (FlaU$^-$); G, SJW2160 (FlaW$^-$); H, SJW2177 (FlaW$^-$); I, SL4055 (FlaR$^-$). The resultant 50-μl suspensions were analyzed by electrophoresis in 10% polyacrylamide gel. The arrows on the right side show the positions of molecular weight markers (see legend to Fig. 1).

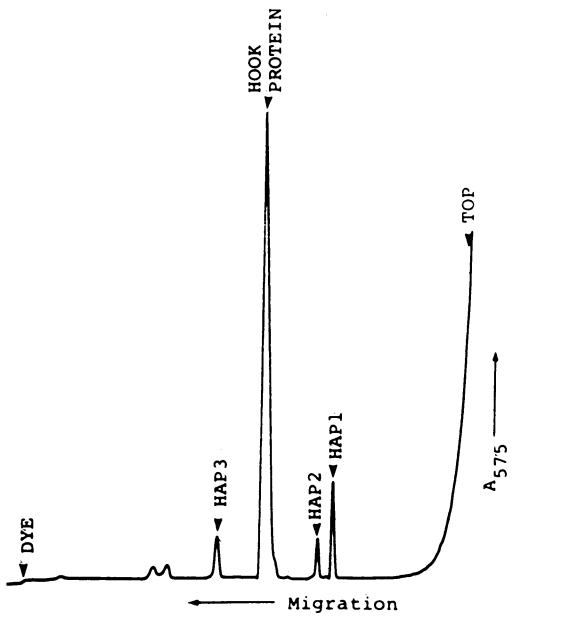

FIG. 6. Absorbance of stained bands on the SDS-PAGE profile of hook purified from the *flaL* mutant.

hook of the *flaL* mutant, as calculated from relative amounts and molecular weights, was 1:10:1.1:0.53.

Electron microscopic observations of the hooks. The hooks from the filamentless mutants were found to have different HAP compositions. Therefore, we examined by electron microscopy whether their protein compositions reflected the structural difference among the hooks from the various filamentless mutants (Fig. 7). Hooks from the *flaL* mutant and the *H1 H2* mutant had one end which was sharp, with a crack at the tip like a claw, and the other end was convex. In hook-basal bodies isolated from the *H1 H2* mutant, the shape of the distal end in the hook was also like a claw (Fig. 8). Therefore, in hooks from the *flaL* mutant as well as from the *H1 H2* mutant, it is likely that the claw-shaped end is the distal end, and the convex end is the proximal end which is attached to the basal body. The claw-shaped ends were not observed in hooks from the *flaV*, *flaU*, or *flaW* mutant. In these hooks, distal ends were rather flat. We could not detect any difference in the shape of these ends among the three mutants. When these results were compared to the protein compositions of the filamentless mutants (Fig. 5), it was concluded that a hook which contains all three HAPs has the claw shape at the distal end, and a defect in any of the HAPs gives the flat end.

The hooks were treated with the antibody against hook protein and observed by electron microscopy. Only the tops of the claw-shaped ends of the *flaL* and *H1 H2* mutants were not covered with the antibody (Fig. 9). Parts which were not covered with the antibody could not be observed in the hooks from the *flaV*, *flaW*, or *flaU* mutants. This result strongly suggests that the claw-shaped end of the hook is a structure made of materials different from hook protein, most probably the HAPs.

DISCUSSION

The hook is structurally more stable than the filament. Conditions of low pH or high temperature, which completely dissociate the filament, have little effect on the hook. Therefore, low pH or heat treatment has been used for the separation of the hook from the filament (2, 5, 7, 23, 31, 33, 34). However, some materials which are supposed to attach to the hook structure might be dissociated from the hook in addition to the filament during this treatment. To avoid this, we chose filamentless mutants, which lack the filament and have hooks attached to the basal body. Thus, it was not necessary to dissociate the filament for the isolation of the hook. It has been reported that the hook in *S. typhimurium* is composed of single polypeptide subunit whose molecular weight is 42,000 (23) and whose structural gene corresponds to a single gene, *flaFV* (28). However, when hooks were prepared from either a *flaL* mutant or a *H1 H2* mutant by the method developed in this study, three protein species (HAPs) were copurified with the hook protein. The failure to detect HAPs in previous studies may be explained by suggesting that the HAPs were released from the hook during the heat or low pH treatment or were overlooked because their amounts are too small to detect as minor components in the hook protein preparation. The present results do not rule out the possibility that intact flagella do not contain HAPs, because the possibility remains that HAPs are similar to scaffolding proteins in bacteriophage,

TABLE 2. Relative amounts of hook protein and HAPs in hooks from filamentless mutants

Strain	Relative amount			
	HAP1	HOOK PROTEIN	HAP3	HAP2
SJW900	1	ND[a]	0.56	0.43
SJW800	1	7.0	0.58	0.49
SJW2149	1	7.1	0.21	
SJW1411	1	ND	0.23	
MH101	1	7.1		

[a] ND, Not determined.

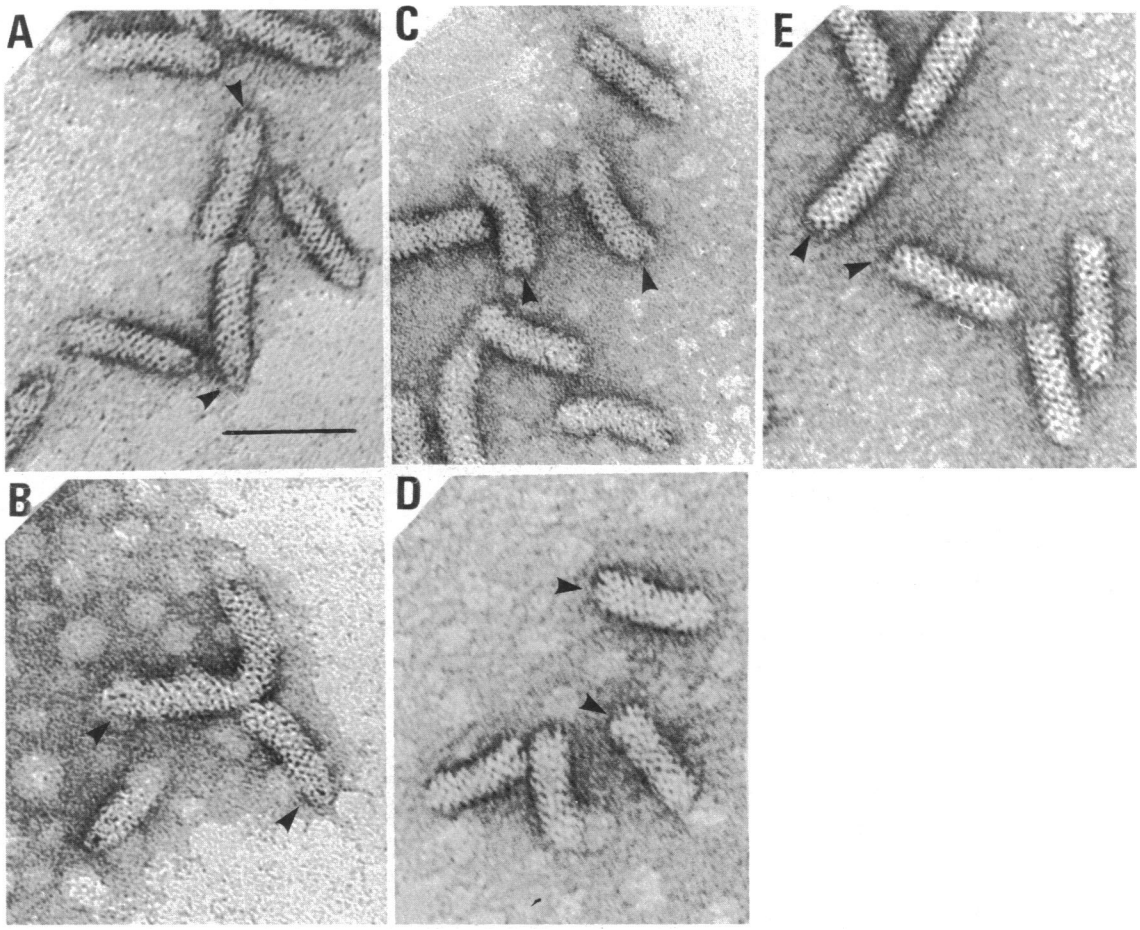

FIG. 7. Electron micrographs of hooks from the filamentless mutants: A, SJW900 ($H1^-$ $H2^-$); B, SJW800 ($FlaL^-$); C, SJW2150 ($FlaV^-$); D, MH101 ($FlaU^-$); E, SJW2160 ($FlaW^-$). Negatively stained with 1% uranyl acetate (pH 4.2). Arrows indicate distal ends of the hook. Bar, 50 nm.

which function catalytically in capsid assembly and are absent from the mature phage (10, 24).

In *Caulobacter crescentus*, the hook was purified from the culture of a filamentless mutant because the hook was released from the cell with the rod during a stage of the cell cycle (35). It was reported that a few kinds of protein were copurified in trace amount with the hook (22, 30). The possibility was discussed that these trace proteins might be components of the rod structure or minor components of the hook. However, it was not clarified whether the *Caulobacter* hook contains proteins like the HAPs which were found in this study.

Genetic studies have shown the presence of more than 25 *fla* genes in *S. typhimurium* (20, 27) and *E. coli* (25, 37). Functional homology between the *fla* genes in these two bacterial species has been examined through intergeneric complementation tests, and it has been shown that the chromosomal alignment of functionally homologous genes is entirely the same in these two bacterial species (27; Kutsukake unpublished data). It was found in this study that *flaV*, *flaU*, *flaW*, and *flaR* of *S. typhimurium* controlled the existence of HAPs in the hook. The correspondence of these *Salmonella* genes to *E. coli* genes has been established: *flaV* to *flbC*, *flaU* to *flaT*, *flaW* to *flaS*, and *flaR* to *flaE* (27; Kutsukake unpublished data). The gene products of *flaS* and *flaT* of *E. coli* have been identified through specific protein synthesis by lambda hybrid phages carrying these *fla* genes, and the molecular weights of their polypeptides as estimated by SDS-PAGE were 60,000 for *flaS* and 35,000 for *flaT* (26). It has been reported that both of them are components of the hook-basal body (13, 14). Therefore, considering that HAP1 and HAP3 are components of the hook, the corresponding

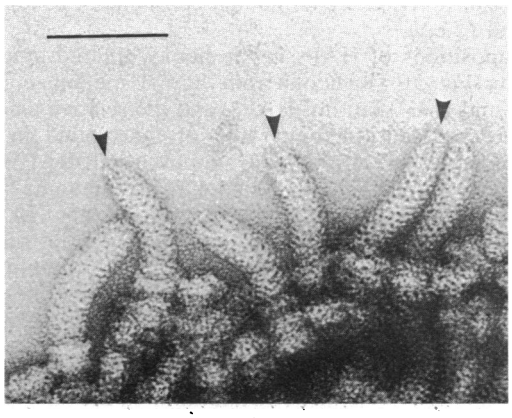

FIG. 8. Electron micrograph of hook basal bodies isolated from the *H1 H2* mutant. Negatively stained with 1% uranyl acetate (pH 4.2). Arrows indicated distal ends of the hook. Bar, 50 nm.

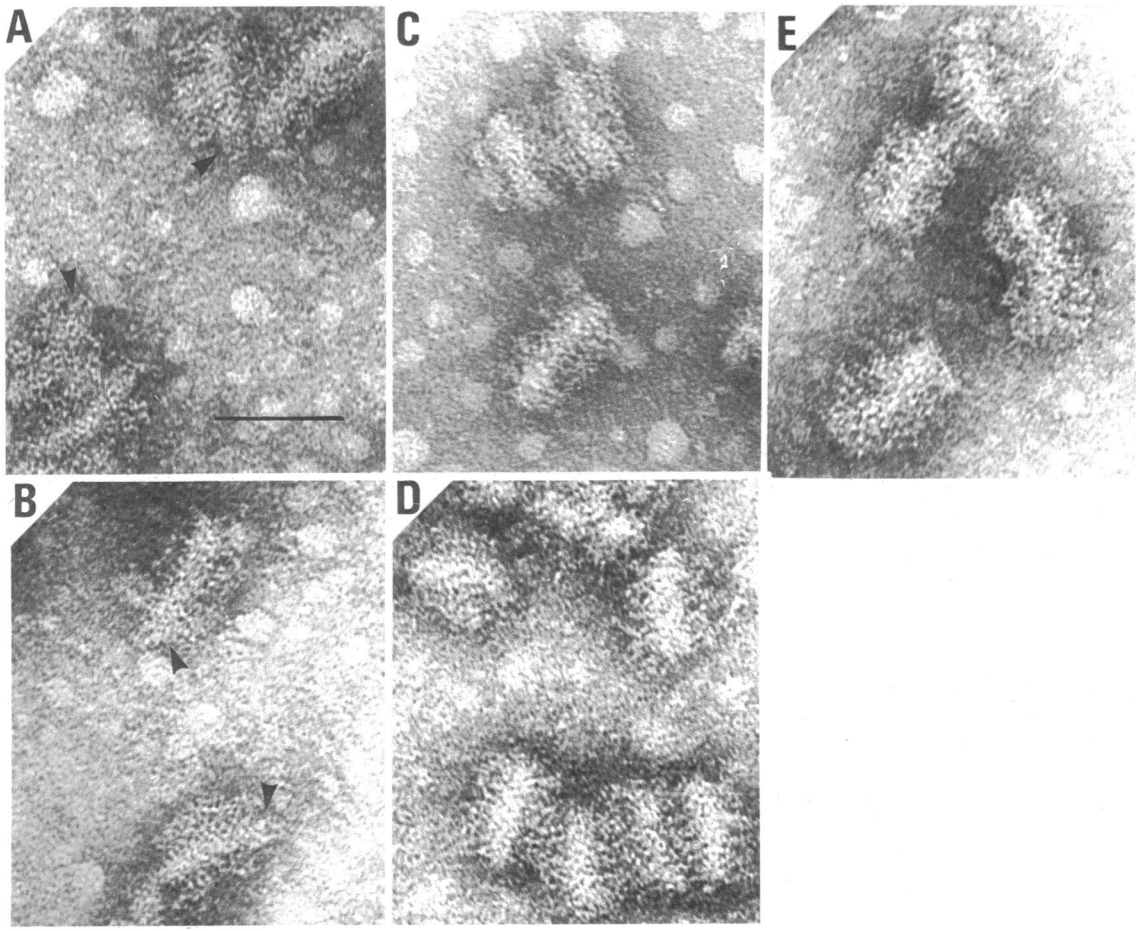

FIG. 9. Electron micrographs of hooks treated with antihook antibody. Hooks were isolated from the filamentless mutants: A, SJW900 ($H1^-$ $H2^-$); B, SJW800 ($FlaL^-$); C, SJW2150 ($FlaV^-$); D, MH101 ($FlaU^-$); E, SJW2160 ($FlaW^-$). Negatively stained with 1% potassium phosphotangstate. Arrows indicate distal ends of the hook. Bar, 50 nm.

Salmonella genes, i.e., *flaU* and *flaW*, might be the structural genes of HAP3 and HAP1, respectively. The estimated molecular weights of these corresponding products of *E. coli* and *S. typhimurium* are different: 59,000 instead of 60,000, and 31,000 instead of 35,000, respectively. These differences may be attributed to differences in the SDS-PAGE conditions employed or to slight differences in the molecular weights of functionally homologus proteins of *S. typhimurium* and *E. coli*.

Compositions of HAPs in the hooks differed among the mutants (Fig. 5). The hooks from the *flaV* mutants contained HAP1 and HAP3 but not HAP2, and the relative amount of HAP3 decreased to about 40% of that found in hooks containing all of HAPs. We suppose that HAP3 is not completely assembled into the hook of *flaV* mutants because of their defect in HAP2. This may show that HAP3 and HAP2 are located close to each other and interact with each other in the hook. The hooks from the *flaU* mutants contained only HAP1. HAP3 may be essential for the attachment of HAP2 to the hook. The hooks from the *flaW* mutants contained a very small amount of HAP3. HAP1 may be essential for the attachment of HAP3 to the hook and the association of HAP3 might be abnormal in the *flaW* mutants in which HAP1 is defective. It is possible to infer the process of hook morphogenesis and the genes responsible for each step as shown in Fig. 10 by assuming that the hook is constructed from simple one to more complex and that *flaV*, *flaU*, and *flaW* control the presence of HAP2, HAP3, and HAP1 in the hook respectively. It is most probable that *flaV*, *flaU*, and *flaW* are the structural genes for HAPs.

Some other proteins in addition to hook protein and HAPs were detected on SDS-PAGE profiles (Fig. 5). Among these proteins, a protein with a molecular weight of 50,000 that appeared in some mutants was thought to depend on a bacteriophage, because this protein was in larger amounts detected when many phage particles were observed by electron microscopy. This protein was separated from the hooks if they were purified by the sucrose density gradient centrifugation (Fig. 4). The proteins of molecular weight about 40,000 are supposed to be the major outer membrane proteins (32), which were not excluded, completely by chance. The other minor proteins may be degradation products of hook protein, minor components less than HAPs of the hook structure, or basal body components joined with the hooks. The *flaR* gene product which controls the hook length, has been thought to be present in the hook (36, 40). However, we could not find such a product in the hook in this study.

The electron micrographs of the hooks from the *flaL* and *H1 H2* mutants, which contained all HAPs, showed that each of the hooks had a sharp tip with crack like a claw in the distal end (Fig. 7). On the other hand, the electron micro-

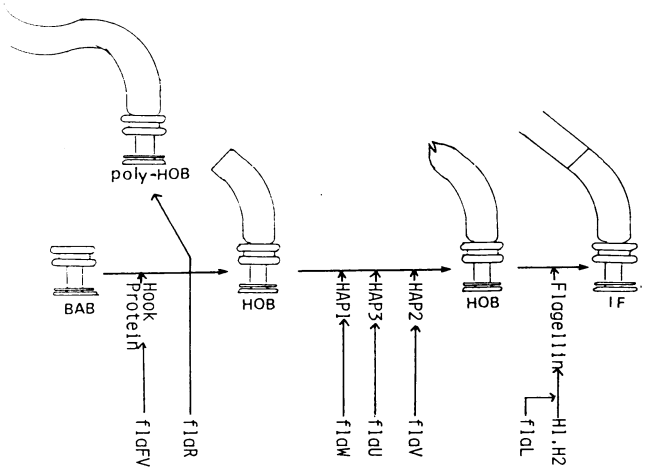

FIG. 10. Hypothetical process of hook morphogenesis. H1 and H2 are the structural genes for flagellin (17), *flaFV* is the structural gene for hook protein (28), *flaR* regulates the length of the hook (33, 40), and *flaL* controls the transcription of mRNA for flagellin (39). Abbreviations: BAB, basal body; HOB, hook-basal body; poly-HOB, polyhook-basal body; IF, intact flagellum. Nomenclature for the component structures has been described previously (5, 41).

graphs of the hooks from the *flaV*, *flaU*, and *flaW* mutants showed that each of them had a rather flat tip in the distal end. Considering the hypothetical process of hook morphogenesis (Fig. 10), absence of HAP2 from the hook is inferred to result in the change of the claw-shaped end to a flat end. HAP2 must be essential to make the claw shape. The electron micrographs of hooks treated with the anti-hook antibody showed that the claw-shaped ends were not covered with the antibody. This result strongly suggests that all HAPs or at least some, most probably HAP2, are located on the distal end. Our presumption based on the above discussions is that all HAPs are located on the distal end of each hook and play an essential role in filament formation. Although hooks from *flaV* mutants contained HAP1 and HAP3, the electron micrographs of the hooks showed no parts which were not covered with the antibody against hook protein. This is perhaps because the parts defective in HAP2 are composed of a smaller amount of protein and are not characteristically claw shaped, and therefore we might not have been able to recognize the parts by electron microscopy under the resolution power of the present experiment. The present results do not completely rule out the possibility that HAP1 or HAP3 or both are cross-reactive with hook protein or that the sites where HAP1 or HAP3 or both are located are not confined to the distal ends.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture in Japan.

LITERATURE CITED

1. **Abram, D., H. Koffler, and A. E. Vatter.** 1965. Basal structure and attachment of flagella in cells of *Proteus vulgaris*. J. Bacteriol. **90**:1337–1354.
2. **Abram, D., J. R. Mitchen, H. Koffler, and A. E. Vatter.** 1970. Differentiation within the bacterial flagellum and isolation of the proximal hook. J. Bacteriol. **101**:250–261.
3. **Abram, D., A. E. Vatter, and H. Koffler.** 1966. Attachment and structural features of flagella of certain bacilli. J. Bacteriol. **91**:2045–2068.
4. **Cohen-Bazire, G., and J. London.** 1967. Basal organelles of bacterial flagella. J. Bacteriol. **94**:458–465.
5. **DePamphilis, M. L., and J. Adler.** 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:384–395.
6. **DePamphilis, M. L., and J. Adler.** 1971. Attachment of flagellar basal bodies to the cell envelope: specific attachment to the outer, lipopolysaccharide membrane and the cytoplasmic membrane. J. Bacteriol. **105**:396–407.
7. **Dimmitt, K., and M. I. Simon.** 1970. Purification and thermal stability of intact *Bacillus subtilis* flagella. J. Bacteriol. **105**:369–375.
8. **Dimmitt, K., and M. I. Simon.** 1971. Purification and partial characterization of *Bacillus subtilis* flagellar hooks. J. Bacteriol. **108**:282–286.
9. **Doetsch, R. N., and R. D. Sjoblad.** 1980. Flagellar structure and function in eubacteria. Annu. Rev. Microbiol. **34**:69–108.
10. **Earmshaw, W. C., and S. R. Casjens.** 1980. DNA packaging by the double-stranded DNA bacteriophages. Cell **21**:319–331.
11. **Emerson, S. U., K. Tokuyasu, and M. I. Simon.** 1970. Bacterial flagella: polarity of elongation. Science **169**:190–192.
12. **Fujita, H., S. Yamaguchi, T. Taira, and T. Iino.** 1981. A simple method for the isolation of flagellar shape mutants in *Salmonella*. J. Gen. Microbiol. **125**:213–216.
13. **Hilmen, M., M. Silverman, and M. Simon.** 1974. The regulation of flagellar formation and function. J. Supramol. Struct. **2**:360–371.
14. **Hilmen, M., and M. Simon.** 1976. Motility and the structure of bacterial flagella, p. 35–45. *In* R. Goddson, T. Pollard, and J. Rosebaum (ed.), Cold Spring Harbor conference on cell proliferation and motility, vol. 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Hoeniger, J. F. M., W. van Iterson, and N. van Zanten.** 1965. Basal bodies of bacterial flagella in *Proteus mirabilis*. J. Cell Biol. **31**:603–618.
16. **Holt, S. C., and E. Canale-parola.** 1968. Fine structure of *Spirochaeta stenostrepta*, a free-living, anaerobic spirochete. J. Bacteriol. **96**:822–835.
17. **Iino, T.** 1969. Genetics and chemistry of bacterial flagella. Bactriol. Rev. **33**:454–475.
18. **Iino, T.** 1969. Polarity of flagellar growth in *Salmonella*. J. Gen. Microbiol. **56**:227–239.
19. **Iino, T.** 1974. Assembly of *Salmonella* flagellin *in vitro* and *in vivo*. J. Supramol. Struct. **2**:372–384.
20. **Iino, T.** 1977. Genetics of structure and function of bacterial flagella. Annu. Rev. Genet. **11**:161–182.
21. **Iino, T., and M. Enomoto.** 1966. Genetical studies of nonflagellate mutants of *Salmonella*. J. Gen. Microbiol. **43**:315–327.
22. **Johnson, R. C., M. P. Walsh, B. Ely, and L. Shapiro.** 1979. Flagellar hook and basal complex of *Caulobacter crescentus*. J. Bacteriol. **138**:984–989.
23. **Kagawa, H., K. Owaribe, S. Asakura, and N. Takahashi.** 1976. Flagellar hook protein from *Salmonella* SJ25. J. Bacteriol. **125**:68–73.
24. **King, J., R. Griffin-Shea, and M. T. Fuller.** 1980. Scaffolding proteins and the genetic control of virus shell assembly. Q. Rev. Biol. **55**:369–393.
25. **Komeda, Y., K. Kutsukake, and T. Iino.** 1980. Definition of additional flagellar genes in *Escherichia coli* K-12. Genetics **94**:277–290.
26. **Komeda, Y., M. Silverman, P. Matsumura, and M. Simon.** 1978. Genes for the hook-basal body proteins of the flagellar apparatus in *Escherichia coli*. J. Bacteriol. **134**:655–667.
27. **Kutsukake, K., T. Iino, Y. Komeda, and S. Yamaguchi.** 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. Mol. Gen. Genet. **178**:59–67.
28. **Kutsukake, K., T. Suzuki, S. Yamaguchi, and T. Iino.** 1979. Role of gene *flaFV* on flagellar hook formation in *Salmonella typhimurium*. J. Bacteriol. **140**:267–275.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lagenaur, C., M. DeMartini, and N. Agabian.** 1978. Isolation and characterization of *Caulobacter crescentus* flagellar hooks. J. Bacteriol. **136**:795–798.

31. **Nauman, R. K., S. C. Holt, and C. D. Cox.** 1969. Purification, ultrastructure, and composition of axial filaments from *Leptospira*. J. Bacteriol. **98**:264–280.
32. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
33. **Patterson-Delafield, J., R. J. Martinez, B. A. D. Stocker, and S. Yamaguchi.** 1973. A new *fla* gene in *Salmonella typhimurium-flaR-* and its mutant phenotype-superhooks. Arch. Microbiol. **90**:107–120.
34. **Raska, I., F. Mayer, C. Edelbluth, and R. Schmitt.** 1976. Structure of plain and complex flagellar hooks of *Pseudomonas rhodos*. J. Bacteriol. **125**:679–688.
35. **Shapiro, L., and J. V. Maizel, Jr.** 1973. Synthesis and structure of *Caulobacter cresentus flagella*. *J. Bacteriol.* **113**:478–485.
36. **Silverman, M., and M. Simon.** 1972. Flagellar assembly mutants in *Escherichia coli*. J. Bacteriol. **112**:986–993.
37. **Silverman, M., and M. Simon.** 1977. Bacterial flagella. Annu. Rev. Microbiol. **31**:397–419.
38. **Smith, H. O., and M. Levine.** 1967. A phage P22 gene controlling integration of prophage. Virology **31**:207–216.
39. **Suzuki, H., and T. Iino.** 1975. Absence of messenger ribonucleic acid specific for flagellin in non-flagellate mutants of *Salmonella*. J. Mol. Biol. **95**:549–556.
40. **Suzuki, T., and T. Iino.** 1981. Role of the flaR gene in flagellar hook formation in *Salmonella* spp. J. Bacteriol. **148**:973–979.
41. **Suzuki, T., T. Iino, T. Horiguchi, and S. Yamaguchi.** 1978. Incomplete flagellar structures in non-flagellate mutants of *Salmonella typhimurium*. J. Bacteriol. **133**:904–915.
42. **Suzuki, T., and Y. Komeda.** 1981. Incomplete flagellar structures in *Escherichia coli* mutants. J. Bacteriol. **145**:1036–1041.
43. **Vaituzis, Z., and R. N. Doetsch.** 1969. Relationship between cell wall, cytoplasmic membrane, and bacterial motility. J. Bacteriol. **100**:512–521.
44. **Vary, P. S. and B. A. D. Stocker.** 1973. Nonsense motility mutants in *Salmonella typhimurium*. Genetics **73**:229–245.
45. **Yamaguchi, S., H. Fujita, T. Kuroiwa, and T. Iino.** 1977. Sensitivity of non-flagellate *Salmonella* mutants to the flagellotropic bacteriophage. J. Gen. Microbiol. **99**:209–214.