

Purification and Characterization of the Flagellar Hook-Basal Body Complex of *Salmonella typhimurium*

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The hook-basal body complex of *Salmonella typhimurium*, a major component of its flagellar apparatus, was subjected to detailed analysis by electron microscopy and gel electrophoresis. The study was facilitated by the development of an improved protocol for isolation of the complexes in high yield and purity. Nine proteins were identified with the structure. These proteins had apparent molecular weights of 65,000 (65K), 60K, 42K, 38K, 32K, 30K, 27K, 16K, and 14K. Small but reproducible shifts in the apparent molecular weights of specific proteins from conditionally nonflagellate mutants indicated the following gene-polypeptide correspondences: *flaFV*, 42K; *flaFVI*, 32K; *flaFVII*, 30K; *flaFIX*, 38K; *flaAII.1*, 65K. Several new morphological features of hook-basal body complexes were recognized, including a clawlike structure on the cytoplasm-proximal M ring and additional material at the cytoplasmic face of the M ring. Based on this study and the work of others, we suggest that the morphological features of the hook-basal body complex correspond to the following proteins: hook-filament junction, 60K; hook, 42K; rod, 30K and 32K; L ring and outer cylinder wall, 27K; P ring, 38K; S ring, unknown; M ring, 65K.

Bacterial taxis is an integrated system of sensory transduction and motor response. Environmental signals generate an intracellular flow of information that modulates the operation of the motor organelles, the flagella, with the result that bacteria migrate toward beneficial environments. Of the considerable number of genes (ca. 50) directly involved in taxis, the majority (ca. 40) are required for motor assembly and function, and the remainder are required for sensory transduction (35, 49).

Bacterial motile behavior is a fairly complex biological phenomenon whose elucidation is a challenging task requiring genetic, structural, biochemical, and physiological approaches. The genetic aspects of the system are currently the best understood in that extensive mutant analysis has probably uncovered most of the relevant genes and established their map location and transcriptional organization; however, the function of these genes is in many cases not known, even to the extent of whether they are structural or regulatory.

The bacterial flagellum is a reversible rotary device (9, 33, 53) driven by proton motive force (38, 40). Partial information is available regarding its morphology, structure, and biochemical composition. Although some correlations among these various aspects are available, the information is still fragmentary; indeed, only for the external flagellar filament has the full correspondence of gene, polypeptide, structure, and function that established.

A major contribution to research in this area is the isolation from cells of an entity called the intact flagellum (15). The structure remaining after depolymerization of the external filament from the intact flagellum is termed the hook-basal body (HBB) complex, the hook being the link to the external filament, and the basal body presumed to

constitute the motor. Although there is now reason to question whether the basal body does, in fact, constitute the entire motor, it undoubtedly represents a major part of the flagellar apparatus and therefore merits careful study. Using λ phage-programmed gene expression, Komeda et al. (29) established several gene-polypeptide correspondences in the HBB complex of *Escherichia coli*. Also, examination by Suzuki and co-workers (54, 55) of electron micrographs of partial HBB structures from flagellar mutants enabled them to generate morphological assembly maps, which have provided valuable clues regarding gene-morphology correlations. Correlations between polypeptides and morphological features of the flagellar apparatus are, with the exception of the filament (6), the hook (27) and a protein or proteins at the hook-filament junction (24), still lacking.

As part of an ongoing study of the flagellar motor of *Salmonella typhimurium*, we report here an improved method of isolating HBB complexes in high yield and purity and the characterization of these complexes by electron microscopy and one- and two-dimensional gel electrophoresis. Further, we describe the identification of four HBB structural genes and confirmation of a fifth on the basis of the properties of temperature-sensitive flagellar (Fla^{Ts}) mutants.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* ST1, a motility-selected derivative of LT2, was used as the wild-type strain in most of the experiments; the cultures expressed flagellin phase 2, yielding the 1,2 antigen (58K). Strain SJW1103 locked in flagellin phase 1 (i antigen [53K]) was also used. Strain SJW880 is a *flaR* mutant used for isolation of polyhook-basal body complexes. Fla^{Ts} mutants isolated by the procedure given below were employed to identify structural genes for basal body components; although about 30 Fla^{Ts} mutants were tested, Table 1 lists only those whose HBB complexes showed differences from wild-type HBB complexes by sodium dodecyl sulfate (SDS) gel electrophoresis. Strain

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TABLE 1. Strains

Species	Strain no.	Relevant genotype and phenotype	Reference
<i>S. typhimurium</i>	ST1	Wild type, 1,2 flagellin	7
	SJW1103	Wild type, i flagellin	61
	SJW880	<i>flaR</i> , polyhook	51
	SJW1368	Δ (<i>flaK-che</i>), nonflagellate	30
	SJW2249	<i>flaFV</i> Fla ^{Ts}	This study
	SJW2263	<i>flaFVI</i> Fla ^{Ts}	This study
	SJW2188	<i>flaFVII</i> Fla ^{Ts}	This study
	SJW2192	<i>flaFVII</i> Fla ^{Ts}	This study
	SJW2238	<i>flaFIX</i> Fla ^{Ts}	This study
	SJW2244	<i>flaFIX</i> Fla ^{Ts}	This study
	SJW2205	<i>flaAII.1</i> Fla ^{Ts}	This study
	SJW2206	<i>flaAII.1</i> Fla ^{Ts}	This study
	SJW2213	<i>flaAII.1</i> Fla ^{Ts}	This study
	<i>E. coli</i>	AW330	Wild type

AW330, a wild-type *E. coli* strain, was used for isolation of HBB complexes from that species.

Isolation of Fla^{Ts} mutants. Flagellotropic phage χ (43) was added at a multiplicity of infection of 1 to a nutrient broth culture of strain SJW1103 containing about 10^8 cells per ml. After overnight incubation at 37°C, the culture was streaked on a semisolid nutrient broth plate (0.3% [wt/vol] agar, 8% [wt/vol] gelatin; see reference 20) to give well-separated colonies, and the plate was incubated overnight at 40°C. Nonflagellate mutants formed compact colonies, whereas motile bacteria that had escaped from χ phage attack produced swarms. Individual compact colonies were then transferred onto two semisolid plates, one of which was incubated overnight at 40°C, and the other of which was incubated overnight at 30°C. Clones which formed compact colonies at 40°C but swarmed at 30°C were regarded as Fla^{Ts} mutants. The mutated gene in each independently isolated clone was identified by P22 phage-mediated complementation analysis, using as recipients mutants representing all the known *fla* complementation groups.

Chemicals. Chemicals were obtained from the following sources. Cesium chloride (grade I), Coomassie brilliant blue R, and Triton X-100 were from Sigma Chemical Co. Acrylamide, *N,N'*-methylenebisacrylamide, and SDS were from Bio-Rad Laboratories. Nonidet P-40 was from Bethesda Research Laboratories; Ampholine was from LKB Instruments, Inc.; silver nitrate was from Aldrich Chemical Co., Inc.; and carrier-free H₂³⁵SO₄ (specific activity, ca. 4,000 Ci/mmol) was from Amersham Corp.

Preparation and purification of HBB complexes. Cells were grown aerobically at 37°C in either nutrient broth, or Vogel-Bonner citrate minimal medium (58) plus 1% [vol/vol] glycerol. Fla^{Ts} mutant cells were grown at a permissive temperature (30°C) in nutrient broth. The cell culture was typically 1 liter, although volumes as small as 200 ml could be used; the remainder of this description is based on a 1-liter volume. Cells were harvested in late logarithmic phase (for wild-type cells, this corresponded to an optical density at 650 nm [OD₆₅₀] of 1.0), centrifuged, suspended in 100 ml of ice-cold sucrose solution (0.5 M sucrose, 0.1 M Tris-hydrochloride [pH 8.0]), and dispersed by gentle stirring. To the suspension was added 5 ml of lysozyme solution (2 mg/ml in distilled water) and 10 ml of 0.1 M EDTA (pH 7.5), and the mixture was incubated on ice with gentle stirring. Although most of the cells were converted into spheroplasts within 10 min, the solution was incubated for ca. 40 min to ensure complete digestion of the peptidoglycan layer.

The resulting spheroplasts were lysed by addition of 10 ml of 10% Triton X-100. After lysis was complete, 10 ml of 0.1 M MgSO₄ was added to the lysate, and the mixture was incubated at 30°C until the viscosity of the solution had decreased greatly, indicating that the cellular DNA had been degraded by endogenous DNase. The process was essentially complete in 30 min. SJW strains were somewhat resistant to lysozyme-EDTA treatment, and the lysis of their spheroplasts did not always proceed as well as with strain ST1. Whenever lysis was poor, the lysate was left at 4°C overnight.

Unlysed cells and cell debris were removed by low-speed centrifugation (4,000 × *g*, 10 min) and the pH of the supernatant raised with 5 N NaOH. As the solution approached a pH of 11, it became translucent, suggesting that outer membrane structures had disintegrated (see below).

The lysate was subjected to high-speed centrifugation (60,000 × *g*, 60 min, polyallomer tubes), and the pellets were suspended in alkaline solution (0.1 M KCl-KOH, 0.5 M sucrose, 0.1% Triton X-100 [pH 11]), recentrifuged, and resuspended in TET buffer (10 mM Tris-hydrochloride, 5 mM EDTA, 0.1% Triton X-100 [pH 8.0]).

The solution was diluted to 90 ml with TET buffer, and 36 g of CsCl was added. The mixture was then centrifuged (Beckman SW27.1 buckets, 55,000 × *g*, 16 h, 15°C). The flagella formed a thick band about 3/4 of the way down the tube, whereas membrane fragments formed a light diffuse band above, and amorphous debris sedimented at the bottom. The flagellar fraction was collected with a Pasteur pipette and dialyzed against TET buffer.

The flagellar filaments were dissociated into monomeric flagellin by suspension in acidic solution (50 mM glycine-hydrochloride, 0.1% Triton X-100 [pH 2.5]). The HBB complexes, which remained intact, were collected by centrifugation (60,000 × *g*, 60 min). Residual supernatant containing flagellin was removed by carefully wiping the wall of each tube with tissue. Because the HBB pellet was difficult to disperse in TET buffer, resuspension in that buffer was only employed for samples for electron microscopy; samples for gel electrophoretic analysis (see below) were resuspended directly in SDS sample buffer.

Isolation of polyhook-basal body complexes. With strain SJW880 (*flaR*), polyhook-basal body complexes were isolated as described above for HBB complexes, except that pH 12 was used instead of pH 11 to dissociate the outer membrane vesicles.

Preparation and purification of filament-hook complexes. *S. typhimurium* ST1 was grown at 37°C in Vogel-Bonner citrate medium (58) plus 1% [vol/vol] glycerol. Cells resuspended in phosphate-buffered saline (13 mM phosphate, 150 mM NaCl [pH 7.0]) were passed several times through a 21-gauge hypodermic needle. After sedimenting the cells at 5,000 × *g*, the sheared filament-hook complexes in the supernatant were collected by centrifugation (60,000 × *g*, 60 min). The material was washed with alkaline solution, resuspended in TET buffer, and subjected to CsCl density gradient centrifugation. Depolymerization of the filament was accomplished by acid treatment as described above.

Partial degradation of HBB complexes. Heat treatment consisted of maintaining an aliquot of purified HBB complexes in a glass tube at the desired temperature (in a controlled temperature block; Temp-Blok Model H2025) for the desired time. After incubation, the sample was chilled on ice and observed by electron microscopy.

For acidic degradation, 100 μ l of purified HBB complexes in TET buffer were diluted into 5 ml of pH 2.0 solution (0.1

M KCl-HCl, 0.1% Triton X-100) and incubated at 30°C for 1 h. The suspension was then centrifuged ($100,000 \times g$, 1 h), and the precipitate was suspended in 100 μ l of TET buffer.

Preparation of 35 S-labeled HBB complexes. Cells were grown at 37°C in Vogel-Bonner citrate medium plus 1% [vol/vol] glycerol but with sulfate at 75 μ M instead of the usual 40 mM. A 5-ml preculture sample was inoculated into 200 ml of medium containing 5 mCi of carrier-free $\text{H}_2^{35}\text{SO}_4$. After 10 h of incubation at 37°C, the cells had reached stationary phase ($\text{OD}_{650} = 0.75$), limited by the availability of sulfur. This is a modification of the protocol described by Hilmen et al. (21). HBB complexes from these 35 S-labeled cells were then prepared as described above.

For autoradiography, gels were washed in 50% methanol for 10 min, sandwiched in cellophane membrane (Bio-Rad) on a glass plate, dried at room temperature, and placed on film (Kodak X-Omat).

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (32) at a polyacrylamide concentration of 12.5%. Two-dimensional electrophoresis was performed as described by O'Farrell (48), using isoelectric focusing (5% polyacrylamide, 2% Ampholine 3.5-10) in the first dimension and SDS electrophoresis through a uniform 12.5% acrylamide gel in the second dimension. Samples were solubilized by the method of Ames and Nikaido (3). One volume of sample was mixed with an equal volume of sample buffer (2% SDS, 5% β -mercaptoethanol, 10 mM Tris-hydrochloride [pH 8.0]) and boiled for 2 min. After being chilled on ice, the solubilized proteins were diluted with an equal volume of sample dilution buffer (9.5 M urea, 2% Ampholine 3.5-10, 5% β -mercaptoethanol, 8% Nonidet P-40). The final urea concentration was achieved by adding solid urea. Immediately after the sample was thus prepared, it was run in the isoelectric focusing gel at 400 V for 12 h and then at 800 V for 30 min. After isoelectric focusing, the tube gels were washed for 2 h with SDS sample buffer lacking β -mercaptoethanol and loaded on the second gel by sealing with agarose also lacking β -mercaptoethanol (see below). Gels were stained either with Coomassie brilliant blue R (henceforth referred to as Coomassie) or with silver (see below).

Silver staining. Silver staining was developed by Merrill et al. (41, 42) and has been modified by various laboratories (44, 60). With the diammine method of Wray et al. (60), we found different extents of staining among the various proteins of the HBB complex. The hook protein did not stain well; on lightly loaded gels, it appeared as a pale brown band, whereas on heavily loaded gels, it actually produced a negative stain density at the center of the band, giving a false impression of the amount of material present. However, from the thickness of the band it was obvious that the hook protein was the most abundant component in the gel. Flagellin had similar anomalous staining properties. Both of these proteins are moderately acidic ($\text{pI} < 5$). Neutral or basic proteins stained more intensely, with black rather than brown coloration. Initially, these anomalies acted as a guide to the correspondences of proteins between one- and two-dimensional gels. In later stages of this study, silver staining was carried out according to the dithiothreitol method of Morrissey (44), which we found gave a more faithful representation of relative amounts of the components within a sample.

Electron microscopy. Samples of intact flagella, HBB complexes, filament/hook complexes, and ring preparations were examined by electron microscopy, usually using 2% phosphotungstate as a negative stain.

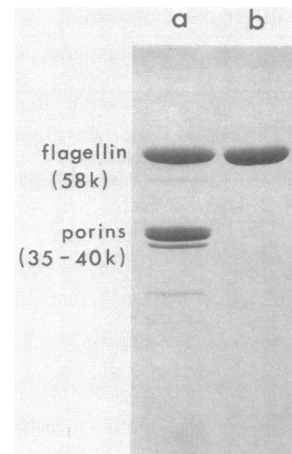


FIG. 1. SDS gels demonstrating effect of alkali treatment on the purity of flagellar preparations. (a) Without alkali treatment, porins were present in approximately the same quantity as that of the flagellar filament protein flagellin. (b) After treatment at pH 11, flagellin was the sole protein evident at the load shown. Although HBB proteins could not be detected in such gels, electron microscopy established that the HBB complex was present at the end of the flagellar filament of almost all particles.

High-intensity dark-field light microscopy. Flagellar preparations were inspected by high-intensity dark-field light microscopy as described before (34, 36, 37).

RESULTS

Purification of HBB complexes. The flagellar basal body of gram-negative bacteria is associated with the inner membrane, the peptidoglycan layer, and the outer membrane (17). It can be freed from the peptidoglycan layer by lysozyme-EDTA treatment and substantially from both inner and outer membranes with the nonionic detergent Triton X-100 (15). However, some outer membrane components resist solubilization and form vesicles which constitute the major contaminant in HBB preparations. We have found conditions under which these vesicles can be solubilized while leaving the flagellar structure intact.

After spheroplast lysis and DNA degradation was complete (see above), the pH of the solution was raised by NaOH addition. At a pH of between 10 and 11, the solution became translucent, suggesting that the outer membrane vesicles responsible for the turbidity of the solution had now dissolved. High-intensity dark-field light microscopy confirmed that samples not subjected to alkaline treatment contained large numbers of light-scattering vesicles, whereas alkali-treated samples revealed only flagella, recognizable by the characteristic helical contour of the filament. Both by light and electron microscopy it was established that flagella could withstand pH values as high as 12 (as has been noted for the flagellar filament [28]). In routine preparations, we employed treatment at pH 11. Flagella were sedimented by high-speed centrifugation and suspended in TET buffer. The solution was very viscous, containing almost pure intact flagella at a high concentration. Coomassie-stained SDS gels of non-alkali-treated flagellar preparations (Fig. 1a) showed that flagellin represented only about 50% of the material, the remainder consisting almost entirely of outer membrane matrix proteins or porins (45, 52), as judged by their size (35K to 40K) and pI (ca. 4.6). After alkali treatment, flagellin was the only component detectable with Coomassie staining (Fig. 1b), even on quite heavily loaded gels. Although the

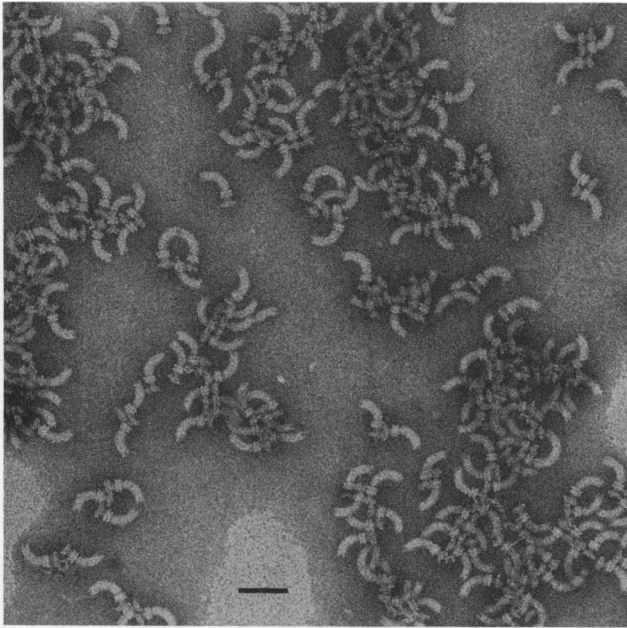


FIG. 2. Low-magnification electron micrograph of HBB preparation. Intact HBB complexes comprised the overwhelming majority of particles in any field. Rings, staples, and detached hooks were occasionally noted in such samples. Bar, 100 nm.

HBB proteins were present in amounts too small to be detected by gel electrophoresis, electron microscopy revealed that most of the flagellar filaments did possess an intact HBB complex at their end.

We tested detergents other than Triton X-100 for solubilization of the outer membrane. In combination with the alkalization step, Nonidet P-40, deoxycholate, and cholate worked as well as did Triton X-100. None was completely effective in the absence of this step.

Because the HBB complex constitutes only about 1% of the mass of an intact flagellum, contaminants at similar levels at this stage of the preparation would, if not removed, have represented a serious problem in the final HBB sample. The flagellar preparation was therefore subjected to CsCl density gradient centrifugation for further purification as described above.

Finally, the flagellar filaments were depolymerized into monomeric flagellin by acidic (pH 2.5) buffer, and the HBB complexes were pelleted by high-speed centrifugation and suspended in TET buffer.

Figure 2 shows an electron micrograph of such a preparation of purified HBB complexes. For the majority of particles, the HBB structure was intact, establishing the validity of the purification procedure. Contamination in the form of outer membrane fragments or flagellar filaments was minor.

Properties of isolated HBB complexes. HBB complexes displayed a characteristic morphology (Fig. 3a and b) consisting of a hook, rod, and four rings, as has been described previously (16). This is illustrated schematically in Fig. 4, which employs the L,P,S, and M nomenclature for the rings

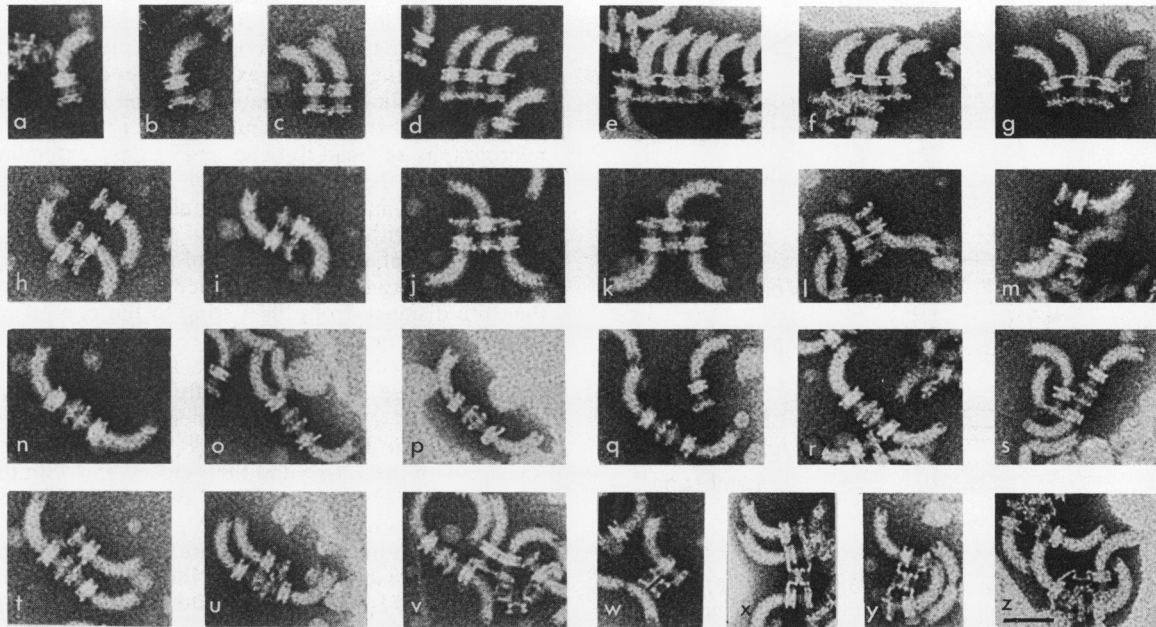


FIG. 3. Morphological and aggregation characteristics of HBB complexes. (a and b) Single HBB complexes revealed the hook, rod, and four-ring structure first described by DePamphilis and Adler (16; see also Fig. 4). The cytoplasm-proximal M ring frequently, as here, exhibited an appearance reminiscent of a claw mount in jewelry. (c to e) Parallel aggregation with in-phase register. (f and g) Parallel aggregation with presumed vestigial outer membrane preventing the hook-proximal L rings from abutting each other directly. (h to k) Antiparallel aggregation, with L and M rings of one HBB complex in register with M and L rings, respectively, of the other. (l and m) Antiparallel aggregation, with L rings in register. (n to r) Head-to-head dimerization. The HBB complexes approach less closely than would be expected from the appearance of single particles, and additional material becomes evident between the M rings of the two HBB complexes. (s to u) The additional distance between M rings is further demonstrated by the register of a third HBB complex aggregating alongside the head-to-head pair. (v to y) Staplelike structures either singly or back to back. These structures commonly demonstrated an affinity for intact HBB complexes, with the open end of the staple in register with the L ring or the M ring. (z) Two HBB complexes linked by a staple and presumed vestigial outer membrane. Bar, 75 nm.

first introduced by DePamphilis and Adler (16). In favorable images, when the M and S rings could be clearly distinguished, the M ring was seen to be of a slightly greater diameter (ca. 35 nm), and often displayed a clawlike appearance at its periphery projecting in the direction that would be toward the cytoplasm in the intact cell.

The basal body is a very hydrophobic structure (17). Both dark-field light microscopy and electron microscopy revealed that isolated intact flagella tended to aggregate through their basal bodies, even in the presence of detergent. HBB complexes likewise showed a pronounced tendency to aggregate via their ring structures. In addition to parallel, in-register alignment (Fig. 3c to e), it was common to note antiparallel alignment, with the L ring of one particle interacting with the M ring of another and vice versa (Fig. 3h to k). Less commonly, L ring-to-L ring antiparallel alignment was noted (Fig. 3l and m). These observations confirm previous conclusions (14, 17) that the L and M rings are primarily responsible for aggregation of HBB complexes. From the plane of the L ring, an extremely sharp extension, which we assume was vestigial outer membrane, was seen on some HBB complexes. This structure could form bridges between the L rings of HBB complexes (Fig. 3f and g), indicating that it had a high affinity but not a covalent attachment, since it is statistically highly unlikely that the HBB complexes were in such close proximity on the intact cell.

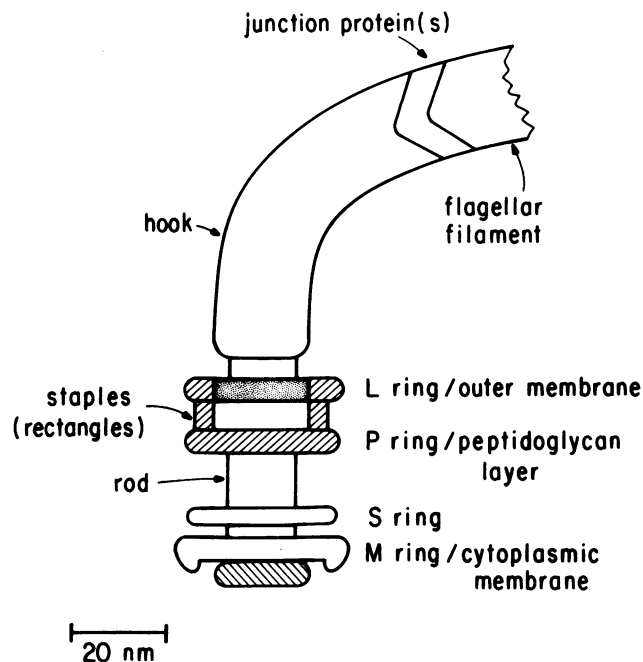


FIG. 4. Schematic diagram of the HBB complex of *S. typhimurium* with the ring nomenclature devised by DePamphilis and Adler (16) for *E. coli*. The juxtapositions with cell surface features deduced from their study are also shown. The staple and rectangle structures that were observed (see text) are believed to be lateral views of detached L ring, P ring, outer cylinder complexes. Staples are indicated here by the right-to-left crosshatched area and rectangles by that area plus the stippled area. The M ring frequently had a characteristic clawlike appearance at its outer radii. Additional material seen at the cytoplasm-proximal face of the M ring when head-to-head dimers of HBB complexes formed is indicated by the left-to-right crosshatched area. For a consideration of the relationship among morphological features of the HBB complex, its protein composition, and its genetic origin, see the text and Table 2.

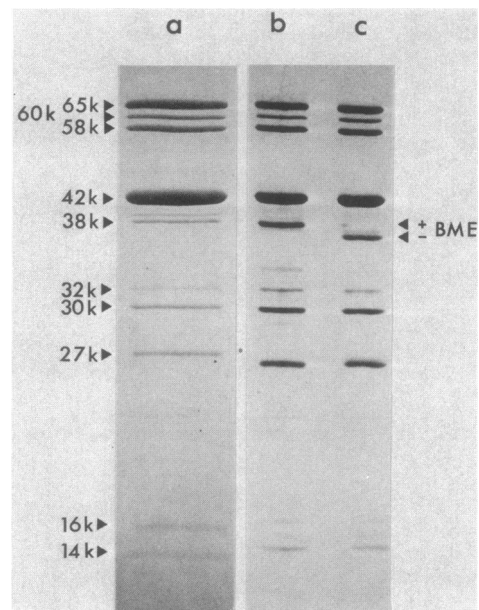


FIG. 5. SDS gels of HBB preparations stained with Coomassie (lane a) or silver (lanes b and c). Those proteins that appeared consistently and were judged to be authentic HBB components are indicated by their apparent molecular weights. The presence (lane b) or absence (lane c) of β -mercaptoethanol (BME) caused a shift in the 38K protein as has been noted previously for *E. coli* (29).

We often encountered pairs of particles in head-to-head alignment, with their M rings facing each other (Fig. 3n to r). The M rings in these cases were separated by a rather reproducible distance of about 10 nm, indicating that there was a part of the structure extending beyond the limit of the M ring as outlined by negative staining of single particles. Hints of this structure could, in fact, be seen in these micrographs of head-to-head aggregates, extending out to about 20 nm, i.e., to about 60% of the M ring diameter. Occasionally, another HBB complex was found in parallel aggregation with a head-to-head aggregate (Fig. 3s to u). Examination of such images reinforced the conclusion that the axial L ring-to-M ring distance was appreciably smaller than the distance from the L ring to the central plane of a head-to-head aggregate.

Staple-shaped structures, with a very characteristic appearance, were seen occasionally (Fig. 3a and v). These structures had a tendency to dimerize (Fig. 3w to y) and to associate with HBB complexes with their open end in register with the L ring and their closed end with the P ring. Figure 3z shows an example of two HBB complexes with their L rings linked via such a staple, directly on one side and via a presumed vestigial outer membrane fragment on the other. We concluded that these staplelike structures were detached L ring and P ring outer cylinder complexes as discussed below.

One-dimensional electrophoretic analysis of HBB complexes. Purified HBB complexes were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. Because cells have only about 5 to 10 flagella each, we initially employed a silver staining method on account of its high sensitivity. Later, as the isolation protocol was improved, it became feasible to use conventional Coomassie staining, and we henceforth employed that for routine purposes. Examples of Coomassie- and silver-stained one-dimensional SDS gels of HBB complexes are shown in Fig. 5.

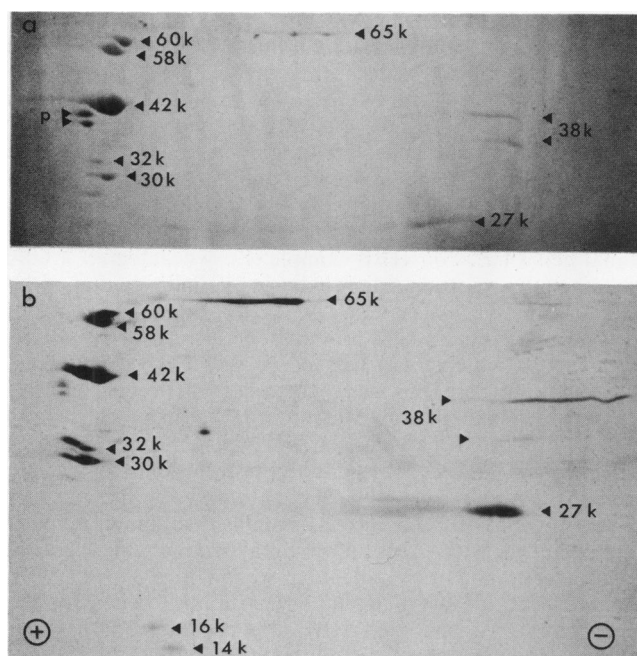


FIG. 6. HBB preparation subjected to isoelectric focusing followed by SDS gel electrophoresis and either (a) Coomassie or (b) silver staining. All of the proteins evident in one-dimensional SDS gels could be seen in such two-dimensional gels. The 16K and 14K proteins were often too faint to be seen by Coomassie staining but could be readily detected by silver staining. Several of the proteins, most notably the 65K protein and the basic 38K protein, showed anomalous isoelectric focusing behavior, appearing as streaks or multiple spots. The two apparent molecular weights exhibited by the 38K protein are a consequence of low β -mercaptoethanol levels carried over from the isoelectric focusing gel (see the text and Fig. 5b and c). Spots marked as p are porins. Acidic and basic ends of the isoelectric focusing gel are indicated by + and -, respectively.

Based on the following criteria, all major bands in our preparations were likely to be components of the HBB complex: the HBB complexes were pure as judged by electron microscopy (Fig. 2); the hook protein was the most abundant component in the gel (Fig. 5a), as was to be expected from its relative size in the structure; and the porins, or outer membrane matrix proteins (45, 52), which were by far the most abundant contaminants in impure preparations (Fig. 1a), were usually minor components in purified preparations of HBB complexes. Hereafter, we describe those proteins reproducibly detectable in Coomassie- or silver-stained one-dimensional SDS gels and refer to these proteins by their apparent molecular weights in such gels (65K, 60K, 42K, 38K, 32K, 30K, 27K, 16K, and 14K). Residual flagellin was also present; no effort was made to remove it entirely, since it acted as a convenient internal standard. It was either 53K or 58K, depending on whether the *H1* (i antigen) or *H2* (1,2 antigen) gene for flagellin was being expressed under the control mechanism that causes flagellin phase variation (62).

The 65K protein was hydrolyzed into smaller fragments if, in preparation for electrophoresis, the sample was boiled for more than 5 min in SDS sample buffer that was acidic as a result of carry-over from the pH 2.5 treatment. A 62K protein, which we originally believed to be an authentic component of the HBB complex, was the initial major species from the hydrolysis; it in turn generated a series of bands in the range 55K to 60K (data not shown). The hook

protein also underwent hydrolysis under the same conditions, giving rise to several fragments of 29K, 25K, and smaller. After we became aware of this artifact, care was taken to ensure that the pH of the HBB preparation in sample buffer was adjusted to neutrality before boiling, in which case no degradation was observed.

A 35K protein was occasionally observed in SDS gels, but it was especially evident in impure preparations and was also found in material isolated when a totally nonflagellate deletion mutant, SJW1368, was subjected to the identical procedure employed for HBB isolation (data not shown).

Two-dimensional electrophoretic analysis of HBB complexes. Samples of purified HBB complexes were analyzed on two-dimensional gels (Fig. 6a and b). Solubilization for isoelectric focusing required boiling in the presence of SDS and β -mercaptoethanol. Without this complete treatment, either diffuseness or disappearance of some spots in the two-dimensional gel resulted.

The major spots were carefully studied from gel to gel in terms of reproducibility of occurrence, appearance, and relative amount. All nine of the proteins listed above for one-dimensional gels were also detected in two-dimensional gels. Their characteristics will be described in turn.

The 65K protein appeared in the region of pH 7 as several adjacent spots, somewhat diffuse and spanning approximately 0.5 pH units. The 60K protein appeared as a single spot at a pI of 5.0. The 42K protein is known to be the hook protein (27) and focused at a pI of 4.7, in agreement with the finding of Kagawa et al. (26). In some gels, it focused as a single spot, but in others, it split into a doublet. The 38K protein was quite basic (pI > 9) and appeared as a diffuse line. Although this protein was always present as a major component of one-dimensional SDS gels, it did not reproducibly enter isoelectric focusing gels, suggesting that it was not readily solubilized even at the high urea concentration used. The 32K and 30K proteins appeared in the acidic region of the gel, with pIs of 4.5 and 4.6, respectively. The 27K protein was reproducibly detected as a diffuse spot at a pI of 7.5. It was difficult to detect low-molecular-weight proteins in two-dimensional Coomassie-stained gels. However, by silver stain, the 16K and 14K proteins could generally be detected as diffuse spots at pIs of 5.4 and 5.5, respectively.

In silver-stained two-dimensional gels, multiple horizontal lines appeared in the region between 60K and 70K, as noted by Merrill et al. (41). These lines disappeared when β -mercaptoethanol was omitted from the buffer for the second-dimension gel (see references 47 and 56). With one exception, the 38K protein, there was no difference between the appearance of proteins with this protocol versus the conventional one. The apparent molecular weight of the 38K protein decreased to 36K in the absence of β -mercaptoethanol (Fig. 5, lanes b and c). There was also a simultaneous presence of both apparent molecular weights in the two-dimensional gels (Fig. 6a and b). Komeda et al. and Matsumura et al. (29, 39) reported that the 38K protein encoded by the *flaM* gene of *E. coli* similarly changed its mobility in SDS gels when solubilized in sample buffer lacking β -mercaptoethanol.

^{35}S -labeled HBB complexes. In a previous study of *E. coli* HBB complexes (29), it was necessary because of low yields to employ a radiolabel to detect the material in electrophoretic gels. We wished therefore to compare the appearance of radiolabeled and stained gels of *S. typhimurium* HBB complexes, and also (see following section) to compare the appearance of gels from the two bacterial species. [^{35}S]methionine backbone-labeled complexes were prepared

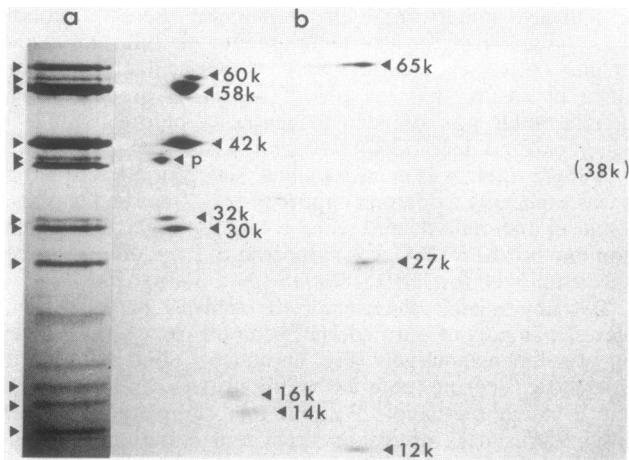


FIG. 7. ^{35}S -labeled HBB preparation subjected to (a) one-dimensional or (b) two-dimensional gel electrophoresis. Results were similar to those obtained with Coomassie or silver stain (Fig. 5 and 6) except that the 38K protein did not enter the isoelectric focusing gel. The spot marked as p is a porin. A 12K protein obscured by the dye front in stained gels is evident here. The polarity of the isoelectric focusing gel is as for Fig. 6.

as described above. Figure 7 shows autoradiograms of one- and two-dimensional gels of such a preparation; there were no major differences in Coomassie- or silver-stained gels. However, a 12K protein, which was obscured by the dye front in stained gels, was now clearly evident, focusing at a pI of 7.3. We are uncertain whether this protein is an authentic component of the HBB complex.

Comparison of HBB complexes from *S. typhimurium* and *E. coli*. The two-dimensional gel patterns of *S. typhimurium* HBB complexes were compared with those of *E. coli* described by Komeda et al. (29). In both species, the acidic proteins (for *S. typhimurium*, 60K, 42K, 32K, 30K, 16K, and

14K; apparent molecular weights for *E. coli* were similar) were found with comparable pI values. Komeda et al. were unable to detect the basic proteins (38K and 27K) in isoelectric focusing gels, although they always detected them in one-dimensional SDS gels. We were able to detect them, but as diffuse lines that suggested incomplete solubility of these proteins even under the extreme conditions used. No counterpart to the 65K protein of the *S. typhimurium* HBB complex is reported for *E. coli* (29). However, in two-dimensional gels of *E. coli* HBB complexes, we detected a 60K protein with a neutral to slightly basic pI similar to that of the 65K *S. typhimurium* protein (A. Castle, unpublished data), as well as the acidic 60K protein seen also in *S. typhimurium*. In one-dimensional gels of *E. coli* HBB complexes, these two 60K proteins were not resolved. Recent genetic studies by Bartlett and Matsumura (8; also see below) further support the conclusion that the 65K protein of *S. typhimurium* and the basic 60K protein of *E. coli* are homologous.

Analysis of HBB complexes from Fla^{Ts} mutants. Even a single amino acid substitution in a protein can cause a change in its apparent molecular weight in SDS gels (13). Therefore, in an effort to identify structural genes for the basal body, we analyzed HBB complexes from flagellar mutants. Because absolute (unconditional) mutants have at most incomplete flagellar structures, we resorted to temperature-sensitive (Fla^{Ts}) mutants isolated as described above. At restrictive temperatures, such mutants were immotile because their flagella were absent or incomplete; at permissive temperatures, they were able to assemble complete flagellar structures and hence were motile.

Mutant strains were grown at a permissive temperature (30°C), and their HBB complexes were isolated and analyzed by SDS gel electrophoresis. We employed silver staining for these gels for two reasons: (i) the samples available were small since we grew cells in small culture volumes to test as many mutants as possible; (ii) Coomassie

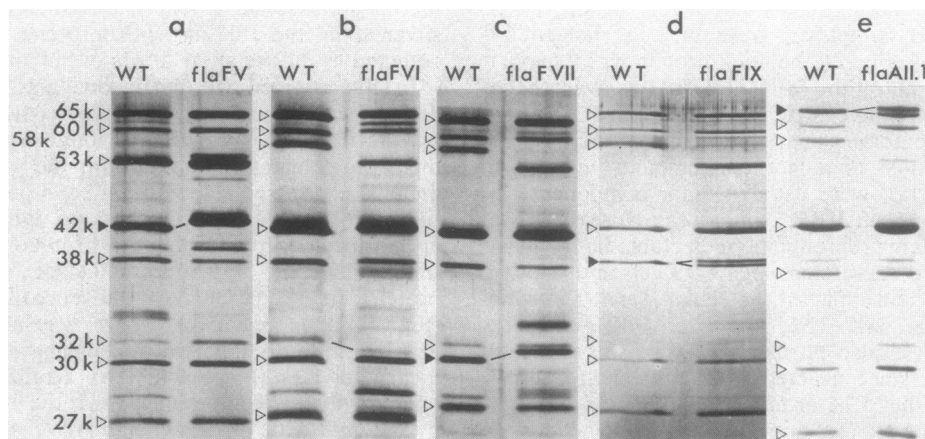


FIG. 8. Use of apparent molecular weights in SDS gels to identify HBB gene products. The left lane of each pair in (a to e) was loaded with wild-type HBB complexes, and the right lane was loaded with HBB complexes from a mutant or mutants defective in a particular flagellar gene. The strains used in the right lanes of each pair were: (a) SJW2249 (*flaFV*), (b) SJW2263 (*flaFVI*), (c) SJW2192 (*flaFVII*), (d) SJW2238 plus SJW2244 (both *flaFIX*), and (e) SJW2205 plus SJW2206 (both *flaAII.1*). A shift in apparent molecular weight between wild-type and mutant was evident in each case for the protein marked with a solid arrowhead. Other HBB proteins are marked with open arrowheads. In lanes d and e, the superposition of material from two mutants defective in the same gene demonstrated a splitting of the gene product to apparent molecular weights above and below that of the wild type. The 58K and 53K proteins are type 1,2 and i flagellins coded for by distinct genes (see the text). The wild-type strain (SJW1103) in the left lane of pair a and the mutants in the right lane of each pair expressed the gene for i flagellin, whereas the wild-type strain (ST1) in the left lanes of pairs b to e expressed the gene for 1,2 flagellin. This difference between wild-type and mutant lanes is not relevant to the *fla* gene under consideration. The decreased apparent molecular weight of the 38K protein in both lanes of e derives from use of a lower β -mercaptoethanol concentration than in the other lanes (see Fig. 5 and the text).

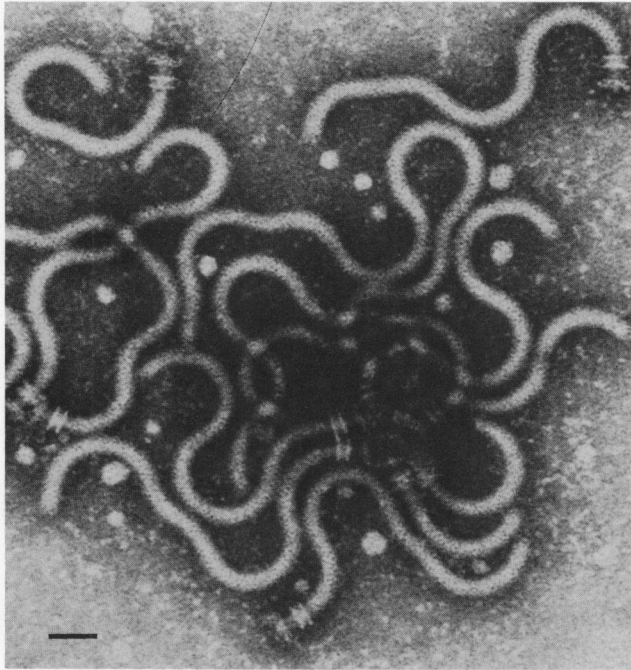


FIG. 9. Polyhook-basal body preparation obtained from a *flaR* (hook length regulation) mutant. The distal end of the polyhooks exhibited a soft rounded appearance, in contrast to the notched appearance (Fig. 3) exhibited by wild-type HBB complexes. Bar, 50 nm.

staining demands a relatively large sample size which can introduce distortions because of overloading of major components such as the hook protein. Such distortions might have obscured subtle mobility differences between mutant and wild-type proteins.

The gel patterns of HBB complexes from Fla^{Ts} -mutants were compared with that of a wild-type strain, either ST1 or SJW1103. Small but reproducible differences were encountered in about 1/5 of the mutants tested. Among the bands of HBB complexes from strain SJW2249 (*flaFV*), the 42K protein had a decreased mobility compared to that of the wild type (Fig. 8a). With complexes from SJW2263 (*flaFVI*), the 32K protein had an increased mobility (Fig. 8b). Two *flaFVII* mutants, SJW2188 and SJW2192, had a 30K protein with a decreased mobility (Fig. 8c [only strain SJW2192 is shown]). Two *flaFIX* mutants, SJW2238 and SJW2244, showed slightly altered mobility of the 38K protein, but in this case in opposite directions; although the mobility difference between the wild-type protein and that of either of the mutants was small, the two mutant proteins were separated sufficiently to be resolved when the respective HBB samples were mixed and run in the same lane (Fig. 8d). A similar situation occurred for the 65K protein of three *flaAII.1* mutants, SJW2205, SJW2206, and SJW2213 (Fig. 8e [only strains SJW2205 and SJW2206 are shown]).

We examined the HBB complexes of several of these Fla^{Ts} mutants by electron microscopy and found them indistinguishable from those of wild-type cells. Furthermore, they were thermally as stable as wild-type complexes, showing no morphological degradation after being maintained at 65°C for 30 min. Finally, Fla^{Ts} cells grown at 30°C retained motility for many hours after transfer to 40°C. These observations all suggest that, once incorporated into

the structure, the mutant proteins are constrained to be as stable as the wild-type protein.

Polyhook-basal body complexes. Polyhook-basal body complexes produced a band in CsCl gradients at about the same position as did HBB complexes and could be purified in a similar fashion. The morphology of their basal bodies appeared normal (Fig. 9). Aside from hook length, the only difference detected was at the distal end of the polyhook, which had, instead of the characteristic V-shaped notch seen with HBB complexes (Fig. 2 and 3), a soft rounded appearance like that noted by Homma et al. (24) for *flaU*, *flaV*, and *flaW* mutants.

Polyhook-basal body complexes (Fig. 10) possessed an approximately 10-fold greater amount of hook protein than did HBB complexes, as expected from the polyhook morphology. The 60K protein was totally absent in these structures, whereas the stoichiometries of all other components were similar to their stoichiometries in HBB complexes.

Filament-hook complexes. Evidence has been presented (24) that several proteins may connect the filament to the hook structure ("mortar" or "junction" proteins; see below). We therefore wished to establish whether any of the proteins found in HBB complexes were present in filament-hook complexes and also whether any additional components were present.

Filament-hook complexes were isolated by shearing as described above, and the filament was depolymerized by acid treatment. SDS gel electrophoresis revealed a 60K protein, as well as hook protein and residual flagellin (Fig. 10). In 2-dimensional gels, this protein focused at the same pI (5.0) as the 60K protein from HBB complexes (data not shown). Thus the 60K protein is an acid-stable component, present at either the proximal or the distal end of the hook. Since it was missing in polyhook-basal body complexes, which presumably have a normal rod/hook junction but might have an abnormal distal end, we conclude that the 60K protein is at the distal end of the hook.

Partial structures from HBB complexes. In our effort to understand correlations between protein components and morphology, we sought treatments which would result in partial but specific degradation of HBB complexes.

HBB complexes were stable, as judged by electron microscopy, to heat treatment at 65°C for 30 min. At 68°C, a general degradation of structure began to occur within 15

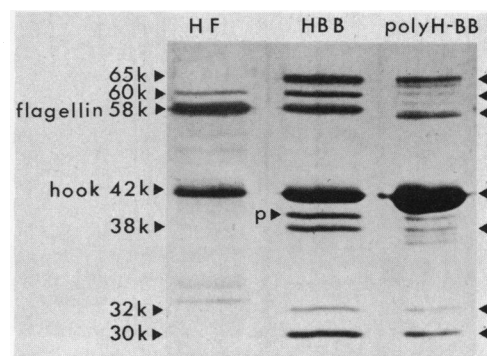


FIG. 10. SDS gels of preparations of hook-filament complexes (HF), HBB complexes, and polyhook-basal body complexes (polyH-BB) isolated from a *flaR* strain (SJW880). HF complexes contained the 60K protein in addition to flagellin and hook protein. Of the flagellar proteins detected in HBB complexes, only the 60K protein was missing from polyH-BB complexes. The band marked p in the HBB lane is a porin.

min, finally resulting in amorphous aggregates. No well-defined partial structures were detected.

Although intact flagella were stable at pH values of up to 12 (see purification protocol above), we found that the filament plays a significant role in conferring this stability. HBB complexes (lacking the filament) proved to be much less stable under these conditions. Although the partial structures that resulted from alkali treatment were better defined than those generated by heat treatment, they were insufficiently homogeneous to justify pursuing biochemical analysis.

Acid treatment was more successful (see reference 16). As described in the purification protocol, the flagellar filament depolymerized at pH 2.5, leaving intact HBB complexes. Isolated hook or polyhook structures can be completely depolymerized by further lowering the pH to 2.0 (1). Under these conditions, we found that HBB complexes underwent a rather specific degradation to yield a preparation consisting almost exclusively of rings (Fig. 11), with no evidence of intact HBB complexes or of anything resembling the rod. Some examples of single or back-to-back staple-shaped structures (described above in the section on HBB morphology) were seen in these preparations. In addition to the open-staple form, closed rectangular structures were noted. The dimensions of the staple and rectangular structures were the same (ca. 16 by 31 nm). They were also the same as those of the outer cylinder ("OCY" in the terminology of Suzuki et al. [54, 55]) that is bounded by the L and P rings in intact HBB complexes. The long dimension of a staple or rectangle was the same as the diameter of the ring structures (Fig. 11).

When ring structures were analyzed by electrophoresis, the 65K, 38K, and 27K proteins were invariably seen in the gel (Fig. 12); no other components were present in significant amounts. The relative amounts of the 38K and 27K proteins were rather constant from preparation to preparation, but the relative amount of the 65K protein was more variable and in general less than that in intact HBB complexes.

DISCUSSION

In this study of the bacterial motor apparatus, we concentrated exclusively on the HBB complex because it is the only

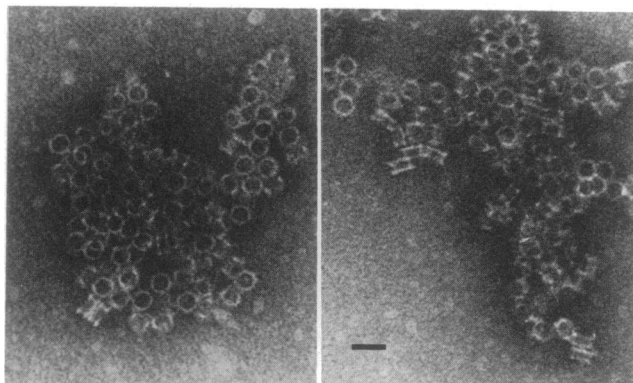


FIG. 11. Two fields of ring preparations obtained from HBB preparations by depolymerization of hook and rod structures at pH 2. Double-staple structures, such as that at the lower left, had the same size and appearance as those in association with HBB complexes (Fig. 3w to y). Single staples and rectangles with the appearance of capped staples can also be seen. The size of rings, and staples or rectangles, was consistent with their being axial and lateral views, respectively, of the same structure. Bar, 50 nm.

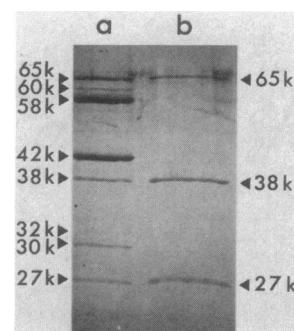


FIG. 12. SDS gels of HBB preparations before (a) and after (b) the pH 2 treatment that yielded the ring structures shown in Fig. 11. Of the proteins present in intact HBB complexes, only the 65K, 38K, and 27K proteins remained.

entity (aside from the helical filament) that is known to be both involved in bacterial motility and recognizable morphologically. Characterization of the HBB complex is important in its own right since it certainly represents a substantial part of the flagellar apparatus. Further, by a process of elimination, its characterization provides a means of establishing which genes and gene products are likely to be involved in other as yet poorly understood parts of the bacterial motor. The present study represents the first detailed examination of the genetic origin and protein composition of the HBB complex of *S. typhimurium*. It complements and extends earlier studies in *E. coli* (16, 29) in a way that reinforces the value of parallel research in these two distinct, yet closely related, enteric bacteria.

From information gained in this and other studies, knowledge of the protein composition of the HBB complex of *E. coli* and *S. typhimurium* is now fairly complete, with a minimum of 9 and perhaps as many as 12 components identified. The structural genes for most of these components are now known, and a number of inferences can be made regarding morphological correspondences.

Improved protocol for isolation of HBB complexes. Without the ability to routinely prepare HBB complexes in high yield and purity, the present study would have been severely hampered. The protocols devised by DePamphilis and Adler (15) and by Simon and co-workers (18, 21) were able to give fairly pure preparations of intact flagella or HBB complexes. However, because of the number of stages involved, yields were poor, and detection of the proteins necessitated use of radiolabeling. In testing these protocols, it became clear to us that the major problem was the rugged character of contaminating outer membrane vesicles, which were resistant to dissolution by a wide range of detergents tested. Since it had been established in previous studies (22, 28) that flagellar structures are capable of withstanding a wide range of pH, we examined the pH dependence of the efficacy of detergents, and found that alkaline pH (11 or higher) gave a rapid and essentially complete dissolution of outer membrane vesicles while leaving the flagellum intact. The addition of this simple step obviated the need for ammonium sulfate fractionation and multiple isopycnic density centrifugations, with the result that a good yield (we estimate from 10 to 40%, depending on culture volume and other factors) of rather pure HBB complexes could be achieved in a 2-day protocol.

Anomalous electrophoretic behavior of HBB proteins. The HBB complex is a very durable structure, able to withstand pH values as acidic as 2.5 and as alkaline as 11 or 12. This

was also reflected in the fact that extreme conditions (SDS, β -mercaptoethanol, and boiling) were necessary to solubilize the sample for isoelectric focusing. Even after this treatment, several of the proteins behaved anomalously, giving rise to streaks or discrete multiple spots in the focusing gel. This may reflect incomplete dissociation into monomers or retention of elements of secondary and tertiary structure.

Morphology of the HBB complex. Our observations of the morphology of the *S. typhimurium* HBB complex conform well to descriptions of the *E. coli* HBB complex by DePamphilis and Adler (16) and Hilmen and Simon (22). However, we noted a few additional features.

The first concerns the shape and size of the S and M rings. Where both could be seen clearly, the S ring was seen as a rather thin, simple disk with no additional features. The M ring, on the other hand, was of slightly greater diameter and thickness; moreover, the thickness was not uniform from inner to outer radii and in the most favorable images gave a distinct impression of additional structure at outer radii that resembled a crown or the claw mount used in setting jewelry.

A further structural feature associated with the M ring only became evident when two HBB complexes were in head-to-head association with each other. The separation between M rings was significantly greater than would have been expected from the appearance of a single particle, and the axial region between the M rings in these HBB dimers did, in fact, reject stain, indicating the presence of additional material. Either this material was invisible in single particles or only a subpopulation of particles possessed the material and underwent dimerization. Whichever explanation is correct, it seems unlikely that the material is amorphous or ill defined since it generated a precise coaxial alignment and a constant separation.

The final feature that we noted was a staplelike structure seen occasionally both in HBB and ring preparations. This structure is probably related to the rectangular structure reported by DePamphilis and Adler (16). Both the dimensions of a staple and its affinity for the outer cylinder of HBB complexes suggest (Fig. 4; see also reference 16) that it is, in fact, a lateral view of an outer cylinder from which the rod has been withdrawn either by breakage, in the case of HBB preparations, or by dissolution, in the case of ring preparations. The appearance suggests a three-dimensional structure somewhat like an open can, with an abrupt right-angle junction between the two surfaces. The apparent thickness of these surfaces was remarkably small (a few nanometers). In some images, especially in ring preparations, the rectangle was closed. We are unable to say whether the difference between closed and open structures represents a real structural difference such as loss of a labile component or whether it is an artifact arising from differences in orientation or staining.

What can be said of the manner in which individual molecular subunits are organized within the HBB complex? All of the major components are proteins of low to medium molecular weight (14K to 65K); for spherical proteins, this would represent diameters in the range of about 3 to 5 nm. The hook was the only HBB component for which any quaternary structural information was available. Like the flagellar filament (46), the hook is a cylinder with close to 5.5 subunits per turn, giving rise to 11 "protofilaments" (2). Three-dimensional reconstruction (59) indicates that the cylindrical diameter (ca. 20 nm) is a result of radial disposition at a tilt angle of about 45° of quite elongated prolate

ellipsoids. The four rings within the basal body were thin, from perhaps 2 to at most 5 nm. It therefore seems likely that each of these rings are radially disposed arrays of subunits of a single protein; the M ring, with its extension at outer radii and the axial material that is apparent in head-to-head dimers, could have additional cytoplasm-proximal components. The rod, which is the transmission shaft of the mechanism, is by our measurement and that of DePamphilis and Adler for *E. coli* (16) less than 15 nm in diameter and must, like the hook and filament, possess an axial channel of about 3 nm for extrusion of external components (hook protein and flagellin). If it has the same type of quaternary organization as do the hook and filament, the subunits are likely to be of considerably lower molecular weight; this is consistent with our suggestion (see below) that the 30K and 32K proteins comprised the rod. The outer cylinder contains, on the basis of our electrophoretic analysis of ring preparations, only two proteins (27K and 38K), although possibly one of the low-molecular-weight components serves to fill in the outer face (L ring) in intact HBB complexes (stippled area of the outer cylinder in Fig. 4) and closed rectangles. What molecular organization generates the remarkable right-angle geometry of the outer cylinder? One possible organization would have the P ring constructed from radially disposed subunits of one protein (probably the 38K protein [see below]) and the cylindrical wall constructed from longitudinally-disposed subunits of the other; the upper lip that creates the appearance of the L ring could be a consequence of a pronounced bend in the subunits at their hook-proximal end.

Apparent molecular weights of proteins from missense mutants can contain valuable information. To identify gene-polyptide correspondences within the HBB complex, we took extensive advantage of a fact that is becoming generally recognized, namely, that the apparent molecular weights of proteins in SDS gels are only approximations of their actual molecular weights. A dramatic example of this phenomenon is the family of methyl-accepting chemotaxis proteins, which undergo substantial shifts in apparent molecular weight as a result of single methyl-esterification events (49). Another example is the reversible shift of the 38K protein of the present study, depending on whether a reducing agent was present. deJong et al. (13) have shown in detailed studies of the mammalian lens protein, α -crystallin, that a single amino acid change and not necessarily one involving a change in charge can cause measurable changes in the mobility of the protein in SDS gels.

The use of spontaneous conditionally nonflagellate mutants with a lesion (presumably a missense mutation) sufficiently minor to permit flagellar assembly at permissive temperatures has enabled us to take advantage of this phenomenon of apparent molecular weight variability. The number of mutants we were able to examine in a given gene varied from 0 to around 10. In each case where we had five or more, at least one displayed a mobility shift, so that the probability of success with any given mutant was fairly high (on the order of 20%).

The method should be generally applicable provided reasonable numbers of mutants are available in the genes of interest. We were forced to resort to conditional mutants to achieve flagellar assembly. However, in systems where absolute mutants can be used, altered protein electrophoretic mobility might be even more common, and the changes might be more pronounced. We restricted our approach to one-dimensional SDS gels because of the poor degree of focusing exhibited by many of the HBB proteins. For

proteins with well-behaved isoelectric focusing properties, the combination of both dimensions might provide an even more powerful technique with a higher probability of detecting a difference in any given mutant.

The most straightforward conclusion that can be drawn from a protein mobility shift in a *fla* mutant is that the protein is an authentic flagellar component, not a contaminant. Can one further conclude that the protein is itself the product of the gene in question? We believe so. An alternative conclusion would be that the protein is posttranslationally modified and that modification is defective in the mutant. However, the flagellum in these Fla^{Ts} mutants is fully functional at the permissive temperature, and it is difficult to imagine a defective modification that would permit function at the permissive temperature yet prevent it at the restrictive temperature. The direct conclusion that the altered protein is the product of the mutated gene is certainly valid for *flaFV*, whose product is the 42K hook protein (27, 31). We regard this as a vindication of our interpretation of the results for the other genes. Finally, several of our identifications are consistent with information from other sources (see below).

Thus far, with the exception of the *flaAII.1* gene, we concentrated on genes in the region I cluster on the genome, because earlier studies (29, 54) had revealed that a number of HBB genes resided there. Extension of the study to include the remaining known flagellar genes is in progress.

Gene-protein-morphology correspondences in the HBB complex. We now review our conclusions regarding correspondences among the genes, gene products, and morphology of the HBB complex (Table 2). The relationship in the hook is straightforward and well known—*flaFV*, 42K protein, and hook structure.

The 60K protein, which we observed in hook-filament complexes and in HBB complexes, but not in polyhook-basal body complexes, was demonstrated by Homma et al. (24) to reside at the distal end of hooks sheared from filamentless mutants. Our observations are in agreement with this conclusion. Homma et al. termed this protein a hook-associated protein (HAP1) and concluded from its absence in certain mutants that it was the product of the *flaW* gene. Komeda et al. (29) assigned a 60K component of the *E. coli* HBB complex to the homologous gene *flaS*. Homma et al. (23) noted that *flaW* mutants secreted flagellin into the medium, suggesting that the hook tip had not undergone a modification needed for filament assembly. Homma et al. also found two other HAPs in hooks from filamentless mutants (HAP2, 53K; HAP3, 35K), which they ascribe to the *flaV* and *flaU* genes, respectively. We have been unable to detect these components in either HBB complexes or hook-filament complexes. It might be that the proteins are labile under the acid conditions we use for depolymerization of the flagellar filament; this explanation would be consistent with the inference from the HAP study that the 60K protein is the first to assemble onto the hook tip. An alternative explanation for the absence of the 53K and 35K proteins would be that they function as scaffolding proteins and are discarded before filament assembly; recall in this regard that Homma et al. reported the presence of HAPs in hooks from filamentless mutants, not at the hook-filament junction of intact flagella. Polyhook-basal body preparations lacked the 60K HAP1 as well as the other HAPs. Like the distal end of hooks in the filamentless structures studied by Homma et al., the distal end of polyhooks was found by us to lack the V-shaped notch that is a characteristic of both the normal hook and flagellar filament. This morphology of "uncapped" hooks is somewhat surprising since it is known (59) that the

TABLE 2. Correspondences among genes, proteins, and morphological features of the HBB complex of *S. typhimurium*^a

Apparent mol wt (10 ³)	pI	Gene	Morphological features, functions, or comments
65	6.8–7.3	<i>flaAII.1</i> ^b	M ring? Base plate for energy-transducing and switching proteins?
60	5.0	<i>flaW</i> ^c	Junction between hook and filament; absent in polyhook-basal body complexes; found as HAP1 in hooks from filamentless mutants
58	4.8	<i>H2</i> ^d	Flagellar filament, 1,2 antigen
53	5.2	<i>H1</i> ^d	Flagellar filament, i antigen
42	4.7	<i>flaFV</i> ^e	Hook protein
38	ca.9	<i>flaFIX</i> ^b	P ring portion of outer cylinder?
32	4.5	<i>flaFVI</i> ^b	Rod?
30	4.6	<i>flaFVII</i> ^b	Rod?
27	7.5	<i>flaFVIII</i> ^f	L ring and wall portion of outer cylinder?
16	5.4	?	?
14	5.5	?	?
12	7.3	?	?
?	?	?	S ring

^a Only proteins detected by us in HBB complexes are shown. Homma et al. (24) reported two additional proteins at the distal end of hooks from filamentless mutants, with sizes of 53K and 35K and which are coded for by the *flaV* and *flaU* genes, respectively (see the text).

^b Gene assignment made in the present study.

^c See reference 24.

^d See references 25 and 62.

^e See reference 30; confirmed in the present study.

^f Status as an HBB protein is uncertain (see the text).

hook subunits themselves are at a tilt angle that would be expected to give a notched appearance, as is the case with the distal end of the flagellar filament (5). Perhaps the hook protein has greater motional freedom at the distal end, and one role of the HAPs is to create a more rigid geometry compatible with that of the filament.

We infer from the data in Fig. 11 and 12 that the 65K, 38K, and 27K proteins are involved in ring structures. Figure 8 shows that the corresponding genes are *flaAII.1* (65K) and *flaFIX* (38K) with the gene for the 27K protein being unassigned. The 38K protein in the HBB complex of *E. coli* was previously established by Komeda et al. (29) as being the product of the *flaM* gene (homologous to *S. typhimurium flaFIX*). That the 38K proteins in the two bacterial species are homologous is further established by their unusual behavior with respect to presence or absence of β -mercaptoethanol in SDS gels (Fig. 5 and 6) (29).

The 38K protein is likely to form the P ring. The *flaFIX* gene was one of two found by Suzuki et al. (54) to be necessary for the P ring to appear in partial HBB structures from *fla* mutants, and we found the 38K protein to be present in ring preparations. The 27K protein may comprise the wall and L ring of outer cylinders as suggested above. The assembly scheme drawn up by Suzuki et al. suggests that the gene responsible for completion of the outer cylinder is *flaFVIII* (their observation was confirmed in the present study by our observation of partial HBB structures lacking the L-ring when a *flaFVIII* mutant was grown at the restrictive temperature; data not shown). Further, the genomic organization with basal-body genes *flaFV*, *flaFVI*, *flaFVII* and *flaFIX* surrounding *flaFVIII* suggests that the latter, too,

may be a basal-body gene. Unfortunately, the one *flaFVIII*(Ts) mutant we isolated failed to show a mobility change in any protein. The homologous gene in *E. coli*, *flaY* (30), was unknown at the time of the study by Komeda et al., and they did not report a protein corresponding to the 27K *S. typhimurium* protein on the basis of their λ programming data. Both they and we did, however, see such a component (reported by them at 20K; in our hands, at 27K) in *E. coli* HBB preparations.

There are several lines of evidence to suggest that the 65K protein is a major component of the M ring. It was present in ring preparations. It is unlikely to be a component of the outer cylinder since the gene for the 65K protein (*flaAII.1*) is a prerequisite for any detectable basal body structure, suggesting that its product is an early component in the assembly process, which is known to proceed from cytoplasmic components outwards (54, 55). Data concerning the susceptibility of the 65K protein to acid hydrolysis (see below) are also consistent with the assignment of the 65K protein to the M ring. Finally, in work carried out before we determined the gene coding for the 65K protein, we obtained a monoclonal antibody that was unambiguously directed against this protein. This antibody decorated the cytoplasmic face of the M ring of HBB complexes (S.-I. Aizawa and J. Countryman, unpublished data). The decoration, however, was not reproducible, and we did not feel justified in making a strong conclusion from it; nonetheless, it is consistent with our suggestion that the M ring is constructed from the 65K protein.

The only major difference between the one-dimensional gel patterns of *S. typhimurium* and *E. coli* HBB complexes was the absence of a 65K protein in the latter. Bartlett and Matsumura (8) have recently established that the *flaB* gene of *E. coli* is, in fact, three genes, with the leading gene, now termed *flaBI*, being homologous with the *flaAII.1* gene of *S. typhimurium*. They also found that the gene product was a protein of 60K. We observed in *E. coli* HBB complexes (see above) a protein of that apparent molecular weight and with a pI similar to that of the *S. typhimurium* 65K protein. Presumably, this 60K HBB protein of *E. coli* is, in fact, the product of the *flaBI* gene.

It is interesting that the adjacent gene to *S. typhimurium* *flaAII.1* is *flaAII.2* (*flaBII* in *E. coli*), which codes for a protein that is known to be necessary for proper motor rotation and switching (11, 19, 57). However, this protein is not a component of the HBB complex; it has an apparent molecular weight of 38K (8) and an acidic pI (S.-I. Aizawa, unpublished data), whereas the only 38K protein in the *S. typhimurium* or *E. coli* HBB complex is the extremely basic *flaFIX* (*flaM*) protein. The *flaAII.2* (*flaBII*) protein is also known to interact with cytoplasmic components of the chemotactic signalling system (8, 50). A reasonable hypothesis therefore would be that the *flaAII.2* protein is a peripheral protein on the cytoplasmic face of an M ring constructed from the *flaAII.1* protein. It is interesting in this regard that all of the Fla^{Ts} mutants (including *flaAII.1* mutants) employed in the present study remained flagellate and motile indefinitely when switched to a restrictive temperature under nongrowth conditions, while a *flaAII.2* mutant with Mot^{Ts} phenotype could be rendered immotile in 0.5 s by an abrupt temperature increase (11), suggesting that the *flaAII.2* protein is in a relatively unconstrained environment.

Although the HBB complex was durable, we found that the primary structure of the 65K protein (and also of the hook protein and flagellin) was susceptible to hydrolysis at acidic pH. This may explain why, in the ring preparations we

obtained at pH 2, the amount of the 65K protein was variable and usually less in relation to the 38K and 27K proteins than in intact HBB complexes. Also, in micrographs such as that shown in Fig. 11, the majority of rings had a thin, unfilled annular appearance, suggesting that they were outer cylinder rings, whereas in samples that had been treated less severely, electron microscopy revealed more debris, but also more examples of rings with a filled appearance.

The 30K and 32K proteins, for which we established the corresponding genes to be *flaFVII* and *flaFVI*, respectively, are not present in ring preparations, and both were found by Suzuki et al. to be essential for the earliest detectable stage of flagellar assembly (54). The most likely structural role for them is the rod. Their relatively low molecular weight would be consistent with the somewhat smaller diameter of the rod compared with either the hook or the flagellar filament. Why should there be two proteins in the rod structure when there is only one in the other major cylindrical structures, the hook and the filament? The answer may be that the rod does not appear to have uniform properties along its length; its interaction with the outer cylinder is confined to the distal end since no axial drift toward the S and M rings was seen in isolated HBB complexes, even though there were no cell surfaces to constrain or impose the axial geometry.

The gene assignments we have made for the 30K and 32K proteins resolves an anomaly in the assignments made earlier in *E. coli* by Komeda et al. (29). They observed two proteins of similar apparent molecular weights (27K and 30K; in our hands, 30K and 32K) and pIs and assigned both of them to the *flaL* gene (homologous to *S. typhimurium* *flaFVII* [30]) with the hypothesis that either multiple translation or posttranslational modification was occurring. More recently, it has been shown that there is an additional gene, *flaX* (homologous to *S. typhimurium* *flaFVI* [30]), adjacent to *flaL*. The hybrid phage that synthesized both proteins presumably contained both genes.

One feature of the HBB complex, the S ring, remains unassigned. It is a quite thin structure, and so presumably is constructed from rather low-molecular-weight subunits. The 16K and 14K proteins are also unassigned, with respect to both their genes and their structural roles. It is possible that one or both of them is a component of the S ring. The 12K protein is another possibility; although it was only detected in ³⁵S-labeled preparations, it did appear to be a well-defined species since it focused at a single pI value.

The HBB complex is not the complete flagellar motor. It is noteworthy that none of the genes that have been identified with HBB structure include those that can give rise to a paralyzed (Mot⁻) or abnormal switching (Che⁻) phenotype. The list of HBB genes now includes the *flaAII.1* gene, which probably codes for the M ring, a component that has often been invoked as the rotor. We suggest instead that the M ring is merely a mounting plate. Indeed, we suggest that the entire HBB complex is a passive structure and that the motor, in the sense of the apparatus for chemomechanical energy transduction and the switch for clockwise-counter-clockwise rotation, is constructed from components that are either integral membrane proteins circumferentially arranged around the HBB complex, as may be the case with the *motA* and *motB* gene products (see recently described physiological experiments by Block and Berg [10] and structural data from Dean et al. [12]), or attached to the cytoplasmic face of the M ring of the HBB complex, as may be the case with the *flaAII.2* (*motC*, *cheV*), *flaN* (*motD*), and *flaQ* (*motE*, *cheC*) gene products. The isolation of a more complete flagellar motor structure that includes these compo-

nents as well as the HBB complex is obviously a desirable goal for the future.

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