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# Isolation and Ultrastructural Study of the Flagellar Basal Body Complex from *Rhodobacter sphaeroides* WS8 (Wild Type) and a Polyhook Mutant PG

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**Filament-Hook-Basal Body (FHBB) complexes were isolated from the purple non-sulphur facultative anaerobic bacterium *Rhodobacter sphaeroides* (WS8) by lysozyme digestion of the cells followed by an alkaline treatment and ultracentrifugation, and they were analysed by electron microscopy. The structure is composed of a filament linked through an enlarged junction to the hook and a basal body composed of L and P rings, a rod, and a less well-defined cytoplasmic ring that has evidence of additional attached structures. Hook-basal body complexes isolated from a mutant (PG) which produces an extended hook but no filament shows basal body structures identical to those of wild-type FHBBs.** © 1997 Academic Press

The bacterial flagellum is composed of a helical filament driven by a 'motor' located in the cell membrane. Rotation of these helical filaments propels the bacteria towards stimulants or away from repellants. The morphology, biochemistry and genetics of this organelle has been extensively studied in *Salmonella typhimurium*, and a vast number of mutations have been characterized that affect the assembly, structure and function of the flagellum (for review see 1,2). This has given us a detailed picture of this organelle, although much remains to be discovered. *S. typhimurium* possesses 5 to 10 flagella per cell, and the filaments rotate in both clockwise and counterclockwise directions. Recent structural studies (3) confirm the presence of four ring groups; the fixed L (lipopolysaccharide) and P (peptidoglycan) rings, and the rotating MS (membrane/supramembrane) and C (cytoplasm) rings. A rod passes from the MS ring through the center of the L and P rings and transmits the rotational force to the hook, which in turn is linked via two hook-associated proteins (HAPS) to the helical filament. The fixed MotA and MotB proteins, which are embedded in the cytoplasmic membrane, form the 'stator' component of the 'motor' and act as part of a channel for protons, which provide the energy to rotate the flagellum (4,5). This complex, minus the filament, is known as the hook-basal body (HBB) complex.

*Rhodobacter sphaeroides* is a motile purple non-sulphur photosynthetic bacterium (6). In contrast to *S. typhimurium* each cell possesses a single flagellum, which rotates only in a clockwise direction (7). There have been several studies on the chemotaxis system of *R. sphaeroides* (8, 9) but very little has been reported on the structure or the genetics of the motor. The only HBB gene characterised is MotA (10), and there is a report of the structure of the HBB and a mutation that appears to eliminate the inner rings (11). In this work we report a simple method of isolating filament-HBB (FHBB) complexes, and an analysis of their structure.

## MATERIALS AND METHODS

*Strains and growth conditions.* *R. sphaeroides* WS8 (wild-type) or PG (polyhook, 12) was grown anaerobically in 1.2L of Sistrom's medium (13) + 2g/l casein acid hydrolysate supplemented with 25mg/L kanamycin for the polyhook strain, for 15h, illuminated by 6 × 100W incandescent bulbs, and reached an $OD_{600}$ between 0.2 and 1.0.

*Purification of FHBB complexes.* Buffer volumes in the preparation are given for a culture $OD_{600}$ of 0.35. Volumes were increased or decreased in direct proportion to the $OD_{600}$ value. Cultures were harvested, washed in 30ml of buffer A (10mM Tris pH 8.0, 50mM NaCl), resuspended in 25ml of buffer B (0.1M Tris pH 8.0, 8mM EDTA, 1% Triton X-100) and treated with lysozyme (0.1mg/ml) for 30min on ice. $MgCl_2$ was added to 10mM and DNase to 10$\mu$g/ml, and the mixture incubated 20 min at 30°C. After a slow-speed centrifugation (4500**g**/10min) the supernatant was passed through a 26-gauge needle 10 times, and the pH raised with 2M NaOH. The FHBB complexes were pelleted at 25 000**g** for 45min at 4°C and resuspended in 0.5ml of TET buffer (10mM Tris pH 8.0, 5mM EDTA, 0.1% Triton X-100).

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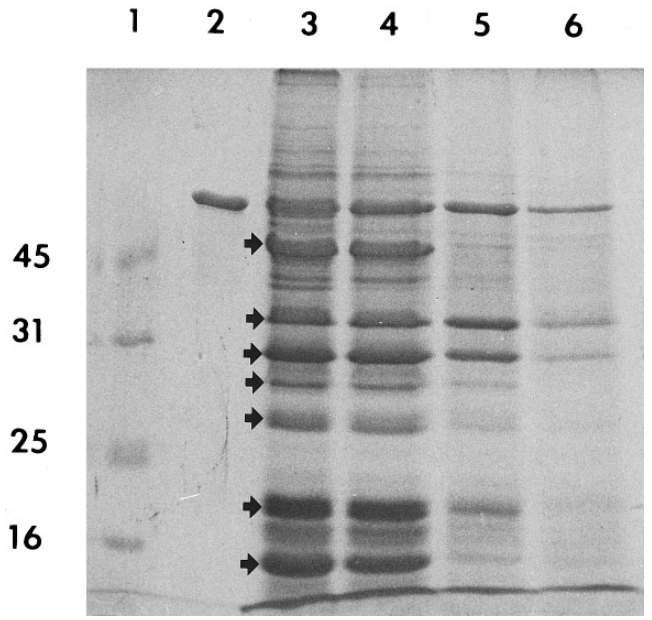


**FIG. 1.** SDS-PAGE of the purification of FHBB complexes. Each lane contains10$\mu$l of samples treated at various pH values. Lane 1, molecular weight markers (kDa); lane 2, purified filament protein (55kDa); lanes 3 to 6, preparations treated at pH 8.0 (3), pH 10.5 (4), pH 11.2 (5), pH 12.0 (6).

*Gel permeation chromatography.* Samples purified by alkaline treatment (200$\mu$l) were loaded on to a Sepharose CL-4B column (10mm $\times$ 150mm) equilibrated in TET buffer. Fractions of 1.2ml were collected, centrifuged 35 000**g**/60min, and resuspended in 100$\mu$l of TET buffer.

*Electron microscopy.* Samples were diluted 1 in 5, applied to carbon-coated copper grids and stained negatively with 1% uranyl acetate or a 1:1 mixture of 1% uranyl acetate:1% phosphotungstic acid and examined at 60 or 80kV in a JEOL 1200EX II electron microscope.

*Image processing.* Composite of the 12 images of figure 2a. were processed using Adobe Photoshop version 4.0. Images were imported from scanned photos, adjusted to the same size using the electron microscope scale bar, aligned on a grid by a horizontal line through the gap between the L and P rings, and a vertical line through the center, and overlaid so that each image contributed equally to the final composite.

*General.* SDS-PAGE was carried out according to the method of Laemmli (14). All chemicals were from Sigma (St Louis MI) or J. T. Baker (México).

## RESULTS AND DISCUSSION

Initially, to isolate the FHBBs we followed the method of Zhao (15), which utilizes shearing at a high concentration of Triton X-100 to remove membrane contaminants. However, the FHBBs we obtained were heavily contaminated by membranes and membrane proteins of the photosynthetic apparatus. An alkaline treatment (see Materials and Methods) greatly improved the purification (Fig. 1). The filament band at 55kDa is by far the major protein of the FHBB complex. During purification, preparations were treated at various alkaline pH values. A disappearance or marked decrease of the arrowed contaminant bands in proportion to the filament band is apparent at higher pH values. The other FHBB proteins do not show in this gel because of their low abundance relative to the filament, but FHBB structures are visible by electron microscopy at all pH values. Fig. 2a shows FHBB complexes isolated at pH 11.5 before (1, 6-10) and after (2-5, 11, 12) passage through a Sepharose CL-4B gel filtration column. The gel filtration step removed some of the contamination remaining after alkaline treatment and gave noticably cleaner electron microscopy preparations. The dimensions of the ring structures were in nearly all cases noticably less at pH 11.5 (Fig. 2a) than at pH 8 (Fig. 2b), probably due to release of adhering contaminating proteins or membrane particles. In addition, two outer rings were discernable, in place of the one wider structure at pH 8.0. *S. typhimurium* HBBs containing the C ring (a large extended structure attached to the MS ring) could be isolated using mild conditions. Similar structures were not seen in *R. sphaeroides* although some preparations were carried out under mild conditions (Fig. 2b), suggesting that this structure either does not exist, or is very delicate and easily lost.

Aggregations of FHBBs via their ring structures were sometimes seen (Fig. 2c) most probably due to hydrophobic interactions between the ring proteins. Hook measurements from 12 micrographs of FHBBs gave a length of 94nm $\pm$ 2% and a diameter of 14.8nm $\pm$ 12%, significantly longer and slightly narrower than the hook of *S. typhimurium*, which is 55nm long and has a diameter of 20nm (16, 17). An unusual feature of the hook-filament junction is a distinct enlargement (arrowed in Figs. 2a and 2b) where the hook-associated proteins (HAPS) are located. No enlargement is seen in this region of the *S. typhimurium* hook-filament junction, suggesting a different organisation of these proteins in *R. sphaeroides*. This may reflect the higher torsional strain that the hook-filament junction is subjected to in this singly flagellated bacterium.

The composite FHBB image constructed with the program Photoshop (Fig. 3a) clearly shows paired outer rings similar to the L and P rings of *S. typhimurium*. The structure of the cytoplasmic ring is less well-defined, although in some of the individual micrographs (Fig.2a, numbers 3, 4, 10, 11, 12) more than one inner ring and evidence of small extensions from this ring can be seen, similar to the MS and C-ring structures of *S. typhimurium*. The overall dimension of the organelle is smaller than that of *S. typhimurium* (compare Figs. 3b and 3c). Paired L-P ring structures are a common feature of HBBs isolated from many bacteria including *S. typhimurium* (18), *Escherichia coli* (19), *Caulobacter crescentus* (20), *Wolinella succinogenes* (21), *Vibrio cholerae, Campylobacter foetus* and

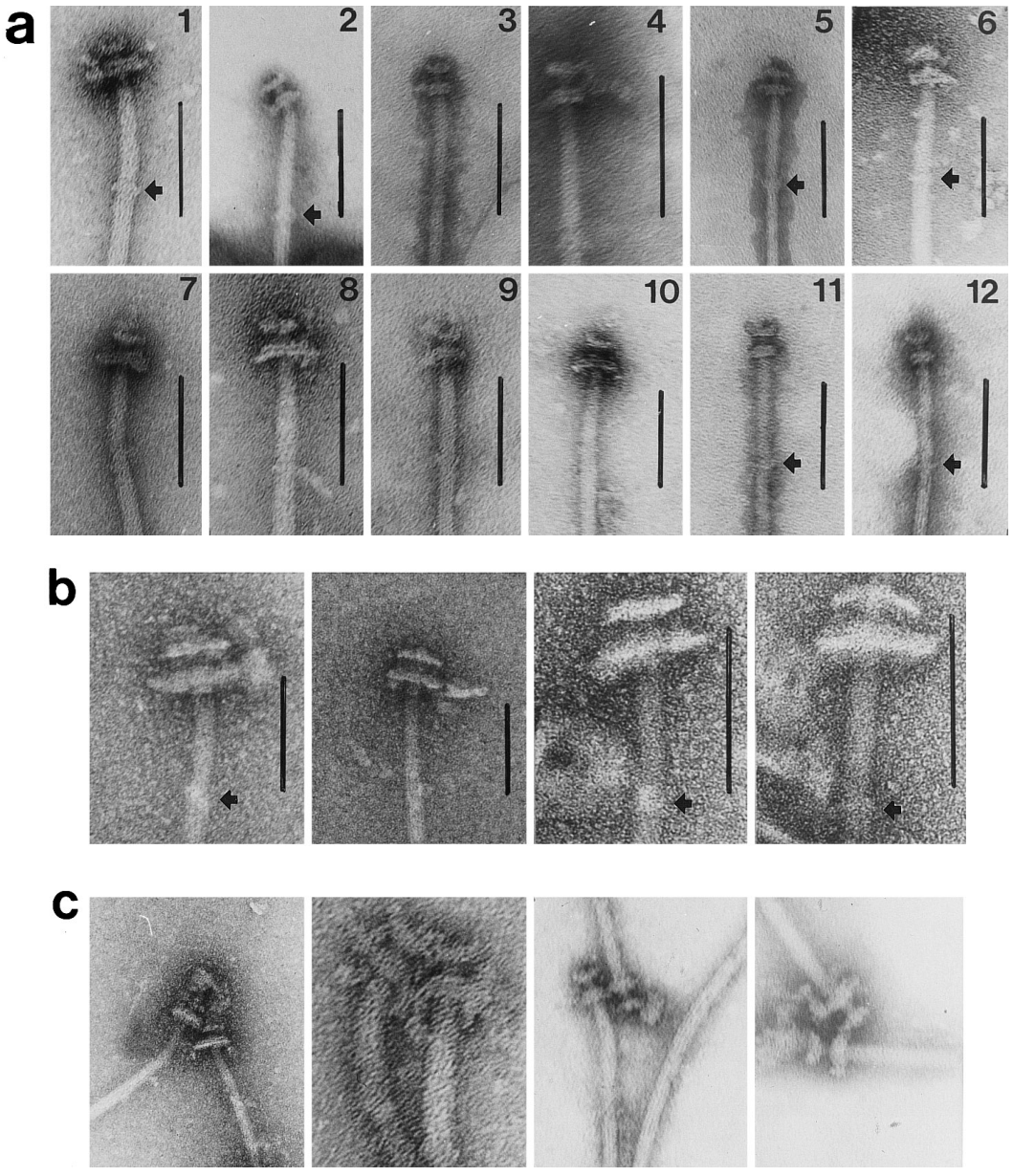


**FIG. 2.** Electron microscopy of FHBB complexes. (**a**) Samples treated at pH 11.5 before (1, 6-10) and after (2-5, 11, 12) chromatography and stained negatively with 1% uranyl acetate (1, 6-10) or a 1:1 mixture of 1% uranyl acetate:1% phosphotungstic acid (2-5, 11, 12). (**b**) Samples treated at pH 8 and stained with 1% uranyl acetate. (**c**) Aggregates of FHBB complexes.

*Aquaspirillum sepans* (22). Their diameters vary from around 20 to 40nm. Inner ring structures of these bacteria show more variability both in number (2 or 3), and in size (11nm to 50nm or more in the case of the C ring of *S. typhimurium*). The dimensions of the HBBs from *R. sphaeroides* are within these ranges.

To analyse the individual proteins of the HBBs it will be necessary to remove more of the contaminating membrane-associated proteins from the FHBB preparations, and eliminate the filament without destroying the delicate HBBs. Elimination is easily achieved in *S. typhimurium* by treatment of the FHBBs at pH 2 to dissociate the filament. Highly pure HBB preparations have been obtained by this method (16) and individual proteins have been identified in SDS-polyacrylamide gels based on calculated molecular weights derived from DNA sequence of the cloned genes. None of this is possible with *R. sphaeroides* because the filament does not dissociate at acid pH (23) and no HBB genes apart from MotA have yet been cloned and sequenced. An alternative way of eliminating the filament is to isolate HBBs from a strain that has a mutation in *fliK*, which codes for a protein that regulates the changeover from hook to filament assembly and produces hooks

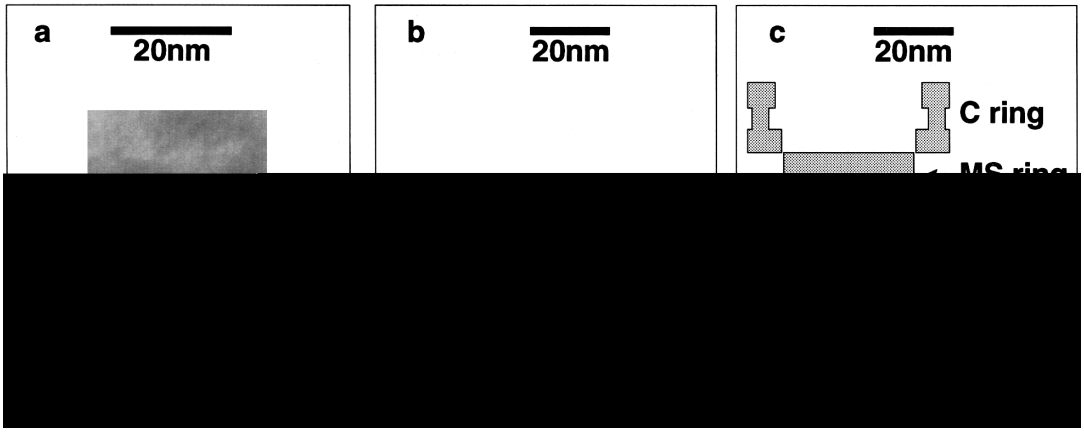


**FIG. 3.** (**a**) Composite of the 12 images of figure 2a. (**b**) Diagram of the HBB complex of *R. sphaeroides* derived from the composite photo. (**c**) Diagram of the HBB complex of *S. typhimurium* based on information from reference 3.

that do not have a filament attached (12). The HBBs that we have obtained from this mutant are shown in Fig. 4. The hook is of variable length, and there is no hook-filament junction, but the basal body structure appears to be identical to that of the wild-type strain in Fig. 2a, indicating that this mutation does not affect the assembly of the basal body components.

Bearing in mind that it was only after extensive development of HBB isolation procedures in *S. typhimurium* that detailed images of structures such as the C ring were obtained, it is highly probable that development of improved purification procedures and more sophisticated image analysis methods for *R. sphaeroides* will reveal many more structural details of the basal

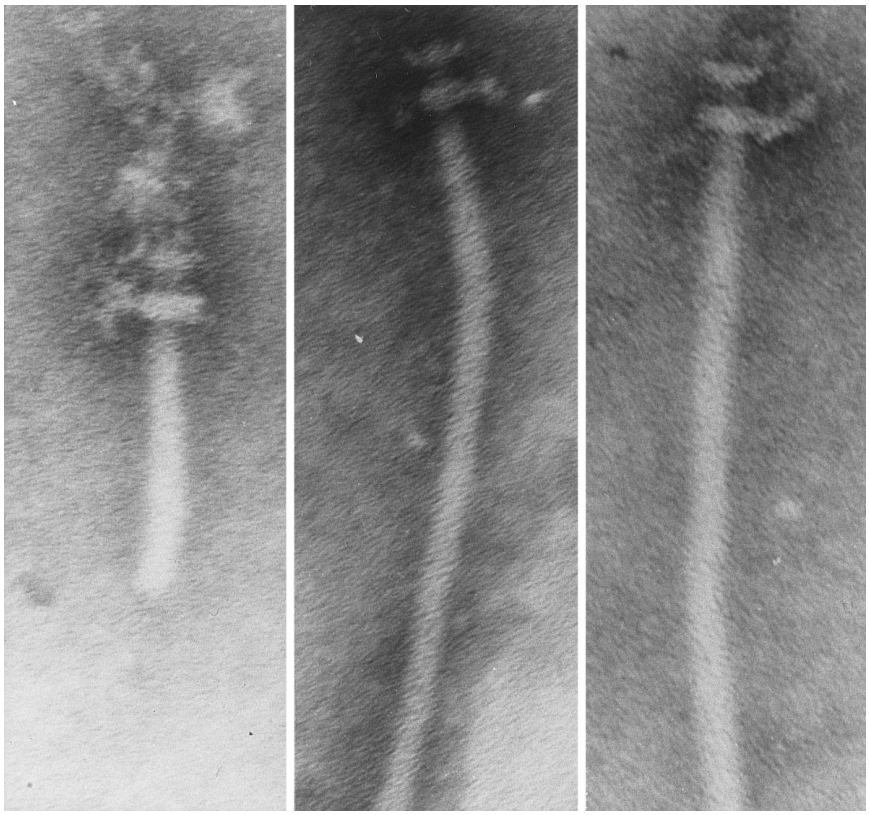

**FIG. 4.** Hook-Basal bodies prepared by the same method as used for FHBB preparations using a pH of 11.5 to remove contamination, without Sepharose CL-4B chromatography, and stained with a 1:1 mixture of 1% uranyl acetate:1% phosphotungstic acid.

body components, especially those of the cytoplasmic rings.

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