Identification of the M-ring protein of the flagellar motor of Salmonella typhimurium
(bacterial flagella/basal body/monoclonal antibody/immunoelectron microscopy)

MICHIO HOMMA, SHIN-ICHI AIZAWA*, GARY E. DEAN†, AND ROBERT M. MACNAB‡
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511
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ABSTRACT The M ring is a substructure of the flagellar basal body of bacteria, which lies in the cytoplasmic membrane and is therefore close to the site where the energy of the transmembrane proton potential is converted into mechanical work of rotation of the motor. The protein from which this ring is constructed has not been identified. Flagellar hook--basal body complexes from Salmonella typhimurium were used as the immunogen for the preparation of monoclonal antibodies. An antibody obtained was directed against a major basal-body component, a 65-kDa protein that from mutant studies has been assigned as the product of the flaH1 gene. By immuno- electron microscopy, the antibody was observed to bind the innermost feature of the basal body: the cytoplasmic-facing surface of the M ring. We conclude that the 65-kDa protein is a component—probably the main component—of this important substructure of the flagellar motor.

Bacterial flagella are rotary devices for motility (1, 2), the energy source being the protonotive force across the cell membrane (3); the flagellar motors switch spontaneously between counterclockwise and clockwise senses of rotation and use this capability to express migrational behavior (4). For review, see ref. 5.

The flagellum consists of at least three parts: the filament, the hook, and the basal body. Of these, only the basal body is relevant to motor function, if we confine the definition of motor function to the generation of torque from protonotive force; the role of the hook and filament is simply to transmit that torque to the medium. The basal body is a structure that is embedded in the cell surface and consists of a pair of outer rings (in Gram-negative bacteria only), a pair of inner rings, and a rod, all in a coaxial arrangement. The outer (L and P) pair of rings and the inner (S and M) pair of rings are associated with the cell wall (outer membrane and peptido- glycan layer) and cytoplasmic membrane, respectively (6).

In Salmonella typhimurium and Escherichia coli, the basal body is constructed from at least eight protein species (7), for which in many cases the structural genes have been identified (7-10). Whereas the proteins comprising the L and P rings have been identified (11), those for the S and M rings remain unknown.

Where precisely does torque generation occur? The inner rings, being located at the cell membrane (6) where the energy source for motor rotation (the protonotive force) resides, are likely to be involved. This is especially true of the M ring, since it actually resides in the cell membrane (the S ring lies just above the membrane surface). There is also substantial genetic evidence that several other non-basal-body components are essential for motor function (7, 8, 12-15). Interaction between these components and the inner rings is likely to be required for motor function.

Identification of the proteins comprising the inner basal-body rings is therefore an important part of the characterization of the flagellar motor. By means of a monoclonal antibody directed against a 65-kDa basal-body protein, we have been able to demonstrate that this protein is a major component of the M ring.

MATERIALS AND METHODS

Bacteria. S. typhimurium strains used were ST1 (16) and SJW1103 (17), both of which synthesize normal flagella, and SJW1135 (18), which carries the flaFI1679 mutation and produces a partial basal-body structure. The E. coli strain used was YK410 (19), which synthesizes normal flagella.

Purification of Hook--Basal Body Complexes from a Wild-Type Strain. HBB complexes were purified as described (7).

Purification of Partial Basal Bodies from flaFI Mutant SJW1135. A crude fraction of partial basal bodies was prepared from SJW1135 as described (fraction BMM; ref. 18). For purification, they were layered onto a 5-30% linear sucrose gradient in 50 mM Tris-HCl, pH 7.8/0.3 M NaCl/2.5 mM EDTA/0.2% Triton X-100 and centrifuged (110,000 × g, 8 hr). Gradient fractions were examined by electron microscopy, and fractions containing partial basal bodies were collected, diluted 1:10 into 10 mM Tris-HCl, pH 7.8/2.5 mM EDTA/0.1% Triton X-100, and centrifuged (100,000 × g, 2 hr). The pellet was then resuspended in the same buffer.

Preparation of Monoclonal Antibody. HBB complexes were prepared, essentially as described (7), from 4 liters of late exponential-phase cells of wild-type S. typhimurium ST1 (total yield of HBB complexes, 270 μg). NZB mice were each immunized with ~20 μg of HBB complexes in Freund’s complete adjuvant by footpad injection; booster injections were given intravenously. Fusion of spleen and SP2/0-Ag14 myeloma cells and cloning of hybridomas were done by conventional procedures (20). Supernatants were screened for binding activity against purified HBB complexes [in phosphate-buffered saline (130 mM NaCl/10 mM phosphate, pH 7.0)/1 mM Tris/0.01% NaDodSO4, boiled for 5 min] applied to microtiter wells; binding was detected by enzyme-linked immunosorbent assay (ELISA) (21), using rabbit anti-mouse IgG and horseradish peroxidase-conjugated protein A, with o-phenylenediamine as the chromogen. Positive clones were amplified by injecting BALB/c mice, tapping the ascites tumor fluid after 12 days, centrifuging (1000 × g, 10 min), treating the supernatant with 45% saturated ammonium sulfate, and dialyzing the precipitated antibody against phosphate-buffered saline.

Abbreviation: HBB, hook--basal body.
*Present address: Erato, Tsukuba, Ibaraki 300-26, Japan.
†Present address: Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45267.
‡To whom reprint requests should be addressed.

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**Polyclonal Antibodies.** Rabbit polyclonal antibodies against hook-associated protein 1 and hook protein had been prepared previously (22, 23).

**Immunoblotting and Immunoprecipitation.** The immunoblotting procedure has been described (24). Immunoprecipitation was performed as described (25), except for the omission of the first NaDodSO4-solubilization step.

**Electron Microscopy.** The procedures for labeling with antibody and for negative staining have been described (23). For labeling with protein A conjugated to 5-nm colloidal gold (protein A-gold; Auroprobe EM protein A G5, Janssen Pharmaceutica, Beerse, Belgium), the sample was placed on a carbon-coated copper-mesh grid, labeled with antibody, and washed with buffer (50 mM Tris-HCl, pH 7.8/0.6 M NaCl/2.5 mM EDTA), and protein A-gold (diluted 1:2 with buffer) was added. After 20 min the sample was washed with buffer and then distilled water, negatively stained with phosphotungstic acid, and examined in a Philips EM201 electron microscope.

**RESULTS**

**Isolation of Monoclonal Antibodies Against a Basal-Body Protein.** Intact HBB complexes from *S. typhimurium* strain ST1 were used as the immunogen for generation of mouse monoclonal antibodies. Two positive clones were obtained. By immunoblotting, it was established that one of the two monoclonal antibodies was directed against a basal-body component (see below). The other was directed against 1,2-flagellin (data not shown), one of two distinct flagellins synthesized by strain ST1; flagellin is usually a minor contaminant in HBB preparations and is a potent antigen.

**Specificity of the Anti-Basal-Body Antibody.** To determine which basal-body component was the antigen for the anti-basal-body antibody, HBB complexes from strain SW1103 were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO4/PAGE) and immunoblotted. The antibody preferentially bound to a basal-body protein of apparent molecular mass 65 kDa (Fig. 1, lane 3); there was minor binding to other HBB components, notably the 42-kDa hook protein, which is the major component. In control blots, the anti-flagellin antibody bound to a small degree to the hook protein, but to no other HBB components (Fig. 1, lane 1). [Although trace amounts of SW1103 flagellin were probably present, it would have been of serotype i (17), not the serotype 1,2 against which the antibody was directed.] A polyclonal antibody directed against hook-associated protein 1 (apparent molecular mass, 59 kDa) bound to that protein (Fig. 1, lane 2). Thus the specificity of binding between the 65-kDa protein and the monoclonal antibody was established. Hereafter we describe the antibody as anti-65K.

**Reactivity Against *E. coli* HBB Complexes.** The 65-kDa protein has been shown indirectly from mutant studies to be the product of the flaAII.1 gene (7); the homologous gene in *E. coli* is flaBI, whose product is a 60-kDa protein (26). Anti-65K failed to precipitate radiolabeled FlabI protein synthesized from a plasmid in minicells (data not shown). In immunoblotting experiments using *E. coli* HBB complexes purified by the same procedure used with *S. typhimurium*, the antibody failed to bind either to the 60-kDa protein or to any other *E. coli* HBB component (Fig. 2, left lane).

**Location of the Antibody Binding Site in the Basal-Body Structure.** Before attempting to use anti-65K to localize the 65-kDa protein within the *S. typhimurium* basal-body structure, we wished to establish whether the relevant epitope (whose accessibility had thus far been established only in denatured samples) was still accessible in the intact HBB complex. To do so, we tested for coprecipitation of a major HBB protein (hook protein) with the 65-kDa protein. Anti-65K, purified *S. typhimurium* HBB complexes, and protein A-Sepharose were mixed, and the precipitate that formed was collected, fractionated by NaDodSO4/PAGE, and immunoblotted with antibody directed against the hook protein; the result was positive, the hook protein being by far the strongest band on the blot (Fig. 3). We conclude that the epitope on the 65-kDa protein remains accessible in the intact basal-body structure.

**Antibody decoration of extensive surfaces, such as those of the hook or flagellar filament, is easily and unambiguously identifiable in electron micrographs as a uniform, continuous "fuzz"; however, where the antigenic surface is relatively small, as with a subfeature of the basal body, and especially where the antibody is monoclonal and therefore can recognize only a single epitope on each subunit of the relevant protein, confirmation that attached material is indeed antibody is desirable. We therefore labeled *S. typhimurium* HBB complexes first with anti-65K and then with protein A-gold. Gold particles were concentrated in the vicinity of the hook.

![Fig. 1](image1.png) Fig. 1. Specificity of a monoclonal antibody (anti-65K) for the 65-kDa component of the HBB complex of *S. typhimurium*. After purified HBB complexes had been subjected to NaDodSO4/PAGE, the proteins were transferred onto a nitrocellulose sheet and incubated with various antibodies, and the bound antibodies were detected by horseradish peroxidase-conjugated protein A. Lane 1: monoclonal antibody against the flagellar filament protein, flagelin. Lane 2: monoclonal antibody against hook-associated protein 1 (HAP1). Lane 3: anti-65K. Lane 4: silver-stained gel of HBB complexes.

![Fig. 2](image2.png) Fig. 2. Test for immunological crossreaction of anti-65K, raised against the HBB complex of *S. typhimurium*, to the homologous structure of *E. coli*. Purified HBB complexes from *E. coli* YK410 (left lane) or *S. typhimurium* SW1103 (right lane) were analyzed by NaDodSO4/PAGE, transferred onto a nitrocellulose sheet, and incubated with anti-65K and a polyclonal antibody raised against *S. typhimurium* hook protein. Bound antibodies were detected by horseradish peroxidase-conjugated protein A.
innermost (M) ring, predominantly at its cytoplasmic face (Fig. 4A). In control samples where the first reaction was with antibody against hook-associated protein 1, gold particles were concentrated at the opposite end of the structure, at the distal end of the hook (Fig. 4B) (cf. ref. 22).

Such second labeling with protein A–gold provides convincing evidence of antibody binding but does not have sufficient spatial resolution to enable the location of the epitopes to be precisely defined. We therefore treated S. typhimurium HBB complexes with anti-65K alone (Fig. 5A). Most particles showed a spray-like decoration of the cytoplasmic face of the M ring, with little evidence of binding to either the side or the upper face of the ring. No decoration of the S ring or of other features of the HBB complex was noted. In an even simpler context—partial basal-body structures consisting only of the inner pair of rings and the rod (produced by a flaF mutant, SJW1135)—similar antibody-decoration patterns were observed (data not shown). Control

![Fig. 3. Ability of antibodies to precipitate intact HBB complexes.](image)

![Fig. 4. Colloidal gold labeling of HBB complexes.](image)

![Fig. 5. Localization of binding of anti-65K within the HBB complex.](image)

**DISCUSSION**

The inner pair of rings of the flagellar basal body have played a prominent role in our thinking about bacterial motility and the flagellar motor; indeed, it sometimes appears to be taken for granted that they are the components that act mutually to...
develop the torque for rotation of the external flagellar filament. While such a specific presumption is not justified by any available evidence, their location at the cell membrane makes it likely either that they themselves are involved in some way in the process of conversion of proton energy into mechanical energy or that they are physically close to components that do. This is especially true of the M ring, which is actually integral to the cell membrane (6). It then becomes important to identify the protein (or proteins) from which these inner rings are constructed and the corresponding structural genes.

In an immunological approach to identifying flagellar components, we used the entire hook–basal body complex of *S. typhimurium* (Fig. 6) as antigen, because a rather simple procedure for purifying large amounts of this HBB complex was available (7); by using the monoclonal antibody approach, we hoped to obtain clones producing antibodies against several HBB components without the need to purify these components individually. However, only one clone producing an antibody against a basal-body component resulted from the screen.

That antibody was directed against a 65-kDa protein (Fig. 1) that had been shown previously to be present in intact HBB complexes and also in a ring-containing subfraction obtained by acid depolymerization of the hook and rod (7). By immunoelectron microscopy, we have shown that the antibody binds to the part of the basal body closest to the cytoplasm (Figs. 4 and 5)—namely, the M ring. Indeed, we were able to conclude that the epitope lies on the cytoplasmic face of the ring, rather than on the edge or the other face. We conclude, therefore, that the 65-kDa protein is a component of the M ring. Although we cannot say that it is the only component, it is probably the major one: NaDodSO₄/polyacrylamide gels (whether stained with Coomassie blue, stained with silver, or autoradiographed) consistently show it as one of the major proteins of the HBB complex, second only to the hook protein (7). Further, the 65-kDa protein remains as one of only three proteins in preparations where the rod and hook structures have been depolymerized and only rings are seen by electron microscopy (7); the other two proteins are known from studies in *E. coli* to reside in the L and P rings (11).

A summary of current knowledge of the ring components of the basal body of *S. typhimurium* is given in Fig. 6. The major proteins of the L, P, and M rings are now known. A notable gap in our knowledge concerns the S ring, a structure whose existence is clear from electron microscopy, but about which no clues have emerged from genetic, biochemical, or physiological studies. For example, the ring fraction described above only contained three proteins, not four, and these have now been identified with the L, P, and M rings. Although in electron micrographs the S and M rings appear as distinct features, we wonder whether they might represent different domains of the same 65-kDa protein; we are not aware of any electron micrographs that convincingly show partial structures with the S ring present and the M ring absent.

What is the structural gene for the 65-kDa protein? Genetic and biochemical evidence has led us to conclude that it is flaAll.1 (7); in the case of several other flagellar genes, assignments made on the same basis have subsequently been vindicated by direct cloning (9, 10). The flaBI gene of *E. coli* (homologous to the flaAll.1 gene of *S. typhimurium*) has been cloned, and its product has been identified as a 60-kDa protein (26) that is presumed to be the same as the 60-kDa protein found in the *E. coli* HBB complex. Had the monoclonal antibody directed against the 65-kDa S. *typhimurium* M-ring protein recognized the 60-kDa *E. coli* protein (Fig. 2), the correspondence between the structural gene and the M-ring protein would have been confirmed directly. Such a confirmation must now await the cloning of the *S. typhimurium* flaAll.1 gene. [The failure of the antibody to crossreact may seem surprising, given the rather strong homologies between the flagellar systems of the two bacterial species, but it should be remembered that the antibody was monoclonal and so was exploring a single epitope; it is clear from the differing apparent molecular masses (65 vs. 60 kDa) that the two proteins do not have identical sequences.]

Knowledge of correspondences between genes, proteins, and structural features is only a prerequisite for the more fundamentally interesting question of how the motor actually works; i.e., how protonmotive force is converted into rotational force. A variety of models have been presented, with the M ring playing a central role in many of them (28–30). It is important, however, to realize that there is as yet no direct experimental evidence implicating any specific feature of the flagellar basal body in torque generation.

Evidence regarding the role of a gene product can often be obtained from mutant phenotype. In this way, five genes have been implicated in motor function; three of them (flaAll.2, flaQ, and flaN) encode proteins that are thought to comprise a complex that functions both in energy transduction and in switching (13, 14, 31), while the other two (motA and motB) encode proteins that appear to function in energy transduction only (12, 32–34). A striking fact about these five proteins is that none of them are present in the basal body.

What then can we infer about the role of the M ring, given the strong presumption that the 65-kDa M-ring protein is encoded by the flaAll.1 gene? The phenotype of all flaAll.1 mutants isolated to date is simple—the absence of any detectable flagellar structure (15). In an extensive search for flagellar and motility mutants (15) with paralyzed or switch-defective phenotype, all of the mutations mapped to the five genes mentioned above. None mapped to flaAll.1. This result is open to two interpretations. (i) The FlaAll.1 protein is involved in energy transduction and switching, but the residues that are critical for these functions are also critical for assembly, and so the potential for paralysis or switching defects is obscured by the more serious defect of lack of flagellation. (ii) The FlaAll.1 protein plays a structural role.

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Fig. 6. Schematic diagram of the filament–hook–basal body complex of *S. typhimurium*. The stippled part depolymerizes at pH 2.5 to leave the HBB complex. Localization of hook-associated proteins (HAPs) has been established by Ikeda et al. (27). The P- and L-ring components have been assigned in *E. coli* by Jones et al. (11). The S ring is known only from electron microscopy and is not characterized at all in terms of gene or protein. The major M-ring component was identified in the present study and is discussed in the text. The filament and hook are not shown to scale, and are in fact much longer. *S. typhimurium* can express either of two genes for flagellin; hence, the two apparent molecular masses (51 kDa and 56 kDa) shown.
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and is not involved in energy transduction or switching—it might be a passive structure onto which a FlaAll.2–FlaQ–FlaN switch complex is mounted, with this complex interacting with the Mot proteins to generate torque; in this hypothesis, the M ring would be a motor component in the broad sense that the energy-transducing interaction could not occur in its absence, but not in the narrow sense of a component within which the actual flow of protons and concomitant generation of force occur. Further studies will be required to distinguish between these possibilities, but in either case the M ring is likely to emerge as an important contributor to motor function.

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