Localization of the Salmonella typhimurium flagellar switch protein Flig to the cytoplasmic M-ring face of the basal body

(bacterial flagellum/rotary motor/Flf)

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ABSTRACT The direction of rotation of the bacterial flagellum is determined by the flagellar switch. We have localized Flig, one of the switch proteins of Salmonella typhimurium, to the cytoplasmic face of the M ring of the flagellar basal body. This localization was made possible by the discovery of two spontaneous mutants in which the flif (M ring) and flig (switch) genes were fused in-frame. In the first mutant, a deletion of 7 base pairs at the 3' end of flif resulted in an essentially full-length fusion protein. In the second mutant, a larger deletion resulted in a fusion in which 56 amino acids from the carboxyl terminus of flig and 35 amino acids from the amino terminus of flif were lost. Both strains were motile and underwent switching; the first strain had a clockwise bias, and the second strain had a counterclockwise bias. Gel electrophoresis and immunoblotting of isolated hook-basal-body complexes verified that they contained the fusion proteins. Electron microscopy revealed additional mass at the cytoplasmic face of the M ring, which could be decorated with anti-Flig antibody. We conclude that the natural location for Flig is at the cytoplasmic face of the M ring and that the stoichiometric ratio between Flif and Flig in wild-type cells is probably 1:1.

The bacterial flagellum contains a switch that determines whether its motor is in counterclockwise or clockwise rotation. Genetic evidence has indicated that in Salmonella typhimurium the switch is a complex containing subunits of three proteins—Flig, Flim, and Flin (1, 2). Based on the fact that some mutants with defects in the flig, flim, and flin genes are paralyzed (2), the switch is also presumed to be needed for the actual process of rotation, along with two other proteins, MotA and MotB (3).

A long-standing question has been the physical location of the switch. A membrane-associated structure called the hook-basal-body (HBB) complex (Fig. 1) has been described, but this complex was not found to contain any of the three switch proteins (4, 9); however, the protocol for isolating this structure is rather harsh and could dissociate the labile components.

If the switch is not part of the basal body, where is it likely to be located? The facts that (i) the direction of motor rotation is modulated by the cytoplasmic protein CheY (10, 11) and (ii) cheY mutations can be suppressed by switch mutations and vice versa (1, 12-14) suggest that the switch should be near the cytoplasmic face of the basal body. The most proximal feature of the basal body is the M ring, which in the intact cell is integral to the cell membrane (15) (Fig. 1). Subunits of the Flif protein are responsible for this feature (5). It has been established recently that the S ring and part of the proximal rod also derive from domains of Flif subunits (T. Ueno, K. Oosawa, and S.-I. Aizawa, personal communication): we shall refer to the overall substructure as the MS ring.

We describe here the serendipitous discovery of two gene-fusion mutants, which provide compelling evidence that one of the switch proteins, Flig, is associated with the MS ring.

MATERIALS AND METHODS

Bacterial Strains. SJW3063-1 is a pseudorevertant of strain SJW3063 (cheY), carrying a second mutation in the vicinity of flig; the second mutation has also been placed in a wild-type cheY background (13). These strains are all derivatives of wild-type strain SJW1103, which is of flagellin serotype i. SJW2334 is a spontaneous switch-bias mutant of SJW806, a wild-type strain of serotype enx (S.Y., unpublished data).

Amplification and Sequencing of the flif-flig Region. Chromosomal DNA was isolated, amplified by the PCR, and sequenced as described (14). The amplified fragment (1.6 kilobase pairs in length) extended from base-pair (bp) 1283 in flif to bp 193 in flig, the gene after flig.

Isolation of HBB Complexes. This isolation was done as described by Aizawa et al. (4), except that pH 10 instead of pH 11 was used to dissociate the outer membrane vesicles. The preparation was made from a 4-liter culture, and the final pellet of HBB complexes was resuspended in 350 μl of buffer.

Antibodies. The monoclonal mouse anti-Flif antibody has been described (5). Polyclonal rabbit anti-Flig antibody was from K. Oosawa and S.-I. Aizawa (Teikyo University, Utsunomiya, Japan).

Electrophoresis and Immunoblotting of HBB Complexes. HBB proteins were separated by SDS/PAGE (10% polyacrylamide) in a Mini-Protean II apparatus (Bio-Rad) and, unless being used for blotting, were stained with Coomassie blue. Electrophoretic transfer was performed by nitrocellulose paper (Schleicher & Schuell) was done in a Mini Trans-Blot cell (Bio-Rad). The paper was blocked with 1% horse serum albumin/phosphate-buffered saline/30 mM sodium azide, washed three times with buffer, and incubated with the primary antibody (diluted 1:1000 and 1:400 for anti-Flig and anti-Flif, respectively) at 4°C on a rocking platform for 12 hr. The blots were then washed in 0.1% Tween 20 and 200 mM NaCl/2.5 mM EDTA. A 3-μl sample was placed on a

Abbreviation: HBB, hook-basal-body.
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carbon-coated grid, rinsed with 5–10 drops of buffer, negatively stained with 2% (wt/vol) uranyl acetate, and recorded with a Philips 301 electron microscope (80 kV, x57,000).

(ii) Samples decorated with antibody. Thirty-eight microliters of diluted stock solution of HBB complexes plus 2 μl of 1:20-diluted anti-FliG antibody were incubated on ice for 10 min. Electron microscopy was done as in i.

(iii) Samples stained with antibody plus gold–protein A. Ten microliters of the sample plus antibody plus 1 μl of (5-nm colloidal gold)-conjugated protein A (Auroprobe EM protein A G5, Janssen Pharmaceutica) diluted 1:2 with buffer were mixed and incubated at room temperature for 20 min. Electron microscopy was done as in i.

RESULTS

Background Concerning the Switch Mutants. We have undertaken an extensive sequence analysis of mutant alleles of the three switch genes—fliG, fliM, and fliN (ref. 14; V.M.I., S.Y., M. Kihara, H. Sockey, and R.M.M., unpublished data). Most of these mutations had been generated as spontaneous suppressors of mutations in the chemotaxis genes cheY and cheZ, but we also analyzed a number of spontaneous first-site mutations from chemotaxis mutants defective in the switch genes.

All of these mutants were motile. In most cases, the mutations proved to be single-amino acid changes, but in two cases these mutations were deletions. These mutations had remarkable implications for the location of the switch, as we now describe.

DNA Sequence and Deduced Amino Acid Sequence of the Switch Mutants. Strain SJW3063-1 is a pseudorevertant of a cheY mutant, SJW3063; the suppressor mutation had been mapped to near the 5′ end of fliG (13). To identify it, we amplified a fragment containing the last 25% of fliF and all of fliG. Comparison of the sequence of this fragment with the wild-type sequence (16, 17) showed that the mutation was actually a deletion of 7 bp very close to the 3′ end of fliF. The two genes overlap so that their reading frames differ by 1 bp (Fig. 2A), with the result that any deletion of 3n + 1 bp in fliF shifts translation into the fliG frame. In the present case, this shift occurs with loss of the last five amino acids of FliF and gain of an isoleucine residue at the fusion site (Fig. 2B). The deduced molecular mass of the fusion is 97 kDa, compared with deduced masses of 61 and 37 kDa for FliF and FliG, respectively. We refer to this protein as the full-length fusion, or FliFG97.

Strain SJW2334 had been isolated as a spontaneous switch mutant (2), and mapping of the mutation indicated it was around the fliF–fliG junction. Sequence analysis revealed a deletion of 445 bp across this junction, which placed the two genes in frame [445 = (3 x 148) + 1]. This result predicts a loss of 56 amino acids from the carboxyl terminus of FliF and 94 amino acids from the amino terminus of FliG, yielding a fusion protein with a molecular mass of 81 kDa. We refer to this protein as the deletion fusion, or FliFG81. The wild-type and the two fusion proteins are shown schematically in Fig. 3.

Flagellation and Motility of the Fusion Mutants. We examined the two mutants by high-intensity dark-field light microscopy (18).

SJW3063-1 cells were well flagellated and vigorously motile, undergoing frequent tumbling and generating polymorphic transitions between the normal and curly helical waveforms. This behavior is characteristic of strains with a moderately high clockwise bias (19) and agrees with an earlier assessment of this strain (13). When tethered to glass using antiflagellar antibody, cells rotated predominantly clockwise, switching frequently for short periods to counterclockwise. A derivative in which the switch mutation had been placed in a wild-type cheY background (13) was even more tumbling than the pseudorevertant, and, when tethered, showed even more pronounced clockwise bias. The enhancement of clockwise bias by CheY is in accord with its known switching effect (10, 11). When SJW3063-1 cells were subjected to a strong-attractant stimulus (1 mM L-serine), no response was seen; however, the cheY mutation is a severe one (14) that may block sensory transduction. With the


The motility of SJW2334 cells ranged from moderately vigorous to rather sluggish, with predominantly smooth swimming (characteristic of counterclockwise bias). Substantial day-to-day variability in motility was noted. The flagellar bundle had a tendency to jam, making it more difficult to detect tumbling episodes. Stimulation with serine, however, definitely increased smooth swimming. Tethered cells rotated predominantly counterclockwise, with some brief clockwise intervals.

Biochemical Characterization of Mutant Basal Bodies. We prepared HBB complexes of the wild-type and mutant strains (4). Identities of most components of the complex are known from previous studies: specifically, the MS-ring protein FliF (deduced molecular mass, 61 kDa) has been identified in gels as a protein with an apparent molecular mass of 65 kDa (4, 5). With wild-type HBB complexes, FliF was seen along with other established components (Fig. 4A, lane 1). With HBB complexes from SJW3063-1 (lane 2), this protein was absent, but another protein was seen with an apparent mass of 100 kDa, in good agreement with the mass predicted for FliFG97. With complexes from SJW2334 (lane 3), the 65-kDa protein was again absent; in this case an 83-kDa protein appeared, consistent with the mass predicted for FliFG81.

To confirm that the additional proteins were the predicted fusions, we transferred the basal-body proteins to nitrocellulose paper and probed with anti-FliF and anti-FliG antibodies. The wild-type FliF protein was only recognized by the anti-FliF antibody (cf. Fig. 4B and C, lane 1). FliFG97 was recognized by both antibodies (Fig. 4B and C, lane 2); the anti-FliF antibody also revealed a fainter band at the position of FliF, suggesting some proteolysis of the fusion protein at the junction may have occurred. FliFG81 was recognized by the anti-FliG antibody (Fig. 4B, lane 3) but not by the anti-FliF antibody (Fig. 4C, lane 3). The latter result presumably means that the relevant epitope in FliF for the monoclonal antibody lies within the deleted carbonyl-terminal sequence, which is highly polar (16) and probably a strong epitope.

DISCUSSION

The switch is a vital part of the bacterial flagellum. Its location has long been suspected to be at the cytoplasmic face of the flagellar basal body (e.g., ref. 1), but direct evidence has been lacking. Whereas the basal body is a very rugged structure, the switch is apparently much more labile. The isolation of coherent fusions between the two structures has enabled us to localize one of the three known switch components, FliG. The evidence that in the mutant, FliG is located at the cytoplasmic face of the basal-body MS ring is conclusive. As we discuss below, this is almost certainly its location in the wild-type cell also.

The discovery of functional FliF-FliG fusions was an accidental one, made possible because the two genes are adjacent on the chromosome. The fact that they are adjacent, however, may not be accidental (cf. ref. 21) because gene order can often reflect functional relationship.

Fusion of the MS-Ring Protein to a Switch Protein Still Permits Function. Remarkably, the phenotype of the fusion mutants was nonflagellate or even paralyzed: flagellar assembly, rotation, and switching all still occurred (although with an alteration of switch bias and, in the deletion-fusion mutant, some impairment of motility).

What does this result imply in terms of the location of the FliG switch protein in a wild-type cell? Both FliF and FliG have to function within a complex multisubunit structure, the flagellum. We think it extremely unlikely that they could function almost normally when forced to be adjacent by covalent linkage were they not similarly arranged in the wild-type structure. Our results, therefore, provide prima facie evidence for association of FliG with the basal body and specifically with FliF, the protein from which the MS-ring substructure is constructed. Independent evidence that FliG is associated with the basal body has recently been obtained from immunoblots of wild-type structures reacted by modified protocols (K. Oosawa and S.-I. Aizawa, personal communication; N.R.F., unpublished data).

Electron microscopy (Fig. 5) reveals that the site of attachment of FliG to the MS ring is at its cytoplasmic face (Fig. 1). For the full-length fusion mutant, the association appears to extend over much of the width of the image (Fig. 5A Center), and so it is not obvious at which radius the covalent junction lies. With the deletion-fusion mutant (Right), the

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**Fig. 4.** Analysis of HBB complexes from wild-type strain SJW1103 and mutant strains SJW3063-1 and SJW2334. The mutants are predicted to synthesize fusion proteins (FliFG97 and FliFG81, respectively) between FliF (the basal-body MS-ring protein) and FliG (one of the switch proteins)(see text and Fig. 3). (A) Proteins from HBB complexes, separated by SDS/PAGE and stained with Coomassie blue. Lane 1, wild-type (wt). The band corresponding to FliF can be seen together with the following other HBB proteins (cf. ref. 4)—flagellin, FliC (present as a contaminant, with the different apparent molecular masses in lanes 1 and 2 vs. lane 3, reflecting the fact that the flagellins are of different serotype and mass); hook protein, FlgE; P-ring protein, FlgJ; rod proteins, FlgF and FlgG; and L-ring protein, FlgH. (Lower molecular mass basal-body proteins (FlgB, FlgC, and FlgE) are not shown in this figure.) Lanes 2 and 3, fusion mutants. The band corresponding to FliF is missing, and additional bands (FliFG97 and FliFG81, respectively) are seen at apparent molecular masses close to the predicted values. Positions of molecular mass standards are shown at right. (B) Immunoblot of the same samples probed with a polyclonal anti-FliF antibody. (C) Immunoblot probed with a monoclonal anti-FliF antibody (see text).
association occurs toward the outer radius of the M ring, with an appearance that suggests a cup-like structure in three-dimensional space. Recent electron microscopic analyses of wild-type basal bodies (refs. 22 and 23; N.R.F., unpublished data) have revealed additional features. These features extend further toward the cytoplasm than the FliG structure seen with the fusion mutants and so presumably contain other as-yet-unidentified components.

Deletion of Terminal Sequence of Both Proteins Still Permits Flagellar Function. A fusion protein lacking 56 carboxyl-terminal residues of FliF and 94 amino-terminal residues of FliG still permits flagellar assembly and rotation. There are other indications that these terminal regions are relatively unimportant: (i) When a mutant version of FliF lacking the carboxyl-terminal 104 amino acids is overproduced, it can still assemble spontaneously into MS-ring complexes in the membrane (T. Ueno, K. Oosawa, and S.-I. Aizawa, personal communication). (ii) In extensive investigations of fliG mutants, we have found no phenotypically significant missense mutations within the amino-terminal region of the protein (V.M.I., S.Y., M. Kihara, H. Sockey, and R.M.M., unpublished data), suggesting that the amino-terminal region does not play a major role in flagellar structure, rotation, or switching.

On the other hand, the carboxyl terminus of FliF and the amino terminus of FliG are (like the rest of the sequence) highly conserved between _Salmonella typhimurium_ and _Escherichia coli_ (16, 17), and so there must be selection pressure for their retention. The variable motility of the deletion-fusion mutant and the altered bias of both mutants also suggest that the presence and conformational freedom of the terminal regions of FliF and FliG are necessary for optimal function.

The Complete Flagellar Switch. Our results concern the location of only one component of the flagellar switch. Genetic data indicate that there are two other components, FliM and FliN, which together with FliG form the complete switch (1, 2). FliM and FliN are, therefore, also probably associated with the basal-body MS ring, although the association might occur indirectly by means of FliG. That _fliM_ and _fliN_ are in a separate operon from the MS-ring gene _fliF_ could be a reflection of such an indirect functional relationship.

Relative Stoichiometries of MS-Ring Protein and FliG Switch Protein. In the fusion strains, the stoichiometric ratio of FliF and FliG is obviously 1:1, and flagellar assembly, rotation, and switching still occur. Although this evidence does not prove that the natural ratio in the wild-type cell is 1:1, it strongly supports that hypothesis. This ratio would
then place the stoichiometry of FliG at ≈26 subunits per basal body, based on estimates for FliF from both quantitative gel electrophoresis (24) and scanning-transmission electron microscopy (25).

Role of MS Ring in Motility. Originally, the M and S rings were thought to function as the rotor and stator of the flagellar motor (e.g., ref. 26). This model cannot be correct because both rings are now known to derive from subunits of the same protein, FliF (T. Ueno, K. Oosawa, and S.-I. Aizawa, personal communication). Further, the MS ring does not appear to contribute to the processes of rotation or switching at all (2). In the present study, we have shown that FliG, a protein that is involved in switching and rotation, binds to the MS ring. Thus, we view the MS ring as a passive mounting plate (Fig. 1) that receives the torque generated by the Mot and switch proteins and mechanically transmits it to the basal-body rod and eventually to the external helical filament that propels the cell.

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