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Mutant MotB Proteins in *Escherichia coli*

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The MotB protein of *Escherichia coli* is an essential component in each of eight torque generators in the flagellar rotary motor. Based on its membrane topology, it has been suggested that MotB might be a linker that fastens the torque-generating machinery to the cell wall. Here, we report the isolation and characterization of a number of *motB* mutants. As found previously for *motA*, many alleles of *motB* were dominant, as expected if MotB is a component of the motor. In other respects, however, the *motB* mutants differed from the *motA* mutants. Most of the mutations mapped to a hydrophilic, periplasmic domain of the protein, and nothing comparable to the slow-swimming alleles of *motA*, which show normal torque when tethered, was found. Some *motB* mutants retained partial function, but when tethered they produced subnormal torque, indicating that their motors contained only one or two functional torque generators. These results support the hypothesis that MotB is a linker.

Cells of *Escherichia coli* and many other motile bacteria are propelled by helical filaments driven at their base by a reversible rotary motor (1, 20); the motor-filament organelle is called a flagellum (for recent reviews, see references 3 and 14). The energy for rotation comes from the movement of protons down an electrochemical gradient across the cytoplasmic membrane, rather than from the hydrolysis of ATP (12, 16). The detailed mechanism underlying this energy conversion is not understood.

The motor apparatus contains about 25 different kinds of proteins (14). Mutations in most of the genes encoding the motor parts causes the flagellum to be assembled incompletely or not at all. Accordingly, these proteins are believed to be important for flagellar structure but probably not for torque generation per se. Five proteins, called MotA, MotB, FliG, FliM, and FliN, can be mutated to give a paralyzed phenotype, in which a normal-looking basal body is built but does not rotate. These proteins are likely to be more directly involved in torque generation. Certain alleles of *fliG*, *fliM*, and *fliN* are nonflagellate, whereas other alleles are defective in switching the direction of motor rotation, so these proteins also are important for flagellar assembly and for chemotaxis (28, 29). Mutations in *motA* and *motB* can impair motility, but none has been found that affects assembly or switching.

Earlier work showed that MotA and MotB are components in each of eight independent torque generators in the motor (4, 6). The MotA and MotB proteins are associated with the cytoplasmic membrane (7, 8, 21, 26, 27). MotA is quite hydrophobic, with four likely membrane-spanning segments (9). Characterization of a number of *motA* mutants indicated that MotA is involved in conducting protons across the cytoplasmic membrane (5). MotB is more hydrophilic and crosses the cytoplasmic membrane only once, with most of the protein located in the periplasmic space. Based on this topology, Chun and Parkinson suggested that MotB is a linker that fastens MotA, or other components of the torque-generating machinery, to the cell wall (8). This linkage is necessary to ensure that the motor components that apply force at the base of the filament remain stationary with respect to the cell as a whole.

We have used hydroxylamine to mutagenize plasmids carrying *motB*, examined the phenotypes of *motB*-deficient and wild-type cells transformed with the mutant plasmids, and determined the corresponding nucleotide base changes. The *motB* mutants were nonmotile or severely impaired; nothing comparable to the slow-swimming alleles of *motA* (5) was found. When tethered, partially functional *motB* mutants appeared to be operating with a relatively small number of torque generators. As seen for *motA*, most of the *motB* mutations were dominant, as expected if nonfunctional MotB molecules can displace functional MotB from the motor. In contrast to *motA* mutations, which caused amino acid substitutions primarily in hydrophobic domains, most of the *motB* substitutions were found in a hydrophilic, periplasmic domain of the protein. These results are discussed, with reference to the hypothesis that MotB links torque-generating elements to the cell wall.

MATERIALS AND METHODS

The *E. coli* strains and plasmids are listed in Table 1. Chemical mutagenesis with hydroxylamine was basically as described by Humphreys et al. (10). A solution of pGM1 in 10 mM Tris (pH 8)–1 mM EDTA was added to 5 volumes of 0.1 M sodium phosphate (pH 6)–1 mM EDTA and added to 5 volumes of 1 M hydroxylamine (pH 6). The mixture was kept on ice for 45 min and then incubated at 70°C for 60 min. The DNA was dialyzed extensively against 10 mM Tris (pH 8)–1 mM EDTA and precipitated by adding NaCl to 0.1 M and then 2 volumes of ethanol.

The mutagenized plasmids were transformed into strain RP3087, which is defective in *motB*. The heat shock recovery period, ordinarily 1 h (15), was shortened to 30 min to minimize replication and consequent duplication of mutants. The cells were added to tryptone broth (1% tryptone, 0.5% NaCl) containing 50 μg of ampicillin per ml, 1 mM isopropyl-β-D-thiogalactopyranoside, and 0.35% dissolved agar. The mixture was poured into plates, allowed to solidify, and then

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
RP437	Wild type for motility and chemotaxis	18
RP3087	*motB*	J. S. Parkinson
BL-19	Δ*motB*	M. Manson
Plasmids		
pGM1	P*lac-motB*$^+$ Ap^r	7
pDFB46	P*mocha-motA*$^+$ Cm^r	4
pSYC62	P*mocha-motB*$^+$ Cm^r	8

incubated overnight at 30°C. Motility mutants grew in colonies that were more compact than the diffuse swarms of the wild type. These colonies were picked and purified on LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) containing ampicillin (50 μg/ml) and then repicked on soft agar to confirm the mutant phenotype. Confirmed mutants were stored at −70°C in 10% dimethyl sulfoxide for further characterization.

The *motB* plasmid was purified from each mutant, and dominance was scored by transforming the wild-type strain RP437. Transformants were cultured to saturation at 30°C in tryptone broth containing ampicillin and isopropyl-β-D-thiogalactopyranoside and then diluted 100-fold into the same medium. Motility was assayed by phase-contrast microscopy after 3 to 4 h of additional growth at 30°C and compared with that of a control strain carrying unmutagenized pGM1. A swarming assay, with plates prepared as described above, was also employed; the two methods gave the same dominance scores.

Cells were tethered to coverslips by their flagellar filaments in a flow cell (2) and videotaped under phase-contrast microscopy. The angular velocities of the cells were determined by timing revolutions with a stopwatch during slow-speed playback (4). To avoid biasing the estimate of mean motor torque, all rotating cells in a field were included in the measurements. The length of each cell and its radius of gyration were measured from the video screen with a ruler that had been calibrated against an objective micrometer. The rotation rate, cell size, and radius of gyration were used to compute the motor torque according to formulas published previously (24, 25).

For measurements of swimming speed, a saturated culture in tryptone broth was diluted 100-fold into fresh broth containing isopropyl-β-D-thiogalactopyranoside and grown for 4 h at 30°C. The cells were pelleted and gently resuspended in a motility medium containing 10 mM potassium phosphate (pH 7), 67 mM NaCl, 10 mM sodium lactate, 0.1 mM EDTA, and 1 μM methionine. Cells were pelleted again, resuspended in motility medium, and mixed with an equal volume of motility medium containing serine and aspartic acid (20 μM each) to promote smooth swimming. The mixture was drawn into the flow cell, and cells near the coverslip were videotaped for several minutes.

Mutations were mapped by recombining different restriction fragments of the *motB* gene with a procedure like that of Mutoh et al. (17). The mapping employed the *Acc*I site at nucleotide 441 in *motB* and a downstream *Sal*I site in the vector or the *Nsi*I site at nucleotide 660 in *motB* and the downstream *Sal*I site in the vector. Together, these mappings permitted the localization of the mutations to one of three segments of the *motB* gene. That segment was then sequenced by using the dideoxy termination method (19) to locate the mutation. All of the mutations were of the kind expected for mutagenesis with hydroxylamine (C to T or G to A).

RESULTS

Plasmid pGM1, which contains the *motB* gene under transcriptional control of the *lac* promoter, was treated with hydroxylamine and transformed into a strain that was defective in *motB* (RP3087, Table 1). Roughly 1% of the ampicillin-resistant clones showed impaired swarming on tryptone plates containing 0.35% agar; these were isolated for further study. Thirty-eight mutants were isolated in all. Of these, 25 did not swarm on 0.35% agar, and 13 swarmed, but more slowly than did the wild type. Representative swarms are shown in Fig. 1A. The plasmids were isolated from each mutant and reintroduced into freshly cultured RP3087. The mutant phenotype was reproduced for all of the nonswarming isolates. However, retransformation with the plasmids from some of the slow-swarming strains yielded some clones with good motility and some that were nonmotile. Sequencing (see below; Table 2) showed that these strains all carried the same mutation and that this mutation reverted rapidly, causing the plasmid preparations to be heterogeneous. Since clones that retained the mutation were nonmotile, these mutants were reclassified as totally nonfunctional. Plasmids from the other slow-swarming strains behaved homogeneously.

Cells of each mutant were cultured in tryptone broth, and their swimming was examined by phase-contrast microscopy. When cultured in liquid broth, the nonswarmers did not swim. Most cells of the slow-swarming strains also did not swim, but writhed and tumbled slowly. Occasionally, a cell harboring allele 8 was seen to swim very slowly. In general, the partially functional *motB* mutants were more severely impaired than the partially functional *motA* mutants characterized previously (5).

If MotB is a structural component of the flagellar motor, as has been suggested (6, 8, 27), then it seems likely that some alleles of *motB* should be dominant, because they encode proteins that are incorrectly incorporated into the motor and therefore exclude wild-type MotB. We tested this proposition by transferring each of the mutant *motB* plasmids into a wild-type strain and assaying its effect on motility. A large proportion of the *motB* mutants (26 out of 38) significantly inhibited the motility of the wild type and are therefore dominant by this criterion. Representative swarms are shown in Fig. 1B. Nonfunctional MotB might inhibit motility by binding to and sequestering other vital components, rather than by binding to the motor itself; since a variety of results suggest that MotB and MotA interact (4, 6, 27), sequestering of MotA seems most likely. We tested this possibility by transforming some of the strains (the wild type, harboring *motB* alleles 1, 3, 6, 7, 8, and 24 on pGM1) with a second plasmid (pDFB46) encoding wild-type *motA*. The motility of the strain harboring *motB* allele 8 was somewhat improved by additional copies of *motA*; however, for all other alleles, the motility remained poor. When additional copies of wild-type *motB* were introduced on a second plasmid (pSYC62), the motility of all strains was significantly improved.

As noted above, some of the mutants swarmed slowly because they contained heterogeneous populations of the plasmid, some copies of which had reverted to the wild type.

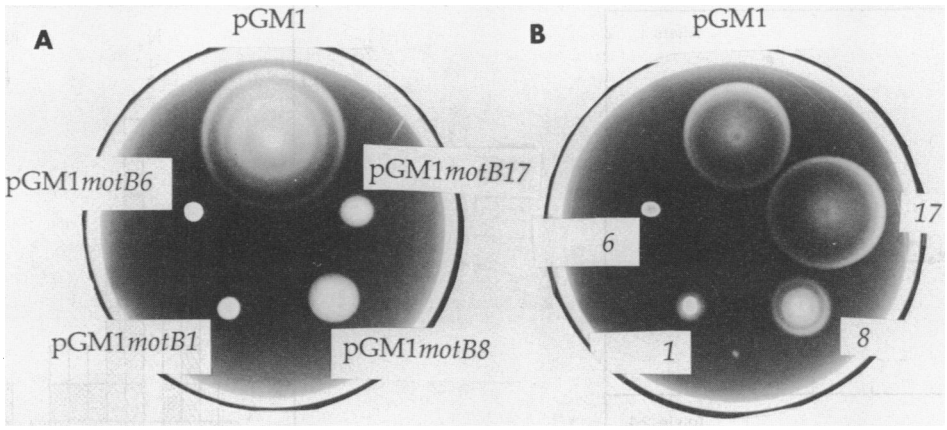

FIG. 1. (A) Swarms of the *motB* strain RP3087 containing pGM1-derived plasmids encoding either wild-type *motB* or representative mutant alleles of *motB*. Plasmids were mutagenized and introduced into RP3087, and transformants were screened and colony purified as described in Materials and Methods. The swarm plate contained tryptone broth, 0.3% agar, ampicillin, and isopropyl-β-D-thiogalactopyranoside. After inoculation with 2 μl of a mid-log-phase culture, the plate was incubated for approximately 10 h at 30°C. (B) Swarms of the wild-type strain RP437 containing the same plasmids, illustrating the dominance of some alleles. This plate was incubated for approximately 7 h at 30°C. The introduction of plasmid-borne *motB* alleles 6, 1, and 8 into the wild type slowed its swarming significantly; in tryptone broth, cells of these strains swam very poorly (see the text).

In some other slow swarmers (alleles 10, 17, 28, and 33), the mutations were recessive, and sequencing showed that they introduced premature termination codons (see below; Table 2). Three of the terminators were very near the amino terminus of the protein (at residues 4, 13, and 26), making it highly unlikely that the truncated proteins retained any function. Evidently, the nonsense mutations in alleles 10, 17, 28, and 33 were partially suppressed, giving rise to the partially functional phenotype. Three dominant, partially functional *motB* mutants were isolated (alleles 1, 8, and 24); sequencing showed that the mutations caused single amino acid substitutions (Table 2). When tethered, cells of these mutant strains rotated much more slowly than did the wild type. Occasionally, a cell changed its rotation speed abruptly. Examples of this behavior are shown in Fig. 2. Cells rotated at two discrete speeds; the higher speed was very nearly double the lower speed. If the rotation rate and cell size are known, then the torque of a motor driving a tethered cell can be computed (24, 25). This was done for several cells of each of the partially functional strains. A histogram of the computed torques is shown in Fig. 3. The torque associated with a single generator, based on earlier work, is also shown. The distribution of torques indicates that most motors contained only one functional torque generator, and some probably contained two. The average torque and the torque increment associated with the speed doublings of the cells in Fig. 2 were close to those of a single wild-type torque generator.

TABLE 2. Nucleotide changes and corresponding amino acid changes in the *motB* mutants

Allele	Nucleotide change	Amino acid change	No. of isolates
Recessive			
33	C-10→T	Gln-4→Stop	2
17	C-37→T	Arg-13→Stop	2
10	G-78→A	Trp-26→Stop	7
28	G-633→A	Trp-211→Stop	1
Dominant, partially functional			
24	C-476→T	Pro-159→Ile	1
8	G-613→A	Glu-205→Lys	1
1	G-719→A	Gly-240→Asp	1
Dominant, non-functional			
30	G-85→A, G-91→A	Ala-29→Thr, Ala-31→Thr	1
5	G-94→A[a]	Asp-32→Asn	8
6	C-116→T	Ala-39→Val	1
39	G-491→A	Gly-164→Asp	1
47	C-587→T	Thr-196→Ileu	1
35	G-589→A	Asp-197→Asn	1
3	C-641→T	Ser-214→Phe	2
40	C-649→T	Arg-217→Trp	1
7	G-665→A	Arg-222→His	1
14	G-724→A	Ala-242→Thr	2
15	C-725→T	Ala-242→Val	2
21	C-772→T	Arg-258→Cys	1
20	G-773→A	Arg-258→His	1

[a] This mutation reverted rapidly (see text).

The *motB* alleles 1, 8, and 24 were dominant, so the mutant proteins are evidently able to displace wild-type MotB from the motor. However, it is conceivable that the mutations render these proteins unable to displace the mutant MotB protein already present in strain RP3087 (which carries a point mutation in *motB*), thus causing the observed speed fluctuations and subnormal torque. To test for an effect of the MotB protein in RP3087, we transformed the mutant plasmids into strain BL-19, which contains a deletion in *motB*. In the deletion background, the motility of allele 8 was significantly improved, whereas the motility of alleles 1 and 24 remained poor.

To define the functionally important domains of the protein, we sequenced the *motB* mutations. The nucleotide base changes and associated amino acid changes are summarized in Table 2. The locations of the mutations in the *motB* gene are shown graphically in Fig. 4, with the hydrophobicity profile of MotB, computed from the sequence by Stader et al. (21), included for comparison. Chun and Parkinson

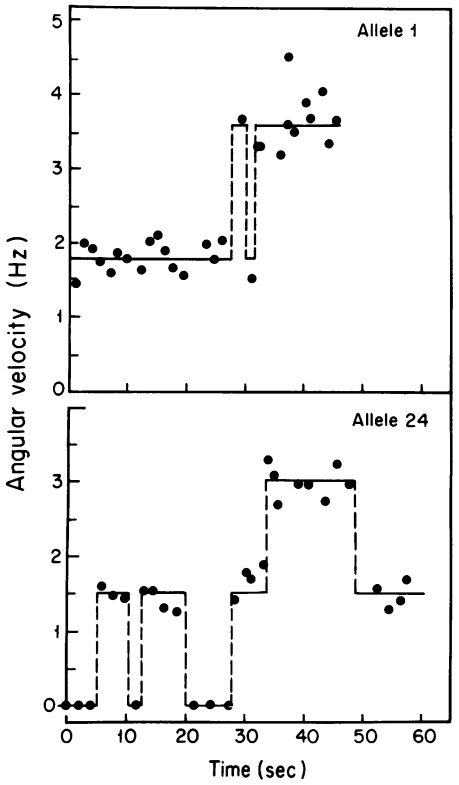

FIG. 2. Angular velocity of tethered cells of the partially functional strains RP3087 (pGM1*motB1*) or RP3087 (pGM1*motB24*) as a function of time, illustrating the sudden speed changes that were occasionally seen. Cells were tethered and videotaped; their speeds were clocked by using a stopwatch during slow-speed playback, as described in Materials and Methods. The higher speed was approximately double the lower speed for both cells. The torque increments associated with the speed increases were 2.5×10^{-12} dyn cm for allele 1 and 1.7×10^{-12} dyn cm for allele 24.

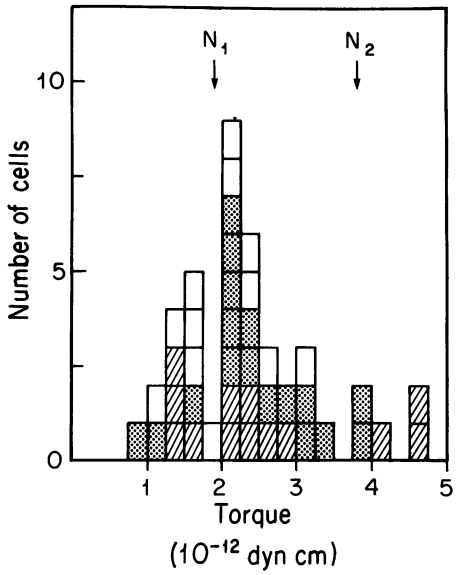

FIG. 3. Histogram of torques of tethered cells of the partially functional strains RP3087 (pGM1*motB1*) (▨), RP3087 (pGM1 *motB8*) (▩), and RP3087 (pGM1*motB24*) (□). The best available estimate for the torque of a single generator (1.91×10^{-12} dyn cm [4]) is indicated by N_1; N_2 indicates twice this value. To compute the mean torques of the *motB* mutants, we assumed that motors producing more than 3.5×10^{-12} dyn cm contained two torque generators. The mean torques, excluding values greater than 3.5×10^{-12} dyn cm, were then (mean ± standard deviation; $\times 10^{-12}$ dyn cm): allele 1, 2.06 ± 0.56 (10 cells); allele 8, 2.28 ± 0.69 (15 cells); allele 24, 1.99 ± 0.58 (12 cells); global mean, 2.13 ± 0.62 (37 cells). The mean for cells with torque greater than 3.5×10^{-12} dyn cm was 4.21 ± 0.36 (5 cells).

showed that MotB crosses the cytoplasmic membrane once near its amino terminus, with most of the molecule situated in the periplasmic space (8). Most of the mutations were found in the periplasmic domain. A few were found within or near the hydrophobic segment that crosses the membrane.

All of the recessive mutations introduced premature termination codons. One of these terminated at codon 211 and should therefore encode a sizable (~23-kDa) protein. This truncated MotB is evidently degraded rapidly, since it was not found when plasmid-encoded proteins were radiolabeled with the T7 polymerase system (22a, 23). On the average, about 1.8 isolates were found for each dominant, nonfunctional allele. We can make a rough estimate of the number of alleles not yet found if we assume that each allele is isolated with equal probability (which is not strictly true, given the evident abundance of particular alleles). By this estimate, about five dominant, nonfunctional alleles remain to be found.

DISCUSSION

A variety of evidence suggests that MotB is part of the flagellar motor. When cells of a *motB* strain were tethered and the synthesis of wild-type MotB (introduced on a plasmid) was induced, torque was restored in a series of equal steps. This result led Block and Berg (6) to suggest that MotB is a component in each of several independent torque generators in the motor. Khan and co-workers have observed rings of particles in the membranes of motile strains of *E. coli*; these rings are disrupted if either the *motA* or *motB* gene is deleted (11). They suggested that MotA and MotB are arranged circumferentially around the basal body in the intact motor. Most of the *motB* mutations examined here were dominant, as judged by their ability to impair the motility of the wild type when introduced on a multicopy plasmid. This result is expected if MotB is a component of the motor; dominance would simply reflect the displacement of functional MotB from the motor by nonfunctional MotB.

The mutant MotB proteins could also impair the motility of the wild type by binding to and sequestering MotA rather than by binding to the motor itself. For several alleles tested, the introduction of additional MotA did not improve motility, ruling out this alternative. Allele 8 was exceptional, since its motility was somewhat improved by additional copies of *motA*. Also in contrast to the other partially functional alleles, allele 8 conferred better motility on a *motB* deletion strain than on a point mutant. Together, these results suggest that the poor motility of allele 8 is caused, at least in part, by a reduction in the affinity of MotB for the motor.

The present results with *motB* present an interesting contrast to our earlier characterization of *motA* mutants (5). Several *motA* mutants swam more slowly than the wild type but nevertheless had normal torque when tethered. These mutants were impaired in proton transfer, which is rate limiting at the high rotation rates of swimming cells (13) but

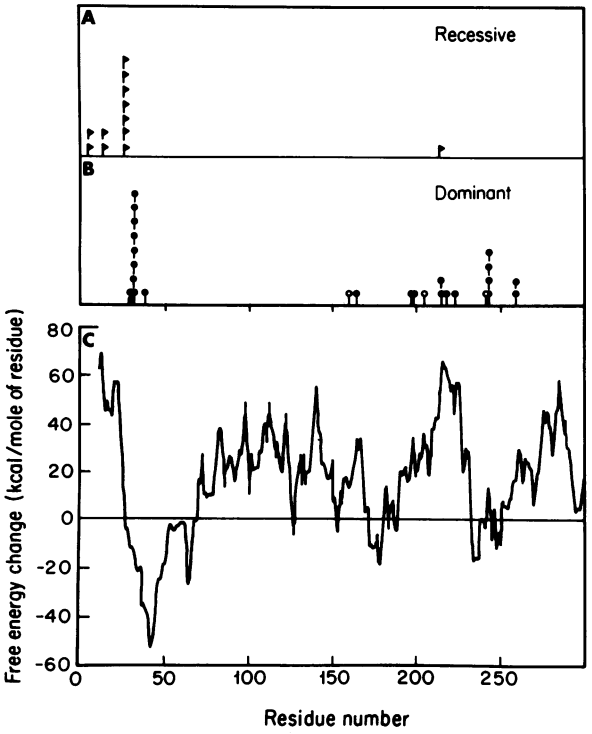

FIG. 4. Positions of the mutations in the *motB* gene and the hydropathy profile of MotB. The hydropathy profile (C) is adapted from reference 21 and reflects the free energy change associated with transferring an 18-residue segment, in α-helical conformation, from water to a nonpolar environment. The deep minimum near residue 45 thus corresponds to a likely membrane spanner. The hydrophilic carboxy-terminal domain of the protein (residues 70 through 308) has been shown to reside in the periplasmic space (8). (A) Positions of recessive mutations: ▶, nonsense (premature termination) mutations. All of the recessive mutations contained premature termination signals. (B) Positions of dominant mutations: ●, missense mutations that abolish function; ○, missense mutations that severely impair function. For exact positions and corresponding amino acid changes, see Table 2.

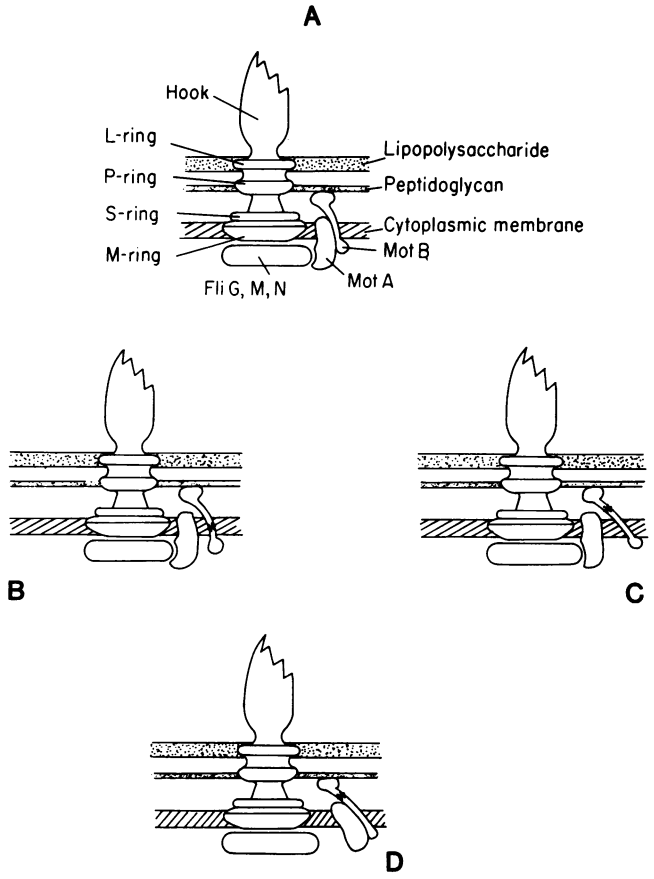

FIG. 5. Suggested role of the MotB protein in flagellar rotation and possible effects of mutation on its function. The cartoon illustrates what is known about the structure of the basal body (the L, P, S, and M rings and the rod that connects them) and its relation to the compound membrane. The shape of the basal body is based upon the image reconstructions of Stallmeyer et al. (22). The locations of MotA, MotB, FliG, FliM, and FliN are speculative, but are as suggested by genetic (28, 29), ultrastructural (11), physiological (4, 6), and biochemical (7, 8, 26, 27) evidence. MotA has been shown to be a transmembrane proton conductor (5); it probably delivers these protons to one or more of the switch-complex proteins FliG, FliM, and FliN. In accordance with the proposal of Chun and Parkinson, MotB is drawn as a linker that fastens MotA, directly or indirectly, to the peptidoglycan layer. We assume that this attachment occurs at a set of specific binding sites (not shown; see the text). The normal arrangement is shown in panel A. In panels B through D, the possible effects of mutations, whose positions are indicated schematically by asterisks, are shown.

not at the slow rotation rate of tethered cells. Nothing comparable to these slow *motA* alleles was found in our collection of *motB* mutants. The loss of function in *motB* mutants was more nearly complete. The three partially functional mutants reported here all swam very poorly or not at all under our standard conditions for assaying motility. When these mutants were tethered, their average motor torques were much less than that of the wild type and were close to that of motors operating with only one torque generator. Some of the mutant cells underwent abrupt doublings in speed; the associated torque increment was close to that of a single torque generator (Fig. 4). These results imply that the partially functional *motB* mutants have a subnormal number of torque generators (usually one and occasionally two) working at any time, but that those working produce normal torque.

Almost all of the *motA* mutations that abolished function mapped to one of four hydrophobic, presumably membrane-spanning domains of that protein (5a), consistent with our suggestion that MotA is a transmembrane proton channel (5). In contrast, most of the dominant *motB* mutations mapped to a very hydrophilic domain located in the periplasmic space (Fig. 4) (8). The periplasmic domain of MotB is evidently critical to its function. A few of the mutations were within or near the single hydrophobic, membrane-spanning segment of MotB, so it is also possible that MotB contributes in some way to forming an efficient transmembrane channel. A variety of circumstantial evidence (4, 6, 27) suggests that MotA and MotB function in close association with each other, but it has proven difficult so far to demonstrate suppression of mutations in one by mutations in the other. This difficulty might reflect the spatial separation of the functionally important domains of MotA, which are in the membrane, from those of MotB, which are mostly in the periplasmic space.

Based on their topological data, Chun and Parkinson suggested that MotB might be a linker that connects the

torque-generating machinery to the cell wall or to some stationary component of the motor. This hypothesis is illustrated in Fig. 5A. We assume that the attachment of MotB to the cell wall occurs at a set of specific binding sites in the vicinity of the motor. If a MotB molecule, which otherwise is nonfunctional, binds normally to such a site, then the mutation will be dominant, because the defective protein will displace the wild-type protein. This specificity could be due to structural features of the peptidoglycan that are unique to this region, to the presence of the P ring, or to other, as yet unidentified molecules that are unique to this region. Although our results do not provide proof that MotB links MotA to the cell wall, they are consistent with this idea in several respects. Unlike MotA, whose main function is to conduct protons (5), MotB cannot be readily mutated to give the slow-swimming phenotype. This suggests that MotB is not closely involved in the processes that govern the motor's kinetics but is consistent with a structural role for MotB as a linker. Some mutations (alleles 30, 5, and 6) are found near the membrane-spanning domain of MotB, where they might directly interfere with its binding to MotA (Fig. 5B). Other mutations are found in the periplasmic domain, where they might cause distortions that separate MotB from MotA (Fig. 5C) or that otherwise disrupt the normal association of motor components (Fig. 5D). Mutations in the periplasmic domain might also disrupt the interaction of MotB with the hypothetical stationary component that anchors it to the cell wall (not illustrated). To discriminate among these possibilities and to test further the idea that MotB is a linker, it will be necessary to measure the affinity of MotB for MotA (and other motor components) directly.

The subnormal torque and speed fluctuations of the partially functional *motB* alleles 1 and 24 suggest that their motors contain one or two torque generators that function normally and several that do not function at all. The subnormal torque does not appear to be a consequence of reduced affinity of MotB for the motor, because these mutations are dominant and because motility was not improved when the mutations were introduced into a *motB* deletion background. It is possible that defective MotB can be incorporated into the motor in a way that causes the torque generators to work intermittently. Intermittent function like that shown in Fig. 2 could be explained if a mutation in MotB caused one of the faulty configurations illustrated in Fig. 5 to be most common but still permitted transient reattachment to give the functional configuration.

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