Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1970–1974, March 1995
Cell Biology

Motility protein interactions in the bacterial flagellar motor

(bacteria/flagella)

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Communicated by Howard C. Berg, Harvard University, Cambridge, MA, December 9, 1994

ABSTRACT **Five proteins (MotA, MotB, FliG, FliM, and FliN) have been implicated in energizing flagellar rotation in *Escherichia coli* and *Salmonella typhimurium*. One model for flagellar function envisions that MotA and MotB comprise the stator of a rotary motor and that FliG, FliM, and FliN are part of the rotor. MotA probably functions as a transmembrane proton channel, and MotB has been proposed to anchor MotA to the peptidoglycan of the cell wall. To study interactions between the Mot proteins themselves and between them and other components of the flagellar motor, we attempted to isolate extragenic suppressors of 13 dominant or partially dominant *motB* missense mutations. Four of these yielded suppressors, which exhibited widely varying efficiencies of suppression. The pattern of suppression was partially allele-specific, but no suppressor seriously impaired motility in a *motB*$^+$ strain. Of 20 suppressors from the original selection, 15 were characterized by DNA sequencing. Fourteen of these cause single amino acid changes in MotA. Thirteen alter residues in, or directly adjacent to, the putative periplasmic loops of MotA, and the remaining one alters a residue in the middle of the fourth predicted transmembrane helix of MotA. We conclude that the MotA and MotB proteins form a complex and that their interaction directly involves or is strongly influenced by the periplasmic loops of MotA. The 15th suppressor from the original selection and 2 *motB* suppressors identified during a subsequent search cause single amino acid substitutions in FliG. This finding suggests that the postulated Mot-protein complex may be in close proximity to FliG at the stator–rotor interface of the flagellar motor.**

The Gram-negative enteric bacterium *Escherichia coli* swims by rotating its flagella (1, 2). The flagellar basal body consists of four rings stacked on a rod. The distal end of the rod connects through a flexible hook to a left-handed helical filament. This entire complex can be isolated as a stable structure (3). A bundle of coalesced, counterclockwise-rotating filaments serves the cell as a propeller. Recent studies have defined a fifth annular structure, the C ring, at the cytoplasmic face of the basal body within the cell (4–6). Bacterial flagella and motility have been extensively reviewed (7–11).

Flagellar rotation is driven by a bidirectional motor at the base of the flagellum. Energy for rotation is provided by the protonmotive force (12–15). The mechanism by which the protonmotive force is converted into rotation is unknown.

Mutations in five genes (*motA, motB, fliG, fliM,* and *fliN*) can lead to the production of paralyzed flagella. The MotA and MotB proteins are not needed for formation of the basal body–hook–filament structure, and they can be added to a preexisting flagellum lacking them to restore rotation (16–18). The FliG, FliM, and FliN proteins form the flagellar "switch–motor complex" (19, 20). Although specific mutations in the "switch–motor" genes lead to paralyzed flagella, other mutations in these genes block flagellar formation or generate flagella with highly skewed rotational biases that disrupt chemotaxis.

MotA and MotB fractionate with the cytoplasmic membrane (21). Based on analysis of its amino acid sequence, MotA is predicted to have four membrane-spanning helices, two short periplasmic loops, and two relatively large cytoplasmic domains (22, 23). Characterization of a set of *motA* mutants by Blair and Berg (24) indicated that the protein conducts H^+ ions across the cytoplasmic membrane. MotB is less hydrophobic than MotA and is predicted to have one N-terminal membrane-spanning helix, with the remainder of the protein extending into the periplasmic space (25, 26). Based on this topology, Chun and Parkinson (26) suggested that MotB anchors MotA or other motor components to the peptidoglycan of the cell wall. Consistent with this proposal is the discovery of a putative peptidoglycan-binding site in the C-terminal half of MotB (27).

Freeze-fracture electron micrographs (28) show an average of 10–12 particles (or "studs") surrounding a doughnut-shaped depression formed by the M ring in the cytoplasmic membrane. The studs disappear when either MotA or MotB is absent, suggesting that the Mot proteins may be distributed in the membrane at the periphery of the M ring. Stolz and Berg (29) constructed a hybrid gene encoding a fusion protein in which the N-terminal 60 residues of MotB are joined to a C-terminal portion of the membrane protein TetA. When this fusion protein is overexpressed with MotA present, cell growth is impaired. Since the growth defect is presumed to result from proton leakage into the cytoplasm, the implication of this result is that this fragment of MotB can activate MotA as a proton-conducting transmembrane channel. However, it has not been demonstrated directly that intact MotB interacts with MotA at the motor.

We have identified extragenic suppressors of four of the *motB* missense mutations described by Blair *et al.* (30). The phenotypes and allele specificity of the suppressed mutants were determined, and a representative selection of the suppressors was identified by DNA sequencing. The majority of the suppressors correspond to single residue changes in the MotA protein, but three suppressors are associated with single residue substitutions in FliG.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain RP437 is wild type for motility and chemotaxis (31). Strain RP6647 was derived from strain RP437 and contains a nonpolar deletion within *motB* (J. S. Parkinson, personal communication). Strain AG64 (*motA*$^+$ Δ*motB*) was constructed by transducing *uvrC279*::Tn*10* (Tet^r) into strain RP6647 and testing for retention of the nonmotile phenotype. Plasmid pGM1 (32) confers ampicillin resistance (Amp^r) and contains *motB* expressed from the *lacUV5* promoter.

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The pMB plasmids (derived from pGM1) carry *motB* missense mutations (30).

Media. Tryptone broth is 1% tryptone (Difco). LB plates contain 1% tryptone, 0.5% yeast extract (Difco), 0.5% NaCl, 20 mM sodium citrate, and 1.5% Difco Bacto-Agar (33). Tryptone swarm plates have 1% tryptone extract, 0.8% NaCl, 20 mM sodium citrate, and 0.35% Difco Bacto-Agar. Mini-swarm plates are tryptone swarm plates in which bacteria are added to molten agar at 50°C before pouring it into Petri plates. Media contained 5 μg of tetracycline or 50 μg of ampicillin per ml as needed and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce plasmid-borne *motB* genes.

Motility Assays. Cells were grown overnight at 30°C in test tubes on a roller drum in 2 ml of tryptone broth containing IPTG and ampicillin. Cultures were then diluted 100-fold into 10 ml of the same medium and grown for 3–4 hr at 30°C in 125-ml flasks with vigorous swirling (final OD_{590} of 0.8). Motility was examined by phase-contrast microscopy. Swarm behavior was assayed by picking fresh overnight colonies into tryptone swarm agar and scoring after 10 hr at 30°C. The diameters of five swarms for each strain were measured and their mean diameter was normalized to the diameter of the swarm formed on the same plate by strain AG64 containing plasmid pGM1 (*motB*$^+$). Motility was quantified as follows: 80–100% of wild type, ++++; 46–80% of wild type, +++; 31–45% of wild type, ++; 16–30% of wild type, +; 5–15% of wild type, ±; <5% of wild type, −.

Isolation of Suppressors. Strain AG64 was mutagenized with ethylmethane sulfonate (EMS) as described by Miller (33). Mutagenized cells were transformed with pMB plasmids, using calcium/rubidium chloride (34). Transformed cells were used to pour mini-swarm plates containing IPTG and ampicillin. After incubation overnight at 30°C pseudorevertants appeared as rare, swarming colonies among thousands of nonmotile colonies. Pseudorevertants were isolated and tested for their swarm phenotypes. Isolates with increased swarm diameters were used to make phage $P1_{vir}$ lysates, which were used to transduce strain RP6647 with or without the appropriate pMB plasmid to Tet^r. Transductants were screened in tryptone swarm agar. Pseudorevertants yielding motile transductants only with the *motB* plasmid-containing recipients were retained.

Mapping of Suppressors. The $P1_{vir}$ cotransduction frequency of each suppressor with *uvrC*::Tn*10* was determined by infecting strain RP6647 containing the appropriate pMB plasmid with $P1_{vir}$ lysates and selecting for Tet^r. Colonies were picked into tryptone swarm plates containing tetracycline and ampicillin. The cotransduction frequency of *uvrC*::Tn*10* was determined to be about 75% with *motA* and between 20% and 25% with *fliG*, *fliM*, and *fliN*. Linkage of the suppressors to the *motB* deletion in strain AG64 was tested by using the lysates to transduce strain RP437 containing the appropriate pMB plasmid to Tet^r and screening the transductants on swarm plates. A low frequency of nonmotile transductants confirmed close linkage between the suppressor and the *motB* deletion.

Allele Specificity of *motB* Suppressors. Each suppressor was transduced into transformants of strain RP6647 containing pGM1 or pMB plasmids. Transductants were tested in tryptone swarm agar to determine to what extent the suppressor restored motility in combination with each of the 13 *motB* missense mutations and how well the suppressors function in combination with wild-type MotB.

Amplification and Sequencing of DNA. DNA fragments containing entire genes were amplified for sequencing using the polymerase chain reaction (PCR). PCRs were run as described by Saiki *et al.* (35) and amplified for 30 cycles in a Perkin-Elmer Cetus thermal cycler. DNA was sequenced by the dideoxynucleotide chain-termination method (36) using the SequiTherm procedure provided by the manufacturer (Epicentre Technologies, Madison, WI).

RESULTS

Properties of *motB* Mutations. The *motB* missense mutations shown in Table 1 were identified by Blair *et al.* (30), who classified them as partially functional or nonfunctional based on the behavior of tethered mutant cells. When their protein products were induced from multicopy plasmids, the mutant alleles displayed various degrees of dominance over a single, chromosomal *motB*$^+$ gene, suggesting that the mutant proteins compete with wild-type MotB for incorporation into the flagellar motor. Overproduction of MotA from a plasmid-borne *motA*$^+$ gene did not overcome this dominance, implying that the mutant MotB proteins do not sequester MotA.

Rationale for Identifying Extragenic Suppressors. The proteins we thought most likely to associate with MotB were those known to be involved in energizing rotation: MotA, FliG, FliM, and FliN. Extragenic suppression analysis was chosen to determine if these proteins interact with MotB. The *motA* gene immediately precedes *motB* and is the first gene in the *mocha* operon, which is located in the FlaII region of the *E. coli* chromosome. The *fliG*, *fliM*, and *fliN* genes are in the FlaIII region. The *uvrC* gene is cotransduced with both regions, so we limited our search to suppressors linked to *uvrC*.

Isolation of *motB* Suppressors. Strain AG64 (Δ*motB uvrC279*::Tn*10*) was used to isolate suppressors (see *Materials and Methods*). Potential suppressors had to pass two tests to warrant further consideration. The first criterion was that they restore motility only in the presence of a pMB plasmid. The second criterion was that the suppressors had to be linked by cotransduction to *uvrC*. Most of the pseudorevertants failed one or both tests. Only 20 independent isolates emerged as viable candidates for extragenic *motB* suppressors mapping in the FlaII or FlaIII region.

Properties of *motB* Suppressors. Four of the 13 *motB* mutations used (Table 1) yielded suppressors. The diameters of the swarms formed by suppressed mutants were compared

Table 1. Suppressor yield with *motB* missense mutations

motB allele	Amino acid change	No. of suppressors identified
Dominant, partially* functional mutation		
motB1	G240D	6
motB24	P159I	5
Dominant, nonfunctional mutation		
motB3	S214F	0
motB6	A39V	3
motB7	R222H	0
motB14	A242T	0
motB20	R258H	0
motB21	R258C	0
motB35	D197N	0
motB39	G164D	6
motB40	R217W	0
motB47	T196I	0
Dominant, nonfunctional double mutation		
motB30	A29T, A32V	0

Suppressors were identified on tryptone mini-swarm plates after EMS mutagenesis and were characterized as described in the text.
*Missense mutations were categorized for function and dominance according to the classification of Blair *et al.* (30). Nonfunctional mutations had a "−" motility phenotype and partially functional mutations had a "±" motility phenotype.

Table 2. Strength and allele specificity of *motB* suppressors

Suppressor allele	*motB*$^+$	*motB1* G240D	*motB6* A39V	*motB24* P159I	*motB39* G164D	Other *motB* mutations
motA$^+$ *fliG*$^+$	++++	±	−	±	−	−
motA T21I (1)	+++	±	[++]	++	−	−
motA G26E (1)	+++	[++]	−	+++	+	−
motA G26V (1)	+++	++	−	[+++]	++	−
motA G189D (1)	+++	+	+	++	[++]	−
motA A191T (3)	+++	[+++]	+++	+++	[++]	−
motA A191V (1)	+++	±	+++	[++]	−	−
motA G199S (4)	++++	±	[++]	[+++]	[++]	−
motA A200V (1)	+++	±	−	[+]	−	−
motA L211F (1)	+++	±	−	++	[+]	−
fliG E108K (1)	+++	±	−	[+]	−	−
fliG R190H (2)	+++	±	−	[+]	−	−

Motility was scored on swarm plates as described in the text. The suppressors were crossed into strain RP6647 containing pMB plasmids with the indicated *motB* alleles. The numbers in parentheses give the number of independent isolates of each suppressor. Brackets enclose the motility scores of each suppressor in combination with the *motB* mutation(s) with which it was isolated.

to the diameters of swarms formed by strain AG64 containing pGM1 (*motB*$^+$) or the parental pMB *motB* plasmid (Table 2). Representative swarms are shown in Fig. 1. Motility of free-swimming individual cells was also examined by phase-contrast microscopy (data not shown); the relative swimming ability of a strain agreed qualitatively with the relative swarm diameter of that strain.

Mapping of the *motB* Suppressors. All but one suppressor were about 70% cotransducible with *uvrC*. This frequency is nearly the same as the 75% linkage found with *motA* (*Materials and Methods*). All of these suppressors were tightly linked to the *motB* deletion, as expected if they were in *motA*. The remaining suppressor did not map near the *motB* deletion, was not cotransducible with the *eda* locus (which is 30% cotransducible with *motA* and on the other side of *motA* from *uvrC*), and was only 15% cotransducible with *uvrC*. Since the *fliG*, *fliM*, and *fliN* genes had cotransduction frequencies with *uvrC* (20–25%) that were close to 15%, we concluded that this last suppressor was probably in the FlaIIIB region.

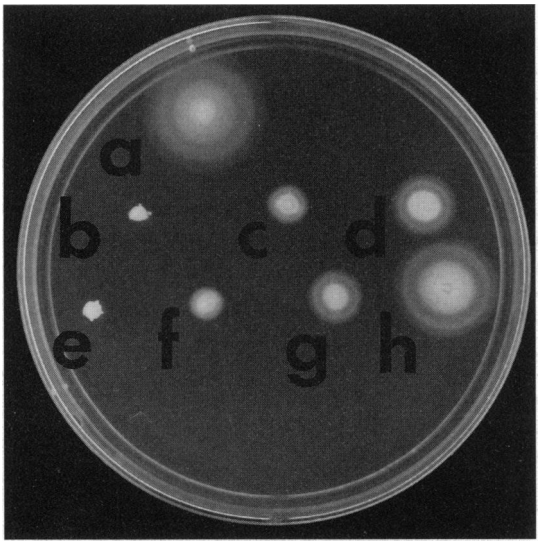

FIG. 1. Swarm behavior of suppressed *motB* mutants. Swarms of plasmid-borne nonfunctional (G164D) and partially functional (P159I) *motB* alleles in the Δ*motB* strain RP6647 containing wild-type and various suppressor *motA* alleles are shown: (a) p*motB*$^+$ with *motA*$^+$, ++++ motility; (b) p*motB*(G164D) with *motA*$^+$, − motility; (c) p*motB*(G164D) with *motA*(L211F), + motility; (d) p*motB*(G164D) with *motA*(G199S), ++ motility; (e) p*motB*(P159I) with *motA*$^+$, ± motility; (f) p*motB*(P159I) with *motA*(A200V), + motility; (g) p*motB*(P159I) with *motA*(A191V), ++ motility; (h) p*motB*(P159I) with *motA*(G26V), +++ motility.

Allele Specificity of the Suppressors. Plasmids carrying the *motB* mutations listed in Table 1 were introduced into strain RP6647, and the transformants were used as transduction recipients for the suppressor alleles. Motility of the transductants was tested on tryptone swarm plates and confirmed by phase-contrast microscopy of swimming cells. The swarm-plate results are given in Table 2. The suppressors did not impair motility in the presence of the *motB*$^+$ plasmid, so their only obvious phenotype was the ability to restore motility to

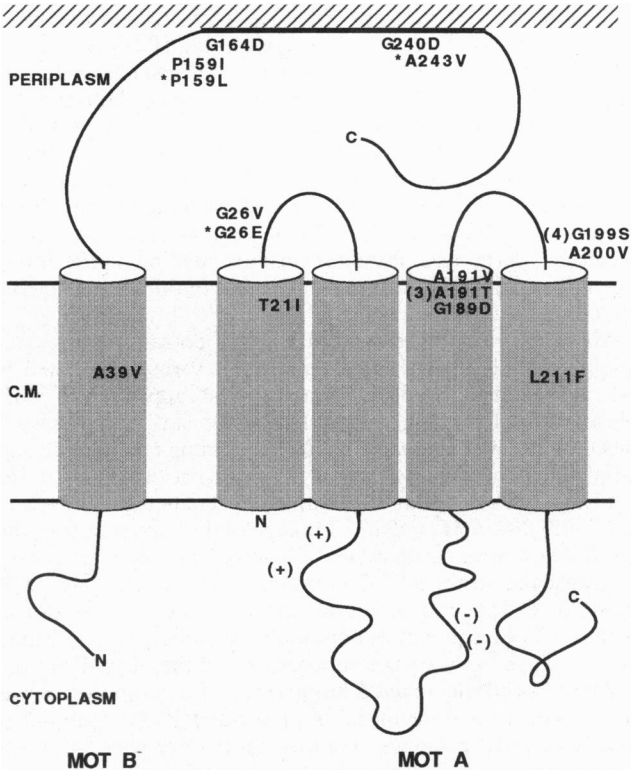

FIG. 2. Location of amino acid substitutions caused by *motB* mutations and their suppressors in *motA*. Molecular cartoons are based on Stader *et al.* (25) and Chun and Parkinson (26) for MotB and on Dean *et al.* (22) and Blair and Berg (23) for MotA. C.M., cytoplasmic membrane. The + and − signs in the cytoplasmic portion of MotA indicate clusters of charged residues. Residues marked with an asterisk were identified previously as *motB* mutation/*motA* suppressor pairs by Chun (37). The extent of the potential peptidoglycan-binding region of MotB (27) is indicated by the broadened line, which is placed adjacent to the hatched structure representing the peptidoglycan layer of the cell wall.

cells making mutant MotB proteins. No suppressor restored motility in combination with the nine *motB* mutations that did not yield suppressors in the original screen. The suppressors gave characteristic patterns of motility restoration with the other four *motB* mutations (Table 2).

Sequence Localization of Suppressors. Fourteen suppressors, chosen on the basis of their allele-specificity patterns, were characterized by sequencing the entire *motA* gene from the suppressor strain. (The remaining 5 suppressors mapping in *motA* were not sequenced because they had allele specificities identical to one or more of the other 14.) Each suppressor corresponded to a single amino acid substitution in MotA. Nine different substitutions affecting seven codons were found (Table 2). We used the topology of MotA proposed by Dean *et al.* (22) to assign the suppressors to particular regions of MotA (Fig. 2).

Identification of the FlaIIIB Region Suppressor. Cotransduction frequencies suggested that one suppressor, which was specific to the P159I substitution in MotB, was located in the FlaIIIB region. Since we thought it likely that the suppressor would affect the switch–motor complex, we sequenced the *fliG, fliM*, and *fliN* genes from this strain. We also sequenced the *fliL* gene, because a knockout of the equivalent gene in *Caulobacter crescentus* abolishes motility (38), although a nonpolar deletion of *fliL* has no obvious phenotype in *E. coli* (39).

No mutations were found relative to the published sequences of the *fliL, fliM*, or *fliN* genes of *E. coli* (40, 41). The *fliG* gene from the suppressor strain had a single base change, corresponding to the substitute R190H. Two additional *fliG* suppressors, isolated subsequently, also were specific for P159I (Table 2). They generate the changes R190H and E108K. [Our sequence of the *fliG*$^+$ gene from strain AG64 diverges in several places from the published *E. coli fliG* sequence (42) and in areas of disagreement corresponds better with the *fliG* sequence of *Salmonella typhimurium* (43). The residues we identified as E108 and R190 based on our sequence correspond to residues E107 and R188 in the protein sequence derived from the *fliG* DNA sequence published for *E. coli.*]

DISCUSSION

Four of the 13 *motB* mutations we examined yielded a total of 20 extragenic suppressors. Nineteen mapped to the *motA* gene. Fourteen suppressors in *motA*, representing the entire range of restored-motility phenotypes, were located by DNA sequencing (Table 2). They comprise nine different substitutions at seven amino acid residues.

Individual suppressors conferred distinct patterns of motility when tested against the collection of 13 *motB* mutations (Table 2). Some suppressors, like G189D and A191T, restored motility with all 4 of the suppressible *motB* mutations. Others, like A200V, restored motility only with the *motB* mutation with which they were isolated. Most suppressors fell between these extremes, restoring motility with 2 or 3 of the suppressible *motB* mutations.

Unique patterns of suppression also appeared among the "intermediate" class of suppressors (Table 2). For example, G26V restored motility with the MotB substitutions P159I, G164D, and G240D, whereas A191V restored motility with A39V and P159I. Furthermore, two suppressors that alter the same residue in MotA (A191T and A191V) exhibited different suppression patterns. However, suppression of G164D was qualitatively similar to, although less complete than, the suppression pattern for P159I (Table 2).

The finding that the *motA–motB* suppression pattern showed partial allele specificity suggests that MotA and MotB form a complex. Stolz and Berg (29), based on data obtained with a different method, reached the same conclusion, which conforms with most models for the function of the Mot proteins. Our data provide genetic evidence that this complex forms in association with the flagellar motor.

We have examined our suppression data with respect to the proposed topologies of MotB (25, 26) and MotA (22, 23). The A39V substitution is in the middle of the N-terminal membrane-spanning helix of MotB, whereas the P159I, G164D, and G240D substitutions are in the C-terminal periplasmic domain of MotB. Of the 14 suppressors we identified in *motA*, 13 alter residues in or immediately adjacent to the periplasmic loops of MotA (Table 2; Fig. 2). A similar result was obtained earlier by Chun (37), who found that the G26E substitution in the first periplasmic loop of MotA suppressed the motility defects caused by P159L and A243V substitutions in MotB. Our remaining suppressor in *motA* alters the L211 residue in the middle of the fourth membrane-spanning helix.

All but one of the residues in MotA affected by the suppressor mutations cluster at the periplasmic surface of the protein. However, we anticipate that few, if any, of the mutation-suppressor pairs we have identified involve residues that come into direct contact at the MotA–MotB interface. One reason for drawing this conclusion is that the suppression, although not totally promiscuous, shows a broad specificity (Table 2). For example, each of the four *motB* mutations is suppressed by at least one mutation altering the first periplasmic loop of MotA and one mutation altering the second periplasmic loop of MotA. A second reason is that the P159, G164, and G240 residues are in or near a region in the C-terminal half of MotB that contains a putative peptidoglycan-binding region (ref. 27; Fig. 2). No experimental evidence firmly links this part of the MotB to the cell wall, but the sequence homology is highly suggestive. This C-terminal region of MotB could contact both MotA and peptidoglycan, but a tight connection of this type seems incompatible with the proposed role of MotB as an elastic linkage to the cell wall and the flagellar motor (44).

Our data provide no evidence for or against the notion that the transmembrane helices of MotB and MotA are in direct contact. A39V (MotB) and L211F (MotA) was the only mutation-suppressor combination in which the altered residues could potentially contact each other within the membrane, but this pairing did not restore motility (Table 2). A selection focused on A39V may discover suppressors that affect the hydrophobic regions of MotA. The residue substitutions generated by such suppressors might help elucidate how the membrane-spanning segments of MotA and MotB interact.

We favor an interpretation of our results that invokes an indirect mechanism of suppression. The P159I, G164D, and G240D mutations may distort the connection between MotB and the cell wall such that the N-terminal portion of MotB, on which MotA may be mounted, positions MotA incorrectly relative to the membrane or switch–motor complex. The contact between MotA and MotB could involve the periplasmic loops of MotA, the transmembrane helices of the two proteins, or both. Suppressors altering the periplasmic loops of MotA may reorient MotA to compensate for the distortion introduced by a *motB* mutation. These suppressors could affect the contact region directly even if the *motB* mutation they suppress does not change a residue at the contact site. Alternatively, these suppressors might shift the position or conformation of one or more of the helices connected by the periplasmic loops. Similarly, the L211F substitution might suppress the P159I and G164D substitutions in MotB by changing the orientation or secondary structure of the membrane segments of MotA or by introducing conformational changes in the helices that could be propagated to the periplasmic loops.

At least two different amino acid changes in FliG, E108K and R190H, can partially suppress the *motB* P159I allele. The same mutations arose as clockwise-biased suppressors of *cheY*

mutations in *S. typhimurium* (45), but we do not know if our *fliG* mutations cause clockwise rotational bias. Cultures of the *motB* P159I mutant contain only a few percent motile cells, which swim sporadically and erratically. Cultures of this mutant that carry the *fliG* R190H allele have 20–30% motile cells, which swim wobbly and slowly but continuously. Thus, this *fliG* suppressor actually improves the motility of the *motB* P159I mutant rather than just conferring a greater amount of tumbling that allows the cells to spread in semisolid agar.

Yamaguchi *et al.* (20) found that a *fliG* Mot$^-$ mutation (a deletion of codons 169–171) in *S. typhimurium* could be weakly suppressed by the L47R substitution in MotB (45). L47R itself, which is near the periplasmic end of the transmembrane helix of MotB, confers a partially paralyzed phenotype (20). Although the data are limited, these findings suggest that the Mot proteins and FliG may be in close proximity at the stator–rotor interface. This conclusion is consistent with the observation that a larger number of Mot$^-$ mutations are found in *fliG* than in *fliM* or *fliN* (19, 45) and with genetic and electron microscopic studies that localize FliG at the circumference of the cytoplasmic face of the innermost ring (the M ring) of the flagellar basal body (5, 6).

MotA may funnel H^+ ions to protonatable residues in the cytoplasmic loop of MotA or on FliG. Mutations altering the orientation of MotB or the geometry of its binding to MotA could interfere with delivery of H^+ ions to these residues. Mutations in *motB* could also misalign regions of MotA and FliG that interact to generate rotation. Compensating changes in either MotA or FliG could partially correct such misalignments and thus partially alleviate the motility defects caused by the *motB* mutations.

We thank David Blair for providing the plasmid-borne *motB* mutations, Sandy Parkinson for the *motB* deletion strain, and Robert Macnab and Howard Berg for critically reading an early draft of the manuscript. Roy Biran, Phil Bronstein, Debbie Salazar, Patricia Valdez, and Jimmy Wohlschlegel were undergraduates who participated enthusiastically in various stages of the project. The Gene Technologies Laboratory of the Institute for Developmental and Molecular Biology at Texas A&M University provided excellent facilities for synthesis of oligonucleotide primers and for DNA sequencing. Lily Bartoszek gave the manuscript a final, thorough proofreading before it was submitted. This work was supported by Grant DAAH04-94-G-0056 from the Army Research Office.

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