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Evidence for Interactions between MotA and MotB, Torque-Generating Elements of the Flagellar Motor of *Escherichia coli*

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Cells that overexpress MotA (encoded on a plasmid derived from pBR322) grow slowly because of proton leakage. We have traced this defect to the coexpression of a fusion protein consisting of 60 amino acids from the N terminus of MotB and 50 amino acids specified by pBR322. Mutations within the N terminus, known to abolish function when present in full-length MotB, reversed the growth defect. Growth also was normal when MotA was coexpressed with wild-type MotB or with a series of MotB N-terminal fragments.

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Escherichia coli is propelled by the rotation of about six flagella that arise at different points on the cell surface. Components that extend out into the external medium include a short proximal hook and a long helical filament. Components embedded in the cell envelope that can be visualized after detergent extraction include four rings mounted on a rod. Other components, lost during detergent extraction, are essential for energy transduction (generation of torque from an electrochemical proton gradient), for control of the direction of rotation (clockwise-counterclockwise switching), and for flagellar synthesis. MotA and MotB are required for torque generation (1, 3, 7, 20), and FliG, FliM, and FliN are required for torque generation, directional control, and flagellar synthesis (26, 27). MotA and MotB are found in the cytoplasmic membrane clustered around the innermost ring (the M-ring), and FliG, FliM, and FliN are thought to reside on the cytoplasmic face of this ring (12, 26). For recent reviews of flagellar structure and function, see references 2, 11, and 14 to 16.

A variety of evidence suggests that MotA is a proton channel. Overexpression of wild-type MotA suppresses growth, presumably by introducing proton leaks into the cytoplasmic membrane (4, 24). Direct evidence for this comes from a comparison of the proton conductance of membrane vesicles prepared from wild-type cells or from dominant nonfunctional MotA mutants: overexpression of the mutant MotA does not suppress growth or enhance proton conductance (4). Structure prediction methods for MotA based on gene sequences show four hydrophobic and possibly two amphipathic α-helices spanning the cytoplasmic membrane (10). Subsequent work favors the four-helix model. Almost all of the amino acid substitutions in the dominant nonfunctional mutants fall within the four hydrophobic domains, and MotA in intact spheroplasts is immune to proteolytic digestion (5). Other mutants have been found that generate normal torque at low speed (when cells are tethered) but abnormally low torque at high speed (when cells are swimming). At high speed, they display altered hydrogen isotope effects, also indicating that MotA is involved in proton transfer (4). These mutants map primarily in the cytoplasmic domain (5).

MotB, on the other hand, probably acts as a linker that connects MotA to some stationary component of the cell wall. The N terminus of MotB spans the cytoplasmic membrane (22), while the bulk of the protein is exposed to the periplasmic space (9). This surmise has been tested by fusing alkaline phosphatase to different N-terminal portions of MotB and confirmed by proteolytic digestion of the protein in intact spheroplasts (9). Dominant mutants of MotB either are completely nonmotile or function with a reduced number of force-generating elements (6).

Circular arrays of particles surrounding a central depression thought to accommodate the M-ring have been seen in freeze-etched membranes of *E. coli* (12). These arrays are absent in strains deleted for *motA* or *motB*. Also, MotB is more stable in the presence of MotA than in its absence (25). Thus, a variety of evidence suggests that MotA and MotB are associated not only with the basal body but also with each other.

In the experiments described here, we overexpressed MotA together with MotB or certain MotB hybrids or fragments. We found that growth impairment requires the coexpression of MotA and a particular MotB hybrid encoded by the plasmid used in the earlier work (4, 24). Point mutations affecting the N-terminal region of this hybrid, which abolish function when present in full-length MotB, reversed the growth defect. Other constructs containing the same N-terminal region of MotB were tested, and all reversed the growth defect. A nonsense mutation that terminates MotB after the third amino acid enabled us to test the effect of the overexpression of MotA without a hybrid protein. Again, growth was normal. Only one truncated product was stable enough to be identified on polyacrylamide gels: an N-terminal fragment of MotB of 291 amino acids. This polypeptide was nonfunctional when expressed in a *motB* strain and recessive in the wild type: it was not able to displace wild-type MotB from the flagellar motor. These results are discussed in terms of a model in which MotB links MotA to a stationary component of the cell wall (2, 18).

Construction of plasmids. The strains and plasmids used are listed in Table 1, and the translation products specified by the final constructs are shown in Fig. 1. All plasmids are derivatives of pBR322. Plasmid pLW3 was constructed by Wilson and Macnab (24) to direct high-level expression of *motA*. It was used by Blair and Berg (4) to compare the effects of high-level expression of wild-type *motA* with that of dominant nonfunctional *motA* alleles.

Plasmid pDFB45 was constructed (17) by ligating a 1.3-kb

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TABLE 1. Strains and plasmids used to construct the plasmids shown in Fig. 1

Strain or plasmid	Relevant genotype or phenotype	Source or reference
RP437	Wild type for motility and chemotaxis	19
MS5037	*motA448*	21
RP3087	*motB580*	J. S. Parkinson
RP6665	Δ(*motA motB*)*m2-13*	J. S. Parkinson
pLW3	p_{trp} *motA*$^+$ Ap^r	24
pGM1	p_{lac} *motB*$^+$ Ap^r	8
pDFB45	p_{trp} *motA*$^+$ *motB*$^+$ Ap^r	D. F. Blair
pGM1*motB6*	p_{lac} *motB6* Ap^r	6
pGM1*motB30*	p_{lac} *motB30* Ap^r	6
pGM1*motB33*	p_{lac} *motB33* Ap^r	6
pDFB36*motA25*	p_{lac} *motA25* *lacI*^{q} Ap^r	5

*Eco*RI-*Mlu*I fragment of pLW3, spanning p_{trp} and the 5′ region of *motA*, with a 5.4-kb *Mlu*I-*Eco*RI fragment of pGM1, encoding the complementary 3′ region of *motA* and the entire *motB* gene. pDFB45 derivatives carrying mutant *motB* alleles were made in the same way, using pLW3 and mutant variants of pGM1 (Table 1). Starting at the *Eco*RI site and proceeding clockwise, pDFB45 encodes p_{trp}, 450 bp of the 3′ end of *cheB*, the first 120 bp of *cheY*, a segment of 20 bp originating from a polylinker, 20 bp preceding the initiation codon of *motA*, all of the *motA* and *motB* genes, and a fragment of *cheA* of 528 bases, with the last 14 bases originating from pBR322.

To ensure that pDFB45 and its derivatives did not differ from pLW3 in some unknown manner that might affect proton leakage, pLW3 was reconstructed by the ligation of an 1.8-kb *Eco*RI-*Sca*I fragment of pDFB45, spanning p_{trp} and the 5′ region of *motB*, and a 2.3-kb *Pvu*II-*Eco*RI fragment of pDFB45, carrying sequences of pBR322. By all of the criteria of which we are aware, this construct (called pBS27; Fig. 1) is identical to pLW3. Derivatives of pBS27 carrying mutant *motB* fragments were constructed in the same way, by ligation of the 1.8- and 2.3-kb fragments of mutant *motB* derivatives of pDFB45. Plasmids pLW3 and pBS27 differ from pDFB45 by the removal of the 2.7-kb *Sca*I-*Pvu*II fragment. Only 181 bases from the 5′ end of *motB* remain. In pLW3 and pBS27, these bases are fused to 149 bases of pBR322, yielding an open reading frame that encodes a protein of 110 amino acids (Fig. 1, B60/50).

Plasmid	Translation product		Increase in generation time upon induction (%)
pDFB45	A295	B308	22
pLW3	A295	B60/50	75
pBS27	A295	B60/50	79
pDFB45*motB33*	A295	B3(Q4ochre)	8
pBS27 *motB6*	A295	B60/50 (A39V)	14
pBS27 *motB30*	A295	B60/50 (A29T,A31T)	12
pBS27 *motB33*	A295	B3(Q4ochre)	20
pBS28 *motA25*	A29 (Q30ochre)	B60/50	11
pBS1	A295	B148/1	2
pBS2	A295	B219	18
pBS3	A295	B291	4
pBS4	A295	B60/4	8
pBS19	A295	B60/100	12

FIG. 1. Schematic representation of the products specified by different plasmids and their effects on generation time. The capital letters refer to protein encoded by *motA* (A) or *motB* (B), and the numbers indicate length in amino acids. Amino acids encoded by nucleotide sequences that are not part of *motA* or *motB* are shown hatched, and their number is indicated after the slash. The notation in parentheses indicates amino acid changes in *motB* alleles. Generation times of wild-type cells carrying the plasmids were compared with and without induction of the *trp* promoter; see the text.

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Plasmid pLW3*motA25* was made by replacing the 0.4-kb *Bam*HI-*Bgl*II fragment of pLW3 with the corresponding *Bam*HI-*Bgl*II fragment of pDFB36*motA25* (Table 1). This plasmid expresses only the first 29 amino acids of MotA (Fig. 1).

Plasmids pBS1 and pBS2 were made in the same way as pDFB45, except that two derivatives of pGM1 (gifts of D. F. Blair) were used in which a nonsense oligonucleotide (*Xba*I linker; New England Biolabs, Inc., Beverly, Mass.) had been inserted into an *Acc*I site and an *Nsi*I site of *motB*, respectively. pBS3 was made from pDFB45 by inserting the nonsense oligonucleotide into a unique *Bsu*36I site in *motB* that was blunt ended by treatment with mung bean nuclease. pBS4 was constructed by ligating a 3.2-kb *Eco*RI-*Nru*I fragment of pDFB45 with a 1.8-kb *Sca*I-*Eco*RI fragment from the same plasmid and linking the dephosphorylated *Nru*I and *Sca*I sites with the nonsense oligonucleotide. pBS19 was made in the same way as pBS4, except that the nonsense oligonucleotide was not inserted. pBS1, pBS2, pBS3, and pBS4 were sequenced with the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio). pBS1 encodes the first 148 amino acids of MotB followed by 1 amino acid (valine) encoded by the linker. pBS2 and pBS3 encode truncated MotB of 219 and 291 amino acids, respectively. pBS4 encodes the first 60 amino acids of MotB followed by 4 amino acids (serine, serine, leucine, and asparagine) encoded by the linker. Finally, pBS19 specifies a larger MotB-pBR322 hybrid of 160 amino acids, arising from the fusion of the first 181 bp of *motB* with 299 bp from the 3′ end of the Tet^r gene of pBR322.

Complementation. The plasmids listed in Fig. 1 were used to transform the *motA* strain MS5037 and the *motB* strain RP3087 (Table 1). In all cases, even without induction, motility was restored by constructs carrying the corresponding wild-type gene, but not the mutant alleles (data not shown).

Generation times. Wild-type cells were transformed with each of the plasmids, and the cells were grown to saturation in Luria-Bertani broth at 34°C in the presence of ampicillin (50 μg/ml), diluted 1:100 into fresh broth plus ampicillin, and grown for an additional 2 to 3 h. The optical density at 600 nm (OD_{600}) was measured, and the cells were diluted to an OD_{600} of 0.01 with fresh medium. After a further 20 min of growth at 34°C, the transcriptional inducer for the *trp* promoter, indole acrylic acid (10 mg/ml in ethanol), was added to 100 μg/ml, and an equivalent amount of ethanol was added to the control samples. The OD_{600} was measured every 20 min until late exponential phase. Generation times were calculated by linear regression analysis of a semilogarithmic plot of these data, with appropriate weightings. The percentages of increase in generation time upon induction are listed in Fig. 1. Generation times for uninduced cells varied from 32 to 35 min (mean ± standard deviation, 33.7 ± 1.2 min).

Growth was markedly retarded only when wild-type MotA was coexpressed with a hybrid protein (B60/50) comprising 60 amino acids from the N terminus of MotB and 50 amino acids specified by pBR322 (plasmids pLW3 and pBS27; Fig. 1). Growth was essentially normal when wild-type MotA was expressed alone (plasmids pDFB45*motB33* and pBS27 *motB33*), when wild-type MotA and MotB were expressed together (plasmid pDFB45; also shown in reference 25), or when wild-type MotA was expressed with a variety of MotB fragments. These included B60/50 hybrids comprising amino acids from the N terminus of mutant MotB specified by dominant nonfunctional *motB* alleles (plasmids pBS27 *motB6* and pBS27*motB30*), B60/4 and B60/100 hybrids (plasmids pBS4 and pBS19), and B148/1, B219, and B291 truncated MotB fragments (plasmids pBS1, pBS2, and pBS3, respectively).

The simplest explanation for these results is that a specific interaction between wild-type MotA and the B60/50 hybrid alters the conformation of MotA and that this change enhances proton leakage. Why this should occur is not apparent. The B60 sequence is given in reference 22. The /50 sequence, SASRVSVMTVKTSDTCSSRRRSQLVCKRMP GADKPVRARQRVLAGVGAQP, inferred from the base sequence of pBR322, is polar and would not be expected to form additional transmembrane segments. The B60/50 hybrid does not act on its own, because growth is normal when it is coexpressed with a MotA fragment (pBS28*motA25*; Fig. 1) or with full-length nonfunctional MotA mutants (4). Its interaction with MotA does not occur when the B60 component is derived from MotB mutants in which alanine 39 is changed to valine or alanines 29 and 31 are changed to threonine (alleles *B6* and *B30*, respectively; see reference 6). These changes are in the putative N-terminal, α-helical, membrane-spanning domain. The corresponding full-length mutant MotB are nonfunctional, but they do bind to the flagellar motor, because the mutations are dominant (6). All of these results are consistent with the proposition that MotA normally interacts with MotB and that this interaction involves the N-terminal α-helical domain.

Protein expression. Cytoplasmic membranes were isolated on sucrose gradients following treatment with EDTA-lysozyme and sonication, as described previously (4). Electrophoresis was carried out as described previously (13), and Coomassie blue-stained gels were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, Calif.). In some cases, in vivo radiolabeling was carried out by using the T7 RNA polymerase/promoter system described previously (23). Genes of interest were subcloned onto plasmid pT7-5 or pT7-6, on which transcription is controlled by a T7 promoter. Plasmids of several transformants of each construct were analyzed by restriction mapping and were then used to transform strain RP437 containing pGP1-2, which encodes the T7 RNA polymerase. Specific labeling of products of the subcloned genes was performed as described previously (23), except that labeling with [^{35}S]methionine was for 15 min. Proteins from whole cells were separated on 15% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate, and the gels were autoradiographed.

MotA and MotB were identified as bands with apparent molecular masses of 31 and 37 kDa, respectively, in agreement with values in the literature (25) (Fig. 2A). When cells were grown to saturation and membranes were isolated, the integrated intensities of these bands were approximately equal (data not shown). However, the only MotB hybrid or fragment that could be identified was that of 291 amino acids encoded by plasmid pBS3 (Fig. 2B, lane 3). In particular, the hybrid B60/50 was not stable enough to be isolated. To rule out the possibility that the mutant hybrid proteins abolished the growth defect by reducing the stability of MotA, we grew cells containing pBS27*motB6* and pBS27*motB30* to late exponential phase and isolated the inner-membrane fractions, as described earlier. The protein concentration of the fractions was determined, and equal amounts were loaded onto 10% polyacrylamide gels. The gels were stained with Coomassie blue, and individual lanes were scanned with the densitometer. The amount of MotA inserted into the cytoplasmic membrane was essentially identical in all cases (data

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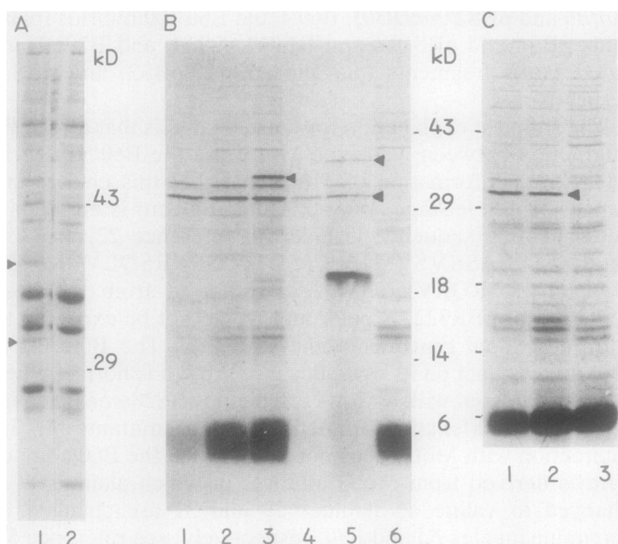

FIG. 2. (A) Polyacrylamide gels (12%) stained with Coomassie blue, showing inner-membrane fractions isolated on sucrose gradients. Cells harboring plasmid pDFB45 were grown under inducing or noninducing conditions (lanes 1 and 2, respectively). Arrowheads indicate the positions of MotA (31 kDa) and MotB (37 kDa). (B and C) Autoradiograms of polyacrylamide gels (15%) showing translation products obtained after programmed expression by using the T7 RNA polymerase/promoter system. The plasmids encoding the proteins visible in each lane are as follows. (B) Lane 1, pBS1; lane 2, pBS2; lane 3, pBS3; lane 4, pBS4; lane 5, pDFB45; lane 6, none (i.e., a control using pT7-6 without an insert). The arrowhead in lane 3 indicates the truncated MotB protein of 291 amino acids. Arrowheads in lane 5 indicate wild-type MotA and MotB. Wild-type MotA also appears in lanes 1 to 4. The band of about 20 kDa in lane 5 is the product of the open reading frame of 528 bases that is located immediately downstream of *motB*; see the text. (C) Lane 1, pLW3; lane 2, pBS1; lane 3, none (as above). The arrowhead in lane 2 indicates wild-type MotA, which also appears in lane 1. The dashes and numbers indicate the positions and molecular masses of proteins of known mass.

not shown). Thus, synthesis of mutant hybrid protein does not change the stability of MotA.

Our failure to identify MotB fragments on gels is disturbing, because it is difficult to see how the hybrid protein encoded by pLW3 and pBS27 can affect the conformation of MotA, unless it is stable enough to interact strongly with MotA. However, we might be dealing with a small number of hybrid molecules interacting strongly with a small number of MotA molecules. In any event, it is gratifying that MotA, when expressed alone or when coexpressed with wild-type MotB, nonfunctional MotB mutants, or other MotB hybrids or fragments, does not impair growth (Fig. 1). If MotA serves as a channel that conducts protons from the periplasmic space to proton-accepting sites on the rotor, then it should not shunt protons directly from the periplasmic space into the cytoplasm. This would be tantamount to uncoupling the motor. When MotA is overexpressed, the amount of protein found in inner-membrane fractions is much larger than the amount that can be accommodated by the flagella (24). The fact that *motA* strains can be resurrected (3, 7, 20) and the existence of dominant nonfunctional *motA* mutants (4, 5) imply that this protein can exchange with MotA bound to the motor. Were the free molecules highly conductive, they would short-circuit the membrane.

The stable MotB construct (encoded by pBS3; Fig. 1) failed to restore motility in a *motB* mutant or to interfere with motility in a wild-type strain. Thus, it appears to be unable to compete for binding sites at the flagellar motor. This raises the intriguing possibility that the C-terminal 17 amino acids of MotB, of which four are prolines, might mediate such binding, anchoring MotB to the cell wall. If the linkage provided by MotB were sufficiently elastic, and the relative motion of MotA and proton-accepting sites on the motor were properly constrained (in a manner dependent on protonation), the resulting ratcheting action could generate the requisite torque (2, 18).

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