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6. AUTHOR(S)
Michael D. Manson

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(ES)
Texas A&M Research Foundation
Box 3578
College Station, TX 77843

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13. ABSTRACT *(Maximum 200 words)*

Rotation of the flagella of *Escherichia coli* is driven by an inward H^+ current maintained by the transmembrane proton motive force. The first objective of the work was to determine, by genetic suppression analysis, interactions between the two proteins (MotA and MotB) that make up the stator of the flagellar motor. The second objective was to determine how the stator interacts with the rotor. We demonstrated that amino acid substitutions affecting portions of MotB thought to contact or lie adjacent to the peptidoglycan layer of the cell wall and that cause severe motility defects can be suppressed by mutations altering the external face or transmembrane regions of MotA. Some *motB* mutations can also be suppressed by mutations in the *fliG* gene, which encodes a component of the rotor. Our overall conclusion is that suppression is achieved by events that affect distant sites within the motor. Thus, a mutation in *motB* that misaligns the stator with respect to the rotor can be corrected by a compensating change in the stator (a *motA* mutation) or in the rotor (a *fliG* mutation). Similar experiments with missense mutations in *motA* produced different results in that suppressors fell chiefly into a defined region encoding residues 136-138 of MotB. In contrast to the suppressors of *motB* mutations, we propose that the residues of MotB identified in this suppression analysis may directly contact MotA and help determine correct alignment or tight binding.

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COUPLING PROTON CONDUCTION TO ROTATION IN THE BACTERIAL FLAGELLAR MOTOR

FINAL PROGRESS REPORT

MICHAEL D. MANSON

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U.S. ARMY RESEARCH OFFICE

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TEXAS A&M UNIVERSITY

STATEMENT OF RESEARCH PROBLEM

Bacteria swim by rotating rigid, helical flagellar filaments. This rotation is driven by a cation current flowing into the cell through a reversible motor located at the base of the flagellum, which is anchored in the cell envelope. Counterclockwise rotation causes the 6-8 left-handed helical filaments per cell to coalesce into a bundle that pushes the cell along a gently curved path (the cell "runs"). Clockwise rotation of the flagella causes the bundle to fly apart and leads to a chaotic motion that randomly reorients the direction of the next run (the cell "tumbles"). Thus, the cell moves in a three-dimensional random walk. Chemotaxis results when signals generated at the chemoreceptors result in runs being extended (by suppressing tumbles) to bias the random walk when the cell swims toward higher concentrations of an attractant or lower concentrations of a repellent.

In most bacteria the energy for flagellar rotation is provided by H^+ ions moving in response to a proton motive force (pmf). In some alkalophilic species, propulsion is provided by the influx of Na^+ ions in response to a sodium motive force. A fundamental question is how these ion currents are coupled to flagellar rotation. In *Escherichia coli* and *Salmonella typhimurium* the MotA and MotB proteins are absolutely essential for motility, but they are dispensable for assembly of a structurally intact basal body and filament. They are thought to form the stator complex of the motor, which has the dual functions of anchoring to the cell wall (primarily through MotB) and forming a transmembrane ion channel (consisting primarily of MotA). The FliG, FliM and FliN proteins, also involved in generating rotation, are required for flagellar assembly. They are apparently mounted on the cytoplasmic face of the basal body and form the flagellar switch-motor complex, which is responsible for controlling the direction of flagellar rotation in response to signals from the chemoreceptors as well as for generating rotation.

We have used intergenic suppression to investigate how the Mot and Fli proteins interact in *E. coli*. The idea was to identify extragenic suppressor mutations that overcome motility defects caused by missense mutations in the *motA* and *motB* genes. We reasoned that identification of genes corresponding to mutant alleles and their suppressors would provide information about which proteins interact directly within the motor. We were also optimistic that the locations of altered amino acids (identified by DNA sequence analysis) within the predicted three-dimensional structures of complementary mutant/suppressor protein pairs would suggest which domains or surfaces of the proteins come into contact. We also anticipated that the mechanical properties (rotational velocity, torque generation, and clockwise/counterclockwise ratio) of the mutant and suppressed-mutant motors relative to those of the wild-type motor would reveal critical functions of particular protein domains or protein-protein interactions.

SUMMARY OF IMPORTANT RESULTS

Three peer-reviewed papers (Garza *et al.*, 1995, 1996a,b) were published under ARO grant DAAH04-94-G-0056 during the three-year funding period (4/1/94 through 3/31/97) with a one-year extension (4/1/97 through 3/31/98). The results contained in those papers and other, as yet unpublished, work accomplished during the funding period are summarized in this section.

1) Extragenic suppressors of dominant and partially dominant *motB* missense mutations. Plasmid-borne missense *motB* alleles were isolated by David Blair, then working as a postdoctoral fellow in Howard Berg's laboratory at Harvard University. When these mutations were introduced

into a motB+ strain they exhibited various degrees of dominance, as assessed by impairment of motility This result suggested that the mutant proteins could compete with wild-type MotB for incorporation into the motor.

We originally identified extragenic suppressors for four of thirteen of these *motB* mutations (Garza *et al.*, 1995). The suppressors showed partial allele specificity, indicating that they were not bypass mutations and that they did not act only in a general way to stabilize the mutant MotB proteins. The majority of the suppressors mapped to *motA* and affected residues at or near the periplasmic face of MotA, demonstrating that this region is important for proper association of MotA with MotB, whether or not it contacts MotB directly. This paper also reported the discovery of two *motB* suppressors located in *fliG*.

Subsequent detailed analysis, focused on pseudorevertants that only moderately restored swimming ability, identified more suppressors for the same four *motB* mutations, but none for the remaining *motB* mutations (Garza *et al.*, 1996a). In addition to many more suppressors in *motA*, five different suppressors in *fliG* and one in *fliM* were found. The majority of the suppressors in *fliG* affected a region predicted from secondary structure analysis to be a flexible linker between the N-terminal (assembly) and C-terminal (motility) domains (D. Blair, personal communication; acknowledged in Garza *et al.*, 1996a).

Motility of the suppressed mutants and of strains containing suppressors but producing wild-type MotB was analyzed on swarm plates and with tethered cells. Some of the suppressors restored swarming ability and rotation of tethered cells equally. However, a significant number, including all of the suppressors in *fliG* or *fliM*, restored swarming better than they restored rotation of tethered cells. Some of the $motB^+$ strains containing suppressors showed this same tendency; they formed wild-type swarms but as tethered cells had mean rotational velocities only half those of fully wild-type cells.

These data were used to develop a model that postulates that the four suppressible *motB* mutations, three of which affect parts of MotB in or near its putative peptidoglycan-binding site, misalign the MotA/MotB stator complexes relative to the rotor. Suppressing mutations in *motA*, *fliG* or *fliM* are proposed to restore a functional alignment, which is unlikely to be exact. Thus, the realigned motors may perform quite well in the low-load, low-torque, high-speed regime in which they operate in swimming cells, including cells swimming through the agar on swarm plates. However, these imperfectly aligned motors may be deficient in generating the high torque needed to rotate tethered cells, whose motors operate in a high-load, low-speed regime.

Some suppressors increased CW flagellar rotation, although increased CW rotation alone was insufficient to restore motility to the *motB* mutants (Garza *et al.*, 1996a). Several of the suppressors in *fliG* and, notably, one of the suppressors in motA, also increased CW rotation in $motB^+$ cells. The latter is the first report of a mutation in a *mot* gene that influences the directional bias of the flagellar motor. One of the suppressors in *fliG*, which results in the G194S substitution, increased both CW rotation and decreased the frequency of switching from CW to CCW or CCW to CW rotation. We speculate that this mutation creates a stiff hinge that increases the energetic barrier to switching. The location of Gly194 in the flexible linker region of FliG discussed previously is consistent with such an interpretation.

2) Extragenic suppressors of dominant and partially dominant *motA* missense mutations. An extensive search employing 31 plasmid-borne *motA* missense mutations isolated by David

Blair identified a number of intergenic suppressors for four of the mutations (Garza *et al.*, 1996b). All but one of the suppressors attributable to single-residue substitutions replaced residues in MotB. The remaining, very weak, suppressor was in *fliG*.

Although the conclusions based on these data were less robust than those for the *motB*-suppressor analysis, an interesting trend emerged. The suppressors in *motB* clustered primarily in codons 136-138, which specify the amino acids Arg-His-Pro. This chemically distinctive sequence lies in a region of MotB between its single N-terminal membrane-spanning helix (residues 28 to 49) and the proposed peptidoglycan-binding motif (residues 161 to 264). Perhaps this region interacts with the periplasmic face of MotA and helps to mount MotA on MotB, or it may hold MotA in the proper orientation to interact with FliG.

3) Growth defects associated with expression of MotB-PhoA fusions. In our 1993 proposal to ARO we reported that certain suppressors of *motB* mutations caused moderate growth defects when they were present in cells over-expressing wild-type MotB (A. Garza, unpublished data). We attributed this inhibition of growth to proton leaks generated by a partial uncoupling of flagellar rotation from proton flow through an altered Mot-protein complex.

Following up on this possibility, postdoctoral student Andreas Tauer set out to identify conditional-lethal mutations in *motA*. The expectation was that such mutations would create massive proton leaks that would dissipate the transmembrane pmf. He used a vector in which he could generate mutations by random mutagenesis in the absence of gene-product expression and then expose the plasmid-bearing strain to inducing conditions that allow the potentially lethal gene product to be expressed. He also established a protocol for detecting proton leaks. The method involves adding glucose to starved, anaerobic cells suspended in lightly buffered medium and recording the rate of acidification of the medium due to the activity of the membrane F_OF_1 ATPase. If the protons that are pumped out of the cells can leak back in, the rate of acidification should be slower.

No *motA* mutations were discovered that conferred a lethal phenotype, and acidification of the medium was not delayed if wild-type or mutant MotA proteins were over-expressed in the presence of wild-type MotB or in the complete absence of MotB. However, over-expression of wild-type MotA severely impaired growth and delayed acidification of the medium if it occurred in the presence of a highly expressed MotB-PhoA* fusion protein. This result was seen previously by Beat Stolz in the laboratory of Howard Berg, who found that MotB-TetA fusion proteins containing only the first 60 residues of MotB seriously impaired growth of a $motA^+$ strain.

4) Identification of mutations that interfere with the assembly of MotB into the motor. The *motB* mutations used in our search for extragenic suppressors were all dominant over a single wild-type copy of *motB* in the chromosome. Presumably, the mutant proteins compete with wild-type MotB for interaction with other motor components. We chose to use dominant mutations in those experiments because we felt that they would give us the best chance of finding suppressors that alter other motor components to restore a productive interaction with mutant MotB proteins.

* PhoA is *E. coli* alkaline phosphatase, which is active only when it is exported to and dimerizes within the periplasmic space. PhoA is normally made with an cleavable leader peptide to direct its export to the periplasm. In fusions of the type described, the PhoA leader peptide is absent, and export of PhoA to the periplasm depends on export signals of the protein to which it is fused.

It seems likely, however, that most mutations that prevent assembly of MotB into the motor

would be recessive. Thus, we probably were not in a position to select for suppressors that compensate for defects in MotB assembly.

Four regions of the protein are of interest in this respect. The first is the N-terminal cytoplasmic and membrane-spanning region of MotB (residues 1-49), which confers a growth defect when hooked up to the TetA protein. This part of MotB may be interacting directly with MotA within the membrane to form the proton channel. Second, our suppressor studies (Garza *et al.*, 1996b) identified a region around residues 136-138 in the periplasmic portion of MotB that seem to be important for interaction with the periplasmic face of MotA. Third, the residues from position 161 to 264 show homology to peptidoglycan-binding proteins and have been proposed to anchor MotB to the cell wall. Finally, we speculate that the region from residues 265 to the end of the protein may be important for positioning MotB around the flagellar basal body.

As a first step in this analysis we have generated a series of site-directed amber (TAG) mutations in plasmid-borne *motB* genes under *tac* promoter control. The mutations have been introduced in parallel into a plasmid containing only the *motB* gene (maintained in the MM5003 strain, which is deleted for *motB* on the chromosome) and both the *motA* and *motB* genes (maintained in the MM5003 strain, which is deleted for the tandem *motA* and *motB* genes on the chromosome). Our choice of sites for amber mutations was dictated by the occurrence of codons for amino acids that can be inserted by amber-suppressor tRNAs (glutamine [Q] by *supE/glnV* [30% efficiency], tyrosine [Y] by *supF/tyrT* [70% efficiency], and serine [S] by *supH/serU* [15% efficiency]). Within the 308 residues of MotB we have thus far replaced codons for Q4, S18, S261, Q268, Q271, Q281, S286, Q296, and S304 with TAG. Mutations at all of the sites through codon 286 produce a completely non-motile phenotype in at least one plasmid and are suppressible to some degree by *supE* and *supF* (we have not yet tested *supH*). The amber mutation at codon 296 is associated with a reduced motility phenotype, whereas the amber mutation at codon 304 has no obvious phenotype. The most 3' nonsense mutation identified by David Blair converted codon 211 of *motB* (TGG [Trp]) to TAG. We do not know why he did not find nonsense mutations later in the gene, but our results substantially extend the portion of MotB that is demonstrably required for function.

All of the mutations displaying an impaired-motility phenotype are recessive in a $motA^+$ $motB^+$ strain, a result which is implicit in their ability to be suppressed by the 30% efficient *supE* allele. These results indicate that we can introduce the desired amber codons (this has been or is being confirmed by DNA sequencing) and demonstrate that all but the most extreme C-terminal tail of MotB is important for its function. Thus, it should be possible to identify recessive missense mutations in the region of *motB* following codon 264 and look for intergenic suppressors of these mutations. The recent availability of MotB antibody from David Blair's laboratory will be a very useful diagnostic tool for determination of levels of mutant and suppressed-mutant proteins.

To check the function of the other regions of MotB described above, we are addressing specific questions by introducing amber mutations at the codons indicated in the following outline. **a)** To test whether the membrane-spanning region alone interferes with growth or has a dominant-negative phenotype for motility, substitute codons for residues S51, Q57, and/or Y61. **b)** To determine the role of the Arg-Pro-His sequence at residues 136-138, substitute codons for the flanking Q124 and/or S128 and Q145 and/or Q156 residues. **c)** To dissect the putative peptidoglycan-binding motif, replace codons for residues S165, Y171, S190, Y201, Y208, S214,

S221, S232, and S248.

5) Mutational analysis of the flexible hinge connecting the two structural domains of FliG. The Blair laboratory has provided compelling evidence that FliG is the switch/motor protein most intimately involved in motility. Their work indicates that FliG contains two distinct structural regions; an N-terminal domain required for flagellar assembly and a C-terminal domain dedicated to flagellar rotation. Preliminary analysis of the secondary structure of FliG predicts that these two domains of FliG are connected by a disordered, flexible sequence that may serve as a hinge. This topology would allow the motility domain to move relative to the assembly domain, which is probably firmly secured to the M-ring. Such movement may be central to switching the direction of flagellar rotation.

A number of suppressors of *motB* mutations localize to the segment of *fliG* that encodes the putative hinge (Garza *et al.*, 1996a). We therefore predict that the hinge plays an important role in aligning the motility domain of FliG properly with the Mot-protein complex. We have targeted random, oligonucleotide-directed mutagenesis to the putative hinge region of *fliG*. We anticipated finding mutations that cause an array of different phenotypes, including: **a)** loss of motility; **b)** reduced motility; **c)** increased CW or CCW bias of flagellar rotation; **d)** elevated or reduced frequencies of motor reversal. We have found many missense mutations affecting the hinge region that moderately to severely impair the ability of colonies to swarm in soft agar. Although the results are still preliminary, different motility phenotypes have been observed and a number of the mutations have been identified by DNA sequence analysis. The most common phenotype is an increasingly CW-biased motility. The next most frequent is the loss or reduction in swimming velocity and/or rotational velocity of tethered cells.

The full value of mutations affecting stator and rotor components will be realized only when the structures of their component proteins are determined. However, some inferences may be drawn even without such information. A misalignment of the stator with the rotor might be revealed in the behavior of swimming or tethered cells. We suggested, on the basis of our suppression analysis (Garza *et al.*, 1996a), that mutations that distort the MotB anchor or the FliG hinge may position the motility domain of FliG too close to or too far away from the stator for optimal motor function. Mutations of the first type should lead to a partially or completely frozen motor that is more resistant to being forced to rotate by an externally applied torque than is a wild-type motor. Mutations of the second type should lead to an overly loose motor that is deficient in generating the high torque required to turn tethered cells and that should be easier to turn with externally applied torque than a wild-type motor. These experiments will be carried out with suitable mutant and suppressed-mutant cells in collaboration with Howard Berg, who has experience with this technique and whose laboratory is outfitted to conduct such measurements.

PEER-REVIEWED PUBLICATIONS ARISING FROM THE PROJECT (listed chronologically)

1) Garza, A.G., L.W. Harris-Haller, R.A. Stoebner, M.D. Manson (1995) Motility protein interactions in the bacterial flagellar motor. Proc. Natl. Acad. Sci. USA **92:** 1970-1975.
2) Garza, A.G., R. Biran, J.A. Wohlschlegel, M.D. Manson (1996a) Mutations in *motB* suppressible by changes in stator or rotor components of the bacterial flagellar motor. J. Mol. Biol. **258:** 270-285.
3) Garza, A.G., P.A. Bronstein, P.A. Valdez, L.W. Harris-Haller, M.D. Manson (1996b) J. Bacteriol. **178:** 6116-6122.

LIST OF PARTICIPATING SCIENTIFIC PERSONNEL (alphabetical by last name)

Tara Anderson (graduate student at Texas A&M, started on project 1/97, dropped out of program 12/97)
Roy Biran (Texas A&M undergraduate, earned B.S., currently in Ph.D. program at University of Utah, Salt Lake City)
Philip A. Bronstein (Texas A&M undergraduate, earned B.S. degree, currently in Ph.D. program at University of Washington, Seattle)
Anthony G. Garza (graduate student, earned Ph.D. in Microbiology from Texas A&M University in 1995, thesis based on work supported by this project grant, currently postdoctoral fellow at University of California, Davis)
Edan Hosking (Ph. D. student, started working on project and supported by grant since 1/98)
Aaron Lee (Texas A&M undergraduate, started working on project 9/98 and is still involved)
Michael D. Manson (Texas A&M Professor and P.I.)
Stefano Millas (Texas A&M undergraduate - worked on project through 7/98)
Andy Tauer (postdoctoral student, Ph.D. from the ETH in Zürich, Switzerland - on project from 9/94 through 8/95)
Susan Van Way (postdoctoral student, Ph.D. from Tulane University- on project from 9/95 through present and continuing for the foreseeable future)
Patricia A. Valdez (Texas A&M undergraduate, earned B.S. degree, currently in Ph.D. program at the University of California, Berkeley)
Jimmy A. Wohlschlegel (Texas A&M undergraduate, earned B.S. degree, currently in Ph.D. program at Harvard University Medical School)

INVENTIONS

None

APPENDICES

Reprints or photocopies of three published articles.