JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6116–6122 Vol. 178, No. 21
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Extragenic Suppression of *motA* Missense Mutations of *Escherichia coli*

ANTHONY G. GARZA,[1]† PHILIP A. BRONSTEIN,[1]‡ PATRICIA A. VALDEZ,[1]§
LARRY W. HARRIS-HALLER,[1,2] AND MICHAEL D. MANSON[1]*

Department of Biology, Texas A&M University, College Station, Texas 77843-3258,[1] and Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Texas A&M University, College Station, Texas 77843-3155[2]

Received 11 June 1996/Accepted 27 August 1996

The MotA and MotB proteins are thought to comprise elements of the stator component of the flagellar motor of *Escherichia coli*. In an effort to understand interactions among proteins within the motor, we attempted to identify extragenic suppressors of 31 dominant, plasmid-borne alleles of *motA*. Strains containing these mutations were either nonmotile or had severely impaired motility. Four of the mutants yielded extragenic suppressors mapping to the FlaII or FlaIIIB regions of the chromosome. Two types of suppression were observed. Suppression of one type (class I) probably results from increased expression of the chromosomal *motB* gene due to relief of polarity. Class I suppressors were partial deletions of Mu insertion sequences in the disrupted chromosomal *motA* gene. Class I suppression was mimicked by expressing the wild-type MotB protein from a second, compatible plasmid. Suppression of the other type (class II) was weaker, and it was not mimicked by overproduction of wild-type MotB protein. Class II suppressors were point mutations in the chromosomal *motB* or *fliG* genes. Among 14 independent class II suppressors characterized by DNA sequencing, we identified six different amino acid substitutions in MotB and one substitution in FliG. A number of the strongest class II suppressors had alterations of residues 136 to 138 of MotB. This particular region within the large, C-terminal periplasmic domain of MotB has previously not been associated with a specific function. We suggest that residues 136 to 138 of MotB may interact directly with the periplasmic face of MotA or help position the N-terminal membrane-spanning helix of MotB properly to interact with the membrane-spanning helices of the MotA proton channel.

The gram-negative enteric bacterium *Escherichia coli* swims by rotating its flagella (2, 48). The flagellar structure was originally isolated as a basal body of four rings stacked on a rod, a flexible coupling called a hook, and a left-handed helical filament that serves as a propeller (16, 17). Recent electron microscopy studies identified additional structures attached to the cytoplasmic face of the basal body (18–20, 27, 29). Khan et al. (27) and Francis et al. (20) have presented evidence that these structures contain the three proteins of the switch-motor complex, FliG, FliM, and FliN (57, 58). Two other proteins involved in generating rotation, MotA and MotB, were not isolated with purified flagella. These proteins seem to be distributed in the membrane around the periphery of the M-ring (28). A number of comprehensive reviews of flagellar structure and function have been published recently (1, 4, 5, 11, 26, 32–34, 36, 44, 45).

Each flagellum is powered by a bidirectional motor located at its base. The energy that drives flagellar rotation is provided by the proton motive force (24, 30, 37, 38). The mechanism by which the proton motive force is converted into rotation is unknown.

Lesions in most flagellar genes affect motility by causing defects in flagellar assembly. Mutations in five genes (*motA*, *motB*, *fliG*, *fliM*, and *fliN*) can cause cells to assemble a nonfunctional flagellum. The role of MotA and MotB in generating motor torque has been demonstrated directly. A strain lacking one or both of the Mot proteins produces normal-looking but paralyzed flagella. A cell tethered by such a flagellum begins to rotate when expression of the missing protein is induced from a plasmid-borne gene under *lac* promoter control (10). The speed of rotation increases in a series of eight equal steps (6), suggesting that there are eight independent force-generating units per flagellar motor. Certain alleles of the switch-motor genes produce strains that are nonflagellate, nonmotile, or nonchemotactic (58). This observation indicates that FliG, FliM, and FliN are necessary for flagellar assembly and that they also participate in flagellar torque generation and switching.

FliG and FliN appear to be more involved in torque generation than FliM is (25, 49). A recent study demonstrated that only FliG has a direct role in flagellar rotation (31), with the C-terminal domain of the protein being dedicated to this function. Two studies have suggested that FliN may also play a role in flagellar export (52, 53). FliM appears to be dedicated primarily to changing the direction of flagellar rotation (49), and in that capacity it may serve as the docking site for phosphorylated CheY protein on the switch-motor complex (54).

MotA and MotB fractionate with the cytoplasmic membrane (41). MotB is predicted to have one N-terminal membrane-spanning segment, with the bulk of the protein extending into the periplasmic space (13, 50). On the basis of this topology, Chun and Parkinson (13) suggested that MotB anchors MotA to the peptidoglycan layer of the cell wall. The recent discovery of a putative peptidoglycan-binding region within the C-termi-

* Corresponding author. Phone: (409) 845-5158. Fax: (409) 845-2891. Electronic mail address (Internet): MIKE@BIOL.TAMU.EDU.

† Present address: Section of Microbiology, University of California, Davis, CA 95616.

‡ Present address: Department of Microbiology, University of Washington, Seattle, WA 98195.

§ Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and phenotype	Source or reference
Strains		
AG70	RP6666 *uvrC279*::Tn*10*; nonmotile Tet^r	This study
DFB225	RP437 Δ*fliG*; nonflagellate	31
RP437	F$^-$ *thr-1*(Am) *leuB6 his-4 metF159*(Am) *eda-50 thi-1 rpsL136 ara-14 lacY1 mtl-1 xyl-1 tonA31 tsx-78*; wild-type motility and chemotaxis	40
RP6647	RP437 Δ*motB* (bp 359 to 781); nonmotile	12
RP6666	RP437 Δ*motA* (bp 366 to 846); nonmotile	12
Plasmids		
pBR322 derivatives		
pDFB36	P*lac-motA*$^+$ *lacI*^{q} Amp^r	8
pMA	P*lac-motA lacI*^{q} Amp^r	8
pACYC184 derivatives		
pRB1	P *tac lacI*^{q} Cam^r	This study
pRB2	pRB1 P*tac-motB*$^+$ *lacI*^{q} Cam^r	This study
pRB3	pRB1 P*tac-fliG*$^+$ *lacI*^{q} Cam^r	This study
pSYC62	P *mocha-motB*$^+$ Cam^r	13

nal periplasmic portion of MotB (15) is consistent with this proposal.

On the basis of its amino acid sequence, MotA was predicted to have four membrane-spanning helices, two short periplasmic loops, and two larger cytoplasmic domains (14). This topology has now been confirmed experimentally (8, 59). Characterization of a set of *motA* missense mutants indicated that MotA forms a channel that conducts hydrogen ions (or H_3O^+ ions) across the cytoplasmic membrane (7, 8).

Stoltz and Berg (51) reported that a fusion polypeptide linking 60 amino acids at the N terminus of MotB to a C-terminal portion of the TetA protein impaired cell growth when it was co-overexpressed with MotA. They hypothesized that the growth defect was caused by proton leakage and that the fragment of MotB activates MotA as an ion channel. Also, when MotB is overproduced, it is unstable unless MotA is overexpressed as well (56). Finally, four of the *motB* missense mutations described by Blair et al. (9) gave rise to extragenic suppressors exhibiting some degree of allele specificity (22, 23). Most of these suppressors were in *motA*, but a few were in *fliG*, and one was in *fliM*. Thus, there is strong evidence that MotA and MotB interact.

We now report the isolation of extragenic suppressors for 4 of 31 *motA* missense mutations described by Blair and Berg (7, 8). The phenotypes and allele specificities of the suppressed mutants were determined, and 18 of the suppressors were identified by DNA sequencing. This analysis led us to conclude that suppression of these *motA* mutations can occur in two different ways.

MATERIALS AND METHODS

Media. Tryptone broth contained 1% tryptone (Difco) and 0.8% NaCl. Motility medium contained 10 mM potassium phosphate, 0.1 M NaCl, 10^{-4} M EDTA, 10^{-5} M L-methionine, 10 mM sodium lactate, and 25 μg of chloramphenicol per ml. LB plates contained 1% tryptone, 0.5% yeast extract (Difco), 0.5% NaCl, 20 mM sodium citrate, and 1.5% agar (39). Tryptone semisoft agar swarm plates contained 1% tryptone, 0.8% NaCl, 20 mM sodium citrate, and 0.35% Difco Bacto Agar. Miniswarm plates were like tryptone swarm plates, except that transformed cells were added to the molten agar at 50°C before it was poured. Media contained 5 μg of tetracycline, 25 μg of chloramphenicol, or 50 μg of ampicillin per ml, as needed. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM to induce plasmid-borne *fliG*, *motA*, or *motB* genes under *lac* or *tac* promoter control.

Bacterial strains and plasmids. The complete list of *E. coli* strains and plasmids used in this study is shown in Table 1. Strain RP437 is wild type for motility and chemotaxis (40). Strains RP6666 and RP6647 are derivatives of RP437 that contain internal deletions within the *motA* and *motB* genes, respectively. In the course of this study, we discovered that these strains are actually deletion-insertions. Base pairs 366 to 846 of *motA* and 359 to 781 of *motB* in the respective strains are replaced with the same 104 bp of phage Mu DNA, presumably because of the way the deletions were constructed from Mud*lacZY* fusions (12). Strain DFB225 contains an in-frame deletion of *fliG* (31). Strain AG70 (*motB*$^+$ Δ*motA*) was constructed by transducing a Tn*10* transposon (Tet^r) inserted into the *uvrC* gene (*uvrC279*::Tn*10*) into strain RP6666 and screening for retention of the nonmotile phenotype.

Plasmid pDFB36 (7) contains the *motA*$^+$ gene expressed from the *lacUV5* promoter. The pMA plasmids (derived from pDFB36) carry *motA* missense mutations (7, 8). The pRB plasmids were derived from pACYC184 and are compatible with the plasmids containing the *motA* alleles (pBR322 derivatives). Plasmids pACYC184 and pJF118HE (21) were digested with *Bam*HI and *Nru*I, yielding 3,647- and 1,300-bp fragments, respectively. The fragments were gel purified, ligated, and used to transform strain RP437. Plasmid minipreps were made from Cam^r Tet^s transformants (35); purified DNA was digested with appropriate endonucleases to confirm its composition. The 4,947-bp plasmid was named pRB1.

Plasmid pRB1 and a PCR fragment containing the *motB*$^+$ gene from strain RP437 were digested with *Bam*HI and *Sal*I. The fragments were gel purified and ligated. Strain RP6647 (Δ*motB*) was transformed with the ligated DNA, and transformed cells were used to pour mini-swarm plates containing chloramphenicol and IPTG. Plasmid minipreps were made from motile, Cam^r colonies; purified DNA was digested with appropriate endonucleases to confirm their composition. The 5,948-bp plasmid containing *motB*$^+$ under control of the *tac* promoter was called pRB2.

Plasmid pRB3 was constructed by inserting a PCR fragment containing the *fliG*$^+$ gene from strain RP437 into the multicloning site (*Bam*HI-*Sal*I) of pRB1 and checking for the ability to restore motility to strain DFB225 (Δ*fliG*). The 5,977-bp plasmid containing the *fliG*$^+$ gene under control of the *tac* promoter was called pRB3.

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 diluted 100-fold into 10 ml of the same medium and grown for 3 to 4 h at 30°C in 125-ml flasks with vigorous swirling (final optical density at 590 nm, 0.8). Motility was examined by phase-contrast microscopy. Swarm behavior was assayed by picking fresh overnight colonies into tryptone semisoft agar and scoring the swarm diameters after 10 h of incubation at 30°C. Twenty swarms were measured for each strain, and their mean diameter was normalized to the mean diameter of 20 swarms formed by strain AG70 containing plasmid pDFB36 (*motA*$^+$). Motility was quantified as follows: ++++, >95% of that of wild type; +++, 46 to 95% of that of wild type; ++, 31 to 45% of that of wild type; +, 16 to 30% of that of wild type; ±, 5 to 15% of that of wild type; −, <5% of that of wild type.

Isolation of *motA* suppressors. The strategy for the isolation of suppressors is outlined in Fig. 1A. Mutagenesis with ethyl methanesulfonate was done as described by Miller (39). Mutagenized cells were transformed with pMA plasmids (*motA*) by the calcium-rubidium chloride method (35). Transformed cells were poured in mini-swarm plates containing IPTG, ampicillin, and tetracycline, with an amount of the transformation mix that yielded several thousand colonies per plate. After incubation overnight at 30°C, pseudorevertants appeared as swarms of various sizes among the nonmotile colonies. Between 50,000 and 100,000 transformants were screened with each pMA plasmid. Potential pseudorevertants were identified as swarming colonies; cells from these colonies were isolated and retested for their swarm phenotypes.

Mapping of *motA* suppressors. Phage P1*vir* lysates made on pseudorevertants were used to determine linkage of the suppressors to *uvrC* (Fig. 1B and 2). To ensure that the suppressing mutation required the parental pMA plasmid to restore motility, lysates were also used to transduce plasmid-free strain RP6666

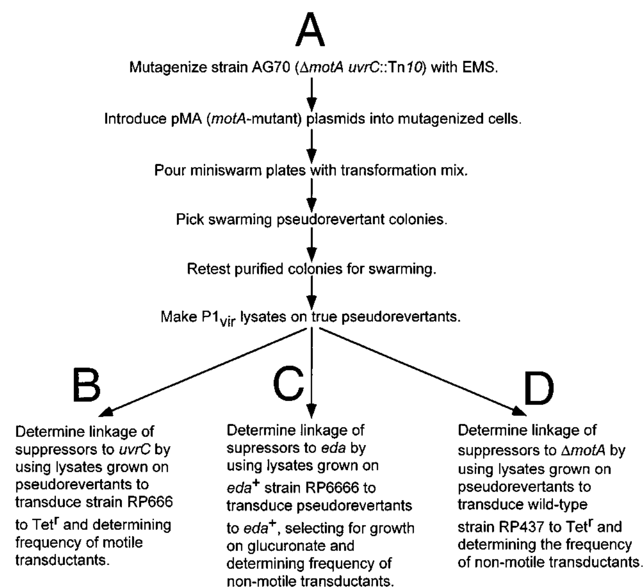

FIG. 1. Outline of scheme for isolation and mapping of *motA* suppressors. (A) Mutagenesis and screening for pseudorevertants; (B) establishing linkage of suppressors to *uvrC*; (C) establishing linkage of suppressors to *eda*; (D) establishing linkage of suppressors to Δ*motA*. See Materials and Methods for details. EMS, ethyl methanesulfonate.

TABLE 2. Suppressor yield with *motA* missense mutations[a]

motA allele and amino acid substitution in MotA	No. of suppressors linked to *uvrC*
Dominant, partially functional mutations[b]	
G23-E	0
E33-K	1
P173-L	2
G176-S	0
V207-M	14
Dominant, nonfunctional mutations[b]	
G6-D, G6-N, G6-S	0
G12-D	0
T131-I	0
G17-D	0
G39-A	0
A40-V	0
S44-L	0
A53-V	0
G169-E, G169-R	0
S171-L	0
A174-V	0
A203-T, A203-V	0
A205-T, A205-V	0
G212-S	1
G212-D	0
A216-V	0
P222-L, P222-S	0
E259-K	0
R262-H	0

[a] Suppressors were identified on tryptone mini-swarm plates after ethyl methanesulfonate mutagenesis and characterized as described in the text.

[b] Missense mutations (8) are given by the amino acid substitution they cause in the *motA* gene product. If more than one substitution was found at a residue, the alternatives are separated by commas within one entry, with the exception of G212, in which the two substitutions are given as separate entries. Alleles were categorized for function and dominance by the method of Blair and Berg (8). We scored the mutations as described in Materials and Methods. All *motA* alleles classified as nonfunctional produced a − phenotype, and all *motA* alleles classified as partially functional produced a ± motility phenotype except the one generating the P173-L substitution, which led to a negative motility phenotype in our hands.

to Tet^r, and the transductants were tested on tryptone semisoft agar to confirm that they were unable to swarm. Cotransduction frequencies of the suppressors with the *eda* locus (Fig. 1C and 2) were determined by using a phage P1*vir* lysate made from an Eda$^+$ derivative of strain RP6666 to transduce motile pseudorevertants to *eda*$^+$ (growth on glucuronate) and then screening the transductants for their swarm phenotypes. To determine linkage of the suppressors to the *motA* deletion (Fig. 1D and 2), P1*vir* lysates from motile pseudorevertants were used to transduce the wild-type strain RP437 containing the parental pMA plasmid to Tet^r, and the transductants were tested for their swarm phenotypes. A low frequency of nonmotile transductants indicates tight linkage between the suppressor and the deletion, since only introduction of the *motA* deletion without the suppressor should give rise to a nonmotile phenotype.

Allele specificity of the suppressors. The suppressors were introduced with P1*vir* into transformants of strain RP6666 containing pDFB36 (*motA*$^+$) or pMA plasmids. Transductants were tested in tryptone swarm plates containing IPTG, tetracycline, and ampicillin to determine whether the suppressor restored motility in combination with the *motA* missense mutations or adversely affected the motility of *motA*$^+$ cells.

Amplification and sequencing of DNA and determination of RNA secondary structure. DNA fragments containing entire genes were amplified for sequencing by the PCR. Primers used in the amplification reaction flanked the 5′ and 3′ ends of the gene. PCRs were run as described by Saiki et al. (42); amplification proceeded for 30 cycles in a Perkin-Elmer Cetus thermal cycler. DNA was sequenced by the dideoxynucleotide chain termination method (43) by using the SequiTherm procedure provided by the manufacturer (Epicentre Technologies, Madison, Wis.). Predictions of RNA structures and the free energies associated with them were performed with the FOLD program of the Wisconsin Genetics Computer Group.

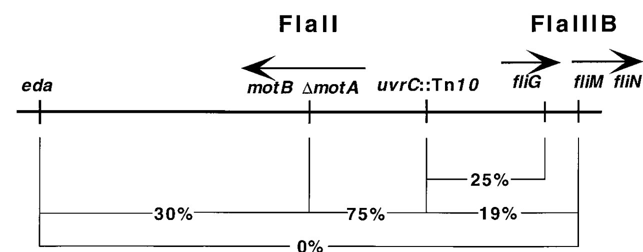

FIG. 2. Linkage of *mot* and *fli* genes to *uvrC* and *eda*. Experimentally determined cotransduction frequencies with phage P1*vir* between *uvrC* or *eda* and reference loci are indicated at the bottom of the figure. Arrows indicate directions of transcription. The approximate map positions of loci on the *E. coli* chromosome are 41.6 min for *eda*, 42.6 min for *motB*, 43.0 min for *uvrC*, 43.4 min for *fliG*, and 43.6 min for *fliM* (3). The map is drawn to scale.

RESULTS

Rationale for identifying suppressors linked to *uvrC*. Five proteins (FliG, FliM, FliN, MotA, and MotB) have been implicated in energizing flagellar rotation. The *motA* gene immediately precedes *motB* 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 FlaIIIB region. The *uvrC* locus is cotransducible by phage P1*vir* with both FlaII and FlaIIIB, whereas the *eda* locus is cotransducible only with the FlaII region. The relative map positions of the loci are given in Fig. 2. Our efforts to identify proteins that interact with MotA focused on suppressors linked to *uvrC*, since a majority of the suppressors of *motB* missense mutations (22, 23) were located in the FlaII or FlaIIIB region.

Properties of parental *motA* mutations. The plasmid-borne *motA* missense mutations listed in Table 2 were identified by Blair and Berg (7, 8). They were classified by the motility

TABLE 3. Strength and allele specificity of suppressors

Strain[a]	Motility[b]						
	motA$^+$	*motA* mutants					
		E33-K	P173-L	G176-S	A203-T	V207-M	G212-S
Wild type (*motB*$^+$ *fliG*$^+$)	++++	±	−	±	−	±	−
Class I suppressors[c]							
Δ[−4 to +96] (1)	++++	++	[++]	+++	±	+++	+
Δ[+29 to +96] + 4 T residues (3)	++++	[++]	[+]	++	−	[+++]	±
Class II suppressors[d]							
In *motB*							
V43-M (1)	++++	±	−	±	−	[++]	−
R136-C (2)	++++	+	−	±	−	[+++]	±
P137-L (2)	++++	+	±	±	−	[++]	±
H138-Y (4)	++++	±	−	±	−	[+++]	±
R173-C (2)	++++	±	−	±	−	[++]	[±]
A180-V (2)	++++	±	−	±	−	[++]	±
In *fliG*							
D96-N (1)	++++	±	−	±	−	[+]	−

[a] To test allele specificity, suppressors were transduced into strain RP6666 containing plasmids with the indicated *motA* alleles. The number of independent isolates of each suppressor is shown in parentheses. Motility was scored on tryptone swarm plates as described in Materials and Methods.

[b] Brackets surround the motility scores for the mutation-suppressor pair in the original suppressed mutants.

[c] The deletions in class I suppressors are designated by the amount they remove of the 104-nucleotide Mu sequence inserted into *motA*. Thus, Δ[−4 to +96] means that four bases of the *motA* sequence preceding the Mu insertion and 96 bases of the Mu sequence were removed; Δ[+29 to +96] plus 4 T means that nucleotides 29 through 96 of the Mu insertion were deleted and that 4 T residues of unknown origin were inserted in their place.

[d] Class II suppressors all correspond to single-base-pair substitutions in *motB* or *fliG* that result in the amino acid changes shown.

phenotypes they generated when mutant cells were tested on tryptone swarm plates, i.e., nonmotile or severely impaired for motility. The *motA* alleles displayed various degrees of dominance in a strain containing a single chromosomal *motA*$^+$ gene, suggesting that the mutant proteins compete with wild-type MotA for incorporation into the flagellar motor or that the mutant MotA proteins sequester other motor components, such as MotB.

Isolation of suppressors. Strain AG70 (Δ*motA uvrC279*::Tn*10*) was used to isolate *motA* suppressors. Only 22 potential pseudorevertants produced larger swarms than their parental strains when retested on tryptone swarm plates. Eighteen isolates contained suppressors that were linked to *uvrC* by cotransduction with phage P1*vir* (Fig. 2). These suppressors restored motility only in the presence of a pMA plasmid, showing that they did not bypass the need for MotA.

Mapping of suppressors. Seventeen of the 18 suppressors were 70% cotransducible with *uvrC*::Tn*10*. This frequency is similar to the 75% linkage found between *uvrC* and *motB* (Fig. 2). All of these suppressors were tightly linked to the *motA* deletion. One suppressor did not map near the *motA* deletion, was not cotransducible with the *eda* locus (Fig. 2), and was 15 to 30% cotransducible with *uvrC*::Tn*10*. Since *uvrC*::Tn*10* had similar cotransduction frequencies with known *fliG*, *fliM*, and *fliN* mutations (Fig. 2), we concluded that this suppressor is in the FlaIIIB region.

Properties of the suppressed mutants. Four of 31 *motA* missense mutations (Table 2) yielded suppressors linked to *uvrC*. The mean diameters of swarms made by the suppressed mutants and unmutagenized strain AG70 containing pDFB36 (*motA*$^+$) or the parental pMA plasmids were compared (Table 3). The level of suppression ranged from very weak (±) for the suppressor of a *motA* mutant with G substituted by S at position 212 (G212-S) to quite strong (+++) for some suppressors of a *motA* mutant with a V207-M substitution. The motility of free-swimming cells was also observed by phase-contrast microscopy. The swimming ability of each strain, estimated from the percentage of swimming cells in the culture and the speed of swimming (data not shown), corresponded qualitatively to its swarming ability.

Allele specificity of suppressors. Plasmids containing mutant or wild-type *motA* alleles were introduced into strain RP6666 (Δ*motA*), and the transformants were used as transduction recipients for the suppressor alleles. The motility of the transductants was tested on tryptone swarm plates and confirmed by phase-contrast microscopy of swimming cells. The results of the swarm plate assays are summarized in Table 3. None of the suppressors impaired the motility of a *motA*$^+$ strain. Their only obvious phenotype was to restore motility to some *motA* mutants.

The suppressors fell into two classes. The four class I suppressors produced a graded series of motility restoration with six of the *motA* mutations, including the four that yielded suppressors in the original search. The relative extent of motility restoration by the class I suppressors with different *motA* mutations was the same, demonstrating that the suppression was not allele specific (Table 3). The fourteen class II suppressors displayed different relative strengths of motility restoration with different *motA* mutations, and most of them restored motility only with a subset of the four *motA* mutations that originally yielded suppressors. No class I or class II suppressor improved the motility of the remaining 25 *motA* mutants.

Sequence localization of suppressors in the FlaII and FlaIIIB regions. The *motB* gene from strains containing the 17 suppressors with 70% linkage to *uvrC* was sequenced. No mutations were found in *motB* from the four strains containing class I suppressors. Instead, these strains contain one of two deletions (Table 3) that remove most of the 104-nucleotide insertion of phage Mu-derived sequence (24a) that replaces nucleotides 366 to 846 of *motA* in strain RP6666.

Thirteen class II suppressors generated single amino acid substitutions in MotB. Six different substitutions were identified (Table 3). Since seven of eight FlaIIIB region suppressors of *motB* mutations identified in another study were in *fliG* (22, 23), we sequenced that gene first for the class II suppressor

TABLE 4. Effect of MotB overproduction on motility of *motA* mutants

Plasmid or suppressor	Motility[a]						
	motA⁺	*motA* mutants					
		E33-K	P173-L	G176-S	A203-T	V207-M	G212-S
Plasmid							
pRB1 (vector)	++++	±	−	±	−	±	−
pRB3 (P *tac-fliG*⁺)	++++	±	−	±	−	±	−
pRB2 (P *tac-motB*⁺)	++++	+++	++	+++	+	+++	+
pSYC62 (P *mocha-motB*⁺)	++++	++	++	+++	±	+++	±
Class I suppressors[b]							
Δ[−4 to +96]	++++	++	++	+++	±	+++	+
Δ[+29 to +96] + 4 T residues	++++	++	+	++	−	+++	±

[a] Motility was scored on tryptone swarm plates as described in Materials and Methods.
[b] The data for the class I suppressors (Mu sequence deletions) are taken from Table 3 and are shown for comparison.

that mapped to the FlaIIIB region. A single mutation, encoding the D96-N substitution, was found.

Overproduction of MotB, but not FliG, rescues motility in *motA* mutants suppressible by class I suppressors. To test whether increased production of MotB could explain how class I suppressors work, we introduced a compatible plasmid (pRB2) carrying the $motB^+$ gene under control of the *tac* promoter into transformants of strain RP6666 carrying each of the six plasmid-borne suppressible *motA* alleles. All of these doubly transformed strains showed improved swarming in tryptone semisoft agar containing 1 mM IPTG. The motility restoration patterns (Table 4) resembled those observed for class I suppressors. In a second test, a compatible plasmid (pSYC62) carrying the $motB^+$ gene under control of its native *mocha* promoter (13) was introduced into strain RP6666 carrying the same six plasmid-borne *motA* mutations. The pattern of motility restoration (Table 4) was similar to that conferred by plasmid pRB2, although less pronounced. Presumably, the levels of suppression reflect different levels of transcription of the $motB^+$ gene from the *tac* and *mocha* promoters, respectively. Overproduction of FliG from plasmid pRB3 did not improve motility with any of the six suppressible *motA* mutations, including the mutation that gave rise to the suppressor in *fliG*.

DISCUSSION

Four of 31 plasmid-borne *motA* mutations examined yielded extragenic suppressors in the FlaII or FlaIIIB region (Table 2). The suppressors fell into two distinct classes. The four class I suppressors corresponded to two different deletions within the phage Mu-derived sequence inserted into the disrupted chromosomal *motA* gene of the RP6666 strain used during isolation of the suppressors. The 14 class II suppressors were single point mutations in *motB* (13 examples) or *fliG* (1 example), each of which generates a single amino acid substitution.

The class I suppressors rescued motility with all four *motA* mutations that originally gave rise to extragenic suppressors plus two additional *motA* mutations (Table 3). The pattern of suppression was similar for both deletions, and it was also similar to the pattern of enhanced motility observed when plasmid-borne $motB^+$ alleles were introduced into these six *motA* mutants (Table 4). Expression of the $motB^+$ gene from the induced *tac* promoter on plasmid pRB2 restored motility better than expression of the $motB^+$ gene from the *mocha* promoter of plasmid pSYC62, presumably because more MotB was made from P *tac.*

These findings suggest that class I suppression is due to increased expression of chromosomally encoded MotB. The transcribed Mu sequence can form an RNA hairpin loop with a perfectly matched 48-bp stem, which should be very stable (−82.6 kcal/mol [ca. −346 kJ/mol]) and could be strongly polar on *motB* transcription or translation. Unfortunately, lack of MotB antiserum prevented us from quantifying MotB levels. Thus, we do not know whether class I suppressors lead to expression of wild-type or of still-higher levels of MotB. The chromosomal *motA* mutation in the strains used to isolate the original plasmid-borne *motA* mutations (7, 8) was not characterized, and it could have residual polarity, so a return to wild-type expression might be sufficient to restore motility. If greater-than-wild-type levels of MotB expression are required for suppression, the combined effects of deleting *motA* and Mu sequences might relieve the natural polarity that keeps the MotA/MotB ratio at 4 to 1 in wild-type cells (55, 56).

Thirteen of 14 independently isolated class II suppressors, causing six different amino acid substitutions, were located in *motB*. Only the V207-M substitution in MotA was strongly suppressed by these *motB* alleles (Table 3). The most generally effective suppressor was the P137-L substitution in MotB, which restored motility to some extent with all four of the *motA* mutations that originally gave rise to suppressors. Two other suppressors affected residues in the same region of MotB (R136-C and H138-Y) and rescued the motility of the V207-M mutant of MotA quite well. The other suppressors in MotB (V43-M, R173-C, and A180-V) were less effective or suppressed fewer *motA* alleles. V43-M and A180-V suppressed only the V207-M mutant.

The specificity patterns of these class II suppressors were not reproduced by increased expression of MotB (Tables 3 and 4). Thus, class II suppression may involve structural changes in MotB that partially compensate for defective MotA proteins. This result complements our earlier finding that a majority of the suppressors of *motB* missense alleles were located in the *motA* gene (22, 23). These genetic suppression studies confirm existing evidence obtained with other methods (28, 47, 51, 56) that shows that the MotA and MotB proteins interact directly.

We have analyzed the sites affected by *motA* mutations and their suppressors in *motB* with respect to the proposed structures of MotA (8, 14, 46, 59) and MotB (13, 47, 50). The mutations that respond to class II suppressors alter residues in three different parts of MotA (Fig. 3). Residue E-33 is in the periplasmic loop that connects the first and second membrane helices. Residue P-173 is in the third membrane-spanning helix, and residues V-207 and G-212 are in the fourth membrane-spanning helix.

Only one suppressor (V43-M) changes in a residue in the N-terminal membrane-spanning segment of MotB. The other

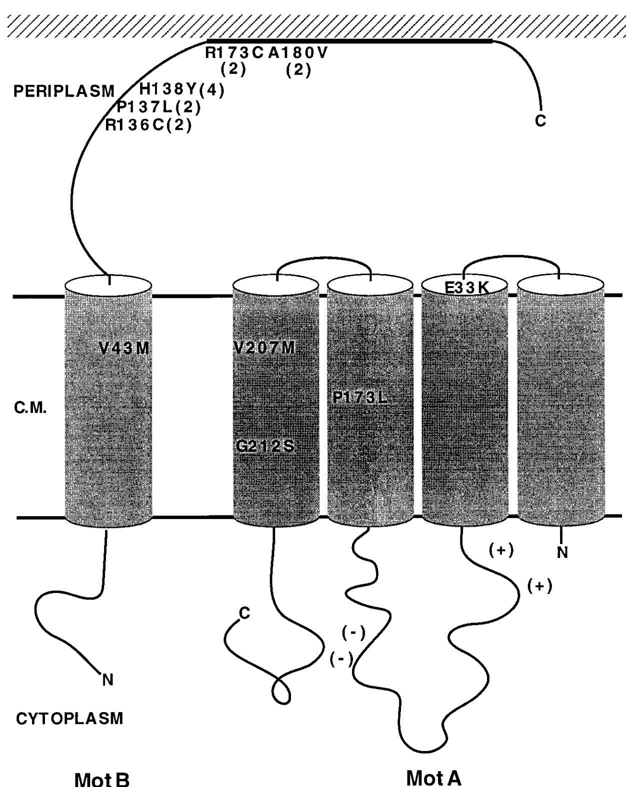

FIG. 3. Locations of amino acid substitutions caused by *motA* mutations and their suppressors in *motB*. Topologies are based on the works of Stader et al. (50) and Chun and Parkinson (13) for MotB and on those of Dean et al. (14), Blair and Berg (8), and Zhou et al. (59) for MotA. C.M., cytoplasmic membrane. The plus and minus signs in the cytoplasmic portion of MotA are included only to indicate that the large cytoplasmic loop of MotA contains many charged residues; their positions were chosen randomly. The extent of the potential peptidoglycan-binding region of MotB (15) is indicated by the broad line that is adjacent to the hatched bar representing the peptidoglycan layer of the cell wall.

five alter residues in the periplasmic region of the protein, including R-173 and A-180 in the putative peptidoglycan-binding region of MotB (15). The R-136, P-137, and H-138 residues of MotB are not in its putative peptidoglycan-binding domain. They could be at the MotA-MotB interface. Suppressors altering MotB residues 136 to 138 may also affect other regions of MotB, such as its single transmembrane helix, that are in contact with MotA. Whatever the function of these residues, their propensity to give rise to suppressors suggests that they may be in a structured region that is important for MotB function.

The V207-M substitution in MotA and the V43-M change in MotB constitute the only mutation-suppressor pair in which the altered residues could touch within the membrane (Fig. 3). V-207 is at the periplasmic end of the fourth membrane-spanning helix of MotA, and V-43 is at the periplasmic end of the MotB transmembrane segment. This situation is the only one we have discovered during extensive second-site suppression analyses (references 22 and 23 and this study) that might represent direct interactional suppression between the MotA and MotB proteins.

No *motA* suppressors were found in *fliM* or *fliN*, and only one very weak suppressor was found in *fliG* (D96-N). Overproduction of wild-type FliG did not mimic the effect of this suppressor. The D-96 residue is in a region of FliG in which there is a cluster of mutations that alter the directional bias of the motor (25). It is more than 10 residues from the most N-terminal site (position 108) affected by *fliG* suppressors of *motB* mutations (22).

We previously found five different suppressors of *motB* mutations in *fliG* and one in *fliM* (22), and Yamaguchi et al. (57), working with *Salmonella typhimurium*, identified a suppressor of a Mot$^-$ *fliG* mutation that mapped to *motB*. Also, the E33-K and G212-S substitutions in MotA suppress a Mot$^-$ *fliG* allele in *S. typhimurium* (34a). A large number of missense motility knockout mutations affect the C terminus of FliG (25, 31), and genetic and electron microscopy studies indicate that FliG localizes to the cytoplasmic face of the innermost ring (the M-ring) of the flagellar basal body (19, 20, 27). Taken together, this information strongly supports the idea that FliG is the component of the switch-motor complex in closest contact with the Mot proteins.

Class I and class II suppression may be explained as follows. Since the mutant MotA proteins examined here confer negative dominance, they probably are not unusually unstable nor have greatly reduced affinity for MotB. They may sequester MotB in complexes that are inefficiently assembled into the motor. The ratio of MotA to MotB in our studies is presumably higher than 4 to 1 (55, 56), since the *motA* alleles are on a multicopy plasmid, and expression from the chromosomal *motB*$^+$ gene may be reduced because of the Mu sequence. Class I suppressors, by making more MotB, may provide enough assembly-competent Mot protein complexes to build a partially functional motor.

The mutations in *motB* and *fliG* that serve as class II suppressors seem unlikely to act by allowing more of the respective gene products to accumulate, although this possibility is not excluded. Furthermore, the pattern of suppression of the suppressors in *motB* is not mimicked by overproduction of wild-type MotB, and overproduction of wild-type FliG does not suppress any of the *motA* mutations. We propose that certain mutant MotA proteins (especially the one with a V207-M substitution) can combine with altered MotB or FliG proteins to restore more productive interactions between the stator and rotor. We draw particular attention to the region around residues 136 to 138 of MotB, which could interact directly with the periplasmic face of MotA or position the N-terminal membrane-spanning helix of MotB to interact with the membrane-spanning helices of MotA.

ACKNOWLEDGMENTS

We thank David Blair for providing the plasmid-borne *motA* mutations. Dana Dixon helped keep us in fresh media and clean glassware. Much of the DNA sequencing was done by Richard A. Stoebner in the Gene Technologies Laboratory of the Institute for Developmental and Molecular Biology at Texas A&M University. Lily Bartoszek gave the manuscript a thorough proofreading before it was submitted.

This work was supported by grant DAAH04-94-G-0056 from the Army Research Office.

REFERENCES

1. **Berg, H. C.** 1995. Torque generation by the flagellar rotary motor. Biophys. J. **68:**163s–167s.
2. **Berg, H. C., and R. A. Anderson.** 1973. Bacteria swim by rotating their flagellar filaments. Nature (London) **245:**380–382.
3. **Berlyn, M. K. B., K. B. Low, and K. E. Rudd.** 1996. Linkage map of *Escherichia coli* K-12, edition 9, p. 1715–1902. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
4. **Blair, D. F.** 1990. The bacterial flagellar motor. Semin. Cell Biol. **1:**75–85.
5. **Blair, D. F.** 1995. How bacteria sense and swim. Annu. Rev. Microbiol. **49:**489–522.
6. **Blair, D. F., and H. C. Berg.** 1988. Restoration of torque in defective flagellar motors. Science **242:**1678–1681.

7. **Blair, D. F., and H. C. Berg.** 1990. The MotA protein of *Escherichia coli* is a proton-conducting component of the flagellar motor. Cell **60:**439–449.
8. **Blair, D. F., and H. C. Berg.** 1991. Mutations in the MotA protein of *Escherichia coli* reveal domains critical for proton conduction. J. Mol. Biol. **221:**1433–1442.
9. **Blair, D. F., D. Y. Kim, and H. C. Berg.** 1991. Mutant MotB proteins in *Escherichia coli*. J. Bacteriol. **173:**4049–4055.
10. **Block, S. M., and H. C. Berg.** 1984. Successive incorporation of force generating units in the bacterial rotary motor. Nature (London) **309:**470–472.
11. **Caplan, S. R., and M. Kara-Ivanov.** 1993. The bacterial flagellar motor. Int. Rev. Cytol. **147:**97–164.
12. **Chun, S.-Y.** 1988. M.S. thesis. University of Utah, Salt Lake City.
13. **Chun, S.-Y., and J. S. Parkinson.** 1988. Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. Science **239:**276–278.
14. **Dean, G. E., R. M. Macnab, J. Stader, P. Matsumura, and C. Burks.** 1984. Gene sequence and predicted amino acid sequence of the *motA* protein, a membrane-associated protein required for flagellar rotation in *Escherichia coli*. J. Bacteriol. **159:**991–999.
15. **DeMot, R., and J. Vanderleyden.** 1994. MicroCorrespondence. Mol. Microbiol. **12:**333–334.
16. **DePamphilis, M. L., and J. Adler.** 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105:**384–395.
17. **Dimmitt, K., and M. Simon.** 1971. Purification and thermal stability of intact *Bacillus subtilis* flagella. J. Bacteriol. **105:**369–375.
18. **Driks, A., and D. J. DeRosier.** 1990. Additional parts associated with the bacterial flagellar basal body. J. Mol. Biol. **211:**669–672.
19. **Francis, N. R., V. M. Irikura, S. Yamaguchi, D. J. DeRosier, and R. M. Macnab.** 1992. Localization of the *Salmonella typhimurium* flagellar switch protein FliG to the cytoplasmic M-ring face of the basal body. Proc. Natl. Acad. Sci. USA **89:**6304–6308.
20. **Francis, N. R., G. E. Sosinsky, D. Thomas, and D. J. DeRosier.** 1994. Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. J. Mol. Biol. **235:**1261–1270.
21. **Furste, J. P., W. Pansegrau, R. Frank, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
22. **Garza, A. G., R. Biran, J. A. Wohlschlegel, and M. D. Manson.** 1996. Mutations in *motB* suppressible by changes in stator or rotor components of the bacterial flagellar motor. J. Mol. Biol. **258:**270–285.
23. **Garza, A. G., L. W. Harris-Haller, R. A. Stoebner, and M. D. Manson.** 1995. Motility protein interactions in the bacterial flagellar motor. Proc. Natl. Acad. Sci. USA **92:**1970–1975.
24. **Glagolev, A. N., and V. P. Skulachev.** 1978. The proton pump is a molecular engine of motile bacteria. Nature (London) **272:**280–282.
24a. **Harris-Haller, L.** Unpublished data.
25. **Irikura, V. M., M. Kihara, S. Yamaguchi, H. Sockett, and R. M. Macnab.** 1993. *Salmonella typhimurium fliG* and *fliN* mutations causing defects in assembly, rotation and switching of the flagellar motor. J. Bacteriol. **175:** 802–810.
26. **Jones, C. J., and S.-I. Aizawa.** 1991. The bacterial flagellum and flagellar motor: structure, assembly and function. Adv. Microbiol. Physiol. **32:**109–172.
27. **Khan, I. H., T. S. Reese, and S. Khan.** 1992. The cytoplasmic component of the bacterial flagellar motor. Proc. Natl. Acad. Sci. USA **89:**5956–5960.
28. **Khan, S., M. Dapice, and T. S. Reese.** 1988. Effects of *mot* gene expression on the structure of the flagellar motor. J. Mol. Biol. **202:**575–584.
29. **Khan, S., I. H. Khan, and T. S. Reese.** 1991. New features of the flagellar basal body in *Salmonella typhimurium* revealed by rapid-freeze electron microscopy. J. Bacteriol. **173:**2888–2896.
30. **Larsen, S. H., J. Adler, J. J. Gargus, and R. W. Hogg.** 1974. Chemomechanical coupling without ATP: the source of energy for motility and chemotaxis in bacteria. Proc. Natl. Acad. Sci. USA **71:**1239–1243.
31. **Lloyd, S. A., H. Tang, X. Wang, S. Billings, and D. F. Blair.** 1996. Torque generation in the flagellar motor of *Escherichia coli*: evidence of a direct role for FliG but not for FliM or FliN. J. Bacteriol. **178:**223–231.
32. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26:**129–156.
33. **Macnab, R. M.** 1995. Flagellar switch, p. 181–199. *In* J. Hoch and T. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, D.C.
34. **Macnab, R. M.** 1996. Flagella and motility, p. 123–145. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
34a. **Macnab, R. M.** Personal communication.
35. **Maniatis, T. E., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Manson, M. D.** 1992. Bacterial motility and chemotaxis. Adv. Microb. Physiol. **33:**277–346.
37. **Manson, M. D., P. Tedesco, H. C. Berg, F. M. Harold, and C. van der Drift.** 1977. A proton motive force drives bacterial flagella. Proc. Natl. Acad. Sci. USA **74:**3060–3064.
38. **Matsuura, B., J.-I. Shoi, and Y. Imae.** 1977. Motility in *Bacillus subtilis* driven by an artificial proton motive force. FEBS Lett. **82:**187–190.
39. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Parkinson, J. S., and S. E. Houts.** 1982. Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. J. Bacteriol. **151:**106–113.
41. **Ridgway, H. F., M. Silverman, and M. I. Simon.** 1977. Localization of proteins controlling motility and chemotaxis in *Escherichia coli*. J. Bacteriol. **132:**657–665.
42. **Saiki, R. K.** 1992. The design and optimization of the PCR, p. 7–16. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. Oxford University Press, New York.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
44. **Schuster, S. C., and S. Khan.** 1994. The bacterial flagellar motor. Annu. Rev. Biophys. Biomol. Struct. **23:**509–539.
45. **Shapiro, L.** 1995. The bacterial flagellum: from genetic network to complex architecture. Cell **80:**525–527.
46. **Sharp, L. L., J. Zhou, and D. F. Blair.** 1995. Features of MotA proton channel structure revealed by tryptophan-scanning mutagenesis. Proc. Natl. Acad. Sci. USA **92:**7946–7950.
47. **Sharp, L. L., J. Zhou, and D. F. Blair.** 1995. Tryptophan-scanning mutagenesis of MotB, an integral membrane protein essential for flagellar rotation in *Escherichia coli*. Biochemistry **34:**9166–9171.
48. **Silverman, M., and M. Simon.** 1974. Flagellar rotation and the mechanism of bacterial motility. Nature (London) **249:**73–74.
49. **Sockett, H., S. Yamaguchi, M. Kihara, V. M. Irikura, and R. M. Macnab.** 1992. Molecular analysis of the flagellar switch protein FliM. J. Bacteriol. **174:**793–806.
50. **Stader, J., P. Matsumura, D. Vacante, G. E. Dean, and R. M. Macnab.** 1986. Nucleotide sequence of the *Escherichia coli motB* gene and site-limited incorporation of its product into the cytoplasmic membrane. J. Bacteriol. **166:**244–252.
51. **Stoltz, B., and H. C. Berg.** 1991. Evidence for interactions between MotA and MotB, torque-generating elements of the flagellar motor of *Escherichia coli*. J. Bacteriol. **173:**7033–7037.
52. **Tang, H., S. Billings, X. Wang, L. Sharp, and D. F. Blair.** 1995. Regulated underexpression and overexpression of the FliN protein of *Escherichia coli* and evidence for an interaction between FliN and FliM in the flagellar motor. J. Bacteriol. **177:**3496–3503.
53. **Vogler, A. P., M. Homma, V. M. Irikura, and R. M. Macnab.** 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. **173:**3564–3572.
54. **Welch, M., K. Oosawa, S.-I. Aizawa, and M. Eisenbach.** 1993. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. Proc. Natl. Acad. Sci. USA **90:**8787–8793.
55. **Wilson, M. L., and R. M. Macnab.** 1988. Overproduction of the MotA protein of *Escherichia coli* and estimation of its wild-type level. J. Bacteriol. **170:**588–597.
56. **Wilson, M. L., and R. M. Macnab.** 1990. Co-overproduction and localization of the *Escherichia coli* motility proteins MotA and MotB. J. Bacteriol. **172:** 3932–3939.
57. **Yamaguchi, S., S.-I. Aizawa, M. Kihara, M. Isomura, C. J. Jones, and R. M. Macnab.** 1986. Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. J. Bacteriol. **168:**1172–1179.
58. **Yamaguchi, S., H. Fujita, A. Ishihara, S.-I. Aizawa, and R. M. Macnab.** 1986. Subdivision of flagellar genes of *Salmonella typhimurium* into regions responsible for assembly, rotation and switching. J. Bacteriol. **166:**187–193.
59. **Zhou, J., R. T. Fazzio, and D. F. Blair.** 1995. Membrane topology of the MotA protein of *Escherichia coli*. J. Mol. Biol. **251:**237–242.