The Complex Flagellar Torque Generator of *Pseudomonas aeruginosa*

Timothy B. Doyle, Andrew C. Hawkins, and Linda L. McCarter*

Department of Microbiology, The University of Iowa, Iowa City, Iowa

Received 10 May 2004/Accepted 24 June 2004

Flagella act as semirigid helical propellers that are powered by reversible rotary motors. Two membrane proteins, MotA and MotB, function as a complex that acts as the stator and generates the torque that drives rotation. The genome sequence of *Pseudomonas aeruginosa* PAO1 contains dual sets of *motA* and *motB* genes, PA1460-PA1461 (*motAB*) and PA4954-PA4953 (*motCD*), as well as another gene, *motY* (PA3526), which is known to be required for motor function in some bacteria. Here, we show that these five genes contribute to motility. Loss of function of either *motAB*-like locus was dispensable for translocation in aqueous environments. However, swimming could be entirely eliminated by introduction of combinations of mutations in the two *motAB*-encoding regions. Mutation of both genes encoding the MotA homologs or MotB homologs was sufficient to abolish motility. Mutants carrying double mutations in nonequivalent genes (i.e., *motA motD* or *motB motC*) retained motility, indicating that noncognate components can function together. *motY* appears to be required for *motAB* function. The combination of *motY* and *motCD* mutations rendered the cells nonmotile. Loss of function of *motAB*, *motY*, or *motAB motY* produced similar phenotypes; although the swimming speed was only reduced to ~85% of the wild-type speed, translocation in semisolid motility agar and swarming on the surface of solidified agar were severely impeded. Thus, the flagellar motor of *P. aeruginosa* represents a more complex configuration than the configuration that has been studied in other bacteria, and it enables efficient movement under different circumstances.

Many bacteria are rapidly propelled through aqueous environments by flagella. Swimming is achieved by rotating a long helical filament that acts as a propeller and is connected to the cell via a flexible hook (reviewed in reference 31). The hook serves as a universal hinge linking the filament to the basal body, which consists of a rod, bushings, and the MS and C rings. The hook-basal body-rod structure acts as a drive shaft in transmitting torque from the rotary motor to the filament. Two cytoplasmic membrane proteins, MotA and MotB, generate the force necessary to drive the propeller. These proteins form a complex that acts as the stator and use membrane potential to conduct ions and supply the torque for rotation (reviewed in references 3 and 28). Flagellar motors can be fueled by protons, as is the case in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, or sodium ions, as in the alkaliphilic *Bacillus* and marine *Vibrio* species (19, 30, 34).

Freeze-fracture electron micrograph images show a circular array of particles embedded in the membrane surrounding the flagellar basal body (24). These stud-like particles are believed to be ~10 MotA/MotB complexes. Gel filtration and cross-linking studies suggest that the stoichiometry of each complex is MotA₄MotB₂ (29, 50). Controlled expression of *motAB* and successive inactivation with covalently bound inhibitors produce stepwise changes in the rotation rate, suggesting that each complex can function independently to generate torque (5, 6, 43). The four transmembrane domains of MotA and the one transmembrane domain of MotB form the channel through which ions are conducted (reviewed in reference 4). MotB is thought to additionally serve as a structural support that anchors the MotA/MotB complex to the cell wall via a pepti-

doglycan-binding domain in the C terminus (9, 11). Another protein with a peptidoglycan-binding domain is required for motor function in some bacteria. MotY was originally discovered as an essential component of the sodium-driven motor in *Vibrio* species (16, 36, 44). However, MotY does not seem to be a peculiarity of the sodium-type motor. More recently, a MotY homolog (LafY) has been shown to be required for proton-driven motility of the lateral flagellar system of *Vibrio parahaemolyticus* (54).

Although the molecular mechanism by which the flagellar motor generates torque is not completely understood, it is known that there is tight coupling of the passage of specific ions through the torque-generating complex formed by the MotA and MotB proteins to the rotation of the flagellum (38). Current evidence suggests a model for torque generation resulting from conformational changes in the stator that occur as protons associate and dissociate from the Asp-32 residue in MotB (reviewed in reference 4).

Pseudomonas aeruginosa is a gram negative, rod-shaped, motile bacterium with a polar flagellum. The flagellar genes and hierarchy of transcriptional control have recently been elegantly elucidated by using a combination of mutant analyses and RNA profiling (10); however, little is known about the flagellar motor in this organism. Surprisingly, the genome sequence contains two gene sets encoding MotA and MotB homologs: PA1460/PA1461 and PA4954/PA4953 (Fig. 1). PA1460 and PA1461 are located within a potential operon encoding chemotaxis proteins, whereas PA4954 and PA4953 are uniquely located in an operon elsewhere on the chromosome. BLAST analyses have shown that these predicted motor proteins are more similar to the motor proteins of other organisms than to each other. For example, the gene products of one locus are quite similar to *Bacillus subtilis* motor proteins; the PA1460 product shows 38% identity and 59% similarity

* Corresponding author. Mailing address: Department of Microbiology, The University of Iowa, Iowa City, IA 52242. Phone: (319) 335-9721. Fax: (319) 335-7679. E-mail: linda-mccarter@uiowa.edu.

($E = 5e-37$) to *B. subtilis* MotA, and the PA1461 product shows 33% identity and 51% similarity ($E = 2e-26$) to *B. subtilis* MotB. The products of PA4954 and PA4953 are quite similar to the *S. enterica* serovar Typhimurium homologs (42% identity and 66% similarity for MotA [$E = 7e-62$] and 38% identity and 58% similarity for MotB [$E = 2e-54$]). In comparison, PA1460 and PA4954 have only 24% identity and 42% similarity ($E = 7e-08$) and PA1461 and PA4953 have 26% identity and 39% similarity ($E = 1e-09$).

Previously published data do not provide clear evidence about the nature of the flagellar motor in *P. aeruginosa*. Kato et al. examined the role of PA1460 and PA1461 by constructing mutations with a kanamycin resistance cassette (23). Their studies suggested that these genes are not required for swimming motility, as the insertion mutants were fully motile as determined by microscopy. Insertion mutants with mutations in the other potential motor-encoding locus were obtained while *P. aeruginosa*-macrophage interactions were being investigated (52). In that study, the workers isolated a mutant with a defect in a gene, designated *rpmA*, that was required for phagocytosis by macrophages. Sequencing revealed that RpmA is the MotA homolog encoded by PA4954. The phagocytosis phenotype of this mutant could be complemented only when both *rpmA* and *rpmB* were supplied in *trans*, suggesting that the two genes comprised an operon. However, no effect on cell motility was detected in a motility plate or by microscopy. It was therefore concluded that the function of *rpmA* in *P. aeruginosa* was different from that of *motA* in enteric bacteria; i.e., *rpmA* was not required for motor function.

Thus, the nature of the flagellar motor and the roles of the Mot homologs in *P. aeruginosa* were not clear. In this work we describe an analysis of mutants with defects in all of the homologs and the contribution of each gene. To test the hypothesis that the dual sets of motor genes have overlapping functions, a series of strains with single and multiple *mot* gene lesions were constructed. Analyses of *mot* mutants were performed with PAK and PAO1 strains, and similar results were obtained.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. Strains were derived from the *P. aeruginosa* PAK (obtained from Pathogenesis Corporation, Seattle, Wash.), PAO1 (obtained from Barbara Iglewski via E. P. Greenberg), or MPAO1 (obtained from C. Manoil). Strains were routinely propagated at 37°C in Luria-Bertani (LB) broth, which contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl. *Escherichia coli* strains S17-1 and SM10 DAP⁻ were used for conjugational transfer to *P. aeruginosa* (55). Tryptone motility medium contained 10 g tryptone per liter and 5 g NaCl per liter and was supplemented with 0.325% (wt/vol) Bacto Agar (Difco) unless indicated otherwise. Swarming plates were prepared by using M9 minimal medium with 0.2% (wt/vol) glucose as the carbon source and 0.05% (wt/vol) glutamate as the nitrogen source (27). The plates were wrapped in Parafilm and incubated at 37°C for ~2 days. The inocula used for swarming were liquid cultures grown overnight in the same minimal medium. PAK strains swarmed best on plates with 0.5% agar, whereas PAO1 strains swarmed best on plates with 0.6% agar. Motility plates were sometimes incubated at lower temperatures (room temperature or 30°C) for photodocumentation. Antibiotics were used at the following concentrations: for *E. coli* ampicillin was used at a concentration of 75 µg/ml, gentamicin was used at a concentration of 40 µg/ml, and tetracycline was used at a concentration of 10 µg/ml; for *P. aeruginosa* carbenicillin was used at a concentration of 300 µg/ml, gentamicin was used at a concentration of 40 µg/ml in rich medium and at a concentration of 100 µg/ml in minimal medium, and tetracycline was used at a concentration of 50 µg/ml. DL-α,ε-Diaminopimelic acid was used at a final concentration of 0.32 mM.

FIG. 1. *P. aeruginosa* flagellar motor genes. Open reading frames are indicated by arrows, and the directions of transcription are indicated by the arrowheads. The solid triangles indicate the positions of the Tn5 (Tet^r) insertions. Restriction sites that were used to create drug resistance cassette mutations are indicated as follows: st, StuI; rv, EcoRV; sm, SmaI; and m, MscI. The *motAB* locus, the *rpmAB* locus (which we renamed *motCD* due to its role in motor function), and the *motY* gene were cloned from strain PAK by using high-fidelity PCR. A gentamicin resistance cassette was inserted into the StuI site to make *motA2::Gen^r*, into the EcoRV site to make *motB2::Gen^r*, into the StuI and EcoRV sites to make *ΔmotAB3::Gen^r*, and into the SmaI sites to make *ΔmotC7::Tet^r*, into SmaI sites to create *ΔmotCD8*, and into MscI sites to create *ΔmotY2::Tet^r*. In-frame deletions that removed the coding regions for *motAB*, *motCD*, and *motY* in strain PAO1 were also created.

DNA manipulations. Plasmid DNA was prepared by using a QIAprep Spin Miniprep kit (QIAGEN, Chatsworth, Calif.). Agarose gel electrophoresis, DNA conjugation, DNA restriction digestion, DNA ligation, Southern blotting, and transformation were performed essentially as previously described (48). Chromosomal DNA was isolated as described by Woo et al. (58).

Mutant construction. Mutant strains were constructed by PCR amplification of the genes of interest by using a high-fidelity polymerase (*Pfu* or *Herculase* from Stratagene, La Jolla, Calif.). PCR products were ligated into the vectors pKV69 (Tet^r Cam^r), pGP704 (Ap^r), and pEX18Gm (Gen^r). The gentamicin cassette was amplified from pGMΩ1 (51), and it was inserted into the StuI site to make *motA2::Gen^r*, into the EcoRV site to make *motB2::Gen^r*, and into the StuI and EcoRV sites to make *ΔmotAB3::Gen^r*. The tetracycline cassette was amplified from pBR322 (New England Biolabs, Beverly, Mass.) and was inserted into the StuI sites to create *ΔmotC7::Tet^r*, into the SmaI sites to create *ΔmotCD8*, and into the MscI sites to create *ΔmotY2::Tet^r*. Mutated genes were introduced into the chromosome by homologous recombination. In-frame deletion mutant strains were constructed by overlap extension PCR as described previously (13). Regions of DNA upstream (963 bp) and downstream (999 bp) of *motAB* were amplified and used as templates for a third PCR amplification. The resulting 1,968-bp product had a 1,638-bp deletion of *motAB*, which left only the ATG of *motA*, an engineered BamHI site, and the TGA codon of *motB*. The in-frame deletion of *motCD* (PA4954-PA4953) was constructed in a similar manner. The upstream (944-bp) and downstream (1,023-bp) products were used as templates for a third PCR amplification. The resulting product had a 1,898-bp deletion of *motCD*, which left only the first three codons of *motC*, a BamHI site, and the last three codons of *motD*. To construct the in-frame deletion of *motY* (PA3526), the upstream (933-bp) and downstream (1,021-bp) PCR products were used to produce a product containing a 939-bp deletion of *motY*, which left only the first eight codons, a BamHI site, and the TGA codon of *motY*. The products with deletions were cloned into the suicide vector pEX18Gm. Suicide plasmids bearing the *mot* mutations were mobilized from *E. coli* into *P. aeruginosa* by conjugation. Recombinants were selected by plating in the presence of antibiotics. Single recombinants derived from the SacB vector were selected for by plating the mating mixture on M63 minimal medium (48) containing 10 mM succinate and 100 µg of gentamicin per ml. Double recombinants were then selected for by plating

TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Parent, source, and/or reference
PAK strains		
PAK	Wild-type <i>P. aeruginosa</i>	Pathogenesis Corp.
LMP1	PAK containing Tn5 (Tet ^r); wild-type swimming and swarming	PAK; Pathogenesis Corp.
LMP2	Gen ^r PAK containing Tn5 <i>lux</i> (Gen ^r); wild-type swimming and swarming	PAK; Pathogenesis Corp.
LMP4	<i>motA1::</i> Tn5 (Tet ^r); Tn5 disrupts gene at position coding for amino acid 229 of MotA	PAK; Pathogenesis Corp. (= 074G05)
LMP5	<i>motA2::</i> Gen ^r ; Gen cassette disrupts gene at position coding for amino acid 93 of MotA	PAK; this study
LMP6	<i>motB3::</i> Gen ^r ; Gen cassette disrupts gene at position coding for amino acid 148 of MotB	PAK; this study
LMP7	<i>motC1::</i> Tn5 (Tet ^r); Tn5 disrupts gene at position coding for amino acid 49 of MotC	PAK; Pathogenesis Corp. (= 028C02)
LMP8	<i>motD2::</i> Tn5 (Tet ^r); Tn5 disrupts gene at position coding for amino acid 310 of MotD	PAK; Pathogenesis Corp. (= 024E04)
LMP9	Δ <i>motAB4::</i> Gen ^r ; deletion of 0.9-kb region coding from amino acid 93 of MotA to amino acid 148 of MotB with insertion of a Gen ^r cassette	PAK; this study
LMP13	Δ <i>motCD6::</i> Gen ^r ; deletion of 1.6-kb region coding from amino acid 49 of MotC to amino acid 310 of MotD with insertion of a Gen ^r cassette	PAK; this study
LMP15	Δ <i>motAB4::</i> Gen ^r <i>motC1::</i> Tn5 (Tet ^r)	LMP7; this study
LMP16	Δ <i>motAB4::</i> Gen ^r <i>motD2::</i> Tn5 (Tet ^r)	LMP8; this study
LMP17	<i>motA2::</i> Gen ^r <i>motC1::</i> Tn5 (Tet ^r)	LMP7; this study
LMP18	<i>motA2::</i> Gen ^r <i>motD2::</i> Tn5 (Tet ^r)	LMP8; this study
LMP19	<i>motB3::</i> Gen ^r <i>motC1::</i> Tn5 (Tet ^r)	LMP7; this study
LMP20	<i>motB3::</i> Gen ^r <i>motD2::</i> Tn5 (Tet ^r)	LMP8; this study
LMP44	Δ <i>motC7::</i> Tet ^r	PAK; this study
LMP47	<i>motA2::</i> Gen ^r Δ <i>motC7::</i> Tet ^r	LMP5; this study
LMP48	Δ <i>motCD8::</i> Tet ^r <i>motA2::</i> Gen ^r	LMP5; this study
LMP49	<i>motB3::</i> Gen ^r Δ <i>motC7::</i> Tet ^r	LMP6; this study
LMP50	<i>motB3::</i> Gen ^r Δ <i>motCD8::</i> Tet ^r	LMP6; this study
LMP54	Δ <i>motY2::</i> Tet ^r	PAK; this study
LMP55	Δ <i>motAB4::</i> Gen ^r Δ <i>motY2::</i> Tet ^r	LMP9; this study
LMP57	<i>flgC1::</i> Tn5 (Tet ^r)	PAK; Pathogenesis Corp. (= 056E02)
LMP60	Δ <i>motCD6::</i> Gen ^r Δ <i>motY2::</i> Tet ^r	LMP13
LMP61	<i>cheB1::</i> Tn5 (Tet ^r)	PAK; Pathogenesis Corp. (= 024A09)
LMP65	Gen ^r Tet ^r PAK; wild-type swimming and swarming motility	PAK; this study
PAO1 strains		
IgPAO1	Wild type	Iglewski via Greenberg
MPAO1	Wild type	20
PAO1000	Δ <i>cheW</i>	13
PAO1001	Δ <i>motAB</i>	MPAO1; this study
PAO1002	Δ <i>motCD</i>	MPAO1; this study
PAO1003	Δ <i>motY</i>	MPAO1; this study
PAO2000	<i>motY::</i> Tn5 (Gen ^r)	IgPAO1; this study
Plasmids		
pEX18Gm	Gen ^r <i>oriT</i> ⁺ <i>sacB</i> ⁺ allelic exchange vector	18
pKV69	Tet ^r Cam ^r allelic exchange vector	K. L. Visick (Loyola University)
pGP704	Amp ^r allelic exchange with R6K origin	39

single recombinants on medium containing 5% sucrose. All strain constructions were confirmed by Southern blot hybridization and/or PCR analysis.

Motility assays. To document swimming motility, plates were incubated for the times indicated below and photographed with a Kodak Company Digital Science imaging system. The average rate of expansion was determined in triplicate by measuring the diameter as a function of time (7). All expansion rates were normalized to the rate of a control wild-type strain inoculated on the same plate.

Swimming speeds were obtained by using a Hobson Tracker, Bacteria Edition (Hobson Tracking Systems Ltd., Sheffield, England). Cells were prepared by subculturing an overnight culture 1:50 and incubating the subculture in LB medium for ~3 h at 30°C. Five microliters of cells was then placed on a microscope slide and viewed. Swimming speed was defined as the length of a run (in micrometers) divided by the duration of the run (in seconds). A value was calculated by adding the incremental distances moved in each frame along the path sampled and dividing by the total time for the track. Sample data were recorded for 30 s, and each sample consisted of at least 10 tracked events. Fifty samples were recorded for each strain analyzed. All events for each strain were averaged, and the standard deviation was calculated.

Chemotaxis assays. Agarose plug assays were carried out as described previously (47). Briefly, the plugs contained 1% low-melting-point agarose (Gibco-BRL, Grand Island, N.Y.) in chemotaxis buffer (40 mM potassium phosphate [pH 7.0]), a few crystals of methylene blue to provide contrast for photography, and either no added attractant or 10 mM L-arginine HCl (Sigma Chemical Co., St. Louis, Mo.) as an attractant. A 10- μ l drop of the melted agarose mixture was placed on a microscope slide, and it was surrounded by two strips cut from a

plastic coverslip and covered with a no. 1 1/2 glass coverslip. The chamber formed in this way was filled with approximately 150 μ l of a suspension of motile bacteria (optical density at 660 nm, ~0.8) in chemotaxis buffer. Chambers were then photographed under phase-contrast lighting conditions at the time intervals specified below. Assays were performed in duplicate on at least two different days, and representative photographs are shown below.

RESULTS

Mutations in either the *motAB* or *motCD* locus affect, but do not abolish, swimming motility. The starting point for these analyses was an observation made with a set of Tn5 (Tet^r) mutants with defects in flagellar genes that were provided by Pathogenesis Corporation. Specifically, insertions in either of the two potential *mot* loci affected swimming in semisolid motility agar compared to the swimming of the parental *P. aeruginosa* PAK strain. Transposon insertions within *motA*, *motC*, or *motD* resulted in mutants with decreased rates of radial expansion in a semisolid motility plate compared to the wild-type PAK strain rates (Fig. 2). These results suggested that both sets of motor genes contribute to motility. To explore this observation and this hypothesis, additional strain PAK mutants

FIG. 2. Transposon insertions in either the *motAB* or *motCD* locus affect movement in semisolid motility agar. The strains and normalized rates of radial expansion were as follows: LMP4 (*motA1::Tn5Tet^r*), 0.66 ± 0.10 ; LMP7 (*motC1::Tn5Tet^r*), 0.51 ± 0.05 ; and LMP8 (*motD2::Tn5Tet^r*), 0.53 ± 0.06 . The rates of expansion were normalized to the rate for reference strain LMP1, which contained Tn5 (*Tet^r*) and showed wild-type swimming motility. Motility plates contained 50 μ g of tetracycline per ml and were incubated at 30°C for 20 h. Similar results were observed in plates without an antibiotic.

were constructed by introducing either a gentamicin resistance cassette to create Δ *motCD6::Gen^r*, *motA2::Gen^r*, *motB3::Gen^r*, and Δ *motAB4::Gen^r* or a tetracycline resistance cassette to create Δ *motC7::Tet^r* and Δ *motCD8::Tet^r*.

Strains with the *motC1::Tn5* (*Tet^r*), *motD2::Tn5* (*Tet^r*), and Δ *motCD6::Gen^r* alleles showed decreased radial expansion in soft agar plates compared to the wild-type parent strain PAK (Fig. 3). Swimming speeds are shown in Fig. 4. Wild-type strain PAK was found to swim at a speed of 41 ± 3 μ m/s. The observed speeds of the three mutants were lower than the speed of the wild type: 28 ± 2 , 27 ± 3 , and 28 ± 2 μ m/s, respectively. Thus, the *motCD* locus contributes to, but is not exclusively responsible for, motor function.

The Tn5 insertion in *motA* in strain LMP4 occurred near the end of the coding region (disrupting the gene at the position coding for amino acid 229 of 246 amino acids). Three additional *mot* alleles were created in the PA1460-PA1461 locus and transferred to the chromosome. The mutations, *motA2::Gen^r*, *motB3::Gen^r*, and Δ *motAB4::Gen^r*, caused severe swimming motility defects in soft agar plates (Fig. 3B). However, the strains were highly motile as determined by light microscopy, and the calculated swimming speeds for *motA2::Gen^r* (40 ± 3 μ m/s) and *motB3::Gen^r* (40 ± 3 μ m/s) were very similar to

the calculated swimming speed for wild-type strain PAK (41 ± 3 μ m/s). The swimming speed for Δ *motAB4::Gen^r* (35 ± 5 μ m/s) was slightly less than that of the wild type.

These results are similar to those observed by Kato et al. (23) and seem to be consistent with the potential for lesions in *motAB* to affect expression of downstream chemotaxis genes. Radial expansion in semisolid agar plates measures chemotaxis as well as motility. The *motAB* mutants were motile as determined by microscopic observations, and yet the *motAB* mutant strains showed severe defects in semisolid motility plates compared to the Δ *motCD* strain or the wild type (Fig. 3C). Even though the gentamicin resistance cassette that was introduced into *motAB* was designed to create a nonpolar mutation (21), chemotaxis genes occur upstream and downstream of *motAB*. It is possible that an imbalance in the specific ratio of chemotaxis signaling components could perturb the chemotaxis phosphotransfer cascade and affect radial expansion in plates. Even though they are competent for swimming (with little or no defect in the observed swimming speed), mutants with defects in the *motAB* locus could be lost in motility plates. However, unlike known chemotaxis mutants (e.g., *cheW* mutants), no profound motor bias that altered swimming patterns was observed by microscopy, and, as demonstrated below, chemotaxis in the *motAB* mutants was not affected.

Disruptions in both *motAB* and *motCD* loci abolish motility.

Since deletion of either set of operons encoding the Mot homologs was not sufficient to abolish swimming, mutants with combinations of *mot* lesions were constructed. Introduction of *motC* and *motD* alleles into the Δ *motAB* strain to make LMP15 and LMP16, respectively, or introduction of *motA* and *motB* into the Δ *motCD* strain to make LMP48 and LMP50 completely abolished motility in semisolid motility plates (Fig. 5, plate A) and motility determined by microscopy (Fig. 4). We next asked whether double mutations that resulted in loss of the homologous genes (i.e., *motA* and *motC* or *motB* and *motD*) were sufficient to disrupt swimming. Like the triple-mutant strains, LMP47 (*motA2::Gen^r motC7::Tet^r*) and LMP20 (*motB3::Gen^r motD2::Tn5*) were nonmotile in plates (Fig. 5, plate B) and in liquid (data not shown). Thus, the two loci appear to encode proteins with overlapping functions; motility was abolished when both sets of *motA*-like or *motB*-like genes were deleted.

Noncognate pairs can work together to generate torque. The additional combinations of alleles that were constructed in-

FIG. 3. Deletion of either the *motAB* or *motCD* locus affects motility in semisolid agar. (A) Motility of strains LMP7, LMP8, LMP13, and wild-type strain PAK. The normalized rates of radial expansion were as follows: LMP7 (*motC1::Tn5Tet^r*), 0.51 ± 0.05 ; LMP8 [*motD2::Tn5* (*Tet^r*)], 0.53 ± 0.06 ; and LMP13 (Δ *motCD6::Gen^r*), 0.55 ± 0.03 . (B) Motility of strains LMP5 (*motA2::Gen^r*), LMP6 (*motB3::Gen^r*), LMP9 (Δ *motAB4::Gen^r*), and LMP2. The reference strain LMP2 contained Tn5 (*Gen^r*) and showed wild-type swimming motility. Radial expansions rates were not calculated because the strains failed to significantly migrate in the motility plate. (C) Motility of strains LMP13, LMP9, and LMP2. The plate in panel A contained no antibiotic and was incubated for 15 h at 30°C. The plates in panels B and C contained 50 μ g of gentamicin per ml and were incubated for 20 h at 30°C.

FIG. 4. Swimming speeds of the wild type and motor mutants. The average swimming rates were determined by using the Hobson Tracker for cells growing exponentially in liquid LB medium. Strains PAK, LMP44 ($\Delta motC7$), LMP7 ($motC1$), LMP8 ($motD2$), LMP13 ($\Delta motCD6$), LMP5 ($motA2$), LMP6 ($motB3$), LMP9 ($\Delta motAB4$), LMP54 ($\Delta motY2$), LMP55 ($\Delta motAB4 \Delta motY2$), LMP60 ($\Delta motCD6 \Delta motY2$), LMP18 ($motA2 motD2$), LMP49 ($motB3 motC7$), LMP17 ($motA2 motC1$), and LMP20 ($motB3 motD2$) were examined.

cluded *motA2* and *motD2* to make LM18 and *motB3* and *motC7* to make LMP49. In motility agar (Fig. 5, plate B), LMP18 was motile (although less motile than the wild type), whereas LMP49 was severely defective. As determined by microscopy, both strain LMP18 and strain LMP49 were motile; the swimming speeds were determined to be 28 ± 2 and 38 ± 2 $\mu\text{m/s}$, respectively, compared to 41 ± 3 $\mu\text{m/s}$ for the wild type (Fig. 4). Thus, motility was retained when one copy of a *motA*-

FIG. 5. Disruption of genes in both *motAB*-like loci abolishes swimming motility. (Plate A) Triple gene knockouts. Strains LMP15 ($\Delta motAB4 motC1$), LMP16 ($\Delta motAB4 motD2$), LMP57 (*flgC1*; nonmotile *Fla*⁻ control), LMP48 ($motA2 \Delta motCD8$), LMP50 ($motB3 \Delta motCD8$), and PAK were included. (Plate B) Double gene knockouts. Strains LMP57 (*flgC1*; nonmotile control), LMP47 ($motA2 \Delta motC7$), LMP20 ($motB3 motD2$), LMP18 ($motA2 motD2$), LMP49 ($motB3 \Delta motC7$), and PAK were included. The motility plates were incubated overnight at room temperature.

Plate A

$\Delta motAB$
 $\Delta motY$
 $\Delta motAB \Delta motY$
 PAK

Plate B

$\Delta motCD$
 $\Delta motY$
 $\Delta motCD \Delta motY$
 PAK

FIG. 6. The fifth motor gene, *motY*. Strains LMP9 ($\Delta motAB$), LMP54 ($\Delta motY$), and LMP55 ($\Delta motAB \Delta motY$) were included in plate A, and strains LMP13 ($\Delta motCD$), LMP54 ($\Delta motY$), and LMP60 ($\Delta motCD \Delta motY$) were included in plate B. The motility plates were incubated overnight at room temperature.

like gene and one copy of a *motB*-like gene remained. These data suggest that the pairs of gene products do not require their cognate partners to work and can function with a partner from the alternate locus.

Discovery of an additional gene required for motor function in *P. aeruginosa*: *motY*. While a transposon mutant library in *P. aeruginosa* strain PAO1 was being analyzed, a mutant that had a severe defect in a motility plate was isolated. The insertion of this mutant was sequenced and was found to disrupt PA3526. The gene product is most similar to the *Vibrio cholerae* *motY* product (30% identity and 51% similarity; $E = 3e-29$). MotY resembles MotB in that it contains a single transmembrane domain and a peptidoglycan interaction domain. A *motY* mutant was constructed in the PAK background and examined. As observed for PAO1, deletion of *motY* in PAK severely affected swimming motility in semisolid motility plates (Fig. 6). The radial expansion rate for LMP54 ($\Delta motY::Tet^r$) normalized to the wild-type rate was 0.22 ± 0.03 . However, in liquid the observed swimming rate for LMP54 was only slightly lower than that of wild-type strain PAK (35 ± 3 $\mu\text{m/s}$ versus 41 ± 3 $\mu\text{m/s}$) and was equivalent to the speed observed for the $\Delta motAB$ strain. Thus, for some reason, the motility defect in *motY* mutants (in PAK and PAO1 backgrounds) appeared to be more profound in a semisolid motility plate than in liquid. The *motY* strain is a prototroph, even though the *motY* coding region is located upstream of and transcribed in the same direction as *argG*, and there are no linked genes that are expected to play additional roles in motility or chemotaxis.

The movement of the *motY* mutant resembled that of the *motAB* mutants in semisolid motility agar and in liquid. To probe the role of *motY*, the $\Delta motY2$ allele was introduced into the $\Delta motAB$ strain to make LMP55 and into the $\Delta motCD$ strain to make LMP60. Strains LMP55 ($\Delta motAB \Delta motY$), LMP9 ($\Delta motAB$), and LMP54 ($\Delta motY$) showed little radial

FIG. 7. Swimming and swarming phenotypes of *mot* mutants in PAK and PAO1 backgrounds. Swimming motility plates contained 0.325% semisolid agar, and solidified swarming plates contained 0.5% agar for PAK strains and 0.6% agar for PAO1 strains. The PAK strains used (top row of plates) were PAK, LMP9 ($\Delta motAB$), LMP13 ($\Delta motCD$), LMP54 ($\Delta motY$), LMP47 ($motA2 motC7$), LMP20 ($motB3 motD2$), LMP57 ($flgC1$), LMP18 ($motA2 motD2$), and LMP49 ($motB3 motC7$). The PAO1 strains used (bottom row of plates) were MPAO1, PAO1001 ($\Delta motAB$ in MPAO1), PAO1002 ($\Delta motCD$ in MPAO1), PAO2000 ($\Delta motY$ in IgPAO1), and IgPAO1. Swimming plates were incubated overnight at room temperature, and swarming plates were incubated for 2 days at 37°C.

expansion in semisolid motility plates. In liquid, the swimming speeds of LMP55, LMP9, and LMP54 were similar and only slightly less than the wild-type speed (i.e., ~85% of the wild-type speed). In contrast, the motility phenotype of LMP60 ($\Delta motCD \Delta motY$) was unlike that of its parental $\Delta motCD$ strain. LMP60 was nonmotile in plates (Fig. 6, plate B) and in liquid (Fig. 4). Thus, mutation of *motY* and *motCD* resulted in a complete loss of motility. Disruption of *motY* caused a phenotype similar to loss of *motAB* or loss of *motAB* and *motY*. Taken together, these results suggest that *motY* is required for MotA/B function, but not for MotC/D function.

Chemotaxis is not affected in the *motAB* mutants. The *motY* gene was unlinked from other potential flagellar or chemotaxis genes. Since *motY* and *motAB* mutants displayed similar phenotypes, which included poor motility on surfaces or in semisolid agar but good motility in dilute aqueous environments, we decided to investigate the *motAB* mutants further. Additionally, we extended our findings by creating in-frame deletions ($\Delta motAB$ and $\Delta motCD$) in another *P. aeruginosa* strain, strain PAO1. The resulting new strains had motility phenotypes comparable to those observed in PAK strains (Fig. 7 and data not shown). Thus, the in-frame deletion of *motAB* in PAO1 caused the same phenotype as the insertion of the (putative) nonpolar gentamicin cassette in PAK. An agarose plug assay was used to examine the chemotaxis phenotypes of these mutants. In this assay, a chemotactic response consisted of the formation of a ring of accumulated bacteria that surrounded an agarose plug containing the chemoattractant argi-

nine. For PAK (Fig. 8A) and PAO1 (Fig. 8B) strains similar rings of cell accumulation were visualized for the wild-type and $\Delta motAB$ strains, and there was no accumulation for strains with known chemotactic defects (LMP61 and PAO1000). Therefore, the radial expansion defect observed for *motAB* strains in semisolid motility agar cannot be attributed to polar effects that disturb chemotaxis.

Effects of motor mutations on swarming: MotA, MotB, and MotY are necessary to power movement on surfaces. Flagellum-assisted motility can be used for locomotion in liquid (called swimming) or over surfaces (called swarming) (reviewed in reference 17). Figure 7 shows the movement of the *mot* mutant strains in the PAK and PAO1 backgrounds over the surface of solidified agar (0.5 and 0.6%, respectively); for comparison, movement within semisolid agar (0.3%) is also shown. Although some differences in the swarming pattern for the three wild-type strains were observed, all motor mutants that showed defects in translocation in semisolid swimming plates showed similar defects on solidified swarming plates. Not all movement was abolished on the surface. This was likely because the strains remained competent for twitching motility, which can also promote movement on surfaces and is powered by a distinctly different mechanism (27, 35).

The mutants with lesions in *motAB* or *motY* were profoundly defective for translocation on surfaces and in semisolid agar, and yet they displayed good motility (i.e., almost wild-type swimming speeds) in liquid (Table 2). These observations suggested that torque generators composed of MotC and MotD

FIG. 8. Chemotactic responses of PAK, PAO1, and isogenic mutants in the presence of an agarose plug containing arginine (ARG) or chemotaxis buffer (CB) containing no chemoattractant. A positive response resulted in a light ring around the dark plug.

worked poorly under high-viscosity conditions. To test this idea, radial expansion in semisolid agar was examined by using a range of agar concentrations. Significant radial expansion was observed for *motAB* or *motY* mutants in motility plates with reduced agar concentrations (Fig. 9). Decreasing the agar concentration also allowed better discrimination of phenotypes. Radial expansion of LMP9 (Δ *motAB*), radial expansion of LMP54 (*motY*), and radial expansion of LMP55 (Δ *motAB motY*) were not identical; LMP54 moved better than LMP9, which moved better than LMP55. Figure 9 also shows that a hybrid MotA/D-type motor does not work well in high-viscosity conditions, whereas a MotB/C-type motor does function well on plates; in liquid the efficiency is reversed and the MotA/D-type motor produces higher speeds than the MotB/C-type motor (38 and 28 μ m/s, respectively) (Fig. 4 and Table 2).

DISCUSSION

Motility is often a key factor in adaptation and survival, and flagella are one of the most widespread and effective means of locomotion for bacteria (2). Acting as propellers, flagella pro-

mote rapid swimming motility in dilute aqueous environments. Flagella allow bacteria to move towards more favorable environments and away from less favorable environments. Flagella can also enable movement through viscous environments and over surfaces (called swarming), as well as biofilm formation and architecture (17, 25, 45). As a result of this multiplicity of roles, it is not surprising that many studies have indicated that flagellar systems are important for pathogenesis of many organisms (reviewed in references 40 and 46), including *P. aeruginosa* (12, 14, 41, 42, 49). In this paper we describe characterization of the *P. aeruginosa* flagellar motor, which is more complex than previously studied motors of other bacteria. The genome contains two sets of homologous *motAB*-like genes. We show here that both loci contribute to motility. These sets of genes are not simply redundant gene sets because the motility phenotypes of mutants are distinct. The products of one locus, MotA-MotB (PA1460-PA1461), require another protein MotY (PA3526) for optimal function. Loss of function of any of the three genes reduces the ability of *P. aeruginosa* to move in semisolid agar or over surfaces, but it does not dramatically reduce swimming

TABLE 2. Comparison of movement in liquid and plates

PAK strain	Relevant genotype ^a	Normalized motility in liquid ^b	Normalized motility in semisolid agar ^c	Motility on solidified agar ^e
PAK	Wild type	1.0	1.0	+
LMP9	Δ <i>motAB</i>	0.85	No expansion ^d	–
LMP13	Δ <i>motCD</i>	0.68	0.55	+
LMP54	<i>motY</i>	0.85	0.22	–
LMP18	<i>motA motD</i>	0.68	0.29	+
LMP49	<i>motB motC</i>	0.93	No expansion ^d	–
LMP47	<i>motA motC</i>	Nonmotile	Nonmotile	–
LMP20	<i>motB motD</i>	Nonmotile	Nonmotile	–
LMP55	Δ <i>motAB motY</i>	0.85	No expansion ^d	–
LMP60	Δ <i>motCD motY</i>	Nonmotile	Nonmotile	–

^a Gene designations correspond to the following PA numbers in the annotated genome: *motA*, PA1460; *motB*, PA1461; *motC*, PA4954; *motD*, PA4953; and *motY*, PA3526.
^b The swimming speeds were normalized to the wild-type value.
^c The Rates of radial expansion in 0.325% semisolid agar motility plates were normalized to the wild-type value.
^d No significant radial migration from the point of inoculation.
^e Motility on the surface of minimal swarming agar (0.5% agar).

FIG. 9. Movement in motility agar solidified with different concentrations of agar. Tryptone motility agar was solidified with the concentrations of agar indicated. Strains LMP57 (*flgC1*), LMP47 (*motA2 motC7*), LMP18 (*motA2 motD2*), LMP49 (*motB3 motC7*), LMP9 (Δ *motAB*), LMP54 (Δ *motY*), LMP55 (Δ *motAB* Δ *motY*), LMP60 (Δ *motCD* Δ *motY*), and PAK were used. The plates were incubated overnight at room temperature.

motility. Although by themselves MotC and MotD do not seem to generate sufficient torque to move bacteria when there is a high external load, they can function well in liquid in the absence of MotA/B. In fact, MotC/D-driven motors seem to outperform MotA/B-driven motors in liquid; *motAB* mutants achieved swimming speeds approaching the wild-type speed. Elimination of MotA or MotB caused no significant change in swimming speed, and elimination of MotY, of MotA and MotB, or of MotA and MotB and MotY caused a reduction to 85% of the wild-type speed. Elimination of MotC and/or MotD reduced the swimming speed to 65% of the wild-type speed and caused a similar reduction in radial expansion in semisolid agar.

Why is there a complex flagellar motor, and how does it work? The products of five genes contribute to flagellar motor function in *P. aeruginosa*, whereas only two proteins are necessary for torque generation in *E. coli*. The analysis of mutants showed that generation of torque by MotA/B/Y or MotC/D is sufficient to promote motility in liquid; however, MotA/B/Y units are necessary for movement in viscous environments and over surfaces. *E. coli* and other peritrichous bacteria solve the problem of motility in the presence of increasing external load by upregulating the number of propellers (17). However, *P. aeruginosa* is polarly flagellated. *V. parahaemolyticus* is also polarly flagellated, and this organism induces a second, peritrichous system under high-viscosity conditions that enables translocation (37). *P. aeruginosa* appears to have solved the problem of motor energization under conditions under which there is a varying load by having a more complex torque-generating system, which may allow the bacterium to effectively function as an all-terrain organism with a single mode of locomotion.

Two scenarios can be imagined for the *P. aeruginosa* motor: dual generators composed of discrete MotA/B/Y and MotC/D torque generators surrounding the rotor and functioning independently and MotA/B/C/D/Y complexes functioning together. We considered the possibility that distinct torque generators are powered differently (i.e., by the proton and sodium motive forces), but the motility phenotypes of the wild type and selected mutants do not change in the presence of the sodium-channel-blocking drug phenamil (80 μ M) (data not shown). Data presented here demonstrate that MotA/MotD or MotB/MotC can interact productively to generate torque necessary to drive the flagellar motor; however, such data simply show

the potential for an interaction in mutant backgrounds and do not address the findings for wild-type cells. The slight difference in plate motility for *motY*, *motAB*, and *motAB motY* mutants, as well as the observed difference in swimming speed between *motA* or *motB* and Δ *motAB* mutants, also suggests that there is potential interaction of the remaining motor components. However, additional experiments are needed to reveal the roles and relationships of these proteins in promoting the generation of torque.

It is intriguing that *motCD* lesions were originally identified as lesions that cause an altered virulence phenotype (52). Specifically, the *motCD* locus was demonstrated to be required for ingestion by macrophages. Although in the original report the workers concluded that there was no effect on motility, we surmise that this phenotype may have been overlooked because it is subtle and different from the predicted and well-characterized *E. coli motAB* phenotype. The discovery that a *motC* mutant was resistant to phagocytosis is consistent with other findings showing that motility and flagella are necessary for phagocytosis (33). Thus, perhaps the dual motors are not coordinately regulated or are differentially functional in the host. Analysis of the microarray data provided by Dasgupta et al. provided evidence that PA1460 (*motA*), PA1461 (*motB*), and PA3526 (*motY*) are coordinately regulated as part of the flagellar biogenesis hierarchy, while PA4594 (*motC*) and PA493 (*motD*) show no change in gene expression compared with the wild type in strains with *rpoN*, *fleQ*, *fleR*, or *fliA* lesions (10).

It seems clear from a number of studies with *P. aeruginosa* and other bacteria that at times it is advantageous for a bacterium to be highly motile, while at other times it is advantageous to be more adhesive and sessile (8, 22, 25, 26). One common means to control motility is stringent transcriptional regulation. Numerous environmental factors and global regulators control the flagellar gene hierarchy (reviewed in references 1 and 53). Moreover, flagellar genes can be down-regulated once infection is established in a host or in biofilms (56, 57). Another known solution controlling motility is mutational loss of flagellation once infection is established (15, 32). However, this work raises an intriguing new possibility. Perhaps a bacterium can choose to be nonmotile by having motors that function or are expressed differentially under certain conditions. Such an option might allow a bacterium to be sessile under one condition and yet poised for motility in response to

changing environments. This may have important implications for the development and maintenance of multicellular communities in biofilms.

ACKNOWLEDGMENTS

This work was supported by a pilot grant from the Iowa Cystic Fibrosis Foundation to L.L.M.

We thank Pathogenesis Corporation and especially Wendy Hufnagle for provision of the transposon insertion mutants in the strain PAK background. We thank Carrie Harwood for support and fruitful discussions. Undergraduate students in the microbial genetics laboratory class at the University of Iowa discovered *motY*.

REFERENCES

- Aldridge, P., and K. T. Hughes. 2002. Regulation of flagellar assembly. *Curr. Opin. Microbiol.* **5**:160–165.
- Armitage, J. P. 1999. Bacterial tactic responses. *Adv. Microb. Physiol.* **41**: 229–289.
- Berg, H. C. 2003. The rotary motor of bacterial flagella. *Annu. Rev. Biochem.* **72**:19–54.
- Blair, D. F. 2003. Flagellar movement driven by proton translocation. *FEBS Lett.* **545**:86–95.
- Blair, D. F., and H. C. Berg. 1988. Restoration of torque in defective flagellar motors. *Science* **242**:1678–1681.
- Block, S. M., and H. C. Berg. 1984. Successive incorporation of force-generating units in the bacterial rotary motor. *Nature* **309**:470–472.
- Boles, B. R., and L. L. McCarter. 2000. Insertional inactivation of genes encoding components of the sodium-type flagellar motor and switch of *Vibrio parahaemolyticus*. *J. Bacteriol.* **182**:1035–1045.
- Boles, B. R., and L. L. McCarter. 2002. *Vibrio parahaemolyticus* *scrABC*, a novel operon affecting swarming and capsular polysaccharide regulation. *J. Bacteriol.* **184**:5946–5954.
- Chun, S. Y., and J. S. Parkinson. 1988. Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. *Science* **239**:276–278.
- Dasgupta, N., M. C. Wolfgang, A. L. Goodman, S. K. Arora, J. Jyot, S. Lory, and R. Ramphal. 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **50**: 809–824.
- De Mot, R., and J. Vanderleyden. 1994. The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both gram-positive and gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. *Mol. Microbiol.* **12**: 333–334.
- Feldman, M., R. Bryan, S. Rajan, L. Scheffler, S. Brunnert, H. Tang, and A. Prince. 1998. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect. Immun.* **66**:43–51.
- Ferrandez, A., A. C. Hawkins, D. T. Summerfield, and C. S. Harwood. 2002. Cluster II *che* genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. *J. Bacteriol.* **184**:4374–4383.
- Fleiszig, S. M., S. K. Arora, R. Van, and R. Ramphal. 2001. FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. *Infect. Immun.* **69**: 4931–4937.
- Garrett, E. S., D. Perlegas, and D. J. Wozniak. 1999. Negative control of flagellum synthesis in *Pseudomonas aeruginosa* is modulated by the alternative sigma factor AlgT (AlgU). *J. Bacteriol.* **181**:7401–7404.
- Gosink, K. K., and C. C. Hase. 2000. Requirements for conversion of the Na⁺-driven flagellar motor of *Vibrio cholerae* to the H⁺-driven motor of *Escherichia coli*. *J. Bacteriol.* **182**:4234–4240.
- Harshey, R. M. 2003. Bacterial motility on a surface: many ways to a common goal. *Annu. Rev. Microbiol.* **57**:249–273.
- Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**:77–86.
- Imae, Y., and T. Asumi. 1989. Na⁺-driven bacterial flagellar motors. *J. Bioenerg. Biomembr.* **21**:705–716.
- Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenther, D. Bovee, M. V. Olson, and C. Manoil. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **100**:14339–14344.
- Jain, S., and D. E. Ohman. 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. *J. Bacteriol.* **180**:634–641.
- Josenhans, C., and S. Suerbaum. 2002. The role of motility as a virulence factor in bacteria. *Int. J. Med. Microbiol.* **291**:605–614.
- Kato, J., T. Nakamura, A. Kuroda, and H. Ohtake. 1999. Cloning and characterization of chemotaxis genes in *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* **63**:155–161.
- Khan, S., R. Zhao, and T. S. Reese. 1988. Architectural features of the *Salmonella typhimurium* flagellar motor switch revealed by disrupted C-rings. *J. Struct. Biol.* **122**:311–319.
- Klausen, M., A. Aes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol. Microbiol.* **50**: 61–68.
- Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol. Microbiol.* **48**: 1511–1524.
- Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* **182**:5990–5996.
- Kojima, S., and D. F. Blair. 2004. The bacterial flagellar motor: structure and function of a complex molecular machine. *Int. Rev. Cytol.* **233**:93–134.
- Kojima, S., and D. F. Blair. 2004. Solubilization and purification of the MotA/MotB complex of *Escherichia coli*. *Biochemistry* **43**:26–34.
- 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.
- Macnab, R. M. 2003. How bacteria assemble flagella. *Annu. Rev. Microbiol.* **57**:77–100.
- Mahenthiralingam, E., M. E. Campbell, and D. P. Speert. 1994. Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect. Immun.* **62**:596–605.
- Mahenthiralingam, E., and D. P. Speert. 1995. Nonopsonic phagocytosis of *Pseudomonas aeruginosa* by macrophages and polymorphonuclear leukocytes requires the presence of the bacterial flagellum. *Infect. Immun.* **63**: 4519–4523.
- Manson, M. D., P. Tedesco, H. C. Berg, F. M. Harold, and C. Van der Drift. 1977. A protonmotive force drives bacterial flagella. *Proc. Natl. Acad. Sci. USA* **74**:3060–3064.
- Mattick, J. S. 2002. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* **56**:289–314.
- McCarter, L. L. 1994. MotY, a component of the sodium-type flagellar motor. *J. Bacteriol.* **176**:4219–4225.
- McCarter, L. L. 2001. Polar flagellar motility of the *Vibrionaceae*. *Microbiol. Mol. Biol. Rev.* **65**:445–462.
- Meister, M., G. Lowe, and H. C. Berg. 1987. The proton flux through the bacterial flagellar motor. *Cell* **49**:643–650.
- Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
- Moens, S., and J. Vanderleyden. 1996. Functions of bacterial flagella. *Crit. Rev. Microbiol.* **22**:67–100.
- Montie, T. C., D. Doyle-Huntzinger, R. C. Craven, and I. A. Holder. 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned-mouse model. *Infect. Immun.* **38**: 1296–1298.
- Montie, T. C., D. Drake, H. Sellin, O. Slater, and S. Edmonds. 1987. Motility, virulence, and protection with a flagella vaccine against *Pseudomonas aeruginosa* infection. *Antibiot. Chemother.* **39**:233–248.
- Muramoto, K., S. Sugiyama, E. J. Cragoe, Jr., and Y. Imae. 1994. Successive inactivation of the force-generating units of sodium-driven bacterial flagellar motors by a photoreactive amiloride analog. *J. Biol. Chem.* **269**:3374–3380.
- Okabe, M., T. Yakushi, M. Kojima, and M. Homma. 2002. MotX and MotY, specific components of the sodium-driven flagellar motor, colocalize to the outer membrane in *Vibrio alginolyticus*. *Mol. Microbiol.* **46**:125–134.
- O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**:295–304.
- Ottemann, K. M., and J. F. Miller. 1997. Roles for motility in bacterial-host interactions. *Mol. Microbiol.* **24**:1109–1117.
- Parales, R. E., J. L. Ditty, and C. S. Harwood. 2000. Toluene-degrading bacteria are chemotactic towards the environmental pollutants benzene, toluene, and trichloroethylene. *Appl. Environ. Microbiol.* **66**:4098–4104.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sato, H., K. Okinaga, and H. Saito. 1988. Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. *Microbiol. Immunol.* **32**:131–139.
- Sato, K., and M. Homma. 2000. Functional reconstitution of the Na⁺-driven polar flagellar motor component of *Vibrio alginolyticus*. *J. Biol. Chem.* **275**: 5718–5722.
- Schweizer, H. D. 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. *BioTechniques* **15**:831–834.

52. **Simpson, D. A., and D. P. Speert.** 2000. RpmA is required for nonopsonic phagocytosis of *Pseudomonas aeruginosa*. *Infect. Immun.* **68**:2493–2502.
53. **Soutourina, O. A., and P. N. Bertin.** 2003. Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS Microbiol. Rev.* **27**:505–523.
54. **Stewart, B. J., and L. L. McCarter.** 2003. Lateral flagellar gene system of *Vibrio parahaemolyticus*. *J. Bacteriol.* **185**:4508–4518.
55. **Watnick, P. L., and R. Kolter.** 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol.* **34**:586–595.
56. **Whiteley, M., M. G. Banger, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg.** 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**:860–864.
57. **Wolfgang, M. C., J. Jyot, A. L. Goodman, R. Ramphal, and S. Lory.** 2004. *Pseudomonas aeruginosa* regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **101**:6664–6668.
58. **Woo, T. H., A. F. Cheng, and J. M. Ling.** 1992. An application of a simple method for the preparation of bacterial DNA. *BioTechniques* **13**:696–698.