

Cloning and Characterization of *motY*, a Gene Coding for a Component of the Sodium-Driven Flagellar Motor in *Vibrio alginolyticus*

ISAO OKUNISHI, IKURO KAWAGISHI,* AND MICHIO HOMMA

Department of Molecular Biology, Faculty of Science, Nagoya University,
Chikusa-ku, Nagoya 464-01, Japan

Received 31 July 1995/Accepted 8 February 1996

The bacterial flagellar motor is a molecular machine that couples proton or sodium influx to force generation for driving rotation of the helical flagellar filament. In this study, we cloned a gene (*motY*) encoding a component of the sodium-driven polar flagellar motor in *Vibrio alginolyticus*. Nucleotide sequence analysis revealed that the gene encodes a 293-amino-acid polypeptide with a single putative transmembrane segment that is very similar (94.5% identity) to the recently described MotY of *V. parahaemolyticus*. Their C-terminal domains were similar to the C-terminal domains of many peptidoglycan-interacting proteins, e.g., *Escherichia coli* MotB and OmpA, suggesting that MotY may interact with peptidoglycan for anchoring the motor. By using the *lac* promoter-repressor system, *motY* expression was controlled in *V. alginolyticus* cells. Swimming ability increased with increasing concentrations of the inducer isopropyl- β -D-thiogalactopyranoside, and the swimming fraction increased after induction. These results are consistent with the notion that MotY is a component of the force-generating unit. *V. alginolyticus motY* complemented the *motY* mutation of *V. parahaemolyticus*. However, *motY* appeared to lack a region corresponding to the proposed *motY* promoter of *V. parahaemolyticus*. Instead, sequences similar to the σ^{54} consensus were found in the upstream regions of both species. We propose that they are transcribed from the σ^{54} -specific promoters.

The flagellar motor rotates the flagellar filament that extends from the cell body to propel the cell (for reviews, see references 8, 21, 29, and 42). The energy source for motor rotation is the electrochemical gradient of a specific ion (proton or sodium ion) across the cytoplasmic membrane. Thus, the flagellar motor is a molecular machine that couples ion flux to force generation. The genes (*motA* and *motB*) that code for the transmembrane components of the proton-driven motors of several species, including *Escherichia coli*, *Bacillus subtilis*, and *Vibrio parahaemolyticus*, have been cloned and sequenced (16, 34, 36, 48). It has been suggested that MotA constitutes a proton-conducting channel (10) and MotB interacts with peptidoglycan (also known as the cell wall or the murine layer) to act as a stator (15). Three other proteins (FliG, FliM, and FliN), which are located peripherally on the cytoplasmic side of the basal body, are involved in motor rotation and switching of the rotational sense (for details, see the reviews cited above). However, the molecular mechanism of energy transduction has not been elucidated.

For investigation of the energy-coupling mechanism, the sodium-driven motor is promising (19), since sodium concentration can be manipulated more readily than proton concentration, and amiloride and its analogs inhibit motor rotation in a fairly specific manner (3, 5, 41, 50). However, this type of motor has been poorly characterized in terms of genetics and biochemistry. We therefore wanted to clone and characterize genes coding for components of the sodium-driven motor.

Certain marine *Vibrio* species are suitable organisms for this purpose. *Vibrio* species have two types of flagella: a single polar flagellum suited for swimming in liquids and numerous lateral flagella suited for swarming over animate or inanimate surfaces (1, 32, 44). The polar flagellar motor is driven by sodium

motive force, and the lateral flagellar motor is driven by proton motive force (4, 23). A single cell possessing two types of motors with two different coupling ions provides an excellent experimental system. Moreover, the polar flagellar motor has the following interesting properties. First, it is one of the fastest biological motors (up to 1,700 rps at 37°C) known to date (28). Second, it functions not only as a locomotive organelle but also as a viscosity sensor that regulates expression of lateral flagellar genes which are induced only under viscous conditions (7, 30).

Using transposon mutagenesis and a cosmid library, McCarter (32, 33) has recently identified two *V. parahaemolyticus* genes (*motX* and *motY*) encoding components of the polar flagellar motor. She has proposed that their gene products, MotX and MotY, each of which has a single membrane-spanning segment, are a channel component and a stator component, respectively. Surprisingly, they are not related to MotA and MotB.

We have already established a reliable procedure for electroporation of *Vibrio alginolyticus* (24), which is closely related to but is less pathogenic than *V. parahaemolyticus* and is well characterized in terms of respiration-coupled primary sodium pumps (47, 51). In this study, we cloned and characterized a *V. alginolyticus* gene coding for a component of the sodium-driven motor, using a mutant defective in polar flagellar motor rotation as a recipient for electroporation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. It should be noted that pSU38 and pSU41 have different multicloning sites. *V. alginolyticus* and *V. parahaemolyticus* cells were cultured at 30°C in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K₂HPO₄, 3% NaCl, 0.2% glucose) or VPG medium (1% polypeptone, 0.4% K₂HPO₄, 3% NaCl, 0.5% glycerol). For compact colony formation and swarm assays, a VC-1.5% agar plate and a VP (0.5% polypeptone, 0.4% K₂HPO₄, 3% NaCl)-0.3% agar plate, respectively, were used. When necessary, chloramphenicol and kanamycin were added at final concentrations of 1.25 and

* Corresponding author. Phone: 81-52-789-2993. Fax: 81-52-789-3001. Electronic mail address: i45406a@nucc.cc.nagoya-u.ac.jp.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description ^a	Reference or source
<i>V. alginolyticus</i> strains		
138-2	Wild type	52
VIK4	138-2 <i>rif</i> (Rif ^r Pof ⁺ Laf ⁺)	This work
VIO5	VIK4 <i>laf</i> (Pof ⁺ Laf ⁻)	This work
VIO542	VIO5 <i>motY</i> (Pom ⁻ Laf ⁻)	This work
<i>V. parahaemolyticus</i> strains		
BB22	Wild type	7
LM4170	<i>lafX313::lux motX118::mini-Mu lac</i> (Tet ^s)	33
LM4171	<i>lafX313::lux motY141::mini-Mu lac</i> (Tet ^s)	32
<i>E. coli</i> strains		
DH5 α	<i>recA1 hsdR17 endA1 supE44 thi-1 relA1 gyrA96 Δ(argF-lacZYA)U169 ϕ80dlacZΔM15</i>	18
XL1-Blue	<i>recA1 hsdR17 endA1 supE44 Δ(lac-proAB) (F'⁺::Tn10 proAB lacF^sZΔM15)</i>	Stratagene
S17-1	<i>recA hsdR thi pro ara RP-4-2-Tc::Mu-Km::Tn7 (Tp^r Sm^r)</i>	46
Plasmids		
pSU21	<i>cat</i> (Cm ^r) P _{lac} <i>lacZα</i>	6
pSU38	<i>kan</i> (Km ^r) P _{lac} <i>lacZα</i> (MCS different from that in pSU21)	6
pSU41	<i>kan</i> (Km ^r) P _{lac} <i>lacZα</i> (MCS same as that in pSU21)	6
pIO2	pSU21 20-kb <i>SalI</i> fragment (<i>motY</i> ⁺)	This work
pIO3	pSU21 0.4- and 6.0-kb <i>HindIII</i> fragments (<i>motY</i> ⁺)	This work
pIO5	pSU21 977-bp <i>HindIII-SphI</i> fragment (<i>motY</i> ⁺)	This work
pIO6	pSU38 1.0-kb <i>HindIII-XbaI</i> fragment (<i>motY</i> ⁺)	This work
pIO7	pSU41 1.0-kb <i>HindIII-XbaI</i> fragment (<i>motY</i> ⁺)	This work
pMMB206	IncQ <i>lacI^s Δbla cat</i> (Cm ^r) P _{lac-lac} <i>lacZα rmb</i>	37

^a Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; MCS, multicloning site; P_{lac}, *lac* promoter; P_{lac-lac}, tandemly located *lac* and *lac* promoters; Rif^r, rifampin resistant; Sm^r, streptomycin resistant; Tet^s, tetracycline sensitive; Tp^r, trimethoprim resistant.

100 μ g/ml, respectively. *E. coli* cells were cultured at 30°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or 2 \times YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl).

Isolation of Laf⁻ and Pom⁻ mutants. An overnight culture of *V. alginolyticus* cells was inoculated into 1.5 ml of VC medium (1:50 dilution) and cultured for 2 h at 30°C with vigorous shaking. Cells were harvested by centrifugation (5,000 \times g for 5 min) at room temperature and washed with 1 ml of ice-cold minimal AV medium [3% NaCl, 1.05% K₂HPO₄, 0.45% KH₂PO₄, 0.1% (NH₄)₂SO₄, 0.05% sodium citrate]. Cells were resuspended in 1 ml of minimal AV, and 15 μ l of ethyl methanesulfonate was added. The suspension was incubated at 30°C for 1 h with gentle shaking. Cells were washed twice with 1 ml of minimal AV medium and resuspended in 10 ml of VC medium. This suspension was divided into 10 test tubes and cultured overnight. About 5 μ l of each culture was spotted at the center of a VC-1.5% agar or VM-0.3% agar plate, and the center of the swarm was picked for single-colony isolation. Single colonies were inoculated on plates to test for swimming or swarming abilities.

Measurement of motility. A fresh overnight culture was inoculated into VPG medium (1:30 dilution) supplemented, if necessary, with 1.25 μ g of chloramphenicol per ml and cultured for 3 h. Cells were harvested and suspended in TNM buffer (50 mM Tris-HCl [pH 7.5], 5 mM glucose, 5 mM MgCl₂, 50 mM NaCl, 250 mM KCl) supplemented with 10 mM L-serine, a chemoattractant. Addition of 10 mM serine suppressed the directional change of swimming for at least 1 min. When the Na⁺ concentration in the medium was varied, KCl was added to keep the salt concentration constant. Swimming speed and motile fraction of the cells were measured by the photographic method described previously (20).

Electroporation. Transformation of *Vibrio* cells by electroporation was carried out as described previously (24). In brief, *Vibrio* cells harvested at late exponential to early stationary phase were subjected to osmotic shock to remove the periplasmic DNase and were washed thoroughly with 10 mM MgSO₄. Plasmids having the P15A replicon and the chloramphenicol or kanamycin resistance gene were used for vectors. Electroporation was performed with a Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo, Japan) at an electric field strength of between 5.0 and 7.5 kV/cm.

DNA sequencing and PCR. The nucleotide sequence was determined by the dideoxy-chain termination method, using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). [α -³²P]dCTP (3 mCi/mmol; Amersham Japan, Tokyo, Japan) was used for radioactive labeling.

Oligonucleotide primers for PCR were synthesized at the Center for Gene Research at Nagoya University. PCR amplification was carried out with the TaKaRa Taq kit (Takara Shuzo, Kyoto, Japan) and Thermal Cycler (Perkin-Elmer Japan, Chiba, Japan) as recommended by the distributors. Reactions were repeated for 25 cycles of 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C. The PCR products were analyzed by 1.7% agarose gel electrophoresis.

Conjugation. Aliquots (20 μ l each) of fresh overnight cultures of *E. coli* S17-1 cells carrying plasmid pMMB206 (donor) and VIO54 cells carrying plasmid pIO6 or pIO7 (recipient) were mixed on a VC-1.5% agar plate and incubated at 30°C overnight. Cells were scraped from the plate and suspended in 100 μ l of VC medium. To select transconjugants, the suspension was plated on a VC-1.5% agar plate supplemented with 1.25 μ g of chloramphenicol per ml and 100 μ g of kanamycin per ml. As a control, the cultures of the donor and recipient were plated separately on VC-1.5% agar plates containing the two antibiotics. Neither VIO542/pIO6, VIO542/pIO7, nor S17-1/pMMB206 cells grew on the selection plate.

Nucleotide sequence accession number. The sequence data reported in this paper have been deposited in the GenBank database.

RESULTS

Cloning of a *pom* gene required for polar flagellar rotation. We isolated *pom* (polar flagellar motility) mutants, which have paralyzed polar flagella, from strain VIO5, which is defective in lateral flagellar formation (Pof⁺ Laf⁻; Pof for polar flagellation and Laf for lateral flagellation). VIO5 cells were mutagenized with ethyl methanesulfonate, and 65 mutants that could not spread in a VP-0.3% agar plate were isolated. We observed these mutant cells by high-intensity dark-field microscopy and identified four *pom* mutants. One of them, strain VIO542, was used as a recipient for shotgun cloning.

Plasmid pSU21 and chromosomal DNA from *V. alginolyticus* 138-2 were digested with *EcoRI*, *SalI*, or *HindIII* and ligated. These DNA libraries were transferred into VIO542 cells by electroporation, and the cells were plated onto a VC-1.5% agar plate supplemented with chloramphenicol. Colonies were then inoculated on a VP-0.3% agar plate containing chloramphenicol. Two colonies that spread in the plates were isolated, and the plasmid DNA from these clones was recovered. They contained the same 20-kb *SalI* fragment. One of them was named pIO2 and used for further analysis. When plasmid pIO2 was introduced into VIO542 cells, the resulting cells spread in a VP-0.3% agar plate as fast as wild-type cells. Therefore, we concluded that the *SalI* fragment contains the *pom* gene that is defective in VIO542.

We then subcloned the *pom*⁺ DNA fragment. Plasmid pIO2 DNA was digested with *HindIII* and ligated. The ligation mixture was introduced into VIO542 cells, and 22 clones that spread in a VP-0.3% agar plate containing chloramphenicol were isolated. Three of the *pom*⁺ clones were found to carry plasmids containing two *HindIII* (5.6- and 0.4-kb) fragments. One of the plasmids was named pIO3 (Fig. 1A). It should be noted that the two *HindIII* fragments and the *HindIII-SalI* fragment may not be contiguous on the *Vibrio* chromosome. Various deletion derivatives were constructed from plasmid pIO3 (Fig. 1A). The complementation assays using the resulting plasmids located the *pom* gene in the 977-bp *HindIII-SphI* fragment (Fig. 1A and B). The plasmid containing the fragment was named pIO5.

VIO542 cells carrying plasmid pIO303 or pIO304 did not

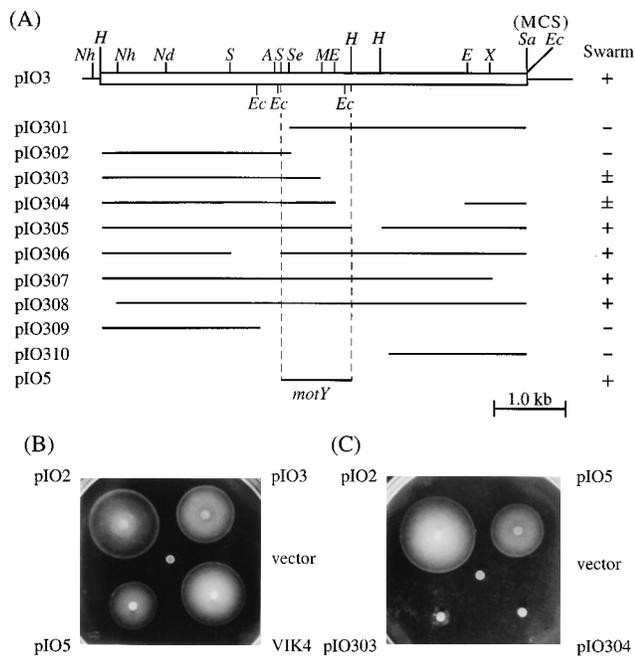


FIG. 1. Deletion mapping of the *pom* gene. (A) Restriction map of plasmid pIO3 and its deletion derivatives. The plasmids carry the fragments indicated by solid lines. Their abilities to complement the defect of strain VIO542 are indicated on the right. Note that the two *Hind*III fragments and the *Hind*III-*Sal*I fragment may not be contiguous on the *Vibrio* chromosome. Symbols: A, *Acc*I; E, *Eco*RV; Ec, *Eco*RI; H, *Hind*III; M, *Mlu*I; Nd, *Nde*I; Nh, *Nhe*I; S, *Sph*I; Sa, *Sal*I; Se, *Spe*I; X, *Xho*I; MCS, multicloning site in vector pSU21; +, swarmed in VP-0.3% agar plates; -, did not swarm; \pm , did not swarm initially but swarms appeared after prolonged incubation. (B and C) Swimming abilities of VIO542 cells carrying various plasmids. Overnight cultures were spotted on VP-0.3% agar plates containing chloramphenicol and incubated at 30°C.

swarm in a VP-0.3% agar plate, but tiny swarm colonies appeared after a few hours of incubation (Fig. 1C). The cells purified from these tiny swarm colonies spread in a VP-0.3% agar plate as fast as wild-type cells (data not shown). These results suggest that plasmids pIO303 and pIO304 do not have intact *pom* genes but have parts of it and that the functional *pom* gene was formed by homologous recombination between the mutant *pom* gene on chromosome and the parts of *pom* gene on the plasmids.

Effects of Na⁺ concentration and inhibitors on the swimming speed of VIO542 cells carrying the *pom*⁺ plasmid pIO5. To characterize the polar flagellar motor of strain VIO542 transformed with the *pom*⁺ plasmid, we examined the effects of sodium ion concentrations and specific inhibitors on the swimming speed of VIO542 cells carrying pIO5. VIO542/pIO5 cells did not swim in the absence of external Na⁺, and their average swimming speed increased as the Na⁺ concentration increased (Fig. 2A). There was no significant difference in the sodium dependency of swimming speed between VIO542/pIO5 and VIO5 (*Pom*⁺ *Laf*⁻) cells. Effects of benzamil (Fig. 2B) and of amiloride and phenamil (data not shown) were also examined. There was little difference, if any, in the effects of the inhibitors between the two strains. These results suggest that strain VIO542/pIO5 has a polar flagellar motor indistinguishable from that of strain VIO5 and that the *pom* gene on the multicopy plasmid pIO5 can function normally in *Vibrio* cells.

Nucleotide sequence of the *pom* gene. We determined the nucleotide sequence of the 977-bp *Hind*III-*Sph*I fragment.

Only one open reading frame of 293 amino acids was found in the region. This gene was named *motY* because the nucleotide sequence was found to be very similar to that of *V. parahaemolyticus motY* (see below). The hydropathy analysis (26) of MotY revealed that there is only one weakly hydrophobic stretch of 21 amino acids very close to the N terminus. This may constitute a membrane-spanning region. The C-terminal region of MotY shows similarities to the C-terminal regions of a number of membrane proteins, such as *E. coli* and *B. subtilis* MotB, *E. coli* OmpA, and *E. coli* Pal (Fig. 3). The C-terminal domains of these proteins are known or presumed to interact with the cell wall peptidoglycan (14, 27). Therefore, the C-terminal domain of MotY may also interact with peptidoglycan to anchor the force-generating components.

While this work was in progress, the nucleotide sequence of *V. parahaemolyticus motY* was published (32). The amino acid sequence of MotY was found to be strikingly similar (94.5% identity) to that of MotY (data not shown). Interestingly, the sequence similarity of the putative transmembrane region between the two proteins is relatively low (71.4% identity). To test whether *V. alginolyticus motY* complements the *motY* mutation of *V. parahaemolyticus*, plasmid pIO5 or the vector pSU21 was introduced into *V. parahaemolyticus* LM4171 (*motY*) and LM4170 (*motX*). LM4171 cells carrying pIO5 formed small swarm colonies in VC 0.3% agar, whereas LM4170/pIO5 cells

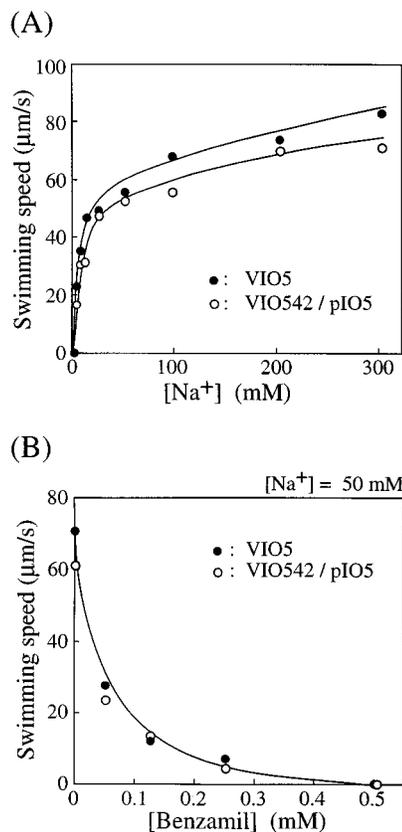


FIG. 2. Effects of sodium concentration and the inhibitor benzamil on swimming speed of VIO542 cells carrying plasmid pIO5. (A) Effect of sodium concentration. VIO542/pIO5 or VIO5 cells were grown at 30°C for 3 h in VPG medium supplemented with chloramphenicol when necessary. Cells were washed and resuspended in TNM buffer containing various concentrations of NaCl. The total salt concentration was kept constant by adjusting the KCl concentration. (B) Effect of benzamil. Cells were suspended in TNM medium containing 50 mM NaCl and 250 mM KCl. Swimming speeds were measured immediately after the addition of benzamil.

```

VAMOTY 199: KRLSQIADYIRHNQDIDLVLVATYTDSTDGKSASQSLSRRAESLRDYFQ
VPMOTY 199: KRLAQIADYVRHNQDIDLVLVATYTDSTDGKSESQSLSRRAESLRTYFE
EMOTB 181: PVLNGI----PNRISLSGHTDDFPYASGEKGYSNWELSADRANASRRELM
BMOTB 165: NLLVINP---PRNIISGHTDNMPIKNS-EFQSNWHLSVMRAVNFMGLLI
BORFB 150: VLLQTI---PNDIQVEGHTDSRNISTY-RYPSNWELSAARASGVIQYFT
EPAL 93: NFLRSNP---SYKVTVEGHADERGTPEY-----NISLGRRANAVKMYLQ
EOMPA 243: SQLSNLD-PKDGSVVVLGYTDRIGSDAY-----NQGLSRRAOSVVDYLI

VAMOTY 249: SLGLPEDRICQVQGYGKRRPIADNGSPIGK-----DKNRRVVISLG
VPMOTY 249: SLGLPEDRICQVQGYGKRRPIADNGTPIGK-----DKNRRVVISLG
EMOTB 227: VGLDSCKVLRVGMAATMRLSDRGPDD-----AVNRRISLVL
BMOTB 211: ENPKLDAKVFSAKGYGEYKPVASNKTAEGR-----SKNRRVEVLIL
BORFB 195: SKEKLPSKRFIAVGYADTKPVKDNKTNEHM-----KENRRVEIVIK
EPAL 135: GKGVSADQI-SIVSGKEKPAVLGHDEAAY-----SKNRRAVLVY-
EOMPA 282: SKGIPADKI-SARGMGESNPVTGNTCDNVKQRALIDCLAPDRRVETEVK

```

FIG. 3. Alignment of the C-terminal regions of MotY, MotY, MotB, OmpA, Pal, and OrfB. Residues identical or similar to those in *V. alginolyticus* MotY are indicated by boldface letters and enclosed by boxes. Abbreviations: VAMOTY, *V. alginolyticus* MotY; VPMOTY, *V. parahaemolyticus* MotY; EMOTB, *E. coli* MotB; BMOTB, *B. subtilis* MotB; BORFB, *B. subtilis* OrfB (a MotB-related protein but not a motor component); EPAL, *E. coli* peptidoglycan-associated protein (Pal); EOMPA, *E. coli* OmpA.

did not spread at all (data not shown). Microscopic observation revealed that LM4171/pIO5 cells swam by polar flagella with notably low tumbling frequency. Although the reason for this smooth-swimming bias is not clear, these results indicate that the *motY* genes of the two species are functionally exchangeable.

Comparison of the 5' upstream sequences of the *motY* coding regions of the two *Vibrio* species. The *V. alginolyticus* *motY* coding region starts with the ATG codon that is preceded by a sequence resembling the *E. coli* ribosome binding site (43) (Fig. 4). The *Hind*III-*Sph*I fragment contains only 70 bp of 5' upstream sequence of the *motY* coding region, within which we could not identify a possible promoter sequence. We therefore determined 648 bp of additional upstream sequence. The *V. alginolyticus* sequence (about 1.6 kb) matched well that of *V. parahaemolyticus*. However, the sequence that was proposed to be the σ^{28} -dependent promoter (TAATN₁₅GCGCTTGA) for *V. parahaemolyticus* *motY* (32) was not identified in the *motY* upstream region of *V. alginolyticus* (Fig. 4). The corresponding region (about 80 bp) seemed to be deleted. Instead, we found a sequence (TGGCGAN₅TTGCA) similar to the σ^{54} -dependent promoter consensus sequence (TGGCACN₅TTGCA) (38) in the -85 to -71 region (when the first residue of the putative initiation codon is numbered +1; Fig. 4). Also in *V. parahaemolyticus*, a similar sequence (TGGCGGN₅TTGCA) was found in the bp -84 to -70 region (Fig. 4).

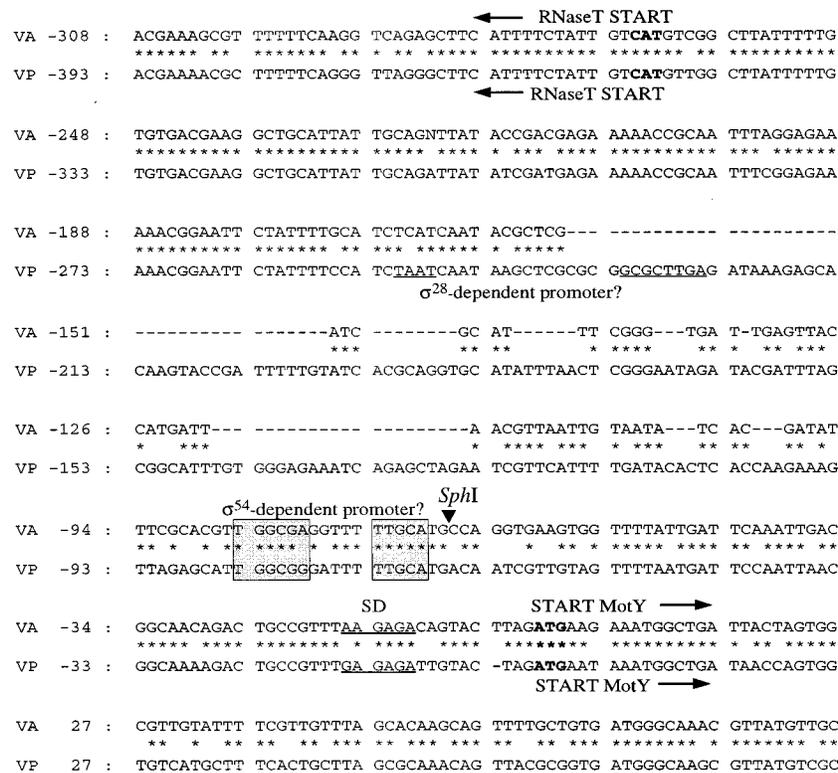
We examined the *motY* upstream region on the chromosomes of the two *Vibrio* species by using PCR. The two PCR primers were designed as shown in Fig. 5. PCR was performed with pIO3 DNA and genomic DNAs of *V. alginolyticus* wild-type strain 138-2 and *V. parahaemolyticus* wild-type strain BB22. The products from pIO3 and genomic DNA of strain 138-2 were the same size (about 820 bp; Fig. 5). This size agreed well with the size (823 bp) predicted from the nucleotide sequence. On the other hand, the product from BB22 DNA was about 920 bp (Fig. 5). Again, the size agreed with the predicted size (908 bp). These results indicate that the 80-bp deletion described above was not a cloning artifact and that *V. alginolyticus* *motY* does not have a sequence similar to that of the σ^{28} -dependent promoter.

PCR products from genomic DNAs of the *motY* mutant and the wild-type strains were the same size (data not shown), indicating that the *motY* mutation is a point mutation or small deletion. The complementation assays described above indi-

cated that a part of *motY* gene on pIO303 but not on pIO302 can recombine with the mutant *motY* on VIO542 chromosome to yield functional *motY* (Fig. 1A and C). Therefore, this mutation site is presumed to be in the 462-bp *Spe*I-*Mlu*I region.

We also attempted to identify MotY protein expressed from plasmids in *V. alginolyticus* and in *E. coli*. However, MotY could not be detected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining (data not shown). The amount of MotY may be too small for detection in this system.

Controlled expression of *motY* in *V. alginolyticus*. To control *motY* expression in *V. alginolyticus*, we constructed plasmid pIO6, in which *motY* is placed downstream of the *lac* promoter, by subcloning the 982-bp *Hind*III-*Xba*I fragment (containing *motY*⁺ *Hind*III-*Sph*I region) of plasmid pIO5 into vector pSU38 carrying the kanamycin resistance gene. As a control, plasmid pIO7, in which *motY* is in the opposite orientation, was constructed by subcloning the same fragment into vector pSU41. The relative positions of the unique *Hind*III and *Xba*I cloning sites of pSU41 are opposite those of pSU38. Strain VIO542 carrying plasmid pIO6 or pIO7 was conjugated with *E. coli* S17-1 carrying plasmid pMMB206 (resistant to chloramphenicol) which contained the *lacI*^q gene. Swimming abilities of the resulting strains, VIO542/pIO6/pMMB206 and VIO542/pIO7/pMMB206, were examined in VP-0.3% agar plates supplemented with chloramphenicol, kanamycin, and various concentrations of isopropyl- β -D-thiogalactopyranoside (IPTG) (Fig. 6). In the absence of IPTG, VIO542/pIO6/pMMB206 cells did not spread well from the position where they were inoculated. As the IPTG concentration increased, the cells spread better. The swimming ability was maximal in the presence of 10 μ M or higher IPTG. However, IPTG did not affect the swimming ability of VIO542/pIO7/pMMB206 cells. Swimming of this strain was rapid at all IPTG concentrations, owing to constitutive expression of *motY* from some promoter on vector pSU41. These results indicate that expression of the *motY* gene in *Vibrio* cells can be artificially controlled by using a *lac* promoter-repressor system. The presence or absence of 1 mM IPTG did not affect growth of VIO542/pIO6/pMMB206 cells significantly (data not shown), indicating that expression of the *motY* gene does not affect cell growth and that the difference in swimming ability described above did not result from the difference in growth rates.



σ²⁸ consensus sequence TAAA-N₁₅-GCCGATAA
 *
V. parahaemolyticus TAAT-N₁₅-GCGCTTGA

σ⁵⁴ consensus sequence TGGCAC-N₅-TTGCA
 *
V. alginolyticus TGGCGA-N₅-TTGCA
 *
V. parahaemolyticus TGGCGG-N₅-TTGCA

FIG. 4. Alignment of 5' upstream regions of *V. alginolyticus* (VA) and *V. parahaemolyticus* (VP) *motY*. The first nucleotide of the initiation codon of each *motY* is numbered +1. ATG and CAT in boldface indicate the initiation codons. Underlines indicate Shine-Dalgarno sequences and the *motY* promoter sequence proposed by McCarter (32). In both species, a gene oriented divergently to *motY* and encoding RNase T is located 5' upstream of *motY* (32). Sequences similar to the consensus sequence for σ⁵⁴-dependent promoters are indicated by boxes. Below the alignment, possible promoter sequences are listed together with the consensus sequences.

We next examined the effect of *motY* expression on free-swimming cells (data not shown). VIO542/pIO6/pMMB206 cells were cultured in the absence of IPTG, and 1 mM IPTG was added at late exponential phase. The motile fraction was monitored before and after IPTG induction. Cells did not swim at all in the absence of IPTG, suggesting that the recombination described above was negligible in this type of experiment. After induction, the motile fraction reached a maximum within 20 min, although many cells remain nonmotile for unknown reasons. At this time, essentially all motile cells swam at maximal speed. In contrast, when IPTG concentration was decreased, at least some fraction of cells was observed to swim at significantly slower speeds. However, we have not done a quantitative analysis in this regard because the relatively low motile fraction of the cells even after full induction of *motY* made it difficult.

DISCUSSION

Using a previously established electroporation protocol (24), we carried out shotgun cloning of a gene complementing a

paralyzed polar flagellum mutation and identified and characterized the gene *motY*, which codes for a putative stator component of the sodium-driven flagellar motor.

The nucleotide sequence of *V. alginolyticus motY* is very similar to that of *V. parahaemolyticus motY*. The 5' upstream regions are also similar, but there is a significant difference. A σ²⁸-dependent promoter sequence is suggested for the latter species (32), but the sequence is not conserved in the 5' upstream region of *motY* in *V. alginolyticus*: the corresponding region is deleted. We demonstrated by PCR that this deletion is not a cloning artifact (Fig. 5). Instead, we found a sequence (TGGCGAN₅TTGCA) that is very similar to the proposed consensus sequence (TGGCACN₅TTGCA) for σ⁵⁴-dependent promoter (38) (Fig. 4). A very similar sequence is found in *V. parahaemolyticus motY* (Fig. 5). Within the consensus, a GG motif is invariant and a GC motif is almost completely conserved in the σ⁵⁴-dependent promoter family (35). Both motifs are perfectly conserved in sequences upstream of *motY* in both species. Furthermore, the *V. alginolyticus* gene (*rpoN*) encoding σ⁵⁴ has recently been cloned as a gene essential for polar

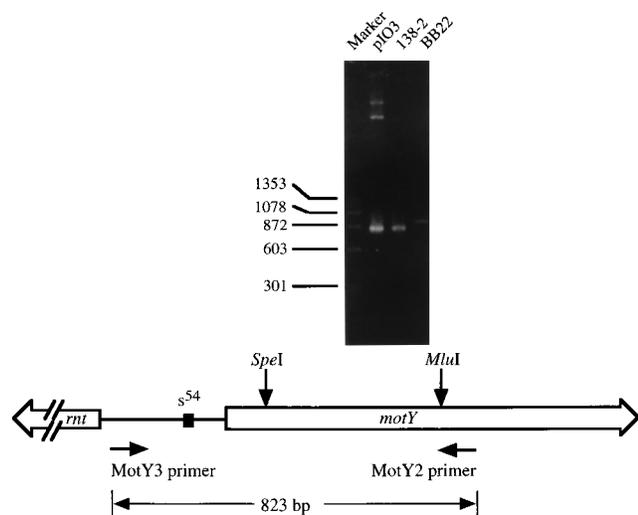


FIG. 5. PCR amplification of the 5' upstream regions of *V. alginolyticus* and *V. parahaemolyticus* *motY* from chromosomal DNAs. PCR primers MotY2 (dA CCTGTCTTCGTAGTGCAAAATCG) and MotY3 (dTGTGTGACGAAG GCTGC) were designed as indicated. This primer pair is presumed to amplify the region containing the proposed promoter sequences and *SpeI* and *MluI* restriction sites. The gene *rnt* encodes a polypeptide similar to *E. coli* RNase T (32; see also Fig. 4). Lanes: Marker, ϕ X174 DNA digested with *HaeIII*; pIO3, PCR products from plasmid pIO3; 138-2, PCR products from chromosomal DNA of *V. alginolyticus* wild-type strain 138-2; BB22, PCR products from chromosomal DNA of *V. parahaemolyticus* wild-type strain BB22.

flagellar synthesis (23a). From the available data, we suggest that *motY* is transcribed from σ^{54} -dependent promoters in both species. In several other polarly flagellated bacteria, such as *Caulobacter crescentus* and *Pseudomonas aeruginosa*, some flagellar genes are transcribed from σ^{54} -dependent promoters (13, 39, 49, 53).

As demonstrated for *V. parahaemolyticus* MotB by McCarter (32), the amino acid sequence of the carboxyl-terminal region of *V. alginolyticus* MotY (about 60 amino acids in length) shows remarkable similarity to sequences of a number of outer membrane proteins known to associate with peptidoglycan, including a major outer membrane protein OmpA and peptidoglycan-associated lipoprotein Pal. The C-terminal portions of OmpA and Pal are believed to be located in the periplasm to interact with peptidoglycan (14, 27). As discussed by De Mont and Vanderleyden (17), the *motB* products of *E. coli* (a

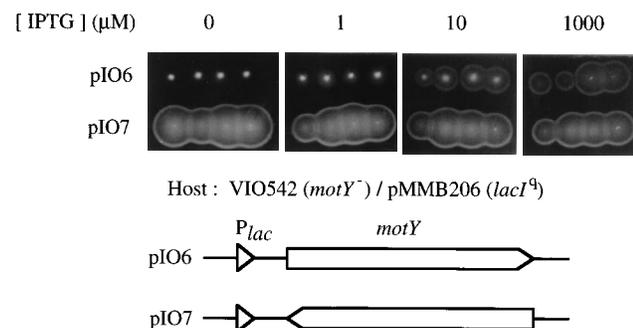


FIG. 6. Controlled expression of *motY* in *V. alginolyticus*. VIO542/pIO6/pMMB206 and VIO542/pIO7/pMMB206 colonies were picked and inoculated onto VP-0.3% agar plates containing chloramphenicol, kanamycin, and various concentrations of IPTG. The plates were incubated at 30°C for 5 h. Orientation of *motY* relative to the *lacUV5* promoter is indicated below the photographs.

gram-negative bacterium) and *B. subtilis* (a gram-positive bacterium), the former of which has been shown to span the cytoplasmic membrane once in the region close to its N terminus and the C-terminal domain is located in the periplasmic space (15), have features in common with this C-terminal domain. The positively charged arginine residues in this domain are postulated to be involved in noncovalent linkage of Pal with peptidoglycan (27) and are essential for MotB function in *E. coli* (11). As in MotB (17), almost all of these residues are conserved in MotY. Therefore, MotY is likely to interact with peptidoglycan to anchor the sodium-driven flagellar motor.

McCarter (33) has identified *motX* encoding another component of the *V. parahaemolyticus* polar flagellar motor that has a single putative transmembrane segment and suggested that MotX forms a sodium channel because expression of *motX* in *E. coli* resulted in growth retardation and this inhibitory effect was reversed by amiloride. We identified by PCR a *motX*-related gene in *V. alginolyticus* (unpublished results).

Why are MotX and MotY so different from MotA and MotB? Difference of the coupling ions may be one reason. However, for example, sodium-translocating F_0F_1 -ATP synthetase of *Propionigenium modestum* is related to the proton-translocating enzyme of *E. coli* (2, 22). Therefore, we may also have to account for the facts that the polar flagellum of *Vibrio* cells is covered by a sheath (1) and that its rotation is very fast (28, 40). These motor components might be evolved to meet with a large mechanical force required for rotation. In this regard, it is interesting that MotX and MotY are similar in membrane topology and have identical four-residue sequences at the corresponding positions (33). These residues are perfectly conserved in MotY.

As an initial step for further investigations of these motor components, we succeeded in regulating *motY* gene expression in *V. alginolyticus* by using a *lac* promoter-repressor system (to our knowledge, this is the first case in which expression of any gene is controlled artificially in marine *Vibrio* cells). Swimming ability increased with increasing concentrations of the inducer IPTG, and swimming fraction increased after addition of IPTG. Using this system, we observed that some cells swam with suboptimal speeds at lower concentrations of IPTG. This finding is consistent with the prediction that MotY may be a constituent of the force-generating unit. To clarify this, it will be necessary to examine in detail the relation between *motY* expression and torque generation. It will be interesting to see whether the rotation rate of a single motor, which can be monitored by a tethered cell method (45) or by laser dark-field microscopy (25, 40), increases stepwise when increasing numbers of MotY subunits are incorporated into a motor, as observed for *E. coli* MotA and MotB (9, 12).

ACKNOWLEDGMENTS

We thank L. McCarter and V. M. Morales for kindly providing strains and plasmids and S. Kudo for helpful discussion. We especially thank R. M. Macnab for critically reading the manuscript.

This work was supported in part by grants-in-aid for scientific research to I.K. from the Ishida Foundation and to I.K. and M.H. from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Allen, R. D., and P. Baumann. 1971. Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. *J. Bacteriol.* **107**:295-302.
- Amann, R., W. Ludwig, W. Laubinger, P. Dimroth, and K. H. Schleifer. 1988. Cloning and sequencing of the gene encoding the beta subunit of the sodium ion translocating ATP synthetase of *Propionigenium modestum*. *FEMS Microbiol. Lett.* **65**:253-260.
- Atsumi, T., Y. Maekawa, H. Tokuda, and Y. Imae. 1992. Amiloride at pH 7.0 inhibits the Na⁺-driven flagellar motors of *Vibrio alginolyticus* but allows cell

- growth. FEBS Lett. **314**:114–116.
4. Atsumi, T., L. McCarter, and Y. Imae. 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. Nature (London) **355**:182–184.
 5. Atsumi, T., S. Sugiyama, E. J. Cragoe, Jr., and Y. Imae. 1990. Specific inhibition of the Na⁺-driven flagellar motors of alkalophilic *Bacillus* strains by the amiloride analog phenamil. J. Bacteriol. **172**:1634–1639.
 6. Bartolomé, B., Y. Jubete, E. Martínez, and F. D. Cruz. 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene **102**:75–78.
 7. Belas, R., M. Simon, and M. Silverman. 1986. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. J. Bacteriol. **167**:210–218.
 8. Blair, D. F. 1990. The bacterial flagellar motor. Semin. Cell. Biol. **1**:75–85.
 9. Blair, D. F., and H. C. Berg. 1988. Restoration of torque in defective flagellar motors. Science **242**:1678–1681.
 10. Blair, D. F., and H. C. Berg. 1990. The MotA protein of *E. coli* is a proton-conducting component of the flagellar motor. Cell **60**:439–449.
 11. Blair, D. F., D. Y. Kim, and H. C. Berg. 1991. Mutant MotB proteins in *Escherichia coli*. J. Bacteriol. **173**:4049–4055.
 12. Block, S. M., and H. C. Berg. 1984. Successive incorporation of force-generating units in the bacterial rotary motor. Nature (London) **309**:470–472.
 13. Brun, Y. V., and L. Shapiro. 1992. A temporally controlled σ -factor is required for polar morphogenesis and normal cell division in *Caulobacter*. Genes Dev. **6**:2395–2408.
 14. Chen, R., W. Schmidmayr, C. Kramer, U. Chen-Schmeisser, and U. Henning. 1980. Primary structure of major outer membrane protein II (OmpA protein) of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **77**:4592–4596.
 15. Chun, S. Y., and J. S. Parkinson. 1988. Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. Science **239**:276–278.
 16. 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.
 17. De Mont, R., and J. Vanderleyden. 1994. The C-terminal sequence conservation between OmpA-related outer membrane protein 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.
 18. Grant, S. N., J. Jessee, F. R. Bloom, and D. Hanahan. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87**:4645–4649.
 19. Imae, Y., and T. Atsumi. 1989. Na⁺-driven bacterial flagellar motors. J. Bioenerg. Biomembr. **21**:705–716.
 20. Imae, Y., H. Matsukura, and S. Kobayasi. 1986. Sodium-driven flagellar motors of alkalophilic *Bacillus*. Methods Enzymol. **125**:582–592.
 21. Jones, C. J., and S.-I. Aizawa. 1991. The bacterial flagellum and flagellar motor: structure, assembly and function. Adv. Microb. Physiol. **32**:109–172.
 22. Kaim, G., W. Ludwig, P. Dimroth, and K. H. Schleifer. 1992. Cloning, sequencing and *in vivo* expression of genes encoding the F₀ part of the sodium-ion-dependent ATP synthetase of *Propionigenium modestum* in *Escherichia coli*. Eur. J. Biochem. **207**:463–470.
 23. Kawagishi, I., Y. Maekawa, T. Atsumi, M. Homma, and Y. Imae. 1995. Isolation of the polar and lateral flagellum-defective mutants in *Vibrio alginolyticus* and identification of their flagellar driving energy sources. J. Bacteriol. **177**:5158–5160.
 - 23a. Kawagishi, I., M. Nakada, and M. Homma. Unpublished results.
 24. Kawagishi, I., I. Okunishi, M. Homma, and Y. Imae. 1994. Removal of the periplasmic DNase before electroporation enhances efficiency of transformation in the marine bacterium *Vibrio alginolyticus*. Microbiology **140**:2355–2361.
 25. Kudo, S., Y. Magariyama, and S.-I. Aizawa. 1990. Abrupt changes in flagellar rotation observed by laser dark-field microscopy. Nature (London) **346**:677–680.
 26. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157**:105–132.
 27. Lazzaroni, J. C., and R. Portaliere. 1992. The *excC* gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). Mol. Microbiol. **6**:735–742.
 28. Magariyama, Y., S. Sugiyama, K. Muramoto, Y. Maekawa, I. Kawagishi, Y. Imae, and S. Kudo. 1994. Very fast flagellar rotation. Nature (London) **371**:752.
 29. Manson, M. D. 1992. Bacterial motility and chemotaxis. Adv. Microb. Physiol. **33**:277–346.
 30. McCarter, L., M. Hilmen, and M. Silverman. 1988. Flagellar dynamometer controls swarmer cell differentiation of *Vibrio parahaemolyticus*. Cell **54**:345–351.
 31. McCarter, L., and M. Silverman. 1990. Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus*. Mol. Microbiol. **4**:1057–1062.
 32. McCarter, L. L. 1994. MotY, a component of the sodium-type flagellar motor. J. Bacteriol. **176**:4219–4225.
 33. McCarter, L. L. 1994. MotX, a channel component of the sodium-type flagellar motor. J. Bacteriol. **176**:5988–5998.
 34. McCarter, L. L., and M. E. Wright. 1993. Identification of genes encoding components of swarmer cell flagellar motor and propeller and a sigma factor controlling differentiation of *Vibrio parahaemolyticus*. J. Bacteriol. **175**:3361–3371.
 35. Merrick, M. J. 1993. In a class of its own—the RNA polymerase sigma factor σ^{54} (σ^N). Mol. Microbiol. **10**:903–909.
 36. Mirel, D. B., V. M. Lustre, and M. J. Chamberlin. 1992. An operon of *Bacillus subtilis* motility genes transcribed by the σ^D form of RNA polymerase. J. Bacteriol. **174**:4197–4204.
 37. Molares, V. M., A. Bäckman, and M. Bagdassarian. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97**:39–47.
 38. Morett, E., and M. Buck. 1989. *In vivo* studies on the interaction of RNA polymerase- σ^{54} with the *Klebsiella pneumoniae* and *Rhizobium meliloti* *nifH* promoters. J. Mol. Biol. **210**:65–77.
 39. Munnich, S. A., and A. Newton. 1987. Promoter mapping and cell cycle regulation of flagellin gene transcription in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **84**:1142–1146.
 40. Muramoto, K., I. Kawagishi, S. Kudo, Y. Magariyama, Y. Imae, and M. Homma. 1995. High-speed rotation and speed stability of the sodium-driven flagellar motor in *Vibrio alginolyticus*. J. Mol. Biol. **251**:50–58.
 41. 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.
 42. Schuster, S. C., and S. Khan. 1994. The bacterial flagellar motor. Annu. Rev. Biophys. Biomol. Struct. **23**:509–539.
 43. Shine, J., and L. Dalgarno. 1974. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
 44. Shinoda, S., and K. Okamoto. 1977. Formation and function of *Vibrio parahaemolyticus* lateral flagella. J. Bacteriol. **129**:1266–1271.
 45. Silverman, M., and M. Simon. 1974. Flagellar rotation and the mechanism of bacterial motility. Nature (London) **249**:73–74.
 46. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1**:784–791.
 47. Skulachev, V. P. 1989. Bacterial Na⁺ energetics. FEBS Lett. **250**:106–114.
 48. 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.
 49. Starnbach, M. N., and S. Lory. 1992. The *flaA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis. Mol. Microbiol. **6**:459–469.
 50. Sugiyama, S., E. J. Cragoe, Jr., and Y. Imae. 1988. Amiloride, a specific inhibitor for the Na⁺-driven flagellar motors of alkalophilic *Bacillus*. J. Biol. Chem. **263**:8215–8219.
 51. Tokuda, H. 1989. Respiratory Na⁺ pump and Na⁺-dependent energetics in *Vibrio alginolyticus*. J. Bioenerg. Biomembr. **21**:693–704.
 52. Tokuda, H., T. Nakamura, and T. Unemoto. 1981. Potassium ion is required for the generation of pH-dependent membrane potential and $\Delta\mu\text{H}$ by the marine bacterium *Vibrio alginolyticus*. Biochemistry **20**:4198–4203.
 53. Totten, P. A., and S. Lory. 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172**:389–396.