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MotX and MotY Are Required for Flagellar Rotation in *Shewanella oneidensis* MR-1▽†

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The single polar flagellum of *Shewanella oneidensis* MR-1 is powered by two different stator complexes, the sodium-dependent PomAB and the proton-driven MotAB. In addition, *Shewanella* harbors two genes with homology to *motX* and *motY* of *Vibrio* species. In *Vibrio*, the products of these genes are crucial for sodium-dependent flagellar rotation. Resequencing of *S. oneidensis* MR-1 *motY* revealed that the gene does not harbor an authentic frameshift as was originally reported. Mutational analysis demonstrated that both MotX and MotY are critical for flagellar rotation of *S. oneidensis* MR-1 for both sodium- and proton-dependent stator systems but do not affect assembly of the flagellar filament. Fluorescence tagging of MotX and MotY to mCherry revealed that both proteins localize to the flagellated cell pole depending on the presence of the basal flagellar structure. Functional localization of MotX requires MotY, whereas MotY localizes independently of MotX. In contrast to the case in *Vibrio*, neither protein is crucial for the recruitment of the PomAB or MotAB stator complexes to the flagellated cell pole, nor do they play a major role in the stator selection process. Thus, MotX and MotY are not exclusive features of sodium-dependent flagellar systems. Furthermore, MotX and MotY in *Shewanella*, and possibly also in other genera, must have functions beyond the recruitment of the stator complexes.

Flagellum-mediated swimming motility is a widespread means of locomotion among bacteria. Flagella consist of protein filaments that are rotated at the filament's base by a membrane-embedded motor (3, 39). Rotation is powered by electrochemical gradients across the cytoplasmic membrane. Thus far, two coupling ions, sodium ions and protons, have been described as energy sources for bacterial flagellar motors (4, 24, 48). Two major components confer the conversion of the ion flux into rotary motion. The first component forms a rotor-mounted ring-like structure at the base of the flagellar basal body and is referred to as the switch complex or the C ring; it is composed of the proteins FliG, FliM, and FliN. The second major component is the stator system, consisting of membrane-embedded stator complexes that surround the C ring (3). Each stator complex is composed of two subunits in a 4:2 stoichiometry. In *Escherichia coli*, MotA and MotB constitute the stator complex by forming a proton-specific ion channel; the Na^+-dependent counterpart in *Vibrio* species consists of the orthologs PomA and PomB (1, 5, 49). MotA and PomA both have four transmembrane domains and are thought to interact with FliG via a cytoplasmic segment to generate torque (2, 50). Stator function is presumably made possible by a peptidoglycan-binding motif located at the C-terminal portion of MotB and PomB that anchors the stator complex to the cell wall (1, 8). In *E. coli*, at least 11 stator complexes can be synchronously involved in driving flagellar rotation (35). However, a single complex is sufficient for rotation of the filament (36, 40). Despite its tight attachment to the peptidoglycan, the stator ring system was found to form a surprisingly dynamic complex. It has been suggested that inactive precomplexes of the stators form a membrane-located pool before being activated upon incorporation into the stator ring system around the motor (13, 45). In *E. coli*, the turnover time of stator complexes can be as short as 30 s (21).

In *Vibrio* species, two auxiliary proteins, designated MotX and MotY, are required for motor function of the Na^+-driven polar flagellar system (22, 23, 28, 31). Recently, it was shown that the proteins associate with the flagellar basal body in *Vibrio alginolyticus* to form an additional structure, the T ring (42). MotX interacts with MotY and the PomAB stator complexes, and both proteins are thought to be crucial for the acquisition of the stators to the motor of the polar flagellum. (29, 30, 42). A MotY homolog is also associated with the proton-dependent motor system of the lateral flagella of *V. alginolyticus* that is induced under conditions of elevated viscosity (41).

We recently showed that *Shewanella oneidensis* MR-1 uses two different stator systems to drive the rotation of its single polar flagellum, the Na^+-dependent PomAB stator and the proton-driven MotAB stator. As suggested by genetic data, the MotAB stator has been acquired by lateral gene transfer, presumably in the process of adaptation from a marine to a freshwater environment (32). The two different stators are recruited to the motor in a way that depends on the sodium ion concentration in the medium. The Na^+-dependent PomAB stator is present at the flagellated cell pole regardless of the sodium ion concentration, whereas the proton-dependent MotAB stator functionally localizes only under conditions of low sodium or in

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the absence of PomAB. It is still unclear how stator selection is achieved and whether additional proteins play a role in this process.

Orthologs of *motX* and *motY* have been annotated in *S. oneidensis* MR-1. We thus hypothesized that MotX and MotY might play a role in stator selection in *S. oneidensis* MR-1. However, the originally published sequence of *motY* harbors a frameshift that would result in a drastically truncated protein lacking a functionally relevant putative peptidoglycan-binding domain at its C terminus (16, 18). This situation seemed inconsistent with a role for MotY in *S. oneidensis* MR-1.

Here we describe a functional analysis of the MotX and MotY orthologs in *S. oneidensis* MR-1. We found that *motY* does not, in fact, contain a frameshift mutation, so that MotY is translated in its full-length form. Both MotX and MotY were essential for Na^+-dependent and proton-dependent motility. Therefore, these proteins have a role in *S. oneidensis* MR-1 that differs from their function in *Vibrio* species. We also used fusions to the fluorescent protein mCherry for functional localization studies of MotX and MotY.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and media. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium at 37°C. For strain WM3064, 2,6-diaminopimelic acid (DAP) was added to a final concentration of 300 μM. *S. oneidensis* strains were cultivated in LB or LM (32) medium as indicated. When necessary, media were solidified using 1.5% (wt/vol) agar and/or supplemented with 10 $\mu g \cdot ml^{-1}$ chloramphenicol, 10 $\mu g \cdot ml^{-1}$ gentamicin, and/or 25 $\mu g \cdot ml^{-1}$ kanamycin. Protein localization depending on sodium concentration was carried out in LM medium with the indicated amount of sodium chloride added. To obtain a similar ionic strength in the medium, KCl was added to yield an overall concentration of 100 mM.

Strain constructions. DNA manipulations were carried out according to standard techniques (37). Kits for isolation of chromosomal DNA, isolation of plasmids, and purification of PCR products were purchased from Qiagen (Hilden, Germany), Sigma Aldrich GmbH (Taufkirchen, Germany), and/or HISS Diagnostics GmbH (Freiburg, Germany). Enzymes were obtained from New England Biolabs (Frankfurt, Germany) and Fermentas (St. Leon-Rot, Germany). Sequencing was conducted using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Darmstadt, Germany), and the reactions were then analyzed on a 3130 genetic analyzer (Applied Biosystems, Darmstadt, Germany).

For resequencing of *motY*, the gene region was amplified from chromosomal DNA and, after treatment with BamHI and XhoI, cloned into the vector pBC SK^+. Five independent clones were sequenced.

In-frame deletion mutations of *motX* and *motY* in *S. oneidensis* MR-1 strains were constructed as described previously (43, 44). DNA fragments that encompassed only short terminal 5′ and 3′ sections of the target genes were amplified by PCR using corresponding primer pairs (see Table S1 in the supplemental material). After purification and treatment with appropriate restriction enzymes, the fragments were ligated to yield an in-frame fusion product encoding a drastically truncated gene product. Subsequently, the fusion product was amplified using the outer primers, digested with appropriate enzymes, and ligated into suicide vector pGP704-Sac28Km. The resulting plasmid was introduced into the corresponding *S. oneidensis* MR-1 strain by conjugative mating using *E. coli* WM3064 as a donor on LB medium containing DAP. Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking DAP. Single colonies were grown overnight in liquid LB without antibiotics and plated on LB containing 10% (wt/vol) sucrose to screen for plasmid excision by double-crossover events. Kanamycin-sensitive colonies were then checked for the targeted deletion by colony PCR using primers bracketing the location of the deletion.

To complement the deletion mutations in *motX* and *motY*, the gene regions were amplified from chromosomal DNA, digested with BamHI and EcoRI, cloned into pBBR1-MCS5, and introduced into *S. oneidensis* MR-1 by electroporation (27).

For C-terminal tagging of proteins to mCherry, the *mCherry* gene was amplified and cloned into the suicide vector pJP5603 after digestion with BamHI and SalI to yield plasmid pJP5603-mCherry. A derivative of *mCherry* that was adapted to the codon usage of *S. oneidensis* MR-1 was used. DNA fragments encoding the C termini of MotX and MotY were amplified and ligated into the EcoRI/BamHI site of pJP5603-mCherry to yield a functional in-frame fusion in which the corresponding target protein is linked to mCherry by a Gly-Ser-Gly-Gly-Gly linker peptide. The resulting constructs were conjugated into *S. oneidensis* MR-1 from *E. coli* WM3064, and kanamycin-resistant colonies were checked for the correct insertion by PCR.

Motility assays. Rapid motility screening was carried out by spotting 3 μl of a liquid culture of the corresponding strain on plates that contained LB medium with an agar concentration of 0.25% (wt/vol). Although these plates are commonly referred to as "swarming plates," the bacterial movement that was actually scored was flagellum-mediated swimming motility. Strains to be directly compared were always spotted onto the same individual plate. Motility was scored after overnight incubation at 30°C.

To monitor swimming motility in liquid media, cell material was directly transferred from fresh overnight LB plates to LB or LM medium to yield an optical density at 600 nm of 0.2 to 0.4 and incubated for 30 min at room temperature. Swimming motility was then determined by light microscopy.

Fluorescence microscopy. Prior to microscopy, cells were immobilized on pads composed of LM medium solidified with 1% agarose. Microscopy was performed with an upright Zeiss Image MI (Oberkochen, Germany) equipped with Cascade 1K camera (Visitron Systems, Puchheim, Germany) and a Zeiss Plan Apochromat 100×/1.4 differential interference contrast (DIC) objective. Image processing was carried out using Metamorph 7.1.2, Adobe Photoshop CS2, and Adobe Illustrator CS2. When polar localization was determined, at least 250 cells per data point were observed.

Flagellum staining. The flagellation state was visualized by the silver impregnation method, which was carried out essentially as described earlier (6). For colocalization of mCherry-tagged stator subunits with the flagellum, the flagellar filaments were visualized by fluorescence labeling using Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen) as previously reported (32). Fluorescence microscopy was carried out as described above. Visualization of the flagellar filament was enhanced by using the "sharpen" function of the Metamorph software.

Immunoblot analysis. To obtain protein lysates for determining the stability of the MotX-mCherry and MotY-mCherry fusions, cells corresponding to an optical density at 600 nm of 0.25 from exponentially growing LB cultures were harvested by centrifugation. The sediments were resuspended in 25 μl sample buffer (20), heated at 99°C for 5 min, and stored at −20°C. Ten microliters of the sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Subsequently, proteins were transferred to polyvinylidene difluoride membranes by semidry transfer. For detection of the proteins, polyclonal antibodies raised against dsRed (Clontech) were used at a dilution of 1:500. Secondary anti-rabbit immunoglobulin G–horseradish peroxidase antibody was used at a dilution of 1:20,000, and signals were detected using the Western Lightning chemiluminescence reagent (Perkin-Elmer LAS, Inc.) followed by exposure to autoradiography film.

RESULTS

***S. oneidensis* MR-1 possesses *motX* and *motY* orthologs.** The *S. oneidensis* MR-1 genome sequence (16) contains two open reading frames that were annotated as *motX* (SO_3936) and *motY* (SO_2754) based on homologies to the corresponding genes in *Vibrio* species. The deduced amino acid sequence of *motX* exhibits 40% identity and 59% similarity to that of MotX of *V. alginolyticus*. As in *Vibrio*, the convergently transcribed gene upstream of *motX* encodes a protein suggested to be involved in purine ribonucleotide synthesis. MotX is predicted to have a molecular mass of 23.2 kDa and to harbor three Sel1 domains, which are thought to mediate protein-protein interactions (15, 26). Sequence analysis using SignalP (12) strongly indicates that MotX has a signal peptide that should be cleaved between amino acids 19 and 20.

The original sequence of *motY* in *S. oneidensis* MR-1 included a frameshift that would create a truncated protein likely to be nonfunctional (16). In order to verify the correctness of

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source or reference
Escherichia coli strains		
DH5α-λpir	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U196 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*	25
WM3064	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA1341*::[erm pir(wt)]	W. Metcalf, University of Illinois, Urbana-Champaign
Shewanella oneidensis strains		
MR-1	Wild type	46
S208	MR-1 Δ*fliF*	This work
S182	MR-1 Δ*motX*	This work
S257	MR-1 Δ*motY*	This work
S256	MR-1 Δ*motX* Δ*motY*	This work
S258	MR-1 Δ*motX* Δ*pomAB*	This work
S255	MR-1 Δ*motY* Δ*pomAB*	This work
S1099	MR-1 Δ*motX* Δ*motAB*	This work
S1100	MR-1 Δ*motY* Δ*motAB*	This work
S305	MR-1 Δ*motX* pBBR-motX, complementation of Δ*motX*, Gm^r	This work
S323	MR-1 Δ*motY* pBBR-motY, complementation of Δ*motY*, Gm^r	This work
S292	MR-1 *motX-mCherry*, C-terminal fusion of *motX* to *mCherry*, Km^r	This work
S293	MR-1 *motY-mCherry*, C-terminal fusion of *motY* to *mCherry*, Km^r	This work
S296	MR-1 Δ*fliF motX-mCherry*, Km^r	This work
S297	MR-1 Δ*fliF motY-mCherry*, Km^r	This work
S295	MR-1 Δ*motX motY-mCherry*, Km^r	This work
S294	MR-1 Δ*motY motX-mCherry*, Km^r	This work
S166	MR-1 *pomB-mCherry*, C-terminal fusion of *pomB* to *mCherry*, Km^r	32
S265	MR-1 Δ*motX pomB-mCherry*, Km^r	This work
S267	MR-1 Δ*motY pomB-mCherry*, Km^r	This work
S266	MR-1 Δ*motXY pomB-mCherry*, Km^r	This work
S1150	MR-1 Δ*motAB* Δ*motXY pomB-mCherry*, Km^r	This work
S165	MR-1 *motB-mCherry*, C-terminal fusion of *motB* to *mCherry*, Km^r	32
S268	MR-1 Δ*pomAB* Δ*motX motB-mCherry*, Km^r	This work
S269	MR-1 Δ*pomAB* Δ*motY motB-mCherry*, Km^r	This work
S291	MR-1 Δ*motX* Δ*motY motB-mCherry*, Km^r	This work
S1151	MR-1 Δ*motY motB-mCherry*, Km^r	This work
S1152	MR-1 Δ*motX motB-mCherry*, Km^r	This work
S1091	MR-1 Δ*pomAB* Δ*motAB motX-mCherry*, Km^r	This work
S1093	MR-1 Δ*pomAB* Δ*motAB motY-mCherry*, Km^r	This work
Plasmids		
pBC SK+	pUC origin, P_{tac} *lacZ*α, Cm^r	Stratagene
pBBR1-MCS5	*ori* pBBR *oriT*. broad-host-range vector, P_{lac}, Gm^r	19
pGP704Sac28Km	*mob*$RP4^+$ *ori*-R6K *sacB*, suicide plasmid for in frame deletions, Km^r	44
pJP5603	*mob*$RP4^+$ *ori*-R6K, suicide plasmid for mutation by plasmid integration, Km^r	33
pBBR-motX	*motX* in pBBR1-MCS5	This work
pBBR-motY	*motY* in pBBR1-MCS5	This work
pGP704Sac28Km-fliF	*fliF* in-frame deletion fragment in pGP704Sac28-Km, Km^r	This work
pGP704Sac28Km-motX	*motX* in-frame deletion fragment in pGP704Sac28-Km, Km^r	This work
pGP704Sac28Km-motY	*motY* in-frame deletion fragment in pGP704Sac28-Km, Km^r	This work
pJP5603-mCherry	Promoterless *mCherry* in pJP5603, Km^r	This work
pJP5603-motX::mCherry	C-terminal fusion of *motX* to *mCherry* in pJP5603, Km^r	This work
pJP5603-motY::mCherry	C-terminal fusion of *motY* to *mCherry* in pJP5603, Km^r	This work
pJP5603-motB::mCherry	C-terminal fusion of *motB* to *mCherry* in pJP5603, Km^r	32
pJP5603-pomB::mCherry	C-terminal fusion of *pomB* to *mCherry* in pJP5603, Km^r	32

[a] Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

that sequence, chromosomal DNA was prepared from an *S. oneidensis* MR-1 culture exhibiting good swimming motility as determined by light microscopy prior to cell harvest. The gene region of *motY* was amplified and cloned. The sequences obtained from five independent clones revealed that the originally published sequence contains an insertion of an additional adenosine at position 420, leading to two consecutive stop codons and early termination of translation. However, the open reading frame of *motY* amplified from motile *S. oneidensis* MR-1 cells encompasses 871 bp, encoding a protein of 289 amino acids with a calculated molecular mass of 33.0 kDa. All further characterizations carried out in this study will refer to that full-length MotY. Similar to the case for MotX, MotY was predicted to harbor a signal peptide with a cleavage site between amino acids 19 and 20. The protein exhibits 44% identity and 66% similarity to MotY of *V. alginolyticus* and is predicted to have a putative peptidoglycan-binding domain at the C terminus. As in *Vibrio* species, the divergently transcribed open

reading frame upstream of *motY* encodes a gene product likely to be involved in RNA processing.

Both *motX* and *motY* are highly conserved in all *Shewanella* species that have been sequenced. Recently, σ^{28}-dependent promoters have been predicted to be located upstream of *motX* and *motY* (38), suggesting that both are part of the flagellar regulatory network in *Shewanella*.

Both MotX and MotY are essential for swimming motility of *S. oneidensis* MR-1. To investigate a possible role for MotX and MotY in the motility of *S. oneidensis* MR-1, in-frame deletion mutations were created in *motX* (Δ*motX*), *motY* (Δ*motY*), and both (Δ*motXY*). To confirm that no further copies of these genes remained in the genome, the deletion was confirmed by Southern analysis (see Fig. S1 in the supplemental material). The mutations did not affect the growth rate (data not shown). Independent of the Na^+ concentration, none of the mutants displayed swimming motility as determined by light microscopy and by swimming assays in semisolid agar (Fig. 1). The motility defects conferred by the single mutations were rescued by ectopic expression of plasmid-borne *motX* and *motY* in the corresponding mutant (see Fig. S1 in the supplemental material).

To rule out that the loss of swimming motility due to deletions in *motX* and *motY* was caused by a defect in flagellum assembly, flagellar filament staining was performed on mutant and wild-type cells (Fig. 1C). All mutants possessed a flagellar filament similar to that of wild type cells. Thus, the nonmotile phenotype is the consequence of an inability of the mutants to rotate the filament.

S. oneidensis MR-1 harbors two stator systems, MotAB and PomAB. Under our experimental conditions, flagellar rotation depends mainly on the Na^+-driven PomAB stator (32). Thus, the observed phenotype could be due to loss of PomAB function or of both PomAB and MotAB function. To specifically determine whether MotX and MotY are required for PomAB- or MotAB-mediated flagellar function, deletions of *motX* and *motY* were introduced into an *S. oneidensis* MR-1 strain lacking *pomAB* (Δ*pomAB* Δ*motX* and Δ*pomAB* Δ*motY*) and *motAB* (Δ*motAB* Δ*motX* and Δ*motAB* Δ*motY*), respectively. All of these strains were nonmotile as determined by microscopic observation and motility in semisolid agar. Based on these results, we conclude that both MotX and MotY are required to support flagellar rotation driven by PomAB and MotAB stators.

Localization of MotX and MotY. For further functional analysis, the localization of MotX or MotY was determined. Signal peptides were predicted for both proteins, and in *V. alginolyticus* it has been demonstrated that both are transported into the periplasm via a Sec-dependent mechanism during which the N-terminal signal sequence is cleaved (30). Therefore, mCherry, which is also functional when folding in the periplasm (7), was fused to the C termini of MotX and MotY. Subsequently, the gene fusions were individually integrated into the native loci by single-crossover events. Stability of the fusion proteins was determined by immunoblot analysis using an antibody specific to mCherry (Fig. 2B). In agreement with the estimated molecular masses of the two fusion proteins, single distinct bands at positions corresponding to ~45 kDa and ~55 kDa were observed for MotX-mCherry and MotY-mCherry, respectively. Minor degradation was found to occur, except for MotX-mCherry in the absence of MotY (Fig. 2B). To determine whether the fusion proteins were still active, strains harboring MotX-mCherry or MotY-mCherry were tested for motility in semisolid agar. Both fusion proteins supported swimming motility at a higher level than the wild-type proteins, suggesting a stabilizing effect (Fig. 2A). From that we concluded that both fusions of MotX and MotY to mCherry are active and stable enough to be used for localization studies of *S. oneidensis* MR-1.

Cells expressing either *motX-mCherry* or *motY-mCherry* were

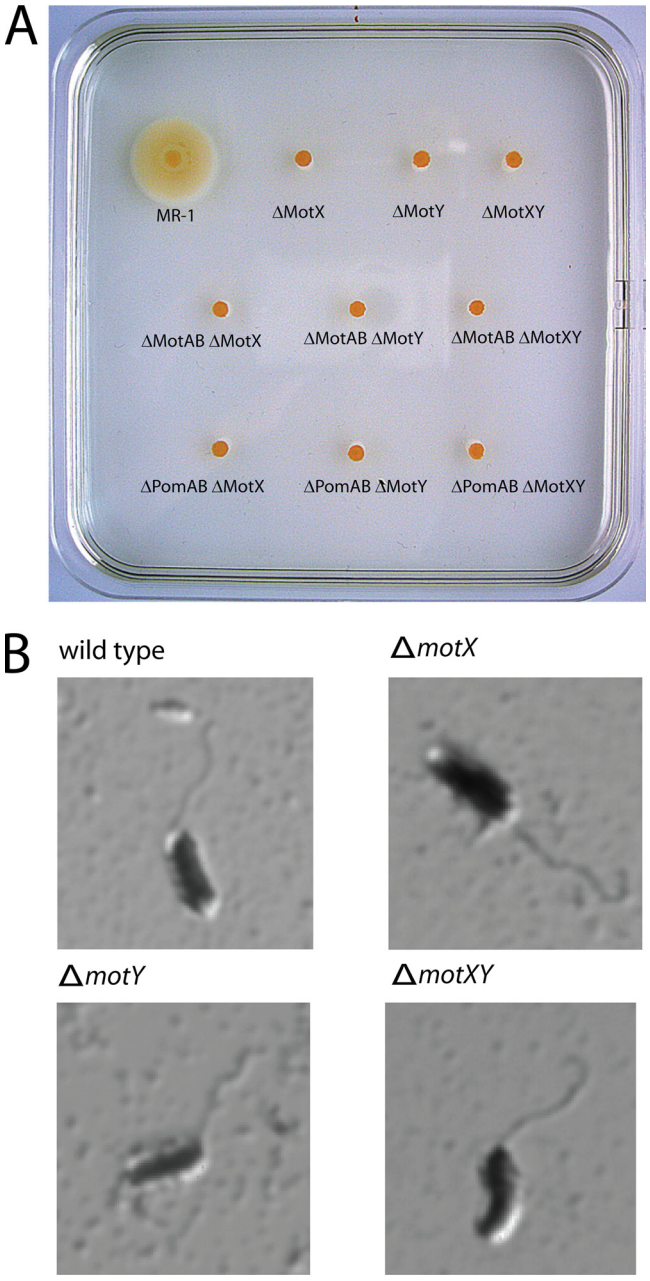

FIG. 1. (A) Swimming phenotype of Δ*motX* and Δ*motY* mutants on a 0.25% semisolid agar plate. The indicated mutants were spotted on soft agar and incubated overnight at 30°C. (B) Flagellation of *motX* and *motY* mutants. Displayed are micrographs of the corresponding strains after flagellar staining.

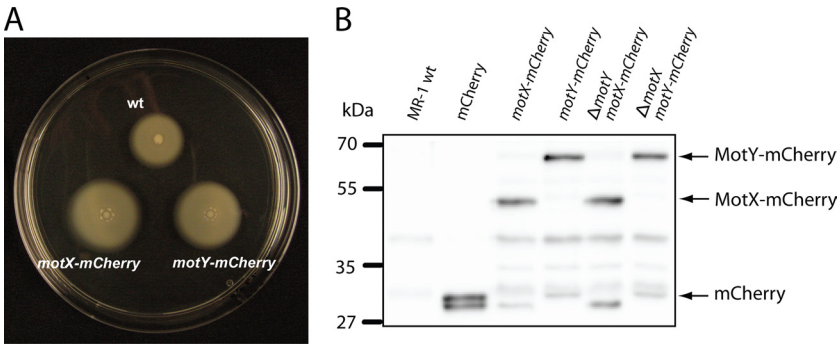

FIG. 2. Properties of MotX and MotY tagged with mCherry. (A) Swimming phenotype of strains harboring *motX-mCherry* and *motY-mCherry*. The strains were spotted on a 0.25% semisolid plate. (B) Detection of MotX-mCherry and MotY-mCherry by immunoblotting using antibodies raised against mCherry. Lane 1, wild type (negative control); lane 2, mCherry (positive control); lane 3, S292 (*motX*::*mCherry*); lane 4, S293 (*motY*::*mCherry*); lane 5, S294 (Δ*motY motX*::*mCherry*); lane 6, S295 (Δ*motX motY*::*mCherry*). Arrows indicate the positions of signals corresponding to MotX-mCherry (54.1 kDa), MotY-mCherry (62.4 kDa), and mCherry (29.3 kDa).

grown to exponential growth phase and examined by fluorescence microscopy (Fig. 3). MotX-mCherry was observed to localize to the flagellated cell pole (Fig. 3), which was identified by simultaneous staining of the flagellar filament by staining with Alexa Fluor 488 succinimidyl ester (Fig. 3). Some fluorescence appeared to be associated with the cell envelope away from the pole. In addition, a diffuse fluorescent signal was detected in the cytoplasm, indicating either that a MotX-mCherry moiety is partly degraded or that not all protein synthesized is transported through the cytoplasmic membrane. MotY-mCherry formed a distinct fluorescent locus at the flagellated cell pole. A significant amount of MotY-mCherry also localized to the cell envelope away from the cell pole.

Since the localization of the MotAB stator in *S. oneidensis* MR-1 depends on the sodium ion concentration in the medium, we then determined whether a similar dependence exists for MotX and MotY. However, both of the fusion proteins localized equally well at all Na^+ concentrations, including in the absence of any added sodium ion (data not shown).

Studies of MotX and MotY of *V. alginolyticus* have suggested that the two proteins form a complex and associate with the flagellar LP ring (42). Therefore, we investigated whether localization of both proteins is mutually dependent in *S. oneidensis* MR-1. An mCherry fusion to MotX was introduced into the chromosome of a Δ*motY* strain (Δ*motY motX-mCherry*), and, correspondingly, an mCherry fusion to MotY was introduced into the chromosome of a Δ*motX* strain (Δ*motX motY-mCherry*). To determine whether localization depends on the presence of the flagellar complex, both fusions were individually introduced into a Δ*fliF* mutant that lacks the flagellar basal body. The corresponding strains were then grown to exponential growth phase, and the cells were visualized by fluorescence microscopy (Fig. 3).

In the absence of MotY, no localization of MotX-mCherry was observed, and a diffuse fluorescence signal was distributed throughout the cell. In contrast, when MotX was absent, localization of MotY-mCherry still occurred at the flagellated cell pole. Localization of both proteins was dependent on the presence of the flagellar basal body. In the Δ*fliF* mutant, MotX-mCherry was distributed as in the Δ*motY* strain. In contrast, MotY-mCherry still formed distinct foci in the cell envelope, but these occurred at random positions in the cell rather than exclusively at the flagellated pole. Thus, both MotX and MotY require the flagellar basal body for normal localization. In addition, MotX also requires the correct localization of MotY to be sequestered at the flagellated cell pole.

Stator localization independence on MotX and MotY. We next determined whether MotX and MotY are crucial for stator recruitment in *S. oneidensis* MR-1 and whether they are involved in the process by which the appropriate stator system is selected at low and high sodium ion concentrations. To this end, genes encoding PomB-mCherry and MotB-mCherry were individually integrated at the corresponding native loci in Δ*motX*, Δ*motY*, and Δ*motXY* strains. We have previously demonstrated that these fusions are stable and functional (32).

The resulting strains were grown in LM medium supplemented with no or 100 mM sodium chloride, and localization of the stators was determined by fluorescence microscopy (Fig. 4). Only minor effects of the deletions on stator localization were observed. PomB-mCherry localized to the flagellated pole in ~70% of the cell population of the wild type and all of the mutants, regardless of the presence or absence of added NaCl. At 100 mM NaCl, localization of MotB-mCherry increased from 12% in the wild type to 22% in cells lacking MotX and/or MotY. In the absence of added NaCl, MotB-mCherry localized to the flagellated pole in more than 50% of the cells in each strain. In a Δ*pom* Δ*motXY* mutant, MotB-mCherry localized in the same pattern as in a Δ*pom* strain, and the same was observed for PomB-mCherry in a Δ*mot* Δ*motXY* mutant compared to a mutant lacking only *motAB* (data not shown). These results indicate that in *S. oneidensis* MR-1, MotX and MotY are not mandatory for stator recruitment or stator selection but might have a stabilizing function.

Protein interaction studies revealed that in *V. alginolyticus*, MotX interacts with MotY and PomB (29); however, in *S. oneidensis* MR-1, MotX and MotY are not crucial for stator acquisition. Thus, polar localization of MotX and MotY might depend on the presence of stator subunits. To test this hypothesis, *motX-mCherry* and *motY-mCherry* were integrated at the corresponding native loci in a strain that lacks both stator systems (Δ*pom* Δ*mot motX-mCherry* and Δ*pom* Δ*mot motY-mCherry*). Subsequently, the localization of MotX-mCherry

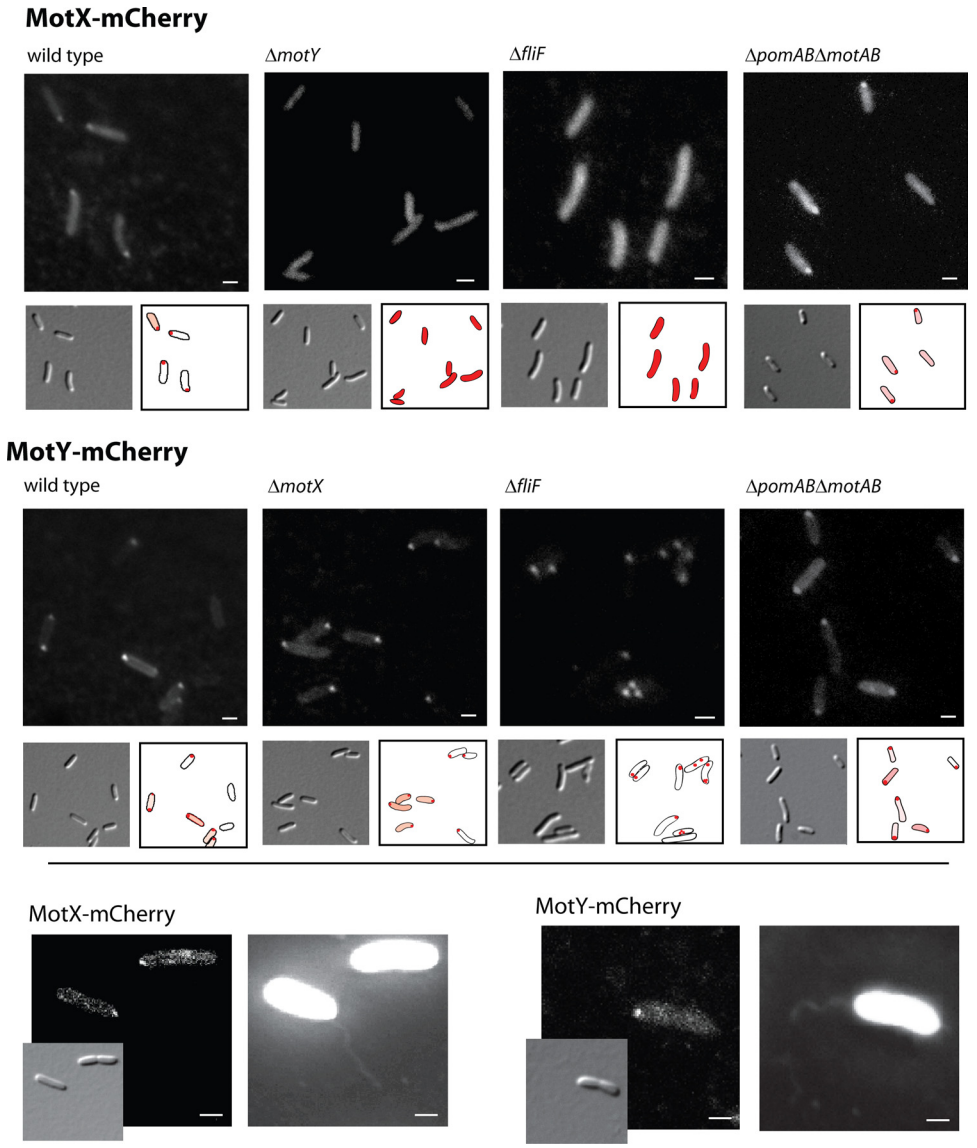

FIG. 3. Localization of MotX-mCherry and MotY-mCherry in *S. oneidensis* MR-1. Displayed are DIC and fluorescence micrographs of cells harboring *motX-mCherry* (upper panel) and *motY-mCherry* (middle panel) in the wild type, Δ*motY* or Δ*motX*, Δ*fliF*, and Δ*pomAB* Δ*motAB* mutant backgrounds (from left to right). To facilitate the visualization, the position of fluorescence signals relative to that of the cells is displayed. The lower panel shows colocalization of the flagellar filament with MotX-mCherry (left) and MotY-mCherry (right). DIC and fluorescence micrographs are displayed. Scale bars, 1 μm.

and MotY-mCherry was determined (Fig. 3). Both proteins localized to the flagellated cell pole just as in wild-type cells. Thus, localization of MotX and MotY to the flagellar motor does not depend on the presence of stator complexes.

DISCUSSION

The typical flagellar rotary motor consists of the switch complex components FliM, FliN, and FliG (3, 39). FliG is directly involved in rotation and physically interacts with the stator elements, PomAB for Na^+-driven motors and MotAB for proton-driven motors. In some bacteria, auxiliary proteins have been implicated in stator function. In *Sinorhizobium meliloti*, MotC and MotE are required for flagellar rotation. MotE is thought to serve as a chaperone for MotC, which in turn binds to the stator subunit MotB. It has been speculated that MotC exerts its function by stabilizing the periplasmic domain of MotB (11, 34). The most comprehensive studies of auxiliary components of the bacterial flagellar motors have been conducted on MotX and MotY of *Vibrio* species. A protein homologous to MotY has also been found associated with the high-speed, proton-driven polar flagellum of *Pseudomonas aeruginosa* PAO1 (10) and the lateral, proton-driven flagella of *Vibrio parahaemolyticus* (41). For both systems, a corresponding MotX partner has not been identified. Thus, ancillary stator components in flagellar rotation are not limited to sodium-dependent or polar flagellar systems. We show here that either sodium-driven or proton-driven flagellar rotation in *S. onei-*

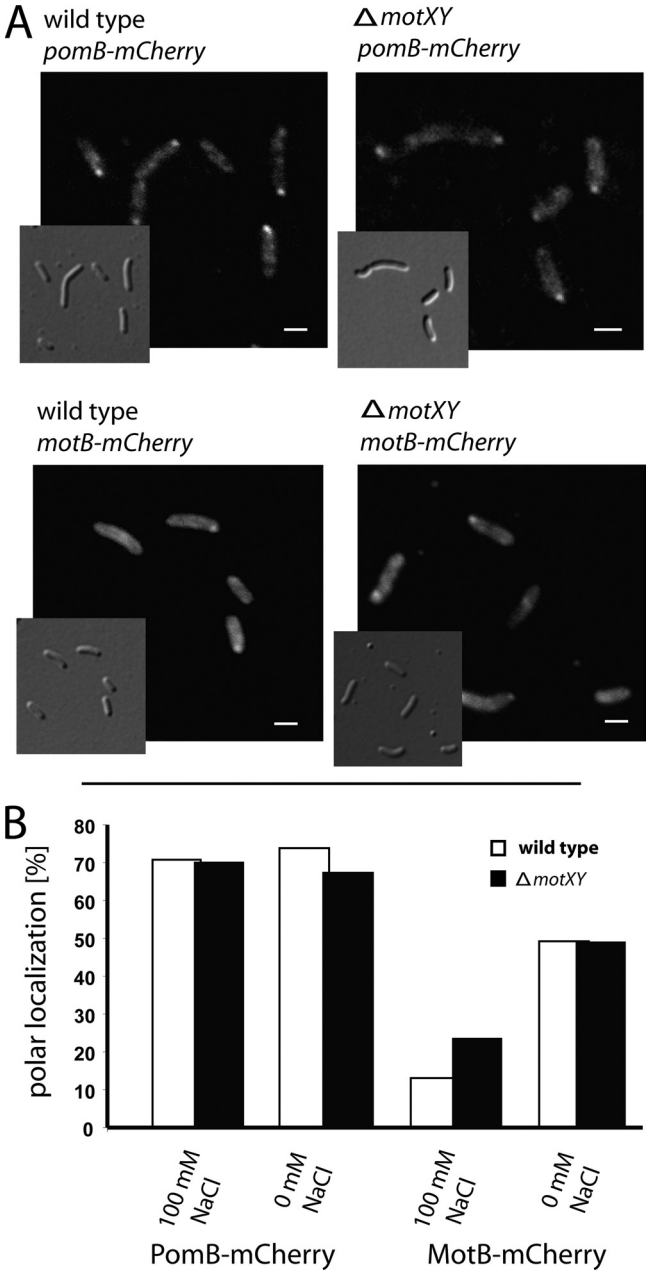

FIG. 4. Localization of PomB-mCherry and MotB-mCherry in the Δ*motX* Δ*motY* mutant. (A) Micrographs (DIC and fluorescence) of cells expressing *pomB-mCherry* (upper image) and *motB-mCherry* (lower image) in wild-type and Δ*motX* Δ*motY* cells. Cells were grown in LM medium with 100 mM NaCl for *pomB-mCherry* and without added NaCl for *motB-mCherry*. Scale bars, 1 μm. (B) Percentages of polarly localized PomB-mCherry and MotB-mCherry in wild-type and Δ*motX* Δ*motY* cells. Each data point represents 250 cells.

densis MR-1 also depends on orthologs of the *Vibrio* proteins MotX and MotY. Both proteins are highly conserved within the genomes of all *Shewanella* isolates sequenced to date. Since all *Shewanella* species also harbor orthologs to the PomAB stator system, it is predicted that, as in *Vibrio*, MotX and MotY would be required to power their single polar flagellum. However, our results provide direct evidence that the alternative proton-conducting MotAB stator elements of *S. oneidensis* MR-1 also require MotX and MotY for normal function.

Localization studies of MotX and MotY in *Vibrio* species were initially carried out with *E. coli*, where they appear to be associated with the outer membrane (22, 23, 29, 30). However, a more recent model suggests that MotX and MotY form a complex that diffuses in the periplasm before interacting with the flagellar basal body (18). We used fluorescent mCherry fusion proteins to monitor the localization of MotX and MotY in *S. oneidensis*. As suggested by the presence of a putative N-terminal signal sequence, and in accordance with the *Vibrio* model, MotY was observed in the cell envelope. However, the fluorescence signal does not distinguish specifically whether the protein is freely diffusing in the periplasmic space or whether it is associated with the inner or outer membrane or the cell wall. As expected, distinct localization of MotY-mCherry occurred at the flagellated cell pole. The signal of MotX-mCherry was more diffuse, and some fluorescence was also detected in the cytoplasmic space. MotX-mCherry localized to the flagellated cell pole, but only when MotY was also present.

In the absence of a flagellar basal body, MotY-mCherry still occurred as distinct fluorescent foci in the cell envelope, indicating unspecific clustering. If MotX and MotY form a precomplex in the periplasm, MotX should also be detected in these clusters, but this was never observed. Instead, in strains lacking the flagellar basal body or MotY, a diffuse fluorescence signal of MotX-mCherry occurred throughout the cell. We conclude that in *S. oneidensis* MR-1, MotX and MotY do not form a precomplex in the periplasm. We propose instead that MotY is exported into the periplasm, forms a pool in the cell envelope, and interacts with the flagellar basal body before MotX is recruited. If MotY is not localized properly, MotX is degraded. This finding is consistent with the observation that, in *Vibrio*, MotX is unstable in the absence of MotY (29, 47). However, it remains unknown whether MotY already stabilizes MotX in the cytoplasm. Since stator elements seem to constitute a dynamic complex (21), the abundance of MotX and particularly MotY in the cell envelope away from the flagellated cell pole could indicate that the MotXY complex also undergoes rapid turnover at the basal body.

The exact functions of MotX and MotY remain unclear. For *Vibrio* species, direct interaction between MotX and PomB has been demonstrated (29), and in *V. alginolyticus*, the PomAB stator complex does not localize correctly to the flagellated cell pole in the absence of MotX or MotY (42). Therefore, in *V. alginolyticus*, a major role of MotXY is to recruit the stators to the flagellar motor. Since this organism also expresses a second lateral flagellar system depending on a different proton-dependent stator, this may help to incorporate the correct stator into the corresponding flagellar motors. In *S. oneidensis* MR-1, two different stator systems are present to drive a single polar flagellum. We have recently demonstrated that selection of the stator system occurs at the level of protein localization in response to the sodium ion concentration (32). At high Na^+ concentrations, both stator systems are present but the flagellar motor is occupied almost exclusively by PomAB. MotAB localizes more efficiently under conditions of low Na^+ concentration or when a mutation eliminates the PomAB system. Regarding their role in *Vibrio*, we hypothesized that in

Shewanella MotX and MotY may be involved in the stator selection process, e.g., by acquisition and incorporation of the stator complexes in response to changing Na^+ levels. Under conditions of high Na^+ concentrations, a slight increase of MotAB localization to the flagellated cell pole was observed in the absence of MotX and MotY. This might indicate that PomAB association with the motor is not as effective in that strain. However, in *S. oneidensis* MR-1, neither MotX nor MotY is absolutely required for localization of PomAB or MotAB to the motor. Hence, in this strain, MotXY must be needed for something involved in generating flagellar rotation after the stator complexes have been recruited.

The well-characterized proton-driven flagella of *E. coli* do not require any stator components beyond the MotAB complex (3). However, when the *E. coli* MotAB complex was ectopically produced in mutants of *V. cholerae* and *V. alginolyticus* lacking PomAB and MotXY, cells became motile using a proton-driven motor (2, 14). When PomA was expressed in *V. alginolyticus* in concert with a chimeric protein containing the cytoplasmic and transmembrane portions of PomB fused to the periplasmic segment of *E. coli* MotB, these two proteins formed an active, Na^+-dependent stator complex that worked independently from MotXY. This same pair of proteins also powered Na^+-dependent rotation of flagella in *E. coli* (2, 40). The overall conclusion is that the requirement for MotXY arises from the periplasmic domain of PomB. An analysis of MotB mutants revealed that its periplasmic region contains a segment that has been proposed to act as a plug to prevent premature proton flow until the MotAB complex encounters a flagellar motor. A conformational change triggered by contact with the basal body might both open the proton-conducting channel and enable tight binding of the periplasmic domain to peptidoglycan in the cell wall (17, 45). Electron microscopy performed on basal complexes isolated from *V. alginolyticus* revealed an additional structure, termed the "T ring," attached below the P and L rings, which function as the bushing for the rotating flagellar rod (9, 42). This structure, which presumably contains MotX and MotY, might be required for conformational changes in the PomAB and MotAB stator complexes of *S. oneidensis* MR-1 that open the ion-conducting channels and enable binding to peptidoglycan.

Prior studies of MotX and MotY have focused almost exclusively on the flagellar motor of *Vibrio* species. Characterization of these two components in *S. oneidensis* MR-1 complements the *Vibrio* studies and suggests possible activities carried out by ancillary motor proteins. Future experiments will be directed toward elucidating those functions.

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REFERENCES

1. **Asai, Y., S. Kojima, H. Kato, N. Nishioka, I. Kawagishi, and M. Homma.** 1997. Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. J. Bacteriol. **179:**5104–5110.
2. **Asai, Y., T. Yakushi, I. Kawagishi, and M. Homma.** 2003. Ion-coupling determinants of Na^+-driven and H^+-driven flagellar motors. J. Mol. Biol. **327:**453–463.
3. **Berg, H. C.** 2003. The rotary motor of bacterial flagella. Annu. Rev. Biochem. **72:**19–54.
4. **Blair, D. F.** 2003. Flagellar movement driven by proton translocation. FEBS Lett. **545:**86–95.
5. **Blair, D. F., and H. C. Berg.** 1990. The MotA protein of *Escherichia coli* is a proton-conducting component of the flagellar motor. Cell **60:**439–449.
6. **Blenden, D. C., and H. S. Goldberg.** 1965. Silver impregnation stain for *Leptospira* and flagella. J. Bacteriol. **89:**899–900.
7. **Chen, J. C., P. H. Viollier, and L. Shapiro.** 2005. A membrane metalloprotease participates in the sequential degradation of a *Caulobacter* polarity determinant. Mol. Microbiol. **55:**1085–1103.
8. **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.
9. **DeRosier, D. J.** 1998. The turn of the screw: the bacterial flagellar motor. Cell **93:**17–20.
10. **Doyle, T. B., A. C. Hawkins, and L. L. McCarter.** 2004. The complex flagellar torque generator of *Pseudomonas aeruginosa*. J. Bacteriol. **186:**6341–6350.
11. **Eggenhofer, E., M. Haslbeck, and B. Scharf.** 2004. MotE serves as a new chaperone specific for the periplasmic motility protein, MotC, in *Sinorhizobium meliloti*. Mol. Microbiol. **52:**701–712.
12. **Emanuelsson, O., S. Brunak, G. von Heijne, and H. Nielsen.** 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. **2:**953–971.
13. **Fukuoka, H., T. Yakushi, A. Kusumoto, and M. Homma.** 2005. Assembly of motor proteins, PomA and PomB, in the Na^+-driven stator of the flagellar motor. J. Mol. Biol. **351:**707–717.
14. **Gosink, K. K., and C. C. Häse.** 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.
15. **Grant, B., and I. Greenwald.** 1996. The *Caenorhabditis elegans* sel-1 gene, a negative regulator of lin-12 and glp-1, encodes a predicted extracellular protein. Genetics **143:**237–247.
16. **Heidelberg, J. F., I. T. Paulsen, K. E. Nelson, E. J. Gaidos, W. C. Nelson, T. D. Read, J. A. Eisen, R. Seshadri, N. Ward, B. Methe, R. A. Clayton, T. Meyer, A. Tsapin, J. Scott, M. Beanan, L. Brinkac, S. Daugherty, R. T. DeBoy, R. J. Dodson, A. S. Durkin, D. H. Haft, J. F. Kolonay, R. Madupu, J. D. Peterson, L. A. Umayam, O. White, A. M. Wolf, J. Vamathevan, J. Weidman, M. Impraim, K. Lee, K. Berry, C. Lee, J. Mueller, H. Khouri, J. Gill, T. R. Utterback, L. A. McDonald, T. V. Feldblyum, H. O. Smith, J. C. Venter, K. H. Nealson, and C. M. Fraser.** 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat. Biotechnol. **20:**1118–1123.
17. **Hosking, E. R., C. Vogt, E. P. Bakker, and M. D. Manson.** 2006. The *Escherichia coli* MotAB proton channel unplugged. J. Mol. Biol. **364:**921–937.
18. **Kojima, S., A. Shinohara, H. Terashima, T. Yakushi, M. Sakuma, M. Homma, K. Namba, and K. Imada.** 2008. Insights into the stator assembly of the *Vibrio* flagellar motor from the crystal structure of MotY. Proc. Natl. Acad. Sci. USA **105:**7696–7701.
19. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. **166:**175–176.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Leake, M. C., J. H. Chandler, G. H. Wadhams, F. Bai, R. M. Berry, and J. P. Armitage.** 2006. Stoichiometry and turnover in single, functioning membrane protein complexes. Nature **443:**355–358.
22. **McCarter, L. L.** 1994. MotX, the channel component of the sodium-type flagellar motor. J. Bacteriol. **176:**5988–5998.
23. **McCarter, L. L.** 1994. MotY, a component of the sodium-type flagellar motor. J. Bacteriol. **176:**4219–4225.
24. **McCarter, L. L.** 2001. Polar flagellar motility of the *Vibrionaceae*. Microbiol. Mol. Biol. Rev. **65:**445–462.
25. **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.
26. **Mittl, P. R., and W. Schneider-Brachert.** 2007. Sel1-like repeat proteins in signal transduction. Cell. Signal. **19:**20–31.
27. **Myers, C. R., and J. M. Myers.** 1997. Replication of plasmids with the p15A origin in *Shewanella putrefaciens* MR-1. Lett. Appl. Microbiol. **24:**221–225.
28. **Okabe, M., T. Yakushi, Y. Asai, and M. Homma.** 2001. Cloning and characterization of *motX*, a *Vibrio alginolyticus* sodium-driven flagellar motor gene. J. Biochem. **130:**879–884.
29. **Okabe, M., T. Yakushi, and M. Homma.** 2005. Interactions of MotX with MotY and with the PomA/PomB sodium ion channel complex of the *Vibrio alginolyticus* polar flagellum. J. Biol. Chem. **280:**25659–25664.
30. **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.
31. **Okunishi, I., I. Kawagishi, and M. Homma.** 1996. Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. J. Bacteriol. **178:**2409–2415.
32. **Paulick, A., A. Koerdt, J. Lassak, S. Huntley, I. Wilms, F. Narberhaus, and K. M. Thormann.** 2009. Two different stator systems drive a single polar flagellum in *Shewanella oneidensis* MR-1. Mol. Microbiol. **71:**836–850.
33. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutants in bacteria. Gene **118:**145–146.
34. **Platzer, J., W. Sterr, M. Hausmann, and R. Schmitt.** 1997. Three genes of a motility operon and their role in flagellar rotary speed variation in *Rhizobium meliloti*. J. Bacteriol. **179:**6391–6399.
35. **Reid, S. W., M. C. Leake, J. H. Chandler, C. J. Lo, J. P. Armitage, and R. M. Berry.** 2006. The maximum number of torque-generating units in the flagellar motor of *Escherichia coli* is at least 11. Proc. Natl. Acad. Sci. USA **103:**8066–8071.
36. **Ryu, W. S., R. M. Berry, and H. C. Berg.** 2000. Torque-generating units of the flagellar motor of *Escherichia coli* have a high duty ratio. Nature **403:**444–447.
37. **Sambrook, K., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. **Song, W., F. S. Juhn, D. Q. Naiman, K. T. Konstantinidis, T. S. Gardner, and M. J. Ward.** 2008. Predicting sigma28 promoters in eleven *Shewanella* genomes. FEMS Microbiol. Lett. **283:**223–230.
39. **Sowa, Y., and R. M. Berry.** 2008. Bacterial flagellar motor. Q. Rev. Biophys. **41:**103–132.
40. **Sowa, Y., A. D. Rowe, M. C. Leake, T. Yakushi, M. Homma, A. Ishijima, and R. M. Berry.** 2005. Direct observation of steps in rotation of the bacterial flagellar motor. Nature **437:**916–919.
41. **Stewart, B. J., and L. L. McCarter.** 2003. Lateral flagellar gene system of *Vibrio parahaemolyticus*. J. Bacteriol. **185:**4508–4518.
42. **Terashima, H., H. Fukuoka, T. Yakushi, S. Kojima, and M. Homma.** 2006. The *Vibrio* motor proteins, MotX and MotY, are associated with the basal body of Na^+-driven flagella and required for stator formation. Mol. Microbiol. **62:**1170–1180.
43. **Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla, Y. Hayakawa, and A. M. Spormann.** 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J. Bacteriol. **188:**2681–2691.
44. **Thormann, K. M., R. M. Saville, S. Shukla, and A. M. Spormann.** 2005. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. J. Bacteriol. **187:**1014–1021.
45. **Van Way, S. M., E. R. Hosking, T. F. Braun, and M. D. Manson.** 2000. Mot protein assembly into the bacterial flagellum: a model based on mutational analysis of the *motB* gene. J. Mol. Biol. **297:**7–24.
46. **Venkateswaran, K., D. P. Moser, M. E. Dollhopf, D. P. Lies, D. A. Saffarini, B. J. MacGregor, D. B. Ringelberg, D. C. White, M. Nishijima, H. Sano, J. Burghardt, E. Stackebrandt, and K. H. Nealson.** 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. Int. J. Syst. Bacteriol. **2:**705–724.
47. **Yagasaki, J., M. Okabe, R. Kurebayashi, T. Yakushi, and M. Homma.** 2006. Roles of the intramolecular disulfide bridge in MotX and MotY, the specific proteins for sodium-driven motors in *Vibrio* spp. J. Bacteriol. **188:**5308–5314.
48. **Yorimitsu, T., and M. Homma.** 2001. Na(+)-driven flagellar motor of *Vibrio*. Biochim. Biophys. Acta **1505:**82–93.
49. **Yorimitsu, T., M. Kojima, T. Yakushi, and M. Homma.** 2004. Multimeric structure of the PomA/PomB channel complex in the Na^+-driven flagellar motor of *Vibrio alginolyticus*. J. Biochem. **135:**43–51.
50. **Zhou, J., S. A. Lloyd, and D. F. Blair.** 1998. Electrostatic interactions between rotor and stator in the bacterial flagellar motor. Proc. Natl. Acad. Sci. USA **95:**6436–6441.