Effect of the MotA(M206I) Mutation on Torque Generation and Stator Assembly in the *Salmonella* H⁺-Driven Flagellar Motor

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ABSTRACT The bacterial flagellar motor is composed of a rotor and a dozen stators and converts the ion flux through the stator into torque. Each stator unit alternates in its attachment to and detachment from the rotor even during rotation. In some species, stator assembly depends on the input energy, but it remains unclear how an electrochemical potential across the membrane (e.g., proton motive force [PMF]) or ion flux is involved in stator assembly dynamics. Here, we focused on pH dependence of a slow motile MotA(M206I) mutant of *Salmonella*. The MotA(M206I) motor produces torque comparable to that of the wild-type motor near stall, but its rotation rate is considerably decreased as the external load is reduced. Rotation assays of flagella labeled with 1- μ m beads showed that the rotation rate of the MotA(M206I) motor is increased by lowering the external pH whereas that of the wild-type motor is not. Measurements of the speed produced by a single stator unit using 1- μ m beads showed that the unit speed of the MotA(M206I) is about 60% of that of the wild-type and that a decrease in external pH did not affect the MotA(M206I) unit speed. Analysis of the subcellular stator localization revealed that the number of functional stators is restored by lowering the external pH. The pH-dependent improvement of stator assembly was observed even when the PMF was collapsed and proton transfer was inhibited. These results suggest that MotA-Met206 is responsible for not only load-dependent energy coupling between the proton influx and rotation but also pH-dependent stator assembly.

IMPORTANCE The bacterial flagellar motor is a rotary nanomachine driven by the electrochemical transmembrane potential (ion motive force). About 10 stators (MotA/MotB complexes) are docked around a rotor, and the stator recruitment depends on the load, ion motive force, and coupling ion flux. The MotA(M206I) mutation slows motor rotation and decreases the number of docked stators in *Salmonella*. We show that lowering the external pH improves the assembly of the mutant stators. Neither the collapse of the ion motive force nor a mutation mimicking the proton-binding state inhibited stator localization to the motor. These results suggest that MotA-Met206 is involved in torque generation and proton translocation and that stator assembly is stabilized by protonation of the stator.

KEYWORDS *Salmonella*, flagellar motor, proton channel, stator assembly, torque generation

The bacterial flagellar motor is a rotary nanomachine driven by the ion motive force (IMF) comprised of membrane potential and a concentration gradient of cation across the cytoplasmic membrane. The coupling ion differs among bacterial species; e.g., *Escherichia coli* and *Salmonella enterica* use proton motive force (PMF) to rotate

Citation Suzuki Y, Morimoto YV, Oono K, Hayashi F, Oosawa K, Kudo S, Nakamura S. 2019. Effect of the MotA(M206I) mutation on torque generation and stator assembly in the *Salmonella* H⁺-driven flagellar motor. *J Bacteriol* 201:e00727-18. <https://doi.org/10.1128/JB.00727-18>.

Editor Conrad W. Mullineaux, Queen Mary University of London

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Received 26 November 2018

Accepted 3 January 2019

Accepted manuscript posted online 14 January 2019

Published 25 February 2019

FIG 1 Arrangement of MotA and MotB. (A) Topology of MotA and MotB and location of important amino acid residues. MotA has four transmembrane segments (TM1, TM2, TM3, and TM4). MotB has a single transmembrane segment (TM) and a peptidoglycan-binding motif (PGB). MotB-Asp33 works as a proton pathway. (B) Arrangement of transmembrane segments of MotA and MotB. The MotA/MotB complex is postulated to have two proton flux pathways, shown as light blue circles. (C) The helical wheel projections showing the arrangement of amino acids in segments forming the proton channel (boxed in panel B) is represented. Hydrophilic residues are represented as circles, hydrophobic residues are shown as diamonds, potentially negatively charged residues are shown as triangles, and potentially positively charged residues are shown as pentagons. Hydrophobicity is color coded as well: the most hydrophobic residue is green, and the amount of green decreases proportionally to the hydrophobicity, with zero hydrophobicity indicated in yellow. Hydrophilic residues are shown in red, with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreases proportionally to the hydrophilicity. The potentially charged residues are shown in purple. The plots were generated using a helical wheel projection (D. Armstrong and R. Zidovetzki, unpublished data).

their peritrichous flagella, and *Vibrio alginolyticus* uses sodium motive force (SMF) for rotation of the polar flagellum (1–3). A dozen stator units formed by four copies of MotA (PomA in *V. alginolyticus*) and two copies of MotB (PomB) reside around the rotor comprising FliF, FliG, FliM, and FliN. Coupling ions are translocated from periplasm to cytoplasm through an ion pathway formed within the stator complex, inducing conformational change of the cytoplasmic domain of MotA. Torque is believed to be generated by electrostatic interaction between the cytoplasmic loop of MotA (e.g., Glu98) and FliG (e.g., Arg281) (4, 5).

A C-terminal region of MotB (MotBc) contains a peptidoglycan binding (PGB) motif (residues 197 to 226) (6, 7), and the stator units are localized around the rotor by being anchored to the peptidoglycan layer via the PGB domain (8). Although a dozen stator units can be incorporated into the flagellar motor (9), subcellular observation of green fluorescent protein (GFP)-tagged stator units has revealed that stator units alternately associate with or dissociate from the rotor, even during rotation (10). Interaction between MotA and FliG is important not only for torque generation but also for stator assembly (11, 12); interaction between MotA-Arg90 and FliG-Asp289 contributes to properly associating MotA/MotB stator units to the rotor (5). In response to changes in input energy and external load, the flagellar motor varies the number of stator units (13–18). Assembly of PomA/PomB stator units of the sodium-driven motor of *V. alginolyticus* is dependent on the sodium concentration in the medium, suggesting that sodium binding to the conserved residue PomB-Asp24 activates the stator units to be assembled to the motor (14). As for proton-driven motors, the *E. coli* MotA/MotB unit requires PMF for localization (13), but in *Salmonella* the stator is assembled to the motor even when PMF is collapsed (11). Thus, the process of stator assembly to the motor somehow involves input energy (i.e., IMF and/or coupling ion flux), but the detailed mechanism remains unclear.

Although many studies have identified the ion conductivity of the stator unit, the precise structures and arrangements of the stator components have not been fully elucidated. In proton-driven motors, biochemical assays have predicted that the proton translocation pathway is formed by transmembrane segments 3 (TM3) and 4 (TM4) of MotA and a single TM of MotB and that two proton pathways are contained in the MotA₄MotB₂ stator unit (Fig. 1A and B) (19, 20). MotB-Asp33 (Asp32 in the *E. coli* MotB)

is believed to be a putative proton-binding site residing in the proton pathway (21). The proton dissociation from MotB-Asp33 to the cytoplasm would be facilitated by conformational change of a cytoplasmic domain of MotA, involving the widely conserved residues Pro173 (TM3) and Tyr217 (TM4) (22). It is known that some mutations of MotB-Ala39 residing on the same side as Asp33 in the proton pathway severely affect motility; e.g., the A39V mutation completely impairs motility and shows a dominant negative effect when it is expressed in the wild-type (WT) *E. coli* (23, 24). Such impairments of motility caused by mutations at MotB-Ala39 are restored by several second mutations at MotA-Met206 (24). MotA-Met206 is thought to be located near the periplasmic side of TM4, facing the proton translocation pathway (20, 25) (Fig. 1C). This suggests the possibility that, in addition to MotB-Asp33, MotA-Pro173, and MotA-Tyr217, MotA-Met206 and MotB-Ala39 are involved in proton translocation. Komatsu et al. have isolated a revertant having the second mutation at MotA-Met206 (M206I) from a *Salmonella* mutant strain, SJW3060 with *fljF* encoding an N-to-T change at position 318 [SJW3060 *fljF*(N318T)], one of more than 400 revertants obtained from this strain (26). The *FljF*(N318T) mutation weakens a physical connection between the rod-hook-filament (RHF) and rotor, resulting in a detachment of the RHF and diminishment of motility on semisolid agar (27). Because the detached RHF was observed under highly viscous conditions where the flagellar motor produced relatively high torque, the reduced motor torque of the revertants could allow the motor to rotate stably, albeit slowly, without releasing the RHF, even in viscous medium. Thus, it is possible that MotA-Met206 is also involved in the torque generation mechanism.

Previously, it has been suggested that MotA-Met206 could be involved in proton translocation and torque generation, leading to the present investigation to seek a deeper understanding of the motor mechanism. In this study, we used a *Salmonella* mutant motor with a MotA(M206I) point mutation. We demonstrated that the M206I mutation reduces the proton conductivity of the stator unit and slows the motor rotation. We found that the mutation reduces the number of stator units docked to the motor in pH 7 medium but that assembly of the mutant stator is improved by lowering the medium pH, thereby increasing the rotation rate of the MotA(M206I) motor labeled with a 1- μ m polystyrene bead (i.e., under a high-load condition). In addition, we examined the effects of pH, PMF, and proton conductivity on subcellular localization of stator units. Our results provide a model for stator assembly that proton binding to stator units stabilizes their localization.

RESULTS

Effect of the MotA(M206I) mutation on the proton conductivity of the unplugged MotA/MotB complex. To evaluate the effect of the M206I mutation on the proton conductivity of the MotAB proton channel complex, we examined growth impairment using *Salmonella* cells expressing stator proteins from plasmids. Although overexpression of the WT stator does not affect cell growth, an in-frame deletion of MotB residues 51 to 70 [MotB(51–70)] in the periplasmic domain causes proton leakage, thereby impairing cell growth. Accordingly, the region MotB(51–70) is believed to act as a “plug” to suppress excess proton translocation while the stators are incorporated into the motor (11, 28). The overexpression of stators lacking this region (unplugged, or Δ plug) and having the M206I mutation caused growth arrest but not at the wild-type level (Fig. 2A). This raises the possibility that the Δ plug stator with the M206I mutation retains the ability to conduct protons to a considerable degree.

We also examined the effect of the MotA(M206I) mutation on the proton conductivity of stator units by measuring the intracellular pH of cells expressing MotA/MotB(Δ plug), MotA(M206I)/MotB(Δ plug), or MotA/MotB(Δ plug D33N) using a ratiometric pH indicator, pHluorin (29). The MotB(D33N) mutation is believed to mimic a protonation state of the stator unit, and the MotA/MotB(D33N) stator therefore lacks proton conductivity, thereby causing a loss of function of a torque generator (30). Figure 2A shows that the growth of cells expressing Δ plug stators gradually recovers from around 3 h after induction as a stress response to a low-pH environment. Thus, we

FIG 2 Effect of MotA(M206I) mutation on proton conductivity. (A) Growth curves of *Salmonella* cells carrying a plasmid expressing *motA motB*, *motA motB(Δplug)*, or *motA(M206I) motB(Δplug)*. Protein expression was induced by the addition of 0.2% arabinose at the time indicated with the arrow. Average values of three independent experiments are shown. (B) Immunoblotting using anti-MotB antibody. Arabinose (0.2%) was added after a 3-h incubation as shown in panel A, and protein expression was induced for 1 h. (C) Intracellular pH measured using pHluorin in the external pH 5.5. Stator proteins were expressed in *E. coli*. Results from more than six independent experiments were averaged; error bars represent standard errors.

induced the expression of the stator proteins for 1 h; Fig. 2B shows no difference in the expression levels among MotA/MotB(Δplug), MotA(M206I)/MotB(Δplug), and MotA/MotB(Δplug D33N). Figure 2C shows that the overexpression of MotA/MotB(Δplug) and MotA(M206I)/MotB(Δplug) decreased the intracellular pH to 5.6 and 5.8, respectively (at external pH 5.5). In contrast, the MotA/MotB(Δplug D33N) stator did not change intracellular pH. The MotA(M206I) mutation slightly reduced the proton channel activity of the unplugged MotAB stator complex, thereby causing a smaller growth defect than that with the unplugged WT stator complex. It is thought that Met206 is located on the periplasmic side of the proton pathway of the stator unit (20), suggesting that the MotA(M206I) mutation may affect water wire formation inside the proton channel of the MotAB stator complex.

Dominant-negative effect of the MotA(M206I)/MotB complex on motility. It is known that a replacement of MotA-Met206 by Ser, Gly, Ala, or Thr does not affect the expression of MotA but slightly decreases that of MotB (24). An immunoblotting test using polyclonal anti-MotA and anti-MotB antibodies showed that the expression level of MotA(M206I)/MotB was approximately equal to that of WT MotA/MotB (Fig. 3C). A motility assay using *E. coli* Δ*motAB* cells showed that MotA(M206I)/MotB retained the motile function, but it is slightly lower than that of WT MotA/MotB (Fig. 3A). The *Salmonella* WT cells carrying an arabinose-inducible plasmid harboring WT or mutant

FIG 3 Effect of MotA(M206I)/MotB overexpression on motility of wild-type (WT) cells. (A) Fresh colonies of transformants of *E. coli* RP6894 carrying pBAD24 (Vector), pYC20 (WT), pYC98 (D33N), and pYS1 (M206I) were inoculated in a soft-agar plate with 0.02% arabinose and incubated at 30°C for 11 h. (B) Motility of *Salmonella* SJW1103 (wild type) carrying the same plasmids in a soft-agar plate with 0.2% arabinose. The plate was incubated at 30°C for 8 h. (C) Immunoblotting of whole-cell proteins prepared from the same strains described for panel B using polyclonal anti-MotA (upper) and anti-MotB (lower) antibodies. The positions of molecular mass markers (in kilodaltons) are shown on the left. Arrowheads indicate the positions of MotA and MotB. (D to F) Arabinose dependence of protein expression, the motile fraction, and swimming speed, as indicated, examined using SJW2241 (Δ*motA motB*) carrying pYC20 (white) and pYS1 (gray). Swimming speeds are average values from more than 30 cells; error bars indicate the standard deviations.

stator genes showed that overexpression of the MotA/MotB(D33N) complex with 0.2% arabinose strongly inhibited motility in soft agar, in agreement with a previous report (11), whereas the inhibition effect of MotA(M206I)/MotB overexpression was subtle (Fig. 3B). To assess the effect of the expression level of stator proteins on stator assembly, we determined the arabinose concentration dependence of the motile fraction and swimming speed. The expression levels of MotA/MotB and MotA(M206I)/MotB proteins increased with increases in the concentration of arabinose added to medium (Fig. 3D). In both strains expressing MotA/MotB and MotA(M206I)/MotB stators, their motile fractions depended on the expression level of each stator protein (Fig. 3E). Swimming speeds were significantly increased by an increase in the expression level of MotA/MotB ($P < 0.05$ for 0.002% versus 0.02%) but not by MotA(M206I)/MotB (Fig. 3F). These results suggest a lower affinity of the MotA(M206I) mutant stator for the rotor that causes a weak dominant negative effect of the mutant stator on motility.

Effect of MotA(M206I) mutation on motor rotation. To examine the effect of the MotA(M206I) mutation on motor rotation, we determined the torque-speed relationship by bead assay at an external pH value of 7.0. We measured rotations of WT and MotA(M206I) motors by labeling sheared flagellar filaments with 1-μm or 0.5-μm polystyrene beads or with 0.1-μm gold nanoparticles in medium with or without Ficoll. Figure 4 shows that the M206I mutation considerably decreased torque in the low-load, high-speed regime, as expected from the data of swimming speeds (Fig. 3F). However, torque produced by the MotA(M206I) motor with a 1-μm bead attached was increased by adding 12% Ficoll, so the maximum torque was almost the same as that of the stall torque of the WT. The effect of the mutation on motility in soft agar (high load) was smaller than that observed in swimming speeds measured in liquid medium (relatively low load) (Fig. 3A and F), which can be explained by the torque-speed curve. These results suggest that the MotA(M206I) mutation does not affect the energy conversion efficiency of the motor near stall (31). The severely reduced rotation rate at low load suggests that the M206I mutation decreases the number of stators as the load is reduced (17) and/or delays the rate of proton translocation (31).

Since rotation rates of *E. coli* and *Salmonella* flagellar motors are altered by intracellular pH but not by extracellular pH, the process of proton dissociation from the

FIG 4 Torque-speed curves of the WT and MotA(M206I) motors. Rotations of the WT (closed symbols) and MotA(M206I) motors (open symbols) were measured using beads with different diameters in motility medium (pH 7.0) with or without Ficoll, indicated as follows: circles, 1.0 μm in 12% Ficoll; triangles, 1.0 μm in medium without Ficoll; squares, 0.5 μm in medium without Ficoll; diamonds, 0.1 μm in medium without Ficoll. Average values and standard deviations are shown.

stator to the cytoplasm is thought to be a rate-limiting step of the torque generation cycle (32, 33). Because Met206 is located near the entrance of the proton channel in the MotA/MotB complex, we investigate whether a change in external pH affects the motor performance of the fully induced MotA(M206I) motor with a 1- μm bead attached. Figure 5A shows that the WT motor was not susceptible to changes in external pH, which is in agreement with a previous report (32). In contrast, the rotation rate of the M206I motor significantly increased as external pH was decreased; the rotation rate measured at pH 6.0 was almost twice that measured at pH 7.0 (Fig. 5A). To change the intracellular pH, we added potassium benzoate to the medium at various pH values as previously described (32, 33); when the external pH is decreased in the presence of benzoate, the intracellular pH is decreased to almost the same value as the external pH because membrane-permeable weak acids can act as proton carriers (34). Thus, the rotation rates of both the WT and M206I motors were decreased as intracellular pH was decreased in the presence of benzoate (Fig. 5B); thus, the proton dissociation from the

FIG 5 Effect of pH changes on motor rotation. Rotation speeds of the WT and MotA(M206I) motors were measured with 1- μm polystyrene beads in the absence (A) or presence (B) of 20 mM potassium benzoate. Average values (circles) and standard deviations (error bars) are shown. No rotation of the M206I motor was observed at pH 5.5 in the presence of benzoate. The right panels show relative values normalized to the rotation speed determined at external pH 7.5.

FIG 6 Effect of pH changes on the speed per stator unit. (A and B) Typical resurrection traces for *Salmonella* motors expressing WT (A) or MotA(M206I)/MotB (B) stator units; right panels are speed histograms obtained from speed-versus-time traces. Rotations were measured at external pH 7.0 (left) and 6.0 (right). Horizontal axes in resurrection traces are times after addition of arabinose. Intervals of horizontal gray lines were determined arbitrarily so as to approximately match resurrection steps in each trace as follows: 8.9 Hz for panel A, left; 9.0 Hz for panel A, right; 4.2 Hz for panel B, left; and 4.0 Hz for panel B, right. (C and D) Speed-versus-resurrection step number for WT (C) or MotA(M206I)/MotB (D) stator unit is also shown. The speed for the first step differed between motors due to variance in the number of docked stators. Slopes of regression lines fitted to data points of each motor indicate the speed produced by a single stator unit.

stator was a rate-limiting step even in the MotA(M206I) motor. These results raise the possibility that the M206I mutation could affect the binding of proton to the proton channel in the MotA/MotB complex but not affect the proton dissociation from the stator. Based on the small dominant negative effect of the MotA(M206I)/MotB stator on motility in soft agar, the pH dependence of the motor rotation raises the following two hypotheses: a change in pH can (i) increase the speed produced by a single stator unit or (ii) restore the stator assembly.

Effect of external pH changes on the speed per stator unit. To verify whether an external pH reduction increases the motor speed produced by MotA(M206I) stator unit, we performed a resurrection test by expressing stator proteins from a plasmid within *Salmonella* Δ motA motB cells. In this experiment, a stepwise restoration of the rotation rate is observed by the induction of protein expression, with the number and size of steps indicating the number of stator units incorporated into the motor and the speed produced by a single stator unit, respectively (9, 35–37). Figure 6A and B show typical resurrection traces of cells carrying a plasmid expressing *motA motB* and one expressing *motA(M206I) motB*. After the addition of 0.075% arabinose at pH 7.0, incorporation of the WT stator to the motor increased the rotation rate with a step size of ~ 9 Hz, whereas expression of the MotA(M206I)/MotB stator restored the motor rotation with a step size smaller than that of the WT stator (~ 4 Hz). The torque-speed curve suggested that the MotA(M206I) mutation does not reduce the energy coupling efficiency of the motor near stall (Fig. 4). Therefore, it is possible that the MotA(M206I)

mutation could affect proton translocation through the proton channel and the energy coupling in a load-dependent manner, thereby decreasing the rotation rate of the motor when external load becomes lower and lower. Induction of stator expression at pH 5.5 extended the time for speed increment and reduced the number of resurrection steps by two or three (see Fig. S1 in the supplemental material) because the expression level of stator proteins at pH 5.5 was dropped to about half of that at pH 7.0 (Fig. S2). This suggests that such acidic conditions affect regulators for gene expression (38). The expression level of stators at pH 6.0 was not much different from that at pH 7.0 (Fig. S2). In agreement with this result, successive speed increments, reaching more than 50 Hz in WT motors, occurred at pH 6.0, and the resurrection step sizes of WT and MotA(M206I) motors appeared to be almost the same as those determined at pH 7.0. The slope of a regression line fitted to rotation rates plotted against the number of resurrection steps indicates the speed produced by a single stator unit (i.e., the speed per stator unit), giving 7.6 ± 0.6 Hz per WT stator unit ($n = 6$ motors) and 4.4 ± 0.2 Hz per MotA(M206I) stator unit ($n = 4$ motors) at pH 7.0 and 7.5 ± 0.3 Hz per WT stator unit ($n = 5$ motors) and 4.6 ± 0.3 Hz per MotA(M206I) stator unit ($n = 7$ motors) at pH 6.0 (Fig. 6C). Thus, the changes in external pH do not affect the unit speed of the MotA(M206I) stator as much as they do the unit speed of the WT stator. The average rotation rate of the MotA(M206I) motor was about 20 Hz at external pH 7.0 and 35 Hz at external pH 6.0 (Fig. 4), suggesting that the number of docked stators would be changed from 5 to 8 by lowering the external pH.

Effect of pH on stator assembly. Another possible cause for the pH-dependent increase in rotation rate of the MotA(M206I) motor is an increase in the number of docked stators around a rotor. We tested the effect of pH on stator assembly by analyzing the subcellular localization of stators using MotB labeled with the fluorescent protein mTurquoise2 (mTurq-MotB) by epifluorescence microscopy. As the fluorescence intensity of mTurquoise2 is not dependent on pH in the range of pH 5 to pH 8 (Fig. S3) (39), we were able to examine the efficiency of stator assembly under a wide range of pH conditions. Fluorescent spots were observed in cells expressing MotA/mTurq-MotB (Fig. 7A, left panel) and MotA(M206I)/mTurq-MotB (Fig. 7A, middle panel). At external pH 7.0, the number of fluorescent spots in MotA(M206I) cells was less than 50% of those in WT cells ($P < 0.001$) (Fig. 7C) although the fluorescence intensities did not show a clear difference (Fig. 7D). Under this experimental condition, the fluorescent spot corresponding to the motor containing a small number of mTurq-MotBs (e.g., 1 or 2 stator units) was not clear. When the number of mTurq-MotB-expressing cells incorporated into the motor increased, obscure spots became clearer and countable. The localization efficiency of stator units in each cell was estimated as the average intensity of fluorescent spots multiplied by the number of fluorescent spots per cell. We found that the localization efficiency of MotA(M206I) stator units was about 40% of that in the WT cells ($P < 0.001$). A decrease in the external pH to 5.5 restored the localization efficiencies of both MotA/mTurq-MotB and MotA(M206I)/mTurq-MotB stator complexes ($P < 0.001$) (Fig. 7C to E, gray bars). These results suggest that the change in speed of the MotA(M206I) motor, which was observed as external pH was lowered (Fig. 5A), would be caused by an increase in the number of docked stators. Improvement of stator incorporation was not observed in resurrection experiments under lower-pH conditions (Fig. 6), suggesting that acidification may affect protein expression level even at pH 6.0.

The recovery of stator assembly with decreasing pH was also observed in the presence of benzoate ($P < 0.001$) (Fig. 7C to E, orange bars), suggesting that the association of the stator with the rotor involves the process of proton uptake rather than that of proton dissociation from the stator. Fluorescent spots from the MotA/MotB(D33N) stators were observed at the WT level in pH 7.0 medium (Fig. 7C to E), as previously described (11). Localization of MotA/mTurq-MotB(D33N) was promoted by lowering the external pH ($P < 0.001$) (Fig. 7C to E). These results suggest that although the binding of proton to Asp33 of MotB may induce a conformational change of the

FIG 7 Effect of pH changes on subcellular localizations of stator units. (A) Epifluorescent images of *Salmonella* cells expressing MotA/mTurquoise2-MotB, MotA(M206I)/mTurquoise2-MotB, or MotA/mTurquoise2-MotB(D33N) obtained at pH 7.0 and pH 5.5. (B) Effect of benzoate or CCCP on the subcellular localization of WT, MotA(M206I), or MotB(D33N) stator; 20 mM potassium benzoate or 50 μ M CCCP was added to medium. The stator localization was quantified from fluorescent images. (C to E) The number of fluorescent spots per cell (C) and fluorescent intensity of each spot (D) were measured; the localization efficiency was evaluated by multiplying the number of spots by the intensity of the spots, representing the total number of docked stators per cell (E). Bars are standard deviations. AU, arbitrary units.

MotAB stator complex to allow the MotAB complex to become an active stator unit around a rotor, proper water wire formation inside the proton channel could stabilize an attachment of the stator complex to the motor. Addition of potassium benzoate collapses the pH differences across the membrane but not membrane potential. Thus, we examined if the collapse of total PMF by the addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to the medium affects the stator localiza-

tions and found that a considerable decrease in total PMF did not impair stator assembly in any of the strains we examined (Fig. 7B to E, light blue bars). This indicates that PMF is not required for stator assembly, as has previously been claimed (11). pH-dependent localization of the MotB(D33N) stator was also observed in the presence of CCCP, suggesting that the stator assembly efficiently occurs in an external-pH-dependent manner but not in either a PMF-dependent or proton translocation-coupled manner. To investigate the differences between *E. coli* and *Salmonella*, we constructed *E. coli* cells expressing *E. coli* MotA and MotB labeled with mTurquoise2 and observed the cells by fluorescence microscopy. Fluorescent spots were observed in *E. coli* cells expressing *E. coli* MotA/mTurq-MotB and MotA/mTurq-MotB(D32N) (Fig. S4). Moreover, addition of CCCP did not significantly affect the stator localization in *E. coli* cells seen in *Salmonella* cells (Fig. S4). These results indicate that both *E. coli* and *Salmonella* stators are incorporated into the motor without PMF.

DISCUSSION

Studies on a turnover of the flagellar stator have shown that stator dynamics depend on the input energy in several bacterial species. Typically, stators of Na⁺-driven motors are incorporated into a motor only in the presence of Na⁺ (14). Here, to clarify the effect of the MotA(M206I) mutation on stator assembly dynamics, we used *Salmonella* cells expressing MotA/MotB(206I) from an arabinose-inducible plasmid and showed that a decrease in external pH increases the probability of assembly of the MotA(M206I)/MotB stator unit to the motor. A previous pH disruption experiment using the light-driven proton pump proteorhodopsin showed PMF-dependent localization of the *E. coli* stator (13). We examined the effect of ΔpH only and total PMF including membrane potential using potassium benzoate and CCCP, respectively, and neither of them changed the efficiency of stator assembly in either *Salmonella* or *E. coli* flagellar motors (Fig. 7). Our result is inconsistent with the previous one, but it may be due to the differences in experimental conditions including PMF control, as Tipping et al. stated (13). Rotation rates of fully induced WT motors are independent of changes in external pH (Fig. 5A), but the fluorescent spot intensity from MotA/mTurq-MotB was increased by lowering the pH (Fig. 7). Reid et al. have analyzed resurrection traces of the *E. coli* motor, showing that speed increments caused when high numbers of stator units are incorporated are smaller than those at low stator numbers and that there were up to 11 increments in resurrection traces (9). Figure 5A shows that rotation rates of the fully induced WT motor reaches 80 Hz at pH 7.5. Since our resurrection experiments showed 7 to 8 Hz per WT stator unit, 10 or 11 stators were incorporated into the fully induced motors, suggesting that further incorporation of stator units would not affect the rotation rate notably. We observed that the WT motor rotating steadily at about 60 Hz (i.e., the number of docked stators was 8 or 9 in the motor) was accelerated up to 80 Hz by lowering external pH to 5.5 (see Fig. S5 in the supplemental material). Therefore, we suggest that the assembly of the MotA/MotB complex to the motor occurs in an external-pH-dependent manner and that the rate of proton translocation through the proton channel coupled with stator-rotor interactions affects the binding affinity of the MotA/MotB complex for the motor.

Biochemical assays (12, 28) and the crystal structure of the periplasmic region of MotB (8, 40) have suggested that FliG-MotA interaction triggers a conformational change in the periplasmic region of MotB, thereby anchoring the stator units to the peptidoglycan layer to induce a detachment of the plug segment from the MotA/MotB proton channel. A charged interaction between MotA-Arg90 and FliG-Asp289 plays a critical role in the arrangement of the stator units around the rotor (5). Taking our results together with these previous reports, we here provide a plausible model: (i) freely diffused stators approach from a membrane pool to the rotor via interactions between MotA-Arg90 and FliG-Asp289; (ii) the MotA-FliG interaction opens the plug; (iii) proton binding to the MotA/MotB complex stabilizes the association of the stator complex with the peptidoglycan layer around the motor. We showed that the MotA/MotB(D33N) stator unit also localized around the rotor in an external-pH-dependent

manner (Fig. 7), suggesting that proton binding to a site other than Asp33 works on the stator assembly. In agreement with this, a recent study by high-speed atomic force microscopy has revealed an Na⁺-induced conformational change of the periplasmic domain of the *Bacillus subtilis* MotP/MotS stator complex, suggesting that the Na⁺ binding to the C-terminal periplasmic region of MotS (MotSc) induces folding of the peptidoglycan binding domain of MotS to be assembled around the rotor (41). The binding of Na⁺ to Asp30 of MotS, which corresponds to Asp33 of *Salmonella* MotB, induces rapid and efficient detachment of a linker region containing the putative plug segment from the transmembrane Na⁺ channel to bind to the peptidoglycan layer to become an active stator unit in the *Bacillus* flagellar motor. In this study, we showed that a decrease in external pH distinctly enhanced the assembly efficiency of the MotA(M206I)/MotB complex into the motor even in the absence of PMF (Fig. 7). Since the M206I mutation does not significantly affect the proton conductivity of the unplugged MotA/MotB complex (lacking the plug segment), this suggests that the M206I mutation may affect plug detachment from the proton channel in the MotA/MotB complex. It has been reported that the MotB(D33E) mutation limits the proton channel activity of the MotA/MotB complex when the external load becomes low enough (42). Since external-pH-dependent stator assembly is also observed in the wild-type MotA/MotB motor, efficient proton binding to MotB-Asp33 may suppress the association of the plug segment with the proton channel in a load-dependent manner.

It has been shown that the MotB(D33E) mutation decreases not only the maximum torque at high load but also the maximum rotation rate at low load, suggesting that MotB-Asp33 directly couples proton translocation through a proton channel of the MotA/MotB complex to torque generation (31). The MotA(P173A) mutation does not affect the stall torque but considerably reduces the maximum motor speed at low load. The prolyl residue in MotA is believed to be responsible for conformational changes of the stator to dissociate protons from the stator to the cytoplasm (20). Thus, the MotA(P173A) mutation could retard conformational dynamics of the proton channel of the stator, thereby reducing the rate of proton translocation (43). The MotA(M206I) mutant motor showed a torque-speed relationship similar to that of the MotA(P173A) motor (Fig. 4). The MotA(M206I) motor produces a stall torque comparable to that of the WT (Fig. 4), indicating that the number of incorporated stators and the energy conversion efficiency of the MotA(M206I) motor are regained to the WT level at high loads. MotA-Met206 can be responsible for mechanosensitivity of the stator unit (15–17). Furthermore, it is possible that the energy conversion efficiency of the M206I motor varies in a load-dependent manner. Thus, we propose that MotA-Met206 may be involved in load-dependent stator assembly and torque generation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. To construct the plasmid carrying *motA* and mTurquoise2-*motB*, *motA-motB*, and mTurquoise2 genes were amplified by PCR from chromosomal DNA of SJW1103 and mTurquoise2-pBAD plasmid (54844; Addgene), respectively. The *E. coli motA-motB* genes were amplified by PCR from chromosomal DNA of W3110. Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis method (Stratagene). For the rotation assay of flagellar motors, YSC2123 carrying pYC20 and YSC2123 carrying pYS1 were used. L-broth, T-broth, and motility medium were prepared, as described previously (33). Potassium benzoate (20 mM) was used to change the intracellular pH, as described previously (32, 33).

Measurements of growth rate. The overnight culture of *Salmonella* cells carrying each plasmid was diluted 1:100 into T-broth containing ampicillin and then incubated at 30°C with shaking. After 3 h, 0.2% arabinose was added to the medium. Optical density of the cell culture was measured once an hour, using a spectrophotometer at a wavelength of 600 nm.

Measurements of proton conductivity. Intracellular pH of *E. coli* BL21(DE3) cells was measured with a ratiometric fluorescent pH indicator protein, pHluorin (29), as described previously (44).

Preparation of whole-cell proteins and immunoblotting. *Salmonella* cells were grown at 30°C in LB medium with shaking. After centrifugation, cell pellets were suspended in an SDS loading buffer and normalized by cell density to give a constant number of cells. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting with polyclonal anti-MotB antibody was carried out as described previously (45).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>Salmonella</i> strains		
SJW1103	Wild type for motility and chemotaxis	47
SJW2241	Δ motA-motB	48
YSC2123	Δ motA-motB Δ cheY Δ fimA Δ flc(Δ 204–292)	5
<i>E. coli</i> strains		
BL21(DE3)	Strain for protein expression	Novagen
RP6894	Δ motA-motB	J. S. Parkinson
Plasmids		
pBAD24	Expression vector	49
pET22b	Expression vector	Novagen
pYC17	pACTrc/pHluorin	50
pYC20	pBAD24/MotA MotB	44
pYC98	pBAD24/MotA MotB(D33N)	42
pYC109	pBAD24/MotA MotB(Δ 52–71)	44
pYC112	pBAD24/MotA MotB(Δ 52–71, D33N)	42
pYS1	pBAD24/MotA(M206I) MotB	This study
pYS1(Δ plug)	pBAD24/MotA(M206I) MotB(Δ 52–71)	This study
pYVM106	pET22b/MotA mTurquoise2-MotB	This study
pYVM107	pET22b/MotA(M206I) mTurquoise2-MotB	This study
pYVM108	pET22b/MotA mTurquoise2-MotB(D33N)	This study
pYVM109	<i>E. coli</i> MotA mTurquoise2-MotB/pET22b	This study
pYVM110	<i>E. coli</i> MotA mTurquoise2-MotB(D33N)/pET22b	This study

Motility assays. Motility in soft agar was tested as described before (11). For measurements of swimming speeds, cells grown at 30°C in LB medium with arabinose for 5 h were diluted 1:10 into motility medium (pH 7.0) and observed under a dark-field microscope at room temperature. Swimming speeds were determined as described before (46).

Bead assays. Bead assays with polystyrene beads with diameters of 1.1 μ m (Invitrogen) were carried out as described previously (9, 31, 32). Arabinose at 0.002% and 0.2% was used to induce expression of the stator protein at chromosomal and overexpression levels, respectively (17). For steady-state experiments, cells were grown in L-broth containing 100 μ g/ml ampicillin for 3.5 h at 30°C with shaking; 0.002% arabinose was added, and the mixture was further incubated for 30 min at 30°C with shaking. The flagellar filaments were sheared by passing cells through a 25-gauge needle before beads were labeled. Bead rotations were observed using a phase-contrast microscope (IX70 microscope with LUCPLFLN 20 $\times$ PH objective; Olympus) and recorded using a high-speed CMOS camera (acA800-510um; Basler) at intervals of 1 ms. For resurrection experiments, cells carrying plasmids were grown in T-broth containing ampicillin and 7.5×10^{-5} % arabinose for 2 h at 30°C; overproduction of the stator proteins was induced by the addition of 0.075% arabinose on the microscope stage. Optics and sampling rate for resurrection experiments were the same as those for steady-state ones. Resurrection traces were obtained by successively determining the power spectra of a 1-s data window for the WT and a 2-s window for the mutant with 0.1-s intervals, giving 1-Hz and 0.5-Hz speed resolution, respectively; because the rotation rate of the mutant motor was about half that of the WT motor, double-speed resolution is useful to determine the step level in resurrection traces of the mutant. Speeds for resurrection steps were determined by multi-Gaussian fitting to speed histograms (Fig. 6A and B, right panels) using Igor Pro software (WaveMetrics).

Fluorescence microscopy. Fluorescence of mTurquoise2 was observed using an inverted fluorescence microscope (IX73; Olympus) with a 100 $\times$ oil immersion objective (model UPLSAPO100XO, numerical aperture [NA] 1.4; Olympus) and a scientific CMOS (sCMOS) camera (Zyla4.2; Andor Technology). mTurquoise2 was excited by a 130-W mercury light source system (model U-HGLGPS; Olympus) with a fluorescence mirror unit (CFP-2432C-OFF; excitation band pass [BP] filter, 438/24 nm; emission BP filter, 483/32 nm) (Semrock). Fluorescence image processing was performed with ImageJ, version 1.51, software (National Institutes of Health) as described previously (5). The data were statistically analyzed by analysis of variance (ANOVA) with Tukey's multiple-comparison test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00727-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We thank Keiichi Namba, Shoichi Toyabe, and Yuta Hanaizumi for technical support, Tohru Minamino for providing the anti-MotA and anti-MotB antibodies, John S. Par-

kinson for the gift of RP6894, and Yong-Suk Che and Yoshiyuki Sowa for provision of unpublished data.

This work was supported by JSPS KAKENHI (grant 24570177 to S.K. and grant 24770141 to S.N.).

We have no conflicts of interest to declare.

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