Full Paper

Suppressor mutants from MotB-D24E and MotS-D30E in the flagellar stator complex of *Bacillus subtilis*

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The bacterial flagellar motor is mainly energized by either a proton (H⁺) or sodium ion (Na⁺) motive force and the motor torque is generated by interaction at the rotor-stator interface. MotA/MotB-type stators use H⁺ as the coupling ion, whereas MotP/MotS- and PomA/PomB-type stators use Na⁺. *Bacillus subtilis* employs both H⁺-coupled MotA/MotB and Na⁺-coupled MotP/MotS stators, which contribute to the torque required for flagellar rotation. In *Escherichia coli*, there is a universally conserved Asp-32 residue of MotB that is critical for motility and is a predicted H⁺-binding site. In *B. subtilis*, the conserved aspartic acid residue corresponds to Asp-24 of MotB (MotB-D24) and Asp-30 of MotS (MotS-D30). Here we report the isolation of two mutants, MotB-D24E and MotS-D30E, which showed a non-motile and poorly motile phenotype, respectively. Up-motile mutants were spontaneously isolated from each mutant. We identified a suppressor mutation at MotB-T181A and MotP-L172P, respectively. Mutants MotB-T181A and MotP-L172P showed about 50% motility and a poorly motile phenotype compared to each wild type strain. These suppressor sites were suggested to indirectly affect the structure of the ion influx pathway.

Key words: *Bacillus subtilis*, flagellar motor, stator, suppressor mutation

Introduction

Many bacteria swim using a rotating flagellum that comprises a basal body (rotary motor), a helical filament

(propeller), and a hook (universal joint). The flagellum is a complex rotary nano-machine made up of multiple copies of at least 25 different proteins (Macnab, 2003; Rajagopala et al., 2007). The energy for the rotation of the flagellar motor is supplied by an H⁺ or Na⁺ gradient across the cytoplasmic membrane. The motor consists of a rotor and some stators that function as an ion channel. The stators are most often called the Mot complex, which is considered to contain four MotA and two MotB subunits. The MotA/MotB complex of *Escherichia coli* is an H⁺-driven flagellar motor. In contrast, alkaliphilic *Bacillus* species or marine bacteria such as *Vibrio* species have MotP/MotS or PomA/PomB complexes that are Na⁺-driven flagellar motors (Manson et al., 1977; Bakeeva et al., 1986; Macnab, 1999; Berg, 2003; Blair, 2003; Li et al., 2011). Dual ion-coupling capacity is found in neutralophilic *Bacillus subtilis* with both MotA/MotB and MotP/MotS (Ito et al., 2004). The *motP/motS* genes are universally located down-stream of the *ccpA* gene, which encodes a central regulator of carbon metabolism in *Bacillus* species and other Gram-positive organisms (Moreno et al., 2001; Ito et al., 2011). As an exception, extremely alkaliphilic *Bacillus alcalophilus* Vedder 1934 has a MotP/MotS type stator which utilizes Na⁺, K⁺ and Rb⁺ as the coupling ions for flagellar rotation (Terahara et al., 2012).

In *E. coli*, a universally conserved aspartic acid residue is present at position 32 (Asp-32) of MotB, which is conserved in all MotB sequences presently known. In a previous random mutational study of MotB in *E. coli*, several other amino acid substitutions for Asp-32 were found to abolish motility, suggesting that Asp-32 functions as the coupling ion binding site. Only a glutamic acid substitution for Asp-32 retained some poor motility (Zhou et al., 1998).

Here we used *B. subtilis*, which employs two types of stators, MotA/MotB and MotP/MotS (Ito et al., 2004, 2005; Terahara et al., 2006). The stator subunits MotB and MotS

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from *B. subtilis* have conserved aspartic acid residues, corresponding to Asp-24 of MotB and Asp-30 of MotS. So far, there is no report whether these amino acids residues are also important for motility and for the coupling ion binding function in *B. subtilis*. In addition, the details of the ion influx pathway of each stator complex have not been characterized in *B. subtilis*. Therefore, first we generated several amino acid residue replacement mutants in Asp-24 of MotB and Asp-30 of MotS and investigated their motility on soft agar plates. Then, we investigated amino acid residues involved in the coupling ion influx pathway of each stator complex by identifying the mutation sites of their up-motile suppressor mutants. In this study, we developed data to elucidate the uncharacterized ion influx pathway of the flagellar stator complex from *B. subtilis*.

Materials and Methods

Bacterial strains, plasmids, growth conditions, and DNA cloning methods. All bacterial strains and plasmid constructs used in this study are listed in Tables 1 and 2, respectively. All primers are listed in Table S1 and their sequence positions are illustrated in Fig. S1. *E. coli* strains were routinely grown at 37°C in Luria Bertani medium (Sambrook et al., 1989). *B. subtilis* 168 strain BR151MA (wild-type) and its derivatives were routinely grown at 37°C in Luria Bertani medium for liquid cultures and different plating media were used for growth in agar. Spore stocks were prepared on agar plates of Difco Nutrient Sporulation Medium and grown on tryptose blood agar base (TBAB). Ampicillin (100 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹), and tetracycline (12.5 µg ml⁻¹) were added as required for

growing plasmid-bearing cells as well as for selecting transformants. Chromosomal DNA was prepared from *B. subtilis* BR151MA and its derivatives by the method of Wilson (Wilson, 2001). Each polymerase chain reaction (PCR) was performed with Phusion DNA polymerase (New England Biolabs, Ipswich, MA). Transformation of *E. coli* strains and all recombinant DNA manipulations were performed using standard methods (Sambrook et al., 1989). The details of each plasmid construction are described below. Recombinant transformants were selected by conventional techniques, and the presence of the insert was confirmed by digestion with appropriate restriction enzymes. All plasmids were confirmed to have the correct sequences. The sequencing was performed by Operon Biotechnologies (Tokyo, Japan), using an ABI PRISM 3100 Genetic Analyzer. All restriction enzymes were purchased from New England Biolabs.

Assays of motility on soft agar. Observations of motility were the basis for formulating Spizizen plating media containing 0.2%–0.25% Noble agar for assessment of motility (Spizizen, 1958; Ito et al., 2004). For MotB-D24 mutants and their derivatives, the medium was adjusted to pH 7.0. Only when screening up-motile candidates from MotB-D24 derivatives was the assay done at pH 6.0. The condition at pH 6.0 with its abundance of protons was expected to yield up-motile mutants compared to the condition at pH 7.0. For MotS-D30 mutants and their derivatives, the medium was adjusted to pH 8.0 with added 200 mM NaCl, if needed. Previously we reported that reducing levels of protons, such as from pH 7.0 to 8.0, facilitates MotPS-dependent motility in the presence of the suboptimal contaminating levels of Na⁺ in the basal medium (Ito et al.,

Table 1. Bacterial strains used in this study.

Strain	Phenotype ^a	Reference
<i>Escherichia coli</i>		
DH5αMCR	<i>F</i> - <i>mcrA</i> Δ1(<i>mrr</i> - <i>hsd</i> RMS- <i>mcr</i> BS) φ80 <i>dlacZ</i> Δ(<i>lacZYA</i> - <i>argF</i>)U169 <i>deoR</i> <i>recA</i> 1 <i>endA</i> 1 <i>supE</i> 44 <i>λ</i> <i>thi</i> -1 <i>gyr</i> -496 <i>relA</i> 1	Stratagene
<i>Bacillus subtilis</i>		
BR151MA	<i>lys</i> 3 <i>trip</i> C2, derived from Marburg 168 (wild type)	(Grundy et al., 1993)
ΔAB	BR151MA Δ <i>motAB</i>	This study
ΔAB ΔPS	BR151MA Δ <i>motAB</i> Δ <i>motPS</i>	This study
MotAB	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> Cm ^r	This study
MotPS	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> Cm ^r	This study
MotB-D24A	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> (MotB-D24A) Cm ^r	This study
MotB-D24A-up	MotB-D24A, spontaneous up-motile variant	This study
MotB-D24N	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> (MotB-D24N) Cm ^r	This study
MotB-D24N-up	MotB-D24N, spontaneous up-motile variant	This study
MotB-D24E	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> (MotB-D24E) Cm ^r	This study
MotB-D24E-up	MotB-D24E, spontaneous up-motile variant	This study
MotB-D24E/T181A	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> (MotB-D24E, MotB-T181A) Cm ^r	This study
MotB-T181A	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motAB</i> (MotB-T181A) Cm ^r	This study
MotS-D30A	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> (MotS-D30A) Cm ^r	This study
MotS-D30A-up	MotS-D30A, spontaneous up-motile variant	This study
MotS-D30N	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> (MotS-D30N) Cm ^r	This study
MotS-D30N-up	MotS-D30N, spontaneous up-motile variant	This study
MotS-D30E	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> (MotS-D30E) Cm ^r	This study
MotS-D30E-up1	MotS-D30E, spontaneous up-motile variant	This study
MotS-D30E-up2	MotS-D30E, spontaneous up-motile variant	This study
MotS-D30E-up3	MotS-D30E, spontaneous up-motile variant	This study
MotS-D30E-up4	MotS-D30E, spontaneous up-motile variant	This study
MotP-L172P/MotS-D30E	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> (MotP-L172P, MotS-D30E) Cm ^r	This study
MotP-L172P	ΔABΔPS <i>amyE</i> ::P _{motAB} - <i>motPS</i> (MotP-L172P) Cm ^r	This study

^aCm^r, chloramphenicol resistant.

Table 2. Plasmids used in this study.

Plasmid	Description ^a	Reference
pUC18	Cloning vector, Ap ^r	TaKaRa Bio
pCm::tet ^R	Cm ^r ::Tc ^r	(Itaya, 1992, Steinmetz and Richter, 1994)
pUC18 Tc	Cloning vector, Ap ^r ::Tc ^r	This study
pUC18 Tc-ΔmotAB	pUC18 Tc + ΔmotAB fragment	This study
pUC18 Tc-ΔmotPS	pUC18 Tc + ΔmotPS fragment	This study
pMW119	Cloning vector, Ap ^r	NIPPON GENE
pGEM7zf(+)	Cloning vector, Ap ^r	Promega
pDG1730	amyE integration vector with Ap ^r , Spe ^r , and Em ^r	(Guerout-Fleury et al., 1996)
pDG-MotAB	pDG1730 + P _{motAB-motAB} (mutation free)	(Ito et al., 2005)
pDG-MotPS	pDG1730 + P _{motAB-motPS} (mutation free)	(Ito et al., 2005)
pDR67	amyE integration vector with Ap ^r and Cm ^r	(Ireton et al., 1993)
pMW119-motB-D24A	pMW119 + P _{motAB-motAB} (motB-D24A)	This study
pMW119-motB-D24N	pMW119 + P _{motAB-motAB} (motB-D24N)	This study
pGEM7zf(+)-motB-D24E	pGEM7zf(+) + P _{motAB-motAB} (motB-D24E)	This study
pGEM7zf(+)-motB-D24E-T181A	pGEM7zf(+) + P _{motAB-motAB} (motB-D24E, T181A)	This study
pDR67-motB-D24A	pDR67 + P _{motAB-motAB} (motB-D24A)	This study
pDR67-motB-D24N	pDR67 + P _{motAB-motAB} (motB-D24N)	This study
pDR67-motB-D24E	pDR67 + P _{motAB-motAB} (motB-D24E)	This study
pDR67-motB-D24E-T181A	pDR67 + P _{motAB-motAB} (motB-D24E, T181A)	This study
pMW119-motS-D30A	pMW119 + P _{motAB-motPS} (motS-D30A)	This study
pMW119-motS-D30N	pMW119 + P _{motAB-motPS} (motS-D30N)	This study
pGEM7zf(+)-motS-D30E	pGEM7zf(+) + P _{motAB-motPS} (motS-D30E)	This study
pGEM7zf(+)-motS-D30E-motP-L172P	pGEM7zf(+) + P _{motAB-motPS} (motS-D30E, motP-L172P)	This study
pDR67-motS-D30A	pDR67 + P _{motAB-motPS} (motS-D30A)	This study
pDR67-motS-D30N	pDR67 + P _{motAB-motPS} (motS-D30N)	This study
pDR67-motS-D30E	pDR67 + P _{motAB-motPS} (motS-D30E)	This study
pDR67-motS-D30E-motP-L172P	pDR67 + P _{motAB-motPS} (motS-D30E, motP-L172P)	This study

^aAp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Spe^r, spectinomycin resistant; Em^r, erythromycin resistant.

2004). Therefore, assay of MotPS-dependent motility was done at pH 8.0. Plates were incubated at 37°C for 16 h and the diameters of the colonies were measured at the indicated times. All experiments were done independently at least three times.

Measurement of swimming speeds. For measurement of swimming speed, MotPS, MotS-D30E, and MotS-D30E up-motile mutant strains and MotP-L172P/MotS-D30E were grown for 5 h at 37°C in Spizizen medium plus 200 mM NaCl, pH 8.0 with shaking. Microscopic observation was carried out immediately by the hanging drop method using a Leica DMLB100 dark field microscope (400×) and Leica DC300F camera, Leica IM50 version 1.20 software (Leica Geosystems, Tokyo, Japan). The speed of 40 individual cells which were swimming (not tumbling) for more than 10 s was measured by 2D movement measurement capture 2D-PTV software (Digimo Co., Ltd., Osaka, Japan). All results shown are the averages of three independent experiments. The swimming speed is the average speed of >40 cells.

Alignment of the stator subunit with homologous proteins of several bacterial species. The amino acid sequences of the Mot subunit of several bacterial species were obtained using the BLASTP algorithm at NCBI (<http://www.ncbi.nih.gov/>). Selected amino acid residues in the alignment were analyzed using ClustalW (<http://align.genome.jp/>).

Construction of plasmid pUC18Tc. For construction of a marker-free mutation plasmid, a tetracycline resistance gene fragment derived from *NotI*- and *XbaI*-double-digested pCm::tetR was cloned into *ScaI*-digested pUC18, yielding pUC18Tc.

Construction of marker-free ΔmotAB and ΔmotPS deleted mutants. The ΔmotAB and ΔmotPS mutations were

constructed by gene splicing via overlap extension as described previously (Horton, 1996). For construction of a fragment between the upstream *motA* gene and downstream *motB* gene, two independent PCR reactions were performed on wild-type DNA with the primer sets MotAB-cm-1 and MotAB-cm-2 (i) and MotAB-cm-3 and MotAB-cm-5 (ii). The PCR products were used as templates for a second PCR with primers motAB-cm-1 and motAB-cm-5. The purified product of this reaction was cloned into *SmaI*-digested pUC18Tc, yielding pUC18Tc-ΔmotAB (Fig. S1A).

For construction of a fragment between the upstream *motP* gene and downstream *motS* gene, two independent PCR reactions were performed on wild-type DNA with the primer sets MotPS-cm-1 and MotPS-cm-2 (i) and MotAB-PS-3 and MotAB-PS-4 (ii). The PCR products were used as templates for a second PCR with primers motPS-cm-1 and motPS-cm-4. The purified product of this reaction was cloned into *SmaI*-digested pUC18Tc, yielding pUC18Tc-ΔmotPS (Fig. S1B).

To construct strain ΔABΔPS, the gene replacement approach that had been modified and developed to reference of personal communication from Dr. H. Kadokura at Kao Corporation was used. Plasmid pUC18Tc-ΔmotAB was used for constructing marker-free ΔmotA/motB mutants (strain ΔAB). The plasmid was directly transformed into competent cells of *B. subtilis* BR151MA, and then 12.5 μg ml⁻¹ tetracycline resistant (Tc^r) colonies were selected. The plasmid was not able to replicate in the *B. subtilis* host. Therefore, Tc-resistant colonies may have occurred by a single-crossover event in the neighborhood of the *motA*/*motB* locus. Each single-crossover candidate was confirmed by PCR reaction and then the mutant was used for isolation of a double-crossover marker-free mutant. The single colony

was inoculated in LB medium at 37°C with shaking overnight. The pre-growth was transferred to 50 ml of fresh LB medium with 1.5 µg ml⁻¹ Tc and the adjusted final OD₆₀₀ of the broth was 0.3, and then cells were grown at 37°C with shaking. During the growth, 50 µl of 300 mg ml⁻¹ ampicillin sodium was added into the broth after 1, 3 and 5 h. Seven hours later, a suitable amount of the broth was diluted by 0.8% NaCl and spread onto LB agar plates and incubated at 37°C overnight. More than 100 colonies was picked and transferred to LB agar plates with/without 12.5 µg ml⁻¹ tetracycline and incubated at 37°C overnight. Tet-sensitive strains were selected and each of the marker-free Δ *motA*/*motB* double-crossover candidates was confirmed by the PCR reaction, yielding strain Δ AB.

Plasmid pUC18Tc- Δ motPS was used for constructing marker-free Δ *motA*/*motB* and Δ *motP*/*motS* mutants (strain Δ AB Δ PS). The plasmid was directly transformed into competent cells of Δ AB, and then selected for 12.5 µg ml⁻¹ tetracycline-resistant (Tc^r) colonies. The strategy for the rest of the procedure was the same as when constructing mutant strain Δ AB. As a result, strain Δ AB Δ PS was obtained.

Strain Δ AB Δ PS was confirmed by PCR to have a deletion of both the *motAB* and the *motPS* regions. For this mutant and each type strain that was constructed using it, the phenotypes of several independent isolates was first assessed on soft agar plate assays to assure that the strain chosen for use in the studies was typical.

Construction of plasmid and point mutations into the *motB* subunit or *motS* subunit of *B. subtilis*. For construction of a fragment MotB-D24A, MotB-D24N and MotB-D24E, two independent PCR reactions were performed on wild-type genomic DNA with the primer sets MotAB-BH-F and MotAB-D24A-R (i), MotAB-BH-F and MotAB-D24N-R (ii), MotAB-BH-F and MotAB-D24E-R (iii), MotAB-D24A-F and MotAB-Sph-R (iv), MotAB-D24N-F and MotAB-Sph-R (v) and MotAB-D24E-F and MotAB-Sph-R (vi). The PCR products of a mixture of (i) and (iv), mixture of (ii) and (v), and mixture of (iii) and (vi) were used as templates for a second PCR with primers MotAB-BH-F and MotAB-Sph-R (Fig. S1A).

For construction of a fragment MotS-D30A, MotS-D30N and MotS-D30E, two independent PCR reactions were performed on strain MotPS genomic DNA for amplification of the front fragment with the primer sets MotAB-BH-F and MotPS-D30A-R (i), MotAB-BH-F and MotPS-D30N-R (ii), MotAB-BH-F and MotPS-D30E-R (iii), and on wild-type genomic DNA for amplification of the rear fragment MotPS-D30A-F and MotPS-Sph-R (iv), MotPS-D30N-F and MotPS-Sph-R (v) and MotPS-D30E-F and MotPS-Sph-R (vi). The PCR products of a mixture of (i) and (iv), mixture of (ii) and (v), and mixture of (iii) and (vi) were used as templates for a second PCR with primers MotAB-BH-F and MotPS-Sph-R (Fig. S1B).

For construction of a fragment MotAB-D24E-M and MotPS-D30E-M, PCR reactions were performed on strain MotAB-D24E-up genomic DNA with the primer sets MotAB-BH-F and MotAB-Sph-R, and on strain MotPS-D30E-up genomic DNA with the primer sets MotAB-BH-F and MotPS-Sph-R (Fig. S1).

Each purified product of these reactions was cloned into *Sma*I-digested pMW119 or pG7EM3zf(+) and it was

confirmed that each DNA sequence was mutation free. Each plasmid was digested with *Bam*HI and *Sph*I and then ligated with *Bam*HI- and *Sph*I-double-digested pDR67. Recombinant transformants were selected by conventional techniques. The presence of the insert with the correct sequence was confirmed.

Integration of selected *mot* genes into the *amyE* loci of particular mutant strains under a *P*_{motAB} promoter A Δ AB Δ PS strain was constructed with each of the selected *mot* mutation genes integrated into the chromosomal *amyE* locus under control of the *P*_{motAB} promoter. Plasmid pDR67 was used for construction of the *P*_{motAB} promoter (Iretton et al., 1993). Plasmid pDR67 contains fragments of the front and back ends of the *amyE* gene flanking a chloramphenicol resistance (Cm^r) gene. Each plasmid was used to transform particular mutants to a chloramphenicol-resistant, amylase-negative phenotype for the pDR67 derivative. Recombinant transformants were selected by conventional techniques, and the presence of the insert was confirmed. The strains used in this study are listed in Table 1. All were confirmed to have the correct sequences.

Visualization of flagella. Flagella staining was carried out as described by Aono et al. (Aono et al., 1992). *Bacillus subtilis* cells were cultured at 37°C in Spizizen I medium and transferred gently to a microscope slide. The sample was air-dried and treated for 2 min with staining solution containing 5% (w v⁻¹) tannic acid, 0.75% (w v⁻¹) FeCl₂, 0.01% NaOH, followed by ammoniac silver nitrate for 30 s. Observations of flagella were made using a Leica DMLB100 bright field microscope and Leica DC300F camera, Leica IM50 version 1.20 software (Leica Geosystems).

Western blot analysis. To investigate the effect of mutation on the expression level of stator protein in membrane fractions, a series of MotB-D24E mutants and a series of MotS-D30E mutant strains were grown in Spizizen I medium and harvested (9,000 × g, 15 min, 4°C) and washed in Tris Buffer (50 mM Tris-HCl pH 8.0). Cells were suspended in the same buffer and a protease inhibitor cocktail (SIGMA) was added. Five microliters of a membrane suspension (total 30 µg of membrane protein) from each *B. subtilis* transformant was used for one-dimensional sodium dodecyl sulfate (SDS)-PAGE analyses on membrane samples. The same volume of SDS loading buffer was added to each sample, after which the proteins were separated on 10% polyacrylamide SDS gels (Bio-Rad, Hercules, CA). The gels were then transferred to nitrocellulose filters (Bio-Rad) electrophoretically by the application of 15 V for 12 h in Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, 20% [v v⁻¹] methanol [pH 8.3]). The MotA, MotP and MotS proteins were detected by anti-MotA antibody, anti-MotP antibody and anti-MotS antibody (Terahara et al., 2006). MotB detectable anti-MotB antibody has not been generated yet; therefore, we did not attempt to detect the MotB protein. The protein concentration of the broken cell suspension was measured by the Lowry method with BSA as a standard (Cheng et al., 1994). Goat anti-rabbit horseradish peroxidase (Bio-Rad) was also used as the second antibody for detection of anti-MotA, anti-MotP or anti-MotS antibodies. ECL Prime solution (Amersham Biosciences, Little Chalfont, UK) was the usual detection reagent. A quantitative imaging system, the ChemiDoc XRS+ imaging system (Bio-Rad),

was used for detection and analysis of chemiluminescence images.

Results

Introduction of a single mutation into the universally conserved aspartic acid residue in both MotB and MotS from *B. subtilis* and analysis of motility of mot mutants

In this paper, we introduced several amino acid mutations into MotB-D24 and MotS-D30 residues from *B. subtilis* BR151MA, which is a derivative strain of Marburg 168. They are conserved in all MotB type subunit sequences. First, each mutant was tested for motility behavior under both liquid and soft agar conditions.

Strains $\Delta AB\Delta PS$, MotB-D24A, MotB-D24N, MotB-D24E, MotS-D30A, and MotS-D30N exhibited loss of motility on soft agar plates (Fig. 1A, B) and in liquid media (data not shown) in all experiments. However, strain MotS-D30E showed poor but detectable motility on Spizizen I medium containing 0.25% Noble agar plus 200 mM NaCl at pH 8.0 (Fig. 1C). This strain exhibited loss of swimming motility in liquid culture (Fig. 2) and on Spizizen I medium containing Noble agar at pH 8.0 (Fig. 1B). Therefore, the up-motile strains MotB-D24A-up, MotB-D24N-up, and MotB-D24E-up as well as MotS-D30A-up, MotS-D30N-up, and MotS-D30E-up were selected by several rounds of transfer of cells from the edge of a colony on Spizizen I medium containing 0.2% Noble agar at pH 6.0 for MotB-D24 derivatives and Spizizen I medium containing 0.2% Noble agar at pH 8.0 for MotS-D30 derivatives.

A panel of several up-motile strains was isolated from strains MotB-D24A, MotB-D24N, MotS-D30A, and

Fig. 1. Motility assay of MotB or MotS subunit mutants and their up-motile strains.

(A) Motility assays on 0.25% soft agar of strains MotAB, $\Delta AB\Delta PS$, MotB-D24 mutant strains, MotB-D24E-up and MotB-D24E/T181A cultured in Spizizen medium, pH 7.0, for 16 h at 37°C. (B) and (C) Motility assays on 0.25% soft agar of strains MotPS, $\Delta AB\Delta PS$, MotS-D30 mutant strains, MotS-D30E up-motile mutant strains and MotP-L172P/MotS-D30E cultured in Spizizen medium, pH 8.0 (B), and in Spizizen medium plus 200 mM NaCl, pH 8.0 (C), for 16 h at 37°C.

Fig. 2. MotS subunit mutants and their up-motile mutant strains measured by the swimming speed assay.

MotPS, MotS-D30E, MotS-D30E up-motile mutant strains and MotP-L172P/MotS-D30E were grown for 5 h at 37°C in Spizizen I medium plus 200 mM NaCl, pH 8.0 with shaking. The swimming speed is the average speed of > 40 cells.

MotS-D30N. However, all mutants were true revertants to the wild type. Because these mutants were generated by introducing a series of single base replacements such as Asp (GAC) to Ala (GCC) or Asn (AAC), we speculated that the single base replacements easily returned to the wild-type motility. Therefore, we constructed a series of double base replacement mutants including Asp (GAC) to Ala (GCA) and Asn (AAT) using the same strategy. As a result, no up-motile mutant could be isolated after more than 50 rounds of transfer of cells from the edge of a colony under the same selective conditions.

It is speculated that the position of MotB-D24 and MotS-D30 is one of the coupling ion binding sites. Therefore, it may be easier to isolate suppressor mutants from strains MotB-D24E and MotS-D30E. In addition to the above, a panel of a MotB-D24E-up strains and four MotS-D30E-up strains was isolated after several rounds of transfer of cells from the edge of a colony under the same selective conditions, and their *motAB* and *motPS* regions were sequenced. We detected a single mutation of MotB-T181A (ACT > GCT) in strain MotB-D24E-up and a single mutation of MotP-L172P (CTG > CCG) in all MotS-D30E-up strains.

The average motilities of the MotB-D24E-up candidate were approximately 46% compared with those of strain MotAB on the Spizizen I medium containing 0.25% Noble agar at pH 7.0 (Fig. 1A). The average flagellar numbers per cell in strains MotAB, MotB-D24E, and MotB-D24E-up were 7.0 ± 2.1 ($n = 33$), 4.21 ± 0.8 ($n = 34$), and 5.0 ± 1.5 ($n = 43$), respectively.

The motilities of the four MotS-D30E-up candidates were approximately 26% compared with those of strain MotPS on the Spizizen I medium plus 200 mM NaCl containing 0.25% Noble agar at pH 8.0 (Fig. 1C). The average flagellar numbers per cell in strains MotPS, MotS-D30E, MotS-D30E-up1, MotS-D30E-up2, MotS-D30E-up3, and MotS-D30E-up4 were 6.1 ± 1.5 ($n = 25$), 4.3 ± 1.6 ($n = 47$), 4.5 ± 1.1 ($n = 44$), 4.4 ± 1.3 ($n = 35$), 4.6 ± 1.4 ($n = 37$), and 4.5 ± 1.5 ($n = 36$), respectively.

The expression levels in the membranes of the MotA protein of up-motile strain MotB-D24E-up and MotP and MotS proteins of up-motile strain MotS-D30E-up, as indicated by Western blot analysis with anti-MotA, anti-MotP and anti-MotS polyclonal antibodies, respectively, were

Fig. 3. Western blot analysis of the expression levels of MotA and MotP proteins in membranes of each mutant strain.

For MotAB and its derivatives, cells were grown in Spizizen I medium (pH 7.0) at 37°C. For MotPS and its derivatives, cells were grown in Spizizen I medium plus 200 mM NaCl (pH 8.0) at 37°C. The procedures are described in the supporting information. The strain is indicated above each lane. M represents the molecular weight marker. A quantitative imaging system, the ChemiDoc XRS+ imaging system (Bio-Rad) was used for detection and analysis of the chemiluminescence image.

almost identical to or more than those of each parental strain (Fig. 3). On the other hand, the expression levels in the membranes of the MotA protein of non-motile strains MotB-D24A, MotB-D24N, MotB-D24E and MotP and MotS proteins of non-motile strains MotS-D30A, MotS-D30N and MotS-D30E were less than 20% of those of each parental strain (Fig. 3).

Complementation analysis

Both strains MotB-D24E-up and MotS-D30E-up were isolated by several rounds of transfer of cells from the edge of a colony on the soft agar plates (Fig. 1). Thus, it is possible that mutations in the chromosome other than in *mot* genes influenced the phenotype. Therefore, we introduced the same mutations (MotB-D24E/T181A and MotP-L172P/MotS-D30E) in the stator gene and integrated them into the *amyE* locus in the chromosome by homologous recombination. The resulting strains were designated as MotB-D24E/T181A and MotP-L172P/MotS-D30E, respectively, and their motility phenotype was determined.

The motility of strain MotP-L172P/MotS-D30E was almost identical to that of strain MotS-D30E-up (Fig. 2). No swimming behavior was observed in either strain MotB-D24E-up or MotB-D24E/T181A (Data not shown). However, the motility of each was observed by the swimming

plate assay (Fig. 1A). The motility of strain MotB-D24E/T181A was approximately 42% compared with that of strain MotB-D24E-up on the Spizizen I medium containing 0.25% Noble agar at pH 7.0 for 16 h (Fig. 1A). The average flagellar numbers per cell in strains MotB-D24E/T181A and MotP-L172P/MotS-D30E were 4.5 ± 1.7 ($n = 35$), and 4.2 ± 1.5 ($n = 40$), respectively.

These results confirmed that the suppressor mutations in strains MotB-D24E-up and MotS-D30E-up were critical for inducing the up-motile phenotype.

Analysis of motility of MotB-T181A and MotP-L172P mutants

To determine whether each suppressor mutation of MotB-T181A and MotP-L172P directly influences the motility, we introduced each mutation in the stator gene and integrated them into the *amyE* locus in the chromosome by homologous recombination. The resulting strains were designated as MotB-T181A and MotP-L172P, respectively, and their motility phenotype was determined.

The motility of strain MotB-T181A was approximately 571% and 80% compared with that of strains MotB-D24E/T181A and MotAB on the Spizizen I medium containing 0.25% Noble agar at pH 7.0 for 16 h (Fig. 1A). The motility of strain MotP-L172P was approximately 34% and 19% compared with that of strains MotP-L172L/MotS-D30E and MotPS on the Spizizen I medium containing 200 mM NaCl and 0.25% Noble agar at pH 8.0 for 16 h (Fig. 1C). The average flagellar numbers per cell in strains MotB-T181A and MotP-L172P were 6.3 ± 1.5 ($n = 43$), and 3.5 ± 0.8 ($n = 40$), respectively.

The intensity of the MotA band was increased 1.4 times in the MotAB and MotB-T181A samples (Fig. 3A), while the levels of MotP and MotS were decreased 0.4 and 0.6 times, respectively, in the MotPS and MotP-L172P samples (Fig. 3B, C).

Discussion

A panel of site-directed mutants in universally conserved aspartic acid residues in MotB and MotS

A panel of site-directed mutants in a conserved aspartic acid residue in MotB and MotS exhibited loss of motility, except for the poorly motile strain MotS-D30E (Fig. 1C). The expression level of the MotA or MotP subunit in each mutant was less than 0.2 of that of the wild type. However, our previous data suggested that the lower expression level of both stator subunits is enough to allow motility (Takahashi and Ito, 2014). We speculate that no motility or poor motility of these mutants may be due to loss of motor function. As another possibility, the expression level of each stator subunit of the up-motile mutants of MotB-D24E and MotS-D30E may also have some relation to the up-motile phenotype of each mutant.

In *B. subtilis*, replacement of aspartic acid with glutamic acid in the *motS* of strain MotS-D30E results in some functional complementation in the presence of added 200 mM NaCl in the medium. The resulting poorly motile phenotype of strain MotS-D30E is similar to that reported for the MotB-D32E mutant of *E. coli* (Zhou et al., 1998), suggesting that the aspartic acid residue is also critical for motility and the function of the coupling cation binding site

in *B. subtilis*.

Failure of isolation of up-motile mutants from MotB-D24A, MotB-D24N, MotS-D30A and MotS-D30N except true revertants may be supportive of the above speculation.

The plate assay of up-motile mutants of MotB-D24E and MotS-D30E showed motility not identical to each wild-type strain; however, each motor was still functional. Therefore, suppressor mutations in the stator complex protein of the up-motile mutant may be related to the coupling ion binding pathway.

Confirmation of mutation sites identified from the up-motile mutants MotB-D24E-up and MotS-D30E-up

The mutation sites in strains MotB-D24E-up and MotS-D30E-up were MotA-T181A (ACT>GCT) and MotP-L172P (CTG>CCG), respectively. The flagellar numbers of each up-motile mutant and their respective parental strains MotB-D24E and MotS-D30E were almost identical. Strain MotP-L172P/MotS-D30E exhibited up-motile phenotypes similar to strain MotS-D30E-up. On the other hand, strain MotB-D24E/T181A also exhibited an up-motile phenotype but the motility activity of strain

MotB-D24E/T181A was lower than that of strain MotB-D24E-up. This may mean strain MotB-D24E-up has more than two effective mutations in the chromosome. One of them is MotB-T181A, and the other mutation is located without the *motA/motB* gene region. In any case, the up-motile phenotype is caused by an additional mutation in the *mot* gene region.

Amino acid residues involved in the ion influx pathway of the flagellar motor stator in up-motile *B. subtilis* mutants

To understand the relationship between the flow of ions and stator-coupling suppressor mutations identified in *B. subtilis*, we first considered the point mutation of MotB-T181 in strain MotB-D24E-up. The crystal structures of the C-terminal region of the MotB subunits have been determined in *Salmonella typhimurium* and *Helicobacter pylori* (O'Neill and Roujeinikova, 2008; Kojima et al., 2009; O'Neill et al., 2011). Therefore, we performed a multiple alignment of MotB amino acid sequences from these strains and those from *B. subtilis* using ClustalW. As shown in Fig. 4A, MotB-T181 is predicted to be located in the peptidoglycan-binding (PGB) motif. In addition, MotB-T181 is located

Fig. 4. Multiple alignments of mutated sites from up-motile mutants and the location of Bs-MotA-L172 in the membrane region of *Bacillus subtilis* MotP/MotS.

(A) Structure-based sequence alignment of the C-terminal region of *Salmonella typhimurium* MotB (St-MotB) and *Helicobacter pylori* MotB (Hp-MotB) with *B. subtilis* MotB (Bs-MotB). Residues indicated by pink boxes in the aligned sequences are conserved in all members of the OmpA family of PGB proteins (Roujeinikova, 2008). Regions of secondary structures are indicated below the corresponding sequences as follows: red line, α -helix; green line, β -strand. The residue indicated by the red box in the Bs-MotB sequence is a mutation site (T181A) in strain MotAB-D24E-up. (B) Multiple sequence alignment between transmembrane (TM) segments 3 and 4 from *Escherichia coli* MotA and *B. subtilis* MotP. The residue indicated by the red box in the Bs-MotA sequence is a mutation site (L172P). (C) and (D) A model for the arrangement of TM segments B, A3, and A4 in the *E. coli* MotA/MotB complex from (C) and TM segments S, P3, and P4 in the *B. subtilis* MotP/MotS complex (D). In all panels, the view is from the periplasmic side of the membrane. Data from Braun et al. (2004) were modified to generate the schematics diagrams of C and D.

next to the aspartic acid residue that is important for association with the peptidoglycan layer in *H. pylori* (Roujeinikova, 2008). This aspartic acid residue is conserved in all of the members of the OmpA family of PGB proteins and is therefore likely to be involved in direct binding to peptidoglycan or in maintaining the fold around the residue recognized by peptidoglycans (Reboul et al., 2011). Further, it is predicted that the identical position of *B. subtilis* MotB-T181 is located between the β 3 and α 4 domains of the PGB motif in the crystal structure of the C-terminal region of MotB from *S. typhimurium* (Kojima et al., 2009)(Fig. 4A).

Blair et al. (1991) reported that the mutation in the *E. coli* MotB-T196I mutant, which resides in a position identical to *B. subtilis* MotB-T181, causes a loss of function. It is predicted that when the stators of *E. coli* and *S. typhimurium* have been incorporated into the flagellar motor, the PGB motif located at the C-terminus of the MotB subunit is fixed to the peptidoglycan layer causing the MotAB complex to open the proton channel. The suppressor mutation MotB-T181A in the up-motile strain MotB-D24E-up may indirectly influence the flow of coupling ions.

Next, we considered the case of a point mutation of MotP-L172P of strain MotS-D30E-up. The crystal structure of the flagellar stator has not been determined, except for the C-terminal hydrophilic region of MotB. Braun et al. (2004) reported that the predicted arrangement of the amino acid residues in the transmembrane segment of the MotA subunit was determined by the cross-linking experiments of the stator MotAB of *E. coli* (Fig. 4C). It was reported that a coupling ion influx pathway is formed by the third and fourth transmembrane segments of the MotA subunit and a single transmembrane segment of the MotB subunit (Braun et al., 2004).

Therefore, we performed a multiple alignment of the amino acid sequences of MotA from *E. coli* and those of MotP from *B. subtilis* using ClustalW. As shown in Fig. 4B, MotP-L172 is predicted to reside near the periplasmic end of the third transmembrane segment of MotP. This position is predicted to face the opposite side of the coupling ion influx pathway to access the pore (Fig. 4D). When proline is introduced into an α -helix, the helix bends slightly because of the lack of the hydrogen bond. The side-chain replacement from leucine to proline may affect the structure of the pore. Therefore, we predict that MotP-L172P indirectly influences the coupling ion pathway to access the pore and may cause MotS-D30E-up to recover motility. Suppressor analysis of a MotB-D33E mutation in *S. enterica* Serovar Typhimurium indicated that the second-site mutations recover a motor torque generation step involving stator-rotor interactions coupled with protonation/deprotonation of Glu-33, but not maximum proton conductivity (Che et al., 2008). The MotB-D33E mutation affects both torque generation and activity for the proton-conducting pathway, suggesting that the torque of the flagellar motor is generated by releasing into the cytoplasm the bound proton in the stator (Kim et al., 2008). However, the mechanism of coupling between the proton-conducting pathway and torque generation remains unknown. To understand this process, we will need to isolate and analyze other types of suppressor mutants and then combine the knowledge gained from their study with the information on mutation sites and torque analysis of

the motor.

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Supplementary Materials

Table S1. Oligonucleotides used in this study.

Fig. S1. Schematic diagram of the motA/motB and motP/motS locus showing the sizes of the predicted open reading frames.

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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