Mutations alter the sodium versus proton use of a *Bacillus clausii* flagellar motor and confer dual ion use on *Bacillus subtilis* motors

Naoya Terahara*, Terry A. Krulwich[†], and Masahiro Ito*[‡]

*Graduate School of Life Sciences and Bio-Nano Electronics Research Center, Toyo University, Oura-gun, Gunma 374-0193, Japan; and [†]Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY 10029

Bacterial flagella contain membrane-embedded stators, Mot complexes, that harness the energy of either transmembrane proton or sodium ion gradients to power motility. Use of sodium ion gradients is associated with elevated pH and sodium concentrations. The Mot complexes studied to date contain channels that use either protons or sodium ions, with some bacteria having only one type and others having two distinct Mot types with different ion-coupling. Here, alkaliphilic *Bacillus clausii* KSM-K16 was shown to be motile in a pH range from 7 to 11 although its genome encodes only one Mot (BCl-MotAB). Assays of swimming as a function of pH, sodium concentration, and ion-selective motility inhibitors showed that BCl-MotAB couples motility to sodium at the high end of its pH range but uses protons at lower pH. This pattern was confirmed in swimming assays of a statorless *Bacillus subtilis* mutant expressing either BCl-MotAB or one of the two *B. subtilis* stators, sodium-coupled Bs-MotPS or proton-coupled Bs-MotAB. Pairs of mutations in BCl-MotB were identified that converted the naturally bifunctional BCl-MotAB to stators that preferentially use either protons or sodium ions across the full pH range. We then identified trios of mutations that added a capacity for dual-ion coupling on the distinct *B. subtilis* Bs-MotAB and Bs-MotPS motors. Determinants that alter the specificity of bifunctional and single-coupled flagellar stators add to insights from studies of other ion-translocating transporters that use both protons and sodium ions.

alkaliphile | MotAB | MotPS | motility | bacteria

The bacterial flagella that support swimming in liquid and on surfaces are rigid, propeller-like structures. Their rotation is powered by electrochemical gradients of either protons or sodium ions across the cytoplasmic membrane (1, 2). Ion channels are part of membrane-embedded stator structures at the base of the flagella. They play a critical role in the transduction of chemiosmotic energy into mechanical energy. The stators of the flagellar motor are most often called Mot complexes. Mot complexes are thought to contain four MotA-like proteins, each of which has four transmembrane segments (TMS), and two MotB-like proteins, each of which has a single TMS (1–3). The MotB-like proteins have a major role in determining ion-coupling specificity (4, 5). Initially, only proton-coupled motility was documented in bacteria, e.g., in *Escherichia coli* (1, 2). However, work of Imae and colleagues in the 1980s showed that extremely alkaliphilic *Bacillus* species use only sodium ion gradients for motility (6, 7). This was followed by evidence that *Vibrio alginolyticus* and *Bacillus subtilis* possess two distinct stators with different ion specificities that make contributions to swimming under particular physical–chemical conditions of pH, salinity, and viscosity (8–12).To date, only a limited number of additional flagellar stators have been directly probed for a capacity to use sodium as the coupling ion. The MotAB-like system of alkaliphilic *Bacillus clausii* KSM-K16 that is described here is an example of a single stator that can use both protons and sodium ions.

B. clausii strains are a group of alkaliphilic bacteria that have received attention because of their use in probiotic formulations (13) and their production of industrially useful enzymes (14, 15). The genome sequence of *B. clausii* KSM-K16 revealed a single set of genes encoding a MotAB-like pair of proteins (GenBank accession no. AP006627). We use the designation BCl-MotAB for this Mot stator. The BCl-MotA and MotB proteins were closely related to the Bs-MotA and MotB that constitute the proton-coupled Mot in *B. subtilis* (identity: 52% and 45%, similarity: 73% and 64%, respectively). They less closely resembled the Bs-MotP and MotS that constitute a sodium-coupled Mot in *B. subtilis* (identity: 32% and 28%, similarity: 57% and 55%, respectively). Motility at very alkaline pH values is expected to require use of a sodium-coupled Mot (6, 16, 17). The presence of the single BCl-MotAB species in *B. clausii* KSM-K16 therefore raised the possibility that either the motility of this alkaliphile strain was lost in the upper pH range for growth or the BCl-MotAB is capable of using both protons and sodium ions so that its coupling pattern changes over its pH range as the relative magnitudes of the transmembrane sodium and proton motive forces change. Preliminary assays showed that *B. clausii* KSM-K16 was motile at pH values from 7 to 11, so we probed the effects of added sodium and ion-specific motility inhibitors on swimming. Because *B. clausii* KSM-K16 is not genetically accessible, we used a statorless (Δ*motAB*Δ*motPS*) mutant strain of *B. subtilis* as the host for experiments in which the functional properties of BCl-MotAB could be compared side by side with those of bona fide proton- and sodium-coupled Mot stators, Bs-MotAB and Bs-MotPS, respectively. The results supported the hypothesis that BCl-AB is bifunctional with respect to ion-coupling capacity. We then identified mutational changes in BCl-MotB that converted the bifunctional BCl-MotAB stator one that prefers either protons or sodium ions at both neutral and highly-alkaline pH and applied an analogous strategy to confer a bifunctional capacity on the ion-specific Bs-MotAB and Bs-MotPS of *B. subtilis*.

Results and Discussion

B. clausii Motility Responds to Added Sodium, Cyanide *m*-Chlorophenyl Hydrazone (CCCP), and 5-(*N*-Ethyl-*N*-Isopropyl)-Amiloride (EIPA) Differently at Neutral and Alkaline pH. Cells of *B. clausii* KSM-K16 had an average of 12 flagellae per cell, with an average length of 6 μm, when grown under several different conditions of pH and
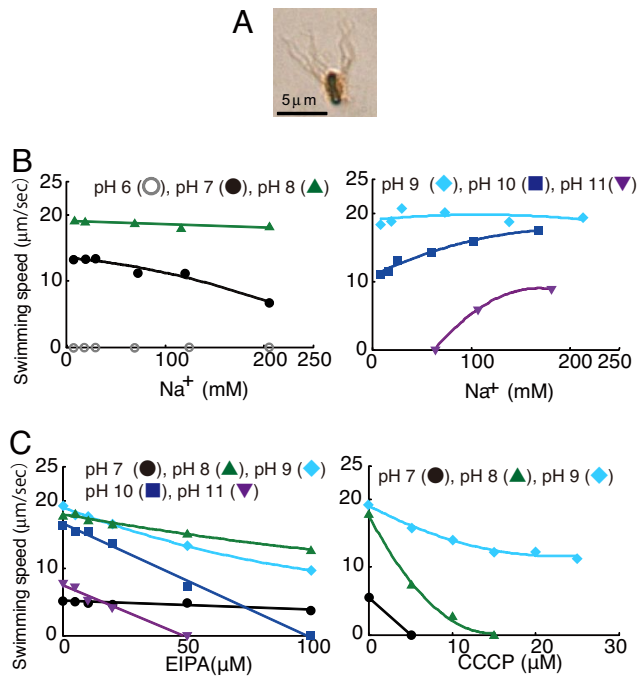
BIOCHEMISTRY

Fig. 1. Properties of the flagella and swimming of *B. clausii* KSM-K16. Cells were grown at 30°C in alkaline complex medium (pH 9.0 and 230 mM Na⁺). (*A*) Stained flagellae of wild-type *B. clausii* KSM-K16. Cells were stained with staining solution that contained 5% (wt/vol) tannic acid as described in *Materials and Methods*. (*B* and *C*) The pH values used in the particular assay are designated by symbols that are indicated. (*B*) The swimming speed of wild-type *B. clausii* in alkaline complex medium as a function of the sodium concentration and pH. The swimming speed was assayed at various concentrations of sodium as described in *Materials and Methods*. (*C*) Effect of EIPA and CCCP on the swimming speed of wild-type *B. clausii*. Swimming speeds were measured at various concentration of EIPA (*Left*) or CCCP (*Right*) in alkaline complex medium containing 230 mM sodium.

sodium concentration [Fig. 1 *A* and supporting information (SI) Fig. S1]. Swimming was observed over a pH range from 7.0 to 11.0 (Fig. 1*B*). The dependence of swimming on the concentration of sodium ions was examined in alkaline complex medium. This medium contained 6 mM sodium. Stimulation of swimming by higher sodium concentrations was observed only at pH 10 and above (Fig. 1*B*). At pH 10, stimulation by sodium was modest, but at pH 11, swimming of *B. clausii* depended on added sodium ions and required a threshold sodium concentration of ≈70 mM. Threshold concentrations at different levels were also observed for sodium-dependent Bs-MotPS at pH 7.0 (5) and pH 8.5 (shown in later figures). Such thresholds have been observed when freely swimming cells of other bacteria were assayed, but their basis is incompletely understood (1).

The sodium channel inhibitor EIPA, an amiloride analogue, did not affect swimming of *B. clausii* at pH 7.0 and affected swimming only modestly at pH 8.0 at inhibitor concentrations up to 100 μM (Fig. 1*C Left*). Complete inhibition of swimming at pH 10.0 and 11.0 was mediated by EIPA at concentrations of 100 and 50 μM, respectively. Conversely, addition of the protonophore CCCP, which dissipates electrochemical proton gradients, completely inhibited swimming at pH 7.0 and 8.0, whereas swimming at pH 9.0 was not inhibited greatly by uncoupler even at CCCP concentrations up to 25 μM (Fig. 1*C Right*). At the same pH, CCCP at 20 μM completely inhibited swimming powered by proton-coupled Bs-MotAB (shown in a later figure). Therefore, the modest inhibition of BCl-MotAB by 20 μM CCCP at pH 9.0 was not due to loss of the capacity of CCCP to uncouple at pH 9.0.
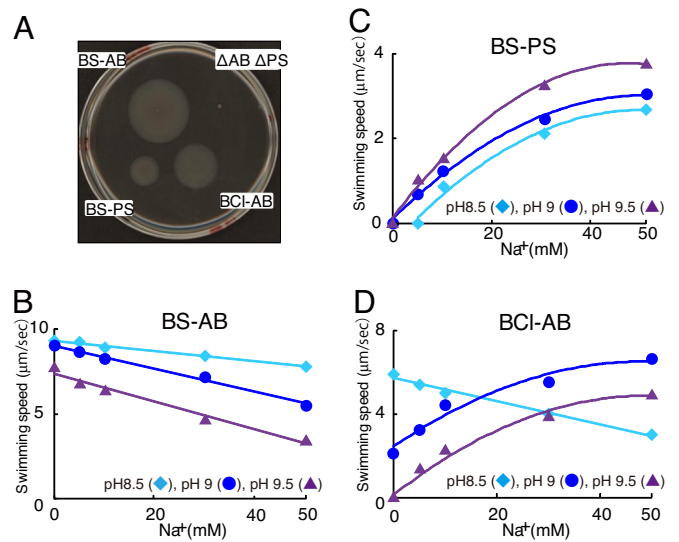

Fig. 2. The motility of a statorless *B. subtilis* mutant (ΔABΔPS) expressing *B. subtilis motAB* (BS-AB), *B. subtilis motPS* (BS-PS), or *B. clausii motAB* (BCl-AB). (*A*) Motility in a soft-agar plate assay. Fresh cells were inoculated into LB medium (22) (pH 7.0) soft agar and incubated at 37°C for 16 h. (*B–D*) Motility in liquid medium. BS-AB (*B*), BS-PS (*C*), and BCl-AB (*D*) cells were grown at 37°C in LB medium (pH 7.0), and the swimming speed was measured at various concentrations of sodium in 1/20 tryptone yeast extract (TY) medium (5) as described in *Materials and Methods*. The pH values of TY/20 medium used in each assay are designated by symbols as indicated.

The results suggested that the BCl-MotAB stator complex used protons with high preference at pH 7.0 and used sodium ions at pH 11. The modest inhibition of swimming by high concentrations of sodium at pH 7.0 (Fig. 1*B*) was observed previously with proton-powered motility of hybrid MotPB stators from *B. subtilis*. Elevated cytosolic sodium was hypothesized to mediate this inhibition via a cytoplasmic binding site for sodium (5). At pH values from 8.0 to 10.0, robust swimming across a range of sodium concentrations suggested that both protons and sodium ions could be used to power motility.

Properties of BCl-MotAB Resemble Those of Bs-MotAB at pH ≤ 8.5 and Resemble Those of Bs-MotPS at pH > 8.5. To further probe the ion-coupling preferences of BCl-MotAB at different pH values, we directly compared the swimming properties conferred by BCl-MotAB with those conferred by proton-coupled Bs-MotAB and sodium-coupled Bs-MotPS. BCl-*motAB* was introduced into the chromosome a *B. subtilis* strain (designated ΔABΔPS) from which both native Bs-*motAB* and Bs-*motPS* were deleted. This produced the mutant strain named BCl-AB that expressed BCl-*motAB* under control of the *B. subtilis motAB* promoter. The *B. subtilis motAB* and *motPS* were each expressed in the same manner in strains of ΔABΔPS designated as BS-AB and BS-PS, respectively. All three stators restored motility to the nonmotile ΔABΔPS strain on soft-agar plates (Fig. 2*A*). Swimming-speed assays in liquid were conducted in tryptone-yeast extract medium (TY) that contained 15 mM sodium and in TY/20 medium that was TY diluted 1:20 with water and contained micromolar concentrations of sodium (5). The richer TY medium supported faster swimming speeds than TY/20. As observed earlier (5, 9), the BS-AB strain expressing proton-coupled Bs-MotAB consistently swam at faster speeds than the BS-PS strain expressing sodium-coupled Bs-MotPS in BS-PS. The swimming speed of BCl-AB was between that of the other two strains. Sodium-coupled swimming in some bacteria occurs with speeds higher than observed for proton-coupled swimming, although this is not

the case with the two *B. subtilis* stators (1, 18). We have hypothesized that in laboratory strains of *B. subtilis*, the interactions of Bs-MotAB are optimized for interactions with the single FliG rotor protein relative to those of Bs-MotPS (5, 9).

The swimming speeds of the *B. subtilis* strains were first studied at pH values from 6.0 to 9.5 in TY medium, in which the BS-AB and BS-PS strains exhibited distinct responses to changes in both pH and sodium ion concentration (5). Overall, the responses of BCl-AB strain resembled those of BS-AB in the pH range from 6 to 8.5 and those of BS-PS at pH 9 and 9.5. However, the sodium content of TY medium supported significant swimming of the completely sodium-coupled BS-PS strain in the absence of added sodium ([Fig. S2](#)). Therefore, detailed comparisons of the sodium-dependence of swimming were conducted on the BS-AB, BS-PS, and BCl-AB strains in TY/20 medium at pH ≥8.5. At this pH, sodium appeared to be the preferred ion for powering the BCl-MotAB stator. In the TY/20 medium, the BS-AB strain exhibited the fastest swimming speed at either pH 8.5 or 9.0. Slower swimming was observed at pH 9.5 and at elevated sodium concentrations (Fig. 2*B*). The basis for inhibitory effects of sodium on the proton-coupled stator has not been investigated. In contrast to the BS-AB swimming pattern, the BS-PS strain exhibited no swimming in the absence of added sodium at either pH 8.5, 9.0 or 9.5, and the fastest swimming was at pH 9.5 (Fig. 2*C*). Swimming of the BCl-AB strain was inhibited rather than stimulated by sodium at pH 8.5, as seen with the proton-coupled BS-AB control strain, but BCl-AB exhibited a sodium-dependent pattern like the sodium-coupled BS-PS strain at pH 9.5 (Fig. 2*D*). At pH 9.0, BCl-AB exhibited evident stimulation by increasing sodium concentrations while still swimming in the absence of added sodium. This suggested that BCl-MotAB uses both protons and sodium ions at pH 9.0 (Fig. 2*D*).

We then examined the effects of added EIPA and CCCP on the swimming speed of the *B. subtilis* strains with the different stators in TY medium containing 63 mM sodium over a range of pH from 8.0 to 9.5 (Fig. 3). The two *B. subtilis* stators functioned as expected (5). The swimming speeds of the sodium-coupled BS-PS strain were extremely sensitive to inhibition by EIPA but insensitive to inhibition by CCCP at every pH value. Swimming of the BS-AB strain, on the other hand, was sensitive to inhibition by CCCP but not by EIPA at every pH value. The BCl-AB strain exhibited a lower swimming speed than BS-AB, but its inhibition pattern resembled that of BS-AB at pH 8.0 and 8.5. Swimming of the BCl-AB strain differed from that of BS-AB in showing modest inhibition by EIPA, as opposed to none, and in requiring a higher concentration of CCCP for full inhibition of swimming. At pH 9.0 and 9.5, the swimming speed of the BCl-AB strain was as sensitive to EIPA inhibition as that of sodium-coupled BS-PS (Fig. 3*A*, bottom two images). Still, there was an indication of continued proton use by BCl-MotAB stator in the BCl-AB strain at pH 9.0. At pH 9.0, swimming of the BCl-AB strain was inhibited 28% by 25 μM CCCP, whereas swimming of the sodium-coupled BS-PS strain was not inhibited at all (Fig. 3*B*, third image in the column). At pH 9.5, the inhibition pattern of the BCl-AB strains was completely comparable with that of BS-PS (Fig. 3 *A* and *B* bottom images). This was consistent with the complete dependence of swimming by the BCl-AB strain on added sodium (Fig. 2*D*).

The comparative swimming assays of BCl-MotAB and control stators of known ion-coupling specificity in the *B. subtilis* setting further supported the conclusion that the MotAB flagellar stator of *B. clausii* is bifunctional. BCl-MotAB primarily uses proton coupling for swimming at pH ≤8.5, primarily sodium coupling for swimming at pH 9.0 and exclusively uses sodium coupling at pH 9.5 in the *B. subtilis* host. The pH at which complete sodium-dependence is observed is lower in the *B. subtilis* host (pH 9.5) than in the native *B. clausii* setting (pH 11), although
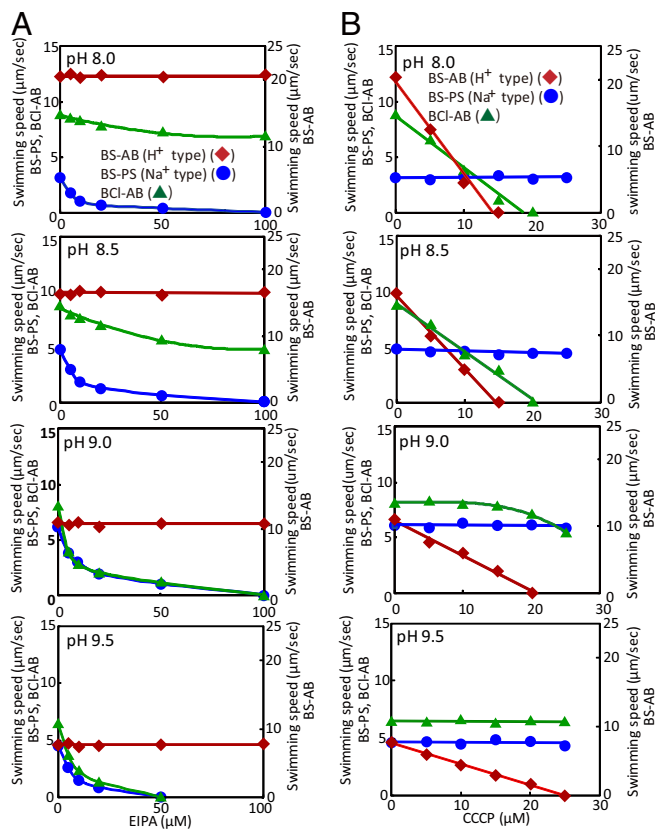

Fig. 3. Effect of EIPA and CCCP on swimming of *B. subtilis* expressing different stators. The swimming speed of BS-AB (filled diamond), BS-PS (filled circle), and BCl-AB (filled triangle) in TY medium containing 63 mM sodium was examined at various concentrations of EIPA (*A*) or CCCP (*B*). The pH values of the TY medium used for the assays were pH 8.0 (*First*), 8.5 (*Second*), 9.0 (*Third*), and 9.5 (*Fourth*). The swimming speed was analyzed as described in *Materials and Methods*.

in both settings, BCl-MotAB increases its use of sodium as the pH becomes increasingly alkaline. Perhaps the different pH for transition to sodium dependence relates to differences in membrane properties or bioenergetic parameters that impact the functional properties of the stator.

Mutations in BCl-MotB Change the Profile of the Bifunctional BCl-MotAB Stator to a Proton-Coupled or Sodium-Coupled Stator Profile. The next challenge was to identify determinants that could change the bifunctional *B. clausii* MotAB system to one that primarily uses either protons or sodium at both neutral and alkaline pH. The MotB-like proteins are major determinants of the ion specificity of the *B. subtilis* MotAB vs. MotPS systems (5). To choose mutations, we focused on the alignment of the BCl-MotB with MotB and MotS from *B. subtilis* and from modestly alkaliphilic *Oceanobacillus iheyensis* HTE831, each of which possesses both stator types (Fig. 4*A*). We noted that membrane protein segments of alkaliphiles that are exposed outside the surface of the cytoplasmic membrane generally have greatly reduced abundance of basic amino acids and increased acidic residues relative to the same segments of neutrophile homologues (17). We expected that a conserved ion-selective domain would be located in or on the surface of the more highly conserved transmembrane segment rather than in these poorly conserved hydrophilic segments further outside the surface.

For construction of a proton-coupled stator (BCl-MotAB-H) and a sodium-coupled stator (BCl-MotAB-Na) from bifunctional BCl-MotAB, we initially made two distinct triple mutants
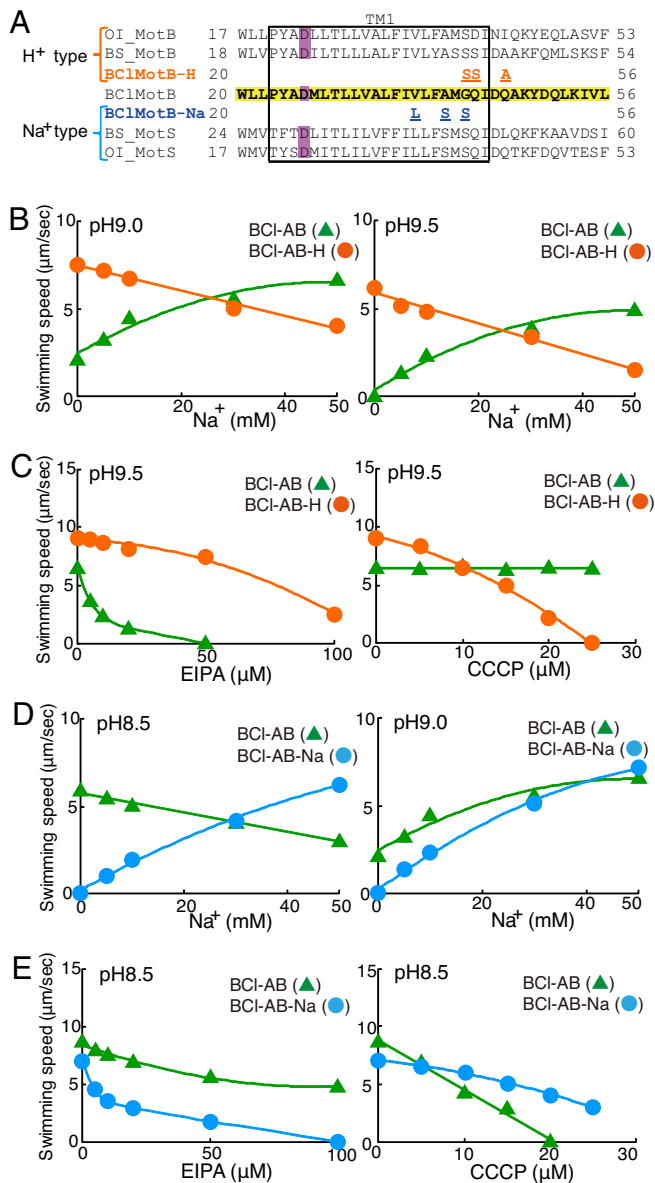
The two triple-mutant BCl-MotB forms were introduced into BCl-MotAB. The properties of the mutant stators BCl-MotAB-H and BCl-MotAB-Na were then studied in *B. subtilis* ΔABΔPS (strains BCl-AB-H and BCl-AB-Na, respectively) in comparison with the wild-type *B. clausii* stator expressed in strain BCl-AB. The BCl-AB-H strain exhibited a BS-AB type of pattern: In TY/20 medium, swimming was best at the lowest sodium concentration at pH 9.0 and 9.5 (Fig. 4*B*); in TY medium with 63 mM sodium at pH 9.5, swimming by the mutant was only partially inhibited by 100 μM EIPA, whereas swimming supported by the parent stator in BCl-AB was completely inhibited by 50 μM EIPA (Fig. 4*C Left*); and conversely, swimming by the BCl-AB strain was not inhibited by CCCP up to 25 μM, whereas swimming supported by the triple-mutant BCl-MotAB-H stator in strain BCl-AB-H was completely inhibited (Fig. 4*C Right*). These results showed that the BCl-MotAB-H stator used protons rather than sodium as the major coupling ion for flagellar rotation at pH 9.0 and 9.5. The BCl-AB-Na strain exhibited a BS-PS type of pattern: Swimming of BCl-AB-Na was sodium dependent at pH 8.5 and 9.0 in TY/20 medium (Fig. 4*D*); swimming at pH 8.5 was completely inhibited at by 100 μM EIPA, whereas the parent BCl-AB was only partially inhibited; and swimming of the BCl-AB-Na strain was more resistant to inhibition by CCCP than swimming of BCl-AB (Fig. 4*E*). We also conducted a set of experiments on the full panel of single-stator strains at pH 7.0 and examined the swimming speed as a function of sodium, EIPA, or CCCP concentration. Again, the pattern of responses of BCl-MotAB-H most closely resembled that of Bs-MotAB, and that of BCl-MotAB-Na most closely resembled that of Bs-MotPS in these assays (Fig. S4). The change in ion-coupling profiles of the two BCl-MotAB mutant forms suggested that the mutational changes in BCl-MotB optimized the binding and/or translocation of one of the coupling ions (e.g., protons for BCl-MotAB-H) so that the other cation (e.g., sodium for BCl-MotAB-H) was no longer used preferentially at pH values where it is favored for coupling of the parent BCl-MotAB.

To investigate the responsible amino acid change(s) in the two triple mutants, each of the mutations of the triple mutants was introduced into BCl-MotB alone or together with one of the other mutations for BCl-MotAB-H or BCl-MotAB-Na and expressed in strain ΔABΔPS. The effect of sodium on swimming at pH 9.0 was tested as an index of sodium-coupling retention in the BCl-MotAB-H stator. The analyses showed that the BCl-MotB-Q43S mutation was critical for loss of sodium coupling and that a combination of the Q43S mutation plus either the G42S or Q46A change was required to achieve loss of sodium use

Fig. 4. Effect of EIPA and CCCP on swimming of *B. subtilis* expressing wild-type BCl-AB or mutant *B. clausii* stators. (*A*) Multiple alignment of the region containing the single transmembrane segment of *Oceanobacillus iheyensis* MotB (OI_MotB) and MotS (OI_MotS), *B. subtilis* MotB (BS_MotB), MotS (BS_MotS), and *B. clausii* MotB (BClMotB). The sequence of *B. clausii* MotB is highlighted in yellow. The red letters show locations of point mutations in *B. clausii* MotB mimicking BS_MotB to yield BClMotB-H. The blue letters show locations of point mutations in *B. clausii* MotB mimicking BS_MotS to yield BClMotB-Na. D26 is critical for flagellar rotation and is highlighted with violet (2). (*B*) The swimming speeds of strains BCl-AB (filled triangles) and BCl-AB-H (filled circles) were measured in TY/20 medium [pH 9.0 (*Left*) and pH 9.5 (*Right*)] at various concentrations of sodium. (*C*) Effect of EIPA and CCCP on swimming speeds of strains BCl-AB (filled triangles) and BCl-AB-H (filled circles). The swimming speeds were measured in TY medium (pH 9.5, 63 mM Na$^+$) at various concentrations of EIPA (*Left*) or CCCP (*Right*). (*D*) The swimming speeds of strains BCl-AB (filled triangles) and BCl-AB-Na (filled circles) were measured in TY/20 medium [pH 8.5 (*Left*) and pH 9.0 (*Right*)] at various concentrations of sodium. (*E*) Effect of EIPA and CCCP on the swimming speeds of strains BCl-AB (filled triangles) and BCl-AB-Na (filled circles). The swimming speeds were measured in TY medium (pH 8.5, 63 mM Na$^+$) at various concentrations of EIPA (*Left*) or CCCP (*Right*).

in which one of the mutations was common to both triple mutants. We hypothesized that replacing the nonconsensus G42 with the conserved serine might reduce flexibility that contrib-
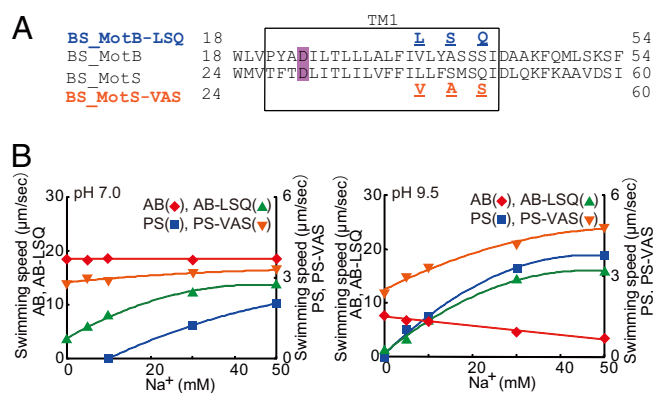
Fig. 5. Mutations that affect the ion-coupling capacities of Bs-MotAB and Bs-MotPS. (*A*) An alignment of a region of Bs-MotB and Bs-MotS (central two lines). The mutational changes introduced into the MotB of Bs-MotAB-LSQ are shown in the top line and those introduced into the MotS of Bs-MotPS are shown in the bottom line. (*B*) Swimming speed of Bs-MotAB, Bs-MotAB-LSQ, Bs-MotPS, and Bs-MotPS-VAS, as designated by symbols, was assayed as a function of sodium concentration at pH 7.0 (*Left*) and 9.5 (*Right*) in TY/20.

(Fig. S5). Q43 is predicted to be located at the border of the TMS and outer surface by the secondary structure prediction programs TMHMM (www.cbs.dtu.dk/services/TMHMM/) and SO-SUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) (Fig. S6). For assessment of the BCl-MotAB-Na type, the single- and double-mutant forms in ΔABΔPS were tested for sodium-dependent swimming at pH 8.5. The results showed that the BCl-MotAB-V37L mutation was critical and that a combination of V37L and either the A40S or the G42S mutation was required for the conversion to sodium coupling at low pH (Fig. S5). V37 is predicted to be located in the middle of the single TMS of BCl-MotB on the same face as the conserved aspartic acid residue, D26, that is required for flagellar rotation (Fig. S6) (2). Thus the mutation in G42 was not necessary for conversion of the bifunctional *B. clausii* to either single-coupling form. There was a critical residue for generating each form, Leu[37] and Val[37], respectively, for proton and sodium preference. In addition there was a requirement for one additional change for which there was some flexibility.

Triple Mutants of Proton-Specific Bs-MotAB and Sodium-Specific Bs-MotPS Gain a Bifunctional Capacity. We were interested in testing whether the general strategy that converted the native bifunctional BCl-MotAB to more single ion-coupled forms could confer a bifunctional capacity on the two native single ion-coupled stators of *B. subtilis*. Based on the alignment of Bs-MotB and Bs-MotS (Fig. 5*A*), we constructed two mutants. In mutant Bs-MotAB-LSQ, the crucial Val of Bs-MotB was mutated to Leu together with A → S and S → Q changes. All three of the changes in Bs-MotB introduced residues corresponding to the MotS sequence in those positions. In mutant Bs-MotPS-VAS, the crucial Leu of Bs-MotS was mutated to Val together with S → A and Q → S changes that changed Bs-MotS residues to the MotB sequence (Fig. 5*A*). When compared with the parent Bs-MotAB in strain BS-AB at pH 7.0, swimming of mutant BS-AB-LSQ at pH 7.0 was clearly stimulated by sodium, although it was not completely dependent as was BS-PS (Fig. 5*B Left*). At pH 9.5, swimming of BS-AB-LSQ depended on sodium and was almost as robust as that of BS-PS (Fig. 5*B Right*). When compared with parent Bs-MotPS in strain BS-PS at pH 7.0, swimming of mutant BS-PS-VAS exhibited very little stimulation by sodium and was more like BS-AB in its response to sodium (Fig. 5*B Left*). At pH 9.5, BS-PS-VAS, unlike the BS-PS strain with the parent stator, exhibited a significant capacity to swim without added sodium but still exhibited stimulation by sodium

(Fig. 5*B Right*). The results suggested that the two mutant forms retained their original ion-coupling capacity but gained a capacity to couple swimming to a new cation. The patterns of inhibition by EIPA and CCCP supported this conclusion (Fig. S7).

A major finding of the current study was the demonstration that a single bacterial flagellar stator of *B. clausii* can couple motility to either protons or sodium ions. This finding may facilitate identification of additional examples of bifunctional cation-coupling capacity among the rapidly growing number of bacteria that exhibit motility in neutral to alkaline pH range and for which genomic sequence is available. We also identified mutations that can convert the bifunctional stator to more specific types and mutations that confer bifunctionality on the single ion-coupled *B. subtilis* stators. Further mutagenesis studies and, ultimately, the correlation of these types of data with high-resolution structural data on these and other transporters that catalyze both proton- and sodium-coupled bioenergetic work will inform detailed models of the cation-binding sites.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. The strains and plasmids used in this study are shown in Table S1. *B. subtilis* 168 strain BR151MA (wild type) and its derivatives were grown at 37°C in LB medium (22). When appropriate, media were supplemented with erythromycin (0.3 μg/ml), neomycin (7.5 μg/ml), or chloramphenicol (5 μg/ml). LB medium containing 0.3% (wt/vol) Noble agar (Difco) was used in assays of motility on soft-agar plates. For assays of swimming speed in liquid, TY medium [1% (wt/vol) Bacto Tryptone and 0.5% (wt/vol) yeast extract] or a 1:20 dilution of TY with water (TY/20) was used at the indicated values of pH and sodium, EIPA, or CCCP (23). The actual final sodium concentration of each assay medium was determined with a flame photometer (Model AMA-175; Tokyo Koden) calibrated with standard sodium solutions of known concentrations. *B. clausii* KSM-K16 cells were grown at 30°C in alkaline complex medium (pH 9.0 and 230 mM sodium) containing 15.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.05 g of $MgSO_4·7H_2O$, 0.34 g of citric acid, 5 g of peptone, 2 g of yeast extract, 5 g of glucose, and 10.6 g of Na_2CO_3 per liter. For swimming speed assays with inhibitors, cells were grown in this medium and then resuspended in fresh medium at the indicated values of pH and sodium content for the assays. For swimming assays over a range of sodium, the cells were grown and harvested as above and then washed and resuspended in alkaline complex medium in which the Na_2CO_3 was replaced by K_2CO_3. Sodium was added as indicated, and adjustments in the potassium content were made to maintain constant ionic strength. The sodium concentration of the medium was determined as described above. *E. coli* cells were grown at 37°C in LB medium. In experiments in which sodium concentrations were varied, constant ionic strength was maintained in the media by adjusting the potassium content of the medium.

Construction of Plasmid Coding *B. clausii* *motAB* Under *B. subtilis* P*motAB* Promoter and Its Point Mutations. All recombinant DNA manipulations were carried out by standard methods. The primers used in this study are shown in Table S2. For cloning of the *B. clausii* *motAB* gene under the *B. subtilis* P*motAB* promoter, we performed gene splicing by the overlap extension (gene SOEing) method based on PCR (24). The primers were synthesized based on sequences from *B. subtilis* and *B. clausii*. The *B. subtilis* P*motAB* promoter was amplified by PCR using *B. subtilis* chromosomal DNA as the template with the set of primers, BSmotAB-BamHI-F designed with a BamHI site and BSPmotAB-BclmotAB-R in which a sequence of N-terminal region of the *B. clausii* *motAB* gene was introduced. The *B. clausii* *motAB* gene was amplified by PCR using *B. clausii* chromosomal DNA as the template with the set of primers BclmotAB-4 and BSPmotAB-BclmotAB-F in which the promoter sequence of *B. subtilis* P*motAB* promoter was introduced. We performed PCR with these two product mixtures as the template to join the *B. subtilis* P*motAB* promoter and the *B. clausii* *motAB* gene. The amplified fragment was digested by BamHI and SphI and cloned into BamHI- and SphI-digested pDR67, yielding pDR-AB. To introduce amino acid substitutions into the *B. clausii* *motB* gene, we used the gene SOEing method. We synthesized pairs of mutant primers to the sense strand of the *B. clausii* *motB* gene, with a mismatch at the mutation site. We amplified the *B. clausii* *motAB* under a *B. subtilis* P*motAB* promoter by PCR with mutant primers and then cloned into BamHI- and SphI-digested pDR67. Mutations that resulted in amino acid substitutions were introduced into the *B. subtilis* *motB* and *motS* genes by using the same method. We amplified the *B. subtilis* *motAB* and *motPS* under a *B. subtilis* P*motAB* promoter by PCR with mutant primers and then cloned the fragment into BamHI- and SphI-digested pDR67. The presence of these mutations was confirmed by DNA sequencing.

Each plasmid was integrated into the *amyE* locus of ΔABΔPS *B. subtilis* host. Recombinant transformants were selected by conventional techniques. The presence of the insert with the correct sequence was confirmed.

Measurement of Swimming Speed. *B. subtilis* cells were cultured at 37°C for 6 h (to an A_{600} nm of 0.5–0.6) in LB medium (pH 7.0) and diluted 50-fold into TY or TY/20 medium at the pH and with additions that are specified for each experiment. In *B. clausii*, alkaline complex medium was used for culture and swimming assays. Cell motility was observed under a dark-field microscope using a Leica DMRE microscope and Progressive 3CCD color video camera system (Model DXC-9000; Sony) and recorded on videotape. Swimming speed was determined by direct tracing of the moving cells on the video monitor. All results shown are the averages of three independent experiments in which the speed of 20 different cells was measured.

Visualization of Flagella. Flagellar staining was carried out as described by Aono *et al.* (25). *B. subtilis* cells were cultured at 37°C for 5 h in LB medium and transferred gently to a microscope slide. The sample was air-dried and treated for 3 min with staining solution containing 5% (wt/vol) tannic acid, 0.75% (wt/vol) $FeCl_2$, and 0.01% NaOH, followed by ammoniac silver nitrate for 1 min. Observations of flagella were made by using a Leica DMLB100 bright-field microscope and Leica DC300F camera, Leica IM50 version 1.20 software (Leica Geosystems). The number of flagellae per cell and flagellar length were measured for 20 cells by using NIH ImageJ version 1.36b software.

1. Berg HC (2003) The rotary motor of bacterial flagella. *Annu Rev Biochem* 72:19–54.
2. Kojima S, Blair DF (2004) The bacterial flagellar motor: Structure and function of a complex molecular machine. *Int Rev Cytol* 233:93–134.
3. De Mot R, Vanderleyden J (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.
4. Asai Y, Kawagishi I, Sockett RE, Homma M (2000) Coupling ion specificity of chimeras between H^+- and Na^+-driven motor proteins, MotB and PomB, in *Vibrio* polar flagella. *EMBO J* 19:3639–3648.
5. Ito M, Terahara N, Fujinami S, Krulwich TA (2005) Properties of motility in *Bacillus subtilis* powered by the H^+-coupled MotAB flagellar stator, Na^+-coupled MotPS or hybrid stators MotAS or MotPB. *J Mol Biol* 352:396–408.
6. Hirota N, Imae Y (1983) Na^+-driven flagellar motors of an alkalophilic *Bacillus* strain YN-1. *J Biol Chem* 258:10577–10581.
7. Sugiyama S (1995) Na^+-driven flagellar motors as a likely Na^+ re-entry pathway in alkaliphilic bacteria. *Mol Microbiol* 15:592.
8. Atsumi T, *et al.* (1996) Effect of viscosity on swimming by the lateral and polar flagella of *Vibrio alginolyticus. J Bacteriol* 178:5024–5026.
9. Ito M, *et al.* (2004) MotPS is the stator-force generator for motility of alkaliphilic *Bacillus* and its homologue is a second functional Mot in *Bacillus subtilis. Mol Microbiol* 53:1035–1049.
10. Kawagishi I, *et al.* (1995) Isolation of the polar and lateral flagellum-defective mutants in *Vibrio alginolyticus* and identification of their flagellar driving energy sources. *J Bacteriol* 177:5158–5160.
11. McCarter LL (2005) Multiple modes of motility: A second flagellar system in *Escherichia coli. J Bacteriol* 187:1207–1209.
12. Terahara N, *et al.* (2006) An intergenic stem-loop mutation in the *Bacillus subtilis ccpA-motPS* operon increases motPS transcription and the MotPS contribution to motility. *J Bacteriol* 188:2701–2705.
13. Senesi S, Celandroni F, Tavanti A, Ghelardi E (2001) Molecular characterization and identification of *Bacillus clausii* strains marketed for use in oral bacteriotherapy. *Appl Environ Microbiol* 67:834–839.
14. Kageyama Y, *et al.* (2007) Intragenomic diversity of the V1 regions of 16S rRNA genes in high-alkaline protease-producing *Bacillus clausii* spp. *Extremophiles* 11:597–603.
15. Kobayashi T, *et al.* (1995) Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. *Appl Microbiol Biotechnol* 43:473–481.
16. Fujinami S, Terahara N, Lee S, Ito M (2006) Na^+ and flagella-dependent swimming of alkaliphilic *Bacillus pseudofirmus* OF4: A basis for poor motility at low pH and enhancement in viscous media in an ''up-motile'' variant. *Arch Microbiol* 187:239–247.
17. Krulwich TA, Hicks DB, Swartz TH, Ito M (2007) in *Physiology and Biochemistry of Extremophiles*, eds Gerday C, Glansdorff N (Am Soc Microbiol Press, Washington, DC), pp 311–329.
18. Magariyama Y, *et al.* (1994) Very fast flagellar rotation. *Nature* 371:752.
19. Meier T, *et al.* (2005) Structure of the rotor ring of F-Type Na^+-ATPase from *Ilyobacter tartaricus. Science* 308:659–662.
20. Murata T, *et al.* (2005) Structure of the rotor of the V-Type Na^+-ATPase from *Enterococcus hirae. Science* 308:654–659.
21. Neumann S, Matthey U, Kaim G, Dimroth P (1998) Purification and properties of the F_1F_0 ATPase of *Ilyobacter tartaricus*, a sodium ion pump. *J Bacteriol* 180:3312–3316.
22. Sambrook J, Frisch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
23. Aagaard A, Brzezinski P (2001) Zinc ions inhibit oxidation of cytochrome *c* oxidase by oxygen. *FEBS Lett* 494:157–160.
24. Horton RM (1996) *In vitro* recombination and mutagenesis of DNA. *Methods Mol Biol* 67:141–149.
25. Aono R, Ogino H, Horikoshi K (1992) pH-dependent flagella formation by facultative alkaliphilic *Bacillus* sp C-125. *Biosci Biotechnol Biochem* 56:48–53.