Extremophiles (2017) 21:3–14
DOI 10.1007/s00792-016-0886-y

SPECIAL FEATURE: REVIEW

11th International Congress on Extremophiles

Nonconventional cation-coupled flagellar motors derived from the alkaliphilic *Bacillus* and *Paenibacillus* species

Masahiro Ito[1,2] · **Yuka Takahashi**[2]

Received: 19 July 2016 / Accepted: 10 October 2016 / Published online: 22 October 2016

Abstract Prior to 2008, all previously studied conventional bacterial flagellar motors appeared to utilize either H^+ or Na^+ as coupling ions. Membrane-embedded stator complexes support conversion of energy using transmembrane electrochemical ion gradients. The main H^+-coupled stators, known as MotAB, differ from Na^+-coupled stators, PomAB of marine bacteria, and MotPS of alkaliphilic *Bacillus*. However, in 2008, a MotAB-type flagellar motor of alkaliphilic *Bacillus clausii* KSM-K16 was revealed as an exception with the first dual-function motor. This bacterium was identified as the first bacterium with a single stator–rotor that can utilize both H^+ and Na^+ for ion-coupling at different pH ranges. Subsequently, another exception, a MotPS-type flagellar motor of alkaliphilic *Bacillus alcalophilus* AV1934, was reported to utilize Na^+ plus K^+ and Rb^+ as coupling ions for flagellar rotation. In addition, the alkaline-tolerant bacterium *Paenibacillus* sp. TCA20, which can utilize divalent cations such as Ca^{2+}, Mg^{2+}, and Sr^{2+}, was recently isolated from a hot spring in Japan, which contains a high Ca^{2+} concentration. These findings show that bacterial flagellar motors isolated from unique environments utilize unexpected coupling ions. This suggests that bacteria that grow in different extreme environments adapt to local conditions and evolve their motility machinery.

Keywords Alkaliphiles · MotPS · Stator · Flagellar motor · Divalent cation

Abbreviations

CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride
pmf	Proton motive force
smf	Sodium motive force

Introduction

"Alkaliphilic bacteria grow well in a highly alkaline environment around pH 10 and generally require sodium ions (Na^+) for growth." Similar descriptions of extremophiles have frequently been included in books over the last 35 years (Horikoshi and Akiba 1982). Dr. Koki Horikoshi, who was committed to developing the study of the basis and application of alkaliphilic microorganisms, was the source of this definition. In the early days of the study of alkaliphilic bacteria, extracellular enzymes produced by these extremophiles were added to detergents to improve the removal of dirt from laundry, and were used for the mass production of cyclodextrin with added value from cheap starch raw material (Horikoshi and Akiba 1982, Horikoshi 1991, 1999, 2016).

A diagrammatic illustration of the pH homeostasis capacity of the Na^+-dependent alkaliphiles and elements of their membrane-associated Na^+ and H^+ translocation pathways is shown in Fig. 1. The extremely alkaliphilic *Bacillus pseudofirmus* OF4 and *Bacillus halodurans* C-125 display robust multi-subunit-type Na^+/H^+ antiport (Mrp system)-dependent

Communicated by S. Albers.

This article is part of a special feature based on the 11th International Congress on Extremophiles held in Kyoto, Japan, September 12–16, 2016.

✉ Masahiro Ito
masahiro.ito@toyo.jp

[1] Faculty of Life Sciences, Toyo University, Oura-gun, Gunma 374-0193, Japan

[2] Bio-nano Electronics Research Center, Toyo University, Kawagoe, Saitama 350-8585, Japan

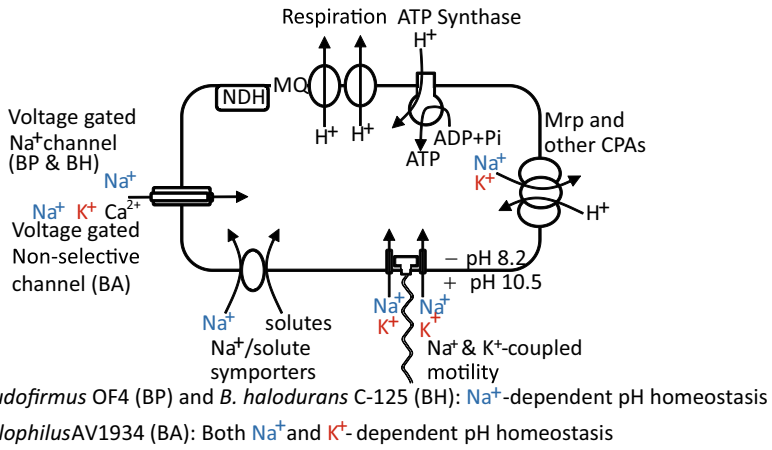

Fig. 1 Potential participation in the Na^+ cycle of alkaliphilic *Bacillus* spp vs. that in the Na^+ and K^+ cycle of alkaliphilic *Bacillus alcalophilus*. An illustration of the pH homeostasis capacity of the Na^+- and Na^+ plus K^+-dependent alkaliphiles and elements of their membrane-associated Na^+, K^+, and H^+ translocation pathways. *NDH* NADH oxidoreductase, *MQ* menaquinone, *Mrp* electrogenic Na^+/H^+ antiporter, *CPAs* cation proton antiporters

pH homeostasis and also contain NaChBac-type voltage-gated sodium channels that participate in Na^+ circulation, which supports pH homeostasis and normal chemotaxis (Fujinami et al. 2007a, 2009; Krulwich et al. 2011). The Mrp antiporter is the dominant electrogenic Na^+/H^+ antiporter, which obtains its energy from the transmembrane electrical potential built as a consequence of the respiratory proton efflux in alkaliphilic *Bacillus* (Krulwich et al. 2011). The work of Krulwich and colleagues in 1979 and the 1980s revealed that alkaliphilic *B. alcalophilus* AV1934 uses electrogenic Na^+/H^+ antiporter activity and a Na^+/solute symporter to facilitate growth at alkaline pH (Guffanti et al. 1979, 1981). Mandel et al. reported the presence of an electroneutral K^+/H^+ antiporter in this bacterium (Mandel et al. 1980). We speculate that *B. alcalophilus* also displays unique K^+-dependent pH homeostasis at high pH. For alkaliphiles, under conditions of a reversed pH gradient where the pH inside the cell is lower than that outside, it is very difficult to utilize a proton motive force for energy production, solute symport, and flagellar rotation under high alkaline pH, compared with the situation for neutrophilic bacteria (Ito 2002; Krulwich et al. 2006; Ito et al. 2011). Instead, alkaliphiles utilize a sodium motive force to survive in such environments. Interestingly, one of the key energy-supplying enzymes, F_oF_1-ATP synthase that performs ATP synthesis by oxidative phosphorylation (OXIPHOS) of alkaliphilic *Bacillus* species utilizes the proton motive force (pmf) rather than the sodium motive force (smf) (Guffanti and Krulwich 1994). There are several hypotheses to explain this mechanism of ATP synthesis, the details of which have been reviewed elsewhere (Krulwich 1995; Krulwich et al. 2011; Preiss et al. 2013, 2015; Goto et al. 2016).

Overview of conventional bacterial flagellar motor and its stator

The bacterial flagellum has many characteristics in common with the type III secretion system (Fig. 2a) (Minamino 2014). On the other hand, the archaeal flagellum, named the archaellum, has many traits in common with the type IV pili (Fig. 2b). For this reason, the bacterial flagellum and archaellum are believed to have different origins (Metlina 2004; Ng et al. 2006). The sources of rotary energy for the bacterial flagellum and archaellum are completely different. The bacterial flagellum utilizes an ion motive force; on the other hand, the archaellum utilizes ATP as a source of rotary energy. The details of the archaellum have been reviewed in detail elsewhere (Albers and Pohlschroder 2009; Albers and Jarrell 2015; Makarova et al. 2016).

The bacterial flagellar motor basically consists of three parts: the basal body, which works as a motor; the external extended flagellar filament; and the hook, which works as a universal joint for connecting the motor and the flagellar filament (Fig. 2a). The motor of Gram-negative bacteria such as *Escherichia coli* has an L and a P ring component, whereas the motor of Gram-positive bacteria such as *Bacillus* species lacks these rings (Fig. 2a) (Fujinami et al. 2009; Minamino and Imada 2015). The motor is divided into a stator part and a rotor part. The MotA-type and MotB-type proteins form the stator complexes, anchored to the cell wall through a putative peptidoglycan-binding (PGB) motif in the periplasmic domain of a MotB-type protein. Mot complexes comprise four copies of MotA-type proteins and two copies of MotB-type proteins, and they are arranged in a ring of membrane-embedded complexes surrounding each flagellum (Fig. 2a).

The stator complex works as an ion channel through which H^+ or Na^+ passes. The MotA-type protein comprises four transmembrane segments with a large cytoplasmic loop between the second and third segments (Fig. 2c, d). The MotB-type protein comprises only one transmembrane segment (Fig. 2c, d).

In *E. coli*, a perfectly conserved aspartic acid residue is present at position 32 (Asp-32) of MotB. This residue is conserved in all MotB, MotS, and PomB sequences known to date (Figs. 2d, 4). In a previous random mutational study of MotB in *E. coli*, several other amino acid substitutions

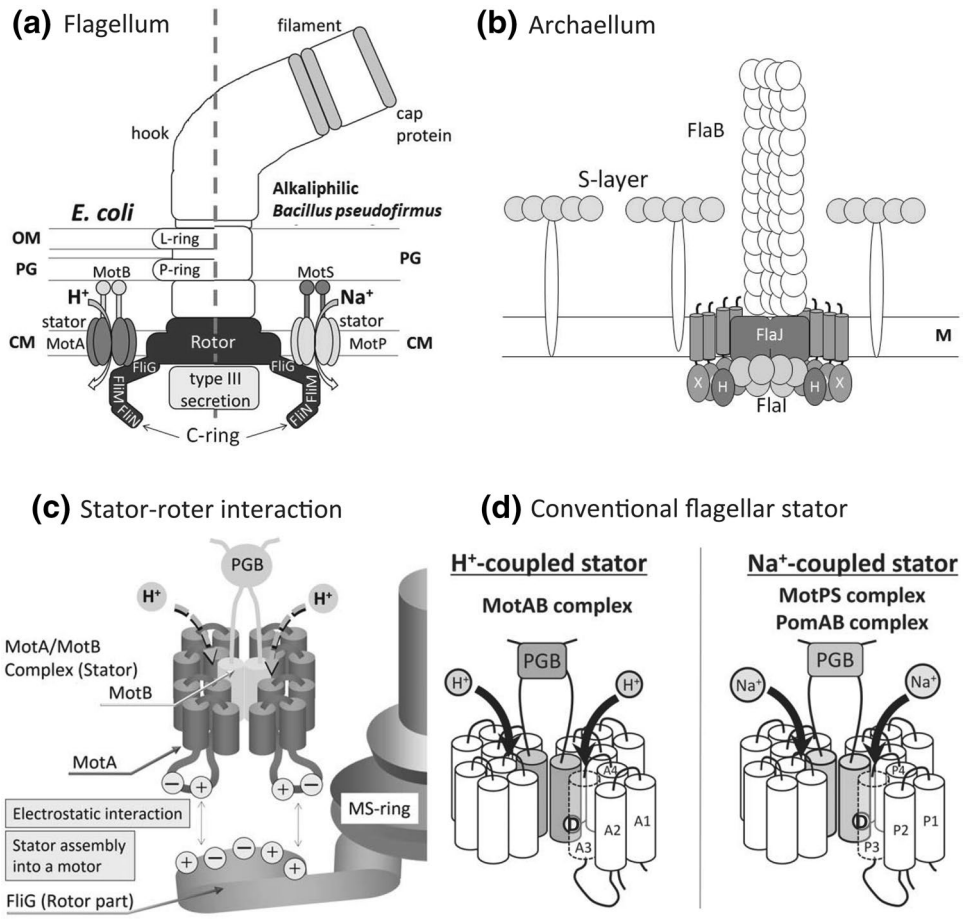

Fig. 2 A schematic of bacterial flagellum and archaellum. **a** The left image shows the proton-driven flagellar motor of *E. coli*, and the right image shows the sodium-driven flagellar motor of alkaliphilic *B. pseudofirmus* OF4. The gram-negative bacteria motor contains additional ring structures, the P-ring and the L-ring. The stator complex is composed of two integral membrane proteins: MotP and MotS for the alkaliphilic *B. pseudofirmus* motor, and MotA and MotB for the *E. coli* motor. The stator is anchored at the PG layer around the rotor via the periplasmic PGB domain of the MotB and MotS subunits. *OM* outer membrane, *CM* cytoplasmic membrane, *PG* peptidoglycan. **b** Schematic diagram of an archaellum. *M* membrane, *X* FlaX, *H* FlaH. **c** Schematic diagram of the proposed electrostatic interactions between rotor and stator interface in the H^+-coupling bacterial flagellar, critical for generating motor torque. *PGB* Peptidoglycan-binding motif. **d** Schematic diagram of conventional flagellar stators using either proton or sodium ions for coupling. *PGB* peptidoglycan-binding motif, *D* an absolutely conserved aspartic acid residue, *A1–A4* transmembrane segments 1–4 of MotA, MotP, or PomA subunit, *P1–P4* transmembrane segments 1–4 of MotP or PomA subunits

for Asp-32 were found to eliminate motility, suggesting that Asp-32 functions as the coupling ion-binding site. Only a glutamic acid substitution for Asp32 retained poor motility (Zhou et al. 1998b). It is proposed that a cytoplasmic hydrophilic loop domain of MotA interacts with the C-terminal domain of the rotor subunit of FliG (Fig. 2c) (Chun and Parkinson 1988; Sharp et al. 1995). In addition, electrostatic interactions between the rotor and the stator interface at the H^+-coupling bacterial flagella are critical for generating motor torque (Zhou et al. 1998a; Kojima and Blair 2001). The electrostatic interaction between the charged amino acid residues of the C-terminal region of FliG and the cytoplasmic loop of the MotA subunit is also important for stator assembly into a motor, as reported in studies performed on *Salmonella* (Morimoto et al. 2010, 2013). Therefore, the cytoplasmic stator part is critical for assembly the stator into a motor and functions as an energy conversion unit for converting an ion motive force to a driving force of flagella.

The stator is not always attached to the motor. On the contrary, each individual stator dynamically moves from the vicinity of the motor to regions not bound to the motor in living cells, as observed using single molecule technology based on GFP-fusion proteins (Leake et al. 2006).

FliG, FliM, and FliN proteins are components of the switch complex, which is configured as a C-ring structure. This configuration has been directly observed using electron microscopy (Fig. 2a) (Marykwas et al. 1996;

Tang et al. 1996). These proteins (FliG, FliM, and FliN) are required for flagellar assembly, torque generation, and control of the flagellar motor rotation [counter clockwise (CCW) or clockwise (CW)] (Yamaguchi et al. 1986a, b; Lloyd et al. 1996).

Escherichia coli, *Salmonella,* and most neutrophilic bacteria utilize a proton motive force (pmf) as a source of rotational energy of the flagella motor, and the stator is a MotA/MotB complex. On the other hand, species of *Vibrio*, *Shewanella*, *Aeromonas*, and alkaliphilic *Bacillus* use a sodium motive force (smf) for the rotary energy (Zhu et al. 2013; Kojima 2015). The Na^+-coupled stator of *Vibrio*, *Shewanella*, and *Aeromonas* is known as PomA/PomB, whereas the stator of the alkaliphilic *Bacillus* is known as MotP/MotS. Both stators are homologues to MotA/MotB.

To date, only alkaliphilic *Bacillus* species and *Vibrio cholera* have been shown to entirely rely on Na^+-coupled motility; however, other alkaline-resistant bacteria have been inferred to possess Na^+- and H^+-driven flagellar motors based on genomic evidence (Matsuura et al. 1977; Atsumi et al. 1992; Hase and Barquera 2001; Takami et al. 2002; McCarter 2004, 2005). For example, a Na^+-driven single polar flagellum has been described from *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, whereas H^+-driven multiple lateral flagella were expressed under certain conditions. Two separate sets of genes encode the polar and lateral flagella of *Vibrio*. Numerous other bacteria have been found to have hybrid motility systems. Moreover, these hybrid systems do not always use different coupling ions. Depending on the environmental conditions, one or the other dual motility system predominates (McCarter 2004, 2005). *Bacillus subtilis* and *Shewanella oneidensis* MR-1 use H^+-coupled MotA/MotB and Na^+-coupled MotP/MotS and PomA/PomB stators to generate the torque required for flagellar rotation (Fujinami et al. 2009; Minamino and Imada 2015; Paulick et al. 2015).

Conventional flagellar stator and motility of alkaliphilic *Bacillus*

In alkaliphilic *Bacillus*, it was shown that the driving force for swimming is not a proton motive force, but a sodium motive force. Hirota et al. first reported that the swimming speed of alkaliphilic *Bacillus* sp. YN-1 cells increased linearly with a logarithmic increase of Na^+ concentration (up to 100 mM) and the optimal pH for motility was approximately 10.5 (Hirota et al. 1981; Hirota and Imae 1983). However, the stator genes were not identified until 2004. Our group identified the stator protein from alkaliphilic *B. pseudofirmus* OF4, which grows optimally above pH 10 and the minimum pH value is approximately 7.5 (Guffanti et al. 1986). We named this stator protein MotP/MotS (pH and sodium) (Ito et al. 2004). Neutrophilic *Bacillus subtilis* has MotP/MotS homologs, YtxD/YtxE, which were originally identified as hypothetical proteins in the chromosome, and these stator proteins are also Na^+-coupled stators (Ito et al. 2004; Terahara et al. 2006). The level of homology between *B. pseudofirmus* MotP and MotS proteins and the sodium-coupled stator subunits, *V. alginolyticus* PomA and PomB, is 36 and 28 % for identity and 62 and 43 % for similarity, respectively. On the other hand, the level of homology between *B. pseudofirmus* MotP and MotS proteins and the proton-coupled stator subunits, *B. subtilis* MotA and MotB, is 40 and 29 % for identity and 62 and 54 % for similarity, respectively. Furthermore, the level of homology between PomA and PomB proteins of *V. alginolyticus* and MotA and MotB proteins in *B. subtilis* is 31 and 22 % for identity and 56 and 45 % for similarity, respectively. The results demonstrate that there is considerable diversity at the protein level among the three stator sub-families within this bacterial stator family (Fig. 3).

Aono et al. reported on the flagellar formation of alkaliphilic *B. halodurans* C-125 (Aono et al. 1992). This alkaliphile showed an increase in the average number of flagella per cell from 0 to 21 when the pH was elevated from 6.9 to 10. The synthesis of flagellin and the formation of flagella were shown to be suppressed during growth at pH 7, which would account for poor motility of cells grown at pH < 8. Therefore, the synthesis of flagellin and the formation of flagella in this bacterium were clearly shown to be regulated by external pH. On the other hand, *B. pseudofirmus* OF4 cells showed one to two flagella per cell, irrespective of the external pH and the level of flagellin expression. These results suggest that the regulation of *B. pseudofirmus* OF4 flagellation differs from that of *B. halodurans* C-125 flagellation and is independent of the external pH (Fujinami et al. 2007b).

The swimming speed of *B. pseudofirmus* OF4 cells changes with external pH and sodium concentration. No motility was observed at pH 6, and little motility was observed at pH 7. However, the swimming speed of *B. pseudofirmus* OF4 cells increased linearly with the logarithmic increase of sodium concentration up to 230 mM. The poor motility of *B. pseudofirmus* OF4 at low pH probably reflects competitive inhibition of Na^+-based motility by high $[H^+]$. Similar suggestions have been made with respect to H^+ sites of the *E. coli* and *Salmonella* rotors (Minamino et al. 2003) and the Na^+ sites of the Na^+-driven rotor of *V. alginolyticus* (Yoshida et al. 1990).

In the following sections, we introduce three nonconventional flagellar motors derived from alkaliphilic *Bacillus* and *Paenibacillus*. The genetic engineering system for each bacterial type has not been developed yet; consequently, our group characterized the properties of each stator in a stator-less mutant of genetically tractable *E. coli* or *B. subtilis*.

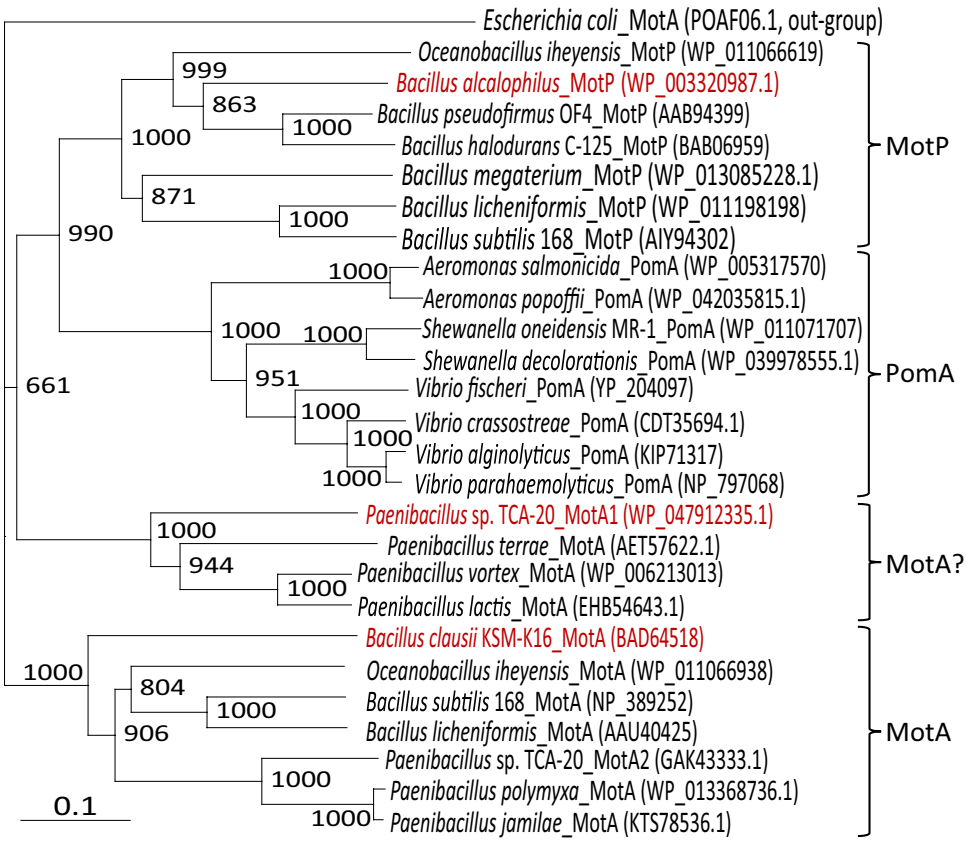

Fig. 3 Phylogenetic tree of the subunits MotA, MotP, and PomA of the flagellar motor from *Aeromonas*, *Bacillus*, *Oceanobacillus*, *Paenibacillus*, *Shewanella*, and *Vibrio* sp. using the neighbor-joining method. The MotA sequence of *E. coli* was used as out-group. The *bar* indicates 0.1 substitutions per amino acid position. The phylogenetic tree was constructed using ClustalW (http://clustalw.ddbj.nig.ac.jp) with 1000 bootstrap samplings. *Bacillus alcalophilus*_MotP, *Paenibacillus* sp. TCA20_MotA1 and *Bacillus clausii* KSM-K16_MotA are shown in red. Functional analysis of the cluster motility containing *Paenibacillus* sp. TCA-20_MotA1 has not yet been experimentally characterized, although each protein was automatically annotated as MotA in the database

Properties of the flagellar stator and motility of alkaliphilic *Bacillus clausii* KSM-K16

Alkaliphilic *B. clausii* KSM-K16 has been noted to produce industrially useful enzymes such as alkaline protease (Kobayashi et al. 1995; Kageyama et al. 2007). Since completion of the genome sequence of *B. clausii* KSM-K16 in 2005, various genes related to the flagellar motor have been identified. Interestingly, the genome sequence of this bacterium revealed a single set of genes encoding a proton-coupled MotA/MotB-like pair of proteins as the stator. *B. clausii* MotA and MotB proteins (BCl-MotA/MotB) were found to be closely related to the proton-coupled stator subunits, MotA and MotB, in *B. subtilis* (identity: 52 and 45 %, similarity: 73 and 64 %, respectively), while they were not as closely related to the sodium-coupled stator subunits, MotP and MotS, in *B. subtilis* (identity: 32 and 28 %, similarity: 57 and 55 %, respectively). As is typically the case in alkaliphilic *Bacillus* species, the flagellar motor is driven by a sodium motive force with MotP/MotS as the stator because a proton motive force is difficult to utilize under highly alkaline pH (Ito et al. 2004). However, only H^+-coupled-type stator genes *motA*/*motB* was identified in this genome. All previously studied flagellar stators utilize either H^+ or Na^+ as coupling ions. This raised the possibility that either the motility of this alkaliphile would be lost in the upper pH range for growth or MotA/MotB are capable of utilizing both a proton and a sodium motive force as a source of rotary energy. This alkaliphile swims well over the range of pH from 7 to 11, and swimming assays suggested that Na^+ could be utilized as a coupling cation for energy for flagellar rotation at high pH (Terahara et al. 2008).

A stator-less *B. subtilis* mutant (Δ*motA/motB,* Δ*motP/motS*) expressing MotA/MotB from *B. clausii* named as BCl-AB was shown that the coupling ion was changed from H^+ to Na^+ with increasing external pH (Fig. 5b). These results support the hypothesis that MotA/MotB from *B. clausii* is a bifunctional stator with respect to ion-coupling capacity. This was also confirmed using the sodium channel inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), an amiloride analogue, and the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which dissipates electrochemical H^+ gradients. At elevated pH, the swimming speeds of BCl-AB were reduced by EIPA, but the results of inhibition by CCCP were reversed. These findings support the hypothesis that the flagellar motor of *B. clausii* MotA/MotB can mainly utilize a proton motive force at a pH below 8.5; on the other hand, it can change to using a sodium motive force above pH 9.0 (Terahara et al. 2008).

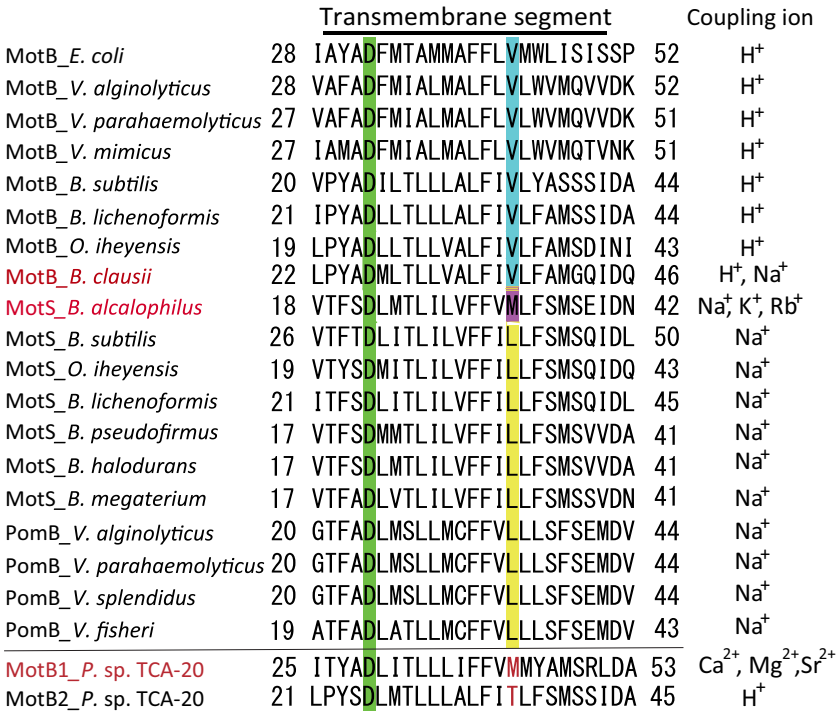

Fig. 4 Alignment with flagellar motor sequences from other bacteria and their coupling ions. Alignments of the region containing the single transmembrane segment of MotB, MotS and PomB. MotB_*B. clausii*, MotS_*B. alcalophilus*, and MotB1_*P.* sp.TCA-20 are shown in *red*. The position of D32 in MotB_*E. coli* is known to be critical for rotation and is highlighted in *green*. The MotAB of *B. clausii* can use Na^+ instead of H^+ to promote flagellar rotation at high pH levels. The position of V43 in MotB_*E. coli* (the *first line*) is conserved among all of the MotB-H^+-type proteins and is highlighted in *light blue*. The position of L41 in MotS_*B. subtilis* (the *10th line* from the *top*) is conserved among all of the MotS- and PomB-Na^+-type proteins, with the exception of MotS_*B. alcalophilus* (*9th line* from the *top*), and is highlighted in *yellow*. The same position in *B. alcalophilus* MotS encodes methionine instead of the conserved leucine residue and is highlighted in *violet*. A MotS-M33L mutation in *B. alcalophilus* exhibits a loss of both K^+ and Rb^+ coupling motility in *E. coli* (Terahara et al. 2012)

Furthermore, we identified whether the bifunctional BCl-MotA/MotB could be modified to a stator that uses either protons or sodium ions at both neutral and alkaline pH. Our previous studies showed that a proton or sodium ion selective domain is present in the MotB or MotS subunits of the Mot complex (Ito et al. 2005). On the basis of the amino acid sequence alignment of *Bacillus* MotB and MotS subunits, we identified some conserved amino acid residues (Fig. 5a). We expected that a conserved ion selective domain would be located in the highly conserved transmembrane segment, because the number of basic residues tends to be greatly reduced in proteins of alkaliphiles facing outside the surface of the cytoplasmic membrane. On the other hand, the same segments present an increased abundance of acidic residues compared to those of neutralophile homologues (Krulwich et al. 2007). To construct a proton-coupled stator and a sodium-coupled stator from bifunctional BCl-MotA/MotB, we initially made two distinct triple mutants (named BCl-MotB-H and BCl-MotB-Na, respectively; shown in Fig. 5). The *B. clausii* MotB introduced mutations (BCl-MotB-H; G42S, Q43S and Q46A) successfully selected for only protons by imitating the MotB subunit of the proton-coupled stator. In contrast, the *B. clausii* MotB mutations (BCl-MotB-Na; V37L, A40S and G42S) successfully selected only for sodium ions by imitating the MotS subunit of the sodium-coupled stator (Fig. 5). These results suggest that these amino acid residues are critical for ion selectivity. Additionally, by analyzing each single and double mutant, the Q43S mutation appears as critical for proton coupling. Furthermore, the Q43S mutation combined with either the G42S or the Q46A mutation was required to achieve the loss of sodium coupling. On the other hand, the V37L mutation was critical for sodium coupling and the V37L mutation combined with either the A40S or the G42S mutation was required to achieve the loss of proton coupling at low pH. The mutation V37 is predicted to be located in the middle of the single TMS of BCl-MotB sequence, on the same face as the conserved aspartic acid residue, D26, critical for flagellar rotation (Figs. 4, 5a) (Kojima and Blair 2004). The mutation L37 is highly conserved among the MotS and PomB components of the sodium-coupled stators of *Bacillus* and *Vibrio* species (Fig. 4). Thus, these results suggest

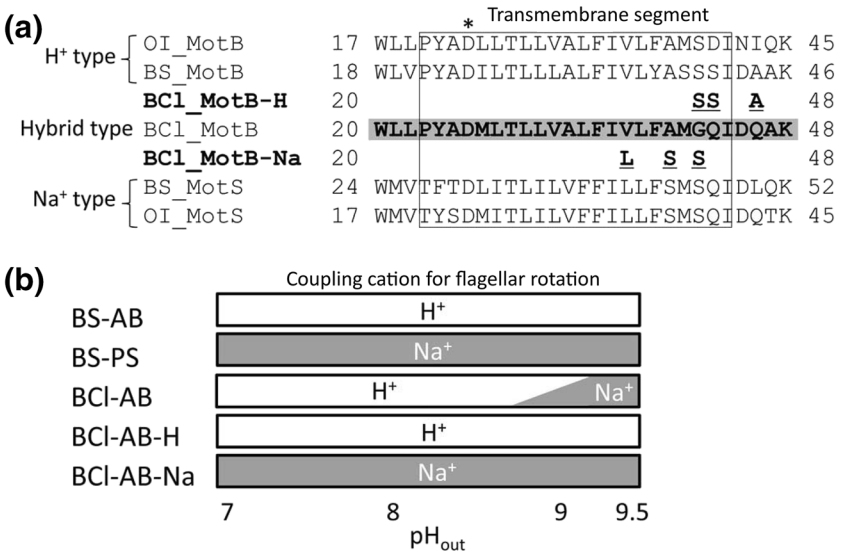

Fig. 5 Multiple alignment of the region containing the single transmembrane segment of MotB and MotS, and the coupling cations of a stator-less *Bacillus subtilis* mutant (ΔABΔPS) expressing *B. subtilis motAB* (BS-AB), *B. subtilis motPS* (BS-PS), *B. clausii motAB* (BCl-AB) and BCl-AB derivative mutants (BCl-AB-H and BCl-AB-Na). **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) and MotS (BS_MotS), and *B. clausii* MotB (BCl_MotB). The sequence of *B. clausii* MotB is highlighted in *gray*. The *black bold letters underlined* represent locations of point mutations in *B. clausii* MotB mimicking BS_MotB (G42S, Q43S and Q46A) to yield BCl_MotB-H, and *B. clausii* MotB mimicking BS_MotS to yield BCl_MotB-Na (V37L, A40S and G42S). The *asterisk* (*) indicates a position of an absolutely conserved aspartic acid residue, critical for flagellar rotation. **b** Relationship between coupling cations and the flagellar rotation to environmental pH (Terahara et al. 2008)

that leucine 37 may function as a sodium-selective filter for these sodium channels.

We also applied the same approach to the distinct H^+- and Na^+-coupled stators of *B. subtilis*, Bs-MotAB and Bs-MotPS, and identified which mutations conferred dual ion-coupling capacity in each of them (Terahara et al. 2008). This is the first report of a bifunctional flagellar stator able to use both Na^+ and H^+ to power motility, changing its preference with the pH.

Properties of the flagellar stator and motility of alkaliphilic *Bacillus alcalophilus* AV1934

Alkaliphilic *B. alcalophilus* AV1934 was one of the first alkaliphilic bacteria to be identified, back in 1934. This alkaliphile was isolated from human feces and found to grow at an alkaline pH (Vedder 1934). This alkaliphile prefers to grow in K^+-rich and Na^+-poor conditions in an extremely alkaline environment. Before the genome sequence of *B. alcalophilus* AV1934 was finished in 2014 (GenBank: ALPT00000000.2) (Attie et al. 2014), the stator genes of this alkaliphile were identified by us in 2012 (Terahara et al. 2012). This bacterium possesses a single set of genes encoding a Na^+-coupled MotP/MotS-like pair of proteins as the stator.

Bacillus alcalophilus MotP and MotS proteins (BA-MotP and BA-MotS) are closely related to the sodium-coupled stator subunits MotP and MotS, in *B. subtilis* (identity: 49 and 48 %, similarity: 75 and 68 %, respectively), while they are not as closely related to the proton-coupled stator subunits, MotA and MotB, in *B. subtilis* (identity: 39 and 29 %, similarity: 61 and 55 %, respectively).

This alkaliphile swims well over the pH range from 7 to 11, and swimming assays in the native host suggested that both Na^+ and K^+ can be utilized as coupling cations to produce the energy for flagellar rotation at high pH. *E. coli* cells expressing BA-MotP/MotS showed both Na^+- and K^+-dependent motility. We identified a key amino acid residue for ion selectivity of K^+ of the MotPS-type stator. The 33rd methionine residue in the single transmembrane segment of the MotS subunit is critical for K^+ selectivity. Generally, this site is conserved as leucine in the MotS subunit in *Bacillus* and *Vibrio* species (Fig. 4). *E. coli* cells expressing BA-MotP/MotS-M33L showed only Na^+-dependent motility and lost K^+-dependent motility completely (Fig. 6).

The swimming speed of *B. alcalophilus* was sensitive to the Na^+-coupled flagellar stator inhibitor EIPA in both Na^+ and K^+ buffers (Terahara et al. 2012). We previously suggested that EIPA-binding sites are present in both the MotP and the MotS subunits and that this inhibitor blocks the passage of K^+ (Ito et al. 2005).

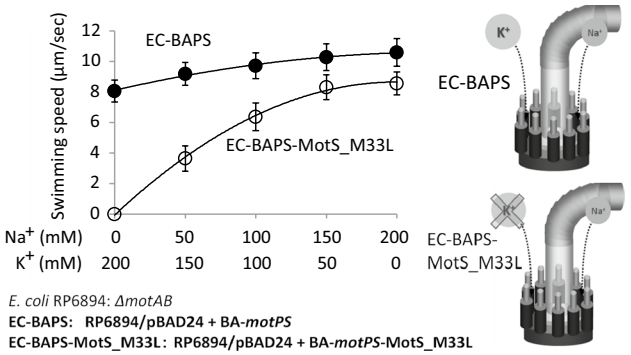

Fig. 6 Effect of KCl and NaCl on swimming speed of *E. coli* strains expressing MotPS from *B. alcalophilus*. The schematic diagrams of both a sodium and potassium ion-coupled flagellar motor (EC-BAPS) and only a sodium ion-coupled flagellar motor (EC-BAPS-MotS_M33L) appear on the right side

In insect larvae, whose gut is known to constitute an alkaline environment, the elevation of gut pH is linked to potassium transport and net accumulation of K_2CO_3 (Dow 1984); in addition, termite-derived alkaliphiles have been shown to exhibit NaCl sensitivity and preference for K^+ over Na^+ during growth at high pH (Ohkuma et al. 2003). Interestingly, a bacterial voltage-gated Na^+ channel (NaChBac) homolog from *B. alcalophilus* AV1934 named Ns_VBA showed a voltage-gated channel that is nonselective among Na^+, Ca^{2+}, and K^+ ions (DeCaen et al. 2014). This is another example of adaptation to the external environment. Therefore, *B. alcalophilus* is assumed to have evolved these properties to adapt to a potassium-rich environment. This is the first report to describe a flagellar motor that can use K^+ and Rb^+ as coupling ions.

Properties of the flagellar stator and motility of alkaline-tolerant bacterium *Paenibacillus* sp. TCA-20

Our group has looked for novel types of flagellar stators from bacteria isolated from diverse environments. There are several kinds of cation-coupled voltage-gated ion channels (Hess et al. 1986; Doyle et al. 1998; Alam and Jiang 2009; DeCaen et al. 2014). However, divalent cation-coupled flagellar motors had not been identified in nature. Therefore, we considered that calcium ions, which are abundant in nature, are another candidate for coupling ions of the bacterial flagellar motor.

The alkaline-tolerant bacterium *Paenibacillus* sp. strain TCA20 which showed Ca^{2+}-dependent growth at pH 7–9 (optimum, pH 8) was isolated from a water sample collected from Tsurumaki Onsen, a well-known hot spring in Kanagawa Prefecture, Japan, which contains a high Ca^{2+} concentration (1740 mg/L). The draft genome sequence of this bacterium was reported in 2014 (GenBank: BBIW00000000.1) (Fujinami et al. 2014).

Adding Ca^{2+} and Mg^{2+} to the medium moderately enhanced growth, which was better than without divalent cations. The swimming behavior of this bacterium showed Ca^{2+}-, Mg^{2+}-, and Sr^{2+}-dependent motility at pH 8.0 (Fig. 7a–c). The draft genome of strain TCA20 shows that it has two sets of MotA/MotB-like genes, named TCA-motA1/motB1 and TCA-motA2/motB2. TCA-MotA1 and MotB1 showed moderate resemblance to *B. subtilis* MotA and MotB, which constitute the H^+-coupled *B. subtilis* stator Mot complex (identity: 37 and 31 %, similarity: 61 and 52 %, respectively), and to *B. subtilis* MotP and MotS, which constitute the Na^+-coupled *B. subtilis* stator Mot complex (identity: 36 and 32 %, similarity: 60 and 51 %, respectively). TCA-MotA2 and MotB2 were shown to be closely related to *B. subtilis* MotA and MotB (identity: 53 and 44 %, similarity: 72 and 64 %, respectively). The phylogenetic features of the two stator proteins suggest that TCA-MotA2/MotB2 function as an H^+-type stator. However, TCA-MotA1/MotB1 belong to a different stator cluster than the H^+-coupled MotAB or Na^+-coupled MotPS complex (Fig. 3).

The function of the motility cluster containing TCA-MotB1 has not yet been experimentally characterized, although each protein was automatically annotated as MotB in the database. TCA-MotA1/MotB1 and TCA-MotA2/MotB2 from strain TCA-20 were integrated into the *lacA* gene locus in the chromosome of *motA/motB* and *motP/motS*-deleted *B. subtilis*. The strains were designated TCA-AB1 and TCA-AB2, respectively. Strains ΔABΔPS carrying *B. subtilis motA/motB* or *B. subtilis motP/motS* were used as individual control strains for H^+- or Na^+-coupling motility. As a result, all three strains exhibited restored motility, and swimming speed was measured in liquid medium.

The swimming speeds of TCA-AB1 and TCA-AB2 cells were measured at several Mg^{2+} concentrations in 10 mM potassium phosphate buffer. TCA-AB1 exhibited no swimming capacity without added Mg^{2+} and stimulated by Mg^{2+} (Fig. 7d). The same experiment was performed using $CaCl_2$. However, the tumbling frequency of all strains was drastically increased and it was very difficult to measure the linear swimming velocity of each strain. Therefore, experiments were performed by the addition of only Mg^{2+}, which indicated that the TCA-AB1 rotor prefers using Mg^{2+} in the heterologous neutrophilic host.

The protonophore CCCP did not affect TCA-AB1 swimming in the presence of Mg^{2+} at inhibitor concentrations up to 25 μM at pH 8.0 (Fig. 7e). Conversely, the Na^+-coupled flagellar stator inhibitor EIPA did not affect the swimming of TCA-AB1 and TCA-AB2 in the presence of 10 mM

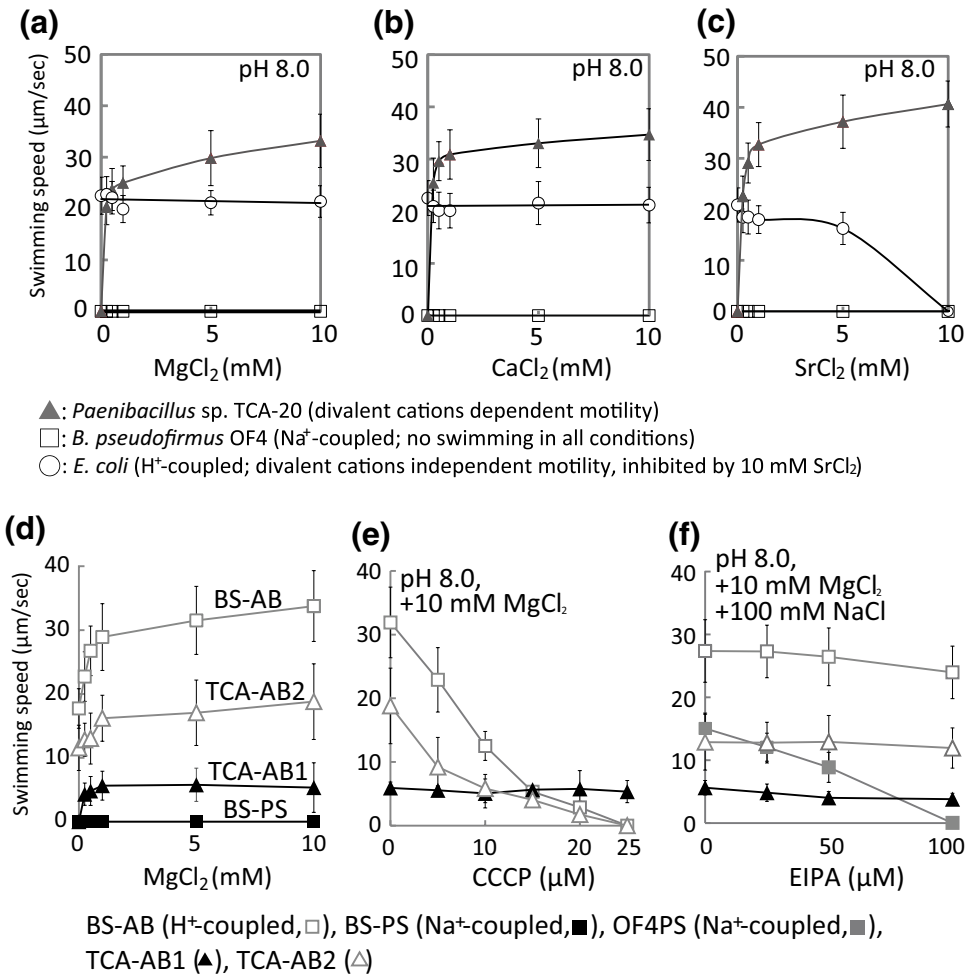

Fig. 7 Effect of divalent cations on swimming speed of *Paenibacillus* sp. TCA20, *Escherichia coli*, and *Bacillus pseudofirmus* OF4, and effect of Mg^{2+}, CCCP, and Na^+ channel inhibitor EIPA on the swimming speed of *Bacillus subtilis* mutant strains. Swimming speeds of *Paenibacillus* sp. TCA20, *E. coli*, and *B. pseudofirmus* OF4 cells were measured in 30 mM Tris–HCl containing several $MgCl_2$ (**a**), $CaCl_2$ (**b**), or $SrCl_2$ (**c**) concentrations. The results represent the average swimming speed of 30 independent cells from three independent experiments. The *error bars* indicate standard deviations. BS-AB, BS-PS, OF4PS, TCA-AB1, and TCA-AB2 mutant strains were grown for 6 h at 37 °C in Spizizen I medium plus 1 mM $MgCl_2$ and 1 % xylose at pH 8.0 with shaking. Cells were suspended in 1 mL of phosphate buffer (pH 8.0) (**e**), plus the indicated amounts of CCCP plus 10 mM $MgCl_2$ (**f**), plus the indicated amounts of EIPA plus 10 mM $MgCl_2$ and 100 mM NaCl (**g**), and then incubated at 37 °C for 10 min. Phosphate buffer contained 10 mM potassium phosphate (pH 8.0), 5 mM glucose, 1 % (w/v) xylose, 10 μg/mL tryptophan, and lysine. The swimming speed is the average speed of >30 cells

$MgCl_2$ and 100 mM NaCl at inhibitor concentrations up to 100 μM (Fig. 7f). These results indicate that the coupling ions of the TCA-MotAB 1 and TCA-MotAB 2 stator complex are Mg^{2+} and H^+, respectively. Additionally, a *B. subtilis* mutant lacking both a stator and a major Mg^{2+} uptake system, expressing TCA-MotAB 1, could complement both growth and motility deficiency under low Mg^{2+} conditions, and exhibited $[Mg^{2+}]_{in}$ identical to that of the wild-type. These results suggest a coupling of flagellar rotation and Mg^{2+} uptake.

The stator protein TCA-MotAB 1 has a universally conserved Asp-33 residue of MotB1 critical for motility, and predicted as a H^+-binding site in *E. coli* (Fig. 4) (Zhou et al. 1998b). A coupling of the ion influx pathway forms by the third and fourth transmembrane segments of the MotA subunit and a single transmembrane segment of the MotB subunit (Braun et al. 2004). There is no further negative charged amino acid residue near Asp-33 of TCA-MotB1 and the third and fourth transmembrane segments of TCA-MotA1. This suggests that divalent cations work as coupling ions for flagellar rotation in the TCA20 strain; however, the predicted coupling ion-binding site was a single negatively charged side chain of an aspartic acid residue. Using divalent cations for flagellar rotation, the membrane potential would be consumed two times faster than using monovalent cations. Therefore, it is interesting that

the motor torque couples with divalent cations as well as with monovalent cations. This is the first report of a flagellar motor that can use Ca^{2+}, Mg^{2+}, and Sr^{2+} as coupling ions (Imazawa et al. 2016).

Conclusions

Previously, bacterial flagellar motors were thought to be driven by H^+ or Na^+. However, various other types of flagellar motors have now been identified. It is interesting that novel motors have been created in the process of microbial adaptation and evolution in the wild. For example, the alkaliphile *B. clausii* KSM-K16 has a MotAB-type flagellar stator, but this stator can utilize Na^+ as a coupling ion as well as H^+ (Terahara et al. 2008). For KSM-K16, utilization of the H^+-type stator is disadvantageous in an alkaline environment. Therefore, this bacterium may have evolutionarily adapted to utilize Na^+ as the coupling ion instead of H^+ in a highly alkaline environment.

Alkaliphilic *B. alcalophilus* AV1934, which was isolated from human feces, has a MotP/MotS-type stator and shows potassium-dependent growth and motility at an alkaline pH (Terahara et al. 2012). In addition, it is believed that *Paenibacillus* sp. TCA-20, which was isolated from hot springs rich in calcium ions, also evolved to utilize calcium ions for flagellar rotation as an adaptation to the environment. This provides an interesting insight into the evolution of movement of the flagellar motor as a new type of adaptation to the environment.

New findings of third and fourth types of flagellar motors, which are coupled to potassium ions and divalent cations, add to the range of diversity identified in flagellar motor research. There are a variety of extreme environments on earth and many extremophiles have been found in such places. If unknown unique microorganisms are isolated from such environments in the future, they are expected to possess novel types of flagellar motor that utilize unexpected coupling ions to produce energy for rotation. In addition, it is expected that the identification of stator genes of microorganisms growing in extreme environments will lead to progress in the application of the flagellar motor as a bioengineered synthetic component.

Acknowledgments We thank Dr. Arthur A. Guffanti for critical discussions and reading of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas No. 24117005 of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MI).

Compliance with ethical standards

Funding This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas No. 24117005 of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MI).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Alam A, Jiang Y (2009) High-resolution structure of the open NaK channel. Nat Struct Mol Biol 16:30–34. doi:10.1038/nsmb.1531

Albers SV, Jarrell KF (2015) The archaellum: how Archaea swim. Front Microbiol 6:23. doi:10.3389/fmicb.2015.00023

Albers SV, Pohlschroder M (2009) Diversity of archaeal type IV pilin-like structures. Extremophiles 13:403–410. doi:10.1007/s00792-009-0241-7

Aono R, Ogino H, Horikoshi K (1992) pH-dependent flagella formation by facultative alkaliphilic *Bacillus* sp. C-125. Biosci Biotechnol Biochem 56:48–53

Atsumi T, McCarter L, Imae Y (1992) Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. Nature 355:182–184

Attie O et al (2014) Draft genome sequence of *Bacillus alcalophilus* AV1934, a Classic alkaliphile isolated from human feces in 1934. Genome Announc. doi:10.1128/genomeA.01175-14

Braun TF, Al-Mawsawi LQ, Kojima S, Blair DF (2004) Arrangement of core membrane segments in the MotA/MotB proton-channel complex of *Escherichia coli*. Biochemistry 43:35–45

Chun SY, Parkinson JS (1988) Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. Science 239:276–278

DeCaen P, Takahashi Y, Krulwich T, Ito M, Clapham D (2014) Ionic selectivity and thermal adaptations within the voltage-gated sodium channel family of alkaliphilic *Bacillus*. Elife 3:e04387. doi:10.7554/eLife.04387

Dow JA (1984) Extremely high pH in biological systems: a model for carbonate transport. Am J Physiol Regul Integr Comp Physiol 246:R633–R635

Doyle DA et al (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280:69–77

Fujinami S et al (2007a) The voltage-gated Na^+ channel NaVBP co-localizes with methyl-accepting chemotaxis protein at cell poles of alkaliphilic *Bacillus pseudofirmus* OF4. Microbiology 153:4027–4038. doi:10.1099/mic.0.2007/012070-0

Fujinami S, Terahara N, Lee S, Ito M (2007b) 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. doi:10.1007/s00203-006-0192-7

Fujinami S, Terahara N, Krulwich TA, Ito M (2009) Motility and chemotaxis in alkaliphilic *Bacillus* species. Future Microbiol 4:1137–1149. doi:10.2217/fmb.09.76

Fujinami S et al (2014) Draft genome sequence of calcium-dependent *Paenibacillus* sp. strain TCA20, isolated from a hot spring containing a high concentration of calcium ions. Genome Announc 2:e00814–e00866. doi:10.1128/genomeA.00866-14

Goto T et al (2016) Contribution of intracellular negative ion capacity to Donnan effect across the membrane in alkaliphilic *Bacillus* spp. J Bioenerg Biomembr 48:87–96. doi:10.1007/s10863-015-9641-9

Guffanti AA, Krulwich TA (1994) Oxidative phosphorylation by ADP + P_i-loaded membrane vesicles of alkaliphilic *Bacillus firmus* OF4. J Biol Chem 269:21576–21582

Guffanti AA, Blanco R, Krulwich TA (1979) A requirement for ATP for b-galactoside transport by *Bacillus alcalophilus*. J Biol Chem 254:1033–1037

Guffanti AA, Cohn DE, Kaback HR, Krulwich TA (1981) Relationship between the Na^+/H^+ antiporter and Na^+/substrate symport in *Bacillus alcalophilus*. Proc Natl Acad Sci USA 78:1481–1484

Guffanti AA et al (1986) Isolation and characterization of new facultatively alkalophilic strains of *Bacillus* species. J Bacteriol 167:766–773

Hase CC, Barquera B (2001) Role of sodium bioenergetics in *Vibrio cholerae*. Biochim Biophys Acta 1505:169–178

Hess P, Lansman JB, Tsien RW (1986) Calcium channel selectivity for divalent and monovalent cations. Voltage and concentration dependence of single channel current in ventricular heart cells. J Gen Physiol 88:293–319

Hirota N, Imae Y (1983) Na^+-driven flagellar motors of an alkalophilic *Bacillus* strain YN-1. J Biol Chem 258:10577–10581

Hirota N, Kitada M, Imae Y (1981) Flagellar motors of alkalophilic *Bacillus* are powered by an electrochemical potential gradient of Na^+. FEBS Lett 132:278–280

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms: a new microbial world. Springer-Verlag, Heidelberg, Tokyo

Horikoshi K (1991) Microorganisms in alkaline environments. VCH Publishers Inc., New York

Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63:735–750

Horikoshi K (2016) Extremophiles: where it all began. Springer, Tokyo

Imazawa R, Takahashi Y, Aoki W, Sano M, Ito M (2016) A novel type bacterial flagellar motor that can use divalent cations as a coupling ion. Sci Rep 6:19773. doi:10.1038/srep19773

Ito M (2002) Aerobic alkaliphiles. In: Bitton G (ed) Encyclopedia of environmental microbiology. Wiley, New York, pp 133–140

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

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

Ito M, Fujinami S, Terahara N (2011) Bioenergetics: Cell motility and chemotaxis of extreme alkaliphiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo, pp 141–162

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

Kobayashi T et al (1995) Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. Appl Microbiol Biotechnol 43:473–481

Kojima S (2015) Dynamism and regulation of the stator, the energy conversion complex of the bacterial flagellar motor. Curr Opin Microbiol 28:66–71. doi:10.1016/j.mib.2015.07.015

Kojima S, Blair DF (2001) Conformational change in the stator of the bacterial flagellar motor. Biochemistry 40:13041–13050

Kojima S, Blair DF (2004) The bacterial flagellar motor: structure and function of a complex molecular machine. Int Rev Cytol 233:93–134

Krulwich TA (1995) Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. Mol Microbiol 15:403–410

Krulwich TA, Hicks DB, Swartz TH, Ito M (2006) Bioenergetic adaptations that support alkaliphily. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM Press, Washington, DC, pp 295–329

Krulwich TA, Hicks DB, Swartz TH, Ito M (2007) Bioenergetic adaptations that support alkaliphily. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM Press, Washington, DC, pp 311–329

Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. Nat Rev Microbiol 9:330–343. doi:10.1038/nrmicro2549

Leake MC, Chandler JH, Wadhams GH, Bai F, Berry RM, Armitage JP (2006) Stoichiometry and turnover in single, functioning membrane protein complexes. Nature 443:355–358. doi:10.1038/nature05135

Lloyd SA, Tang H, Wang X, Billings S, Blair DF (1996) Torque generation in the flagellar motor of *Escherichia coli*: evidence of a direct role for FliG but not for FliM or FliN. J Bacteriol 178:223–231

Makarova KS, Koonin EV, Albers SV (2016) Diversity and evolution of type IV pili systems in archaea. Front Microbiol 7:667. doi:10.3389/fmicb.2016.00667

Mandel KG, Guffanti AA, Krulwich TA (1980) Monovalent cation/proton antiporters in membrane vesicles from *Bacillus alcalophilus*. J Biol Chem 255:7391–7396

Marykwas DL, Schmidt SA, Berg HC (1996) Interacting components of the flagellar motor of *Escherichia coli* revealed by the two-hybrid system in yeast. J Mol Biol 256:564–576. doi:10.1006/jmbi.1996.0109

Matsuura S, Shioi J, Imae Y (1977) Motility in *Bacillus subtilis* driven by an artificial proton motive force. FEBS Lett 82:187–190

McCarter LL (2004) Dual flagellar systems enable motility under different circumstances. J Mol Microbiol Biotechnol 7:18–29. doi:10.1159/000077866

McCarter LL (2005) Multiple modes of motility: a second flagellar system in *Escherichia coli*. J Bacteriol 187:1207–1209. doi:10.1128/JB.187.4.1207-1209.2005

Metlina AL (2004) Bacterial and archaeal flagella as prokaryotic motility organelles. Biochemistry (Mosc) 69:1203–1212

Minamino T (2014) Protein export through the bacterial flagellar type III export pathway. Biochim Biophys Acta 1843:1642–1648. doi:10.1016/j.bbamcr.2013.09.005

Minamino T, Imada K (2015) The bacterial flagellar motor and its structural diversity. Trends Microbiol 23:267–274. doi:10.1016/j.tim.2014.12.011

Minamino T, Imae Y, Oosawa F, Kobayashi Y, Oosawa K (2003) Effect of intracellular pH on rotational speed of bacterial flagellar motors. J Bacteriol 185:1190–1194

Morimoto YV, Nakamura S, Kami-ike N, Namba K, Minamino T (2010) Charged residues in the cytoplasmic loop of MotA are required for stator assembly into the bacterial flagellar motor. Mol Microbiol 78:1117–1129. doi:10.1111/j.1365-2958.2010.07391.x

Morimoto YV, Nakamura S, Hiraoka KD, Namba K, Minamino T (2013) Distinct roles of highly conserved charged residues at the MotA-FliG interface in bacterial flagellar motor rotation. J Bacteriol 195:474–481. doi:10.1128/JB.01971-12

Ng SY, Chaban B, Jarrell KF (2006) Archaeal flagella, bacterial flagella and type IV pili: a comparison of genes and posttranslational modifications. J Mol Microbiol Biotechnol 11:167–191. doi:10.1159/000094053

Ohkuma M et al (2003) An alkaliphilic and xylanolytic *Paenibacillus* species isolated from the gut of a soil-feeding termite. Microbes Environ 18:145–151

Paulick A et al (2015) Dual stator dynamics in the *Shewanella oneidensis* MR-1 flagellar motor. Mol Microbiol 96:993–1001. doi:10.1111/mmi.12984

Preiss L et al (2013) The c-ring stoichiometry of ATP synthase is adapted to cell physiological requirements of alkaliphilic *Bacillus pseudofirmus* OF4. Proc Natl Acad Sci USA 110:7874–7879. doi:10.1073/pnas.1303333110

Preiss L, Hicks DB, Suzuki S, Meier T, Krulwich TA (2015) Alkaliphilic bacteria with impact on industrial applications, concepts of early life forms, and bioenergetics of ATP synthesis. Front Bioeng Biotechnol 3:75. doi:10.3389/fbioe.2015.00075

Sharp LL, Zhou J, Blair DF (1995) Tryptophan-scanning mutagenesis of MotB, an integral membrane protein essential for flagellar rotation in *Escherichia coli*. Biochemistry 34:9166–9171

Takami H, Takaki Y, Uchiyama I (2002) Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. Nucleic Acids Res 30:3927–3935

Tang H, Braun TF, Blair DF (1996) Motility protein complexes in the bacterial flagellar motor. J Mol Biol 261:209–221. doi:10.1006/jmbi.1996.0453

Terahara N, Fujisawa M, Powers B, Henkin TM, Krulwich TA, Ito M (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. doi:10.1128/JB.188.7.2701-2705.2006

Terahara N, Krulwich TA, Ito M (2008) Mutations alter the sodium versus proton use of a *Bacillus clausii* flagellar motor and confer dual ion use on *Bacillus subtilis* motors. Proc Natl Acad Sci USA 105:14359–14364. doi:10.1073/pnas.0802106105

Terahara N, Sano M, Ito M (2012) A *Bacillus* flagellar motor that can use both Na^+ and K^+ as a coupling ion is converted by a single mutation to use only Na^+. PLoS One 7:e46248. doi:10.1371/journal.pone.0046248

Vedder A (1934) *Bacillus alcalophilus* n. sp.; benevens enkele ervaringen met sterk alcalische voedingsbodems. Antonie Van Leeuwenhoek 1:141–147

Yamaguchi S, Aizawa S, Kihara M, Isomura M, Jones CJ, Macnab RM (1986a) Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. J Bacteriol 168:1172–1179

Yamaguchi S, Fujita H, Ishihara A, Aizawa S, Macnab RM (1986b) Subdivision of flagellar genes of *Salmonella typhimurium* into regions responsible for assembly, rotation, and switching. J Bacteriol 166:187–193

Yoshida S, Sugiyama S, Hojo Y, Tokuda H, Imae Y (1990) Intracellular Na^+ kinetically interferes with the rotation of the Na^+-driven flagellar motors of *Vibrio alginolyticus*. J Biol Chem 265:20346–20350

Zhou J, Lloyd SA, Blair DF (1998a) Electrostatic interactions between rotor and stator in the bacterial flagellar motor. Proc Natl Acad Sci USA 95:6436–6441

Zhou J et al (1998b) Function of protonatable residues in the flagellar motor of *Escherichia coli*: a critical role for Asp 32 of MotB. J Bacteriol 180:2729–2735

Zhu S, Kojima S, Homma M (2013) Structure, gene regulation and environmental response of flagella in *Vibrio*. Front Microbiol 4:410. doi:10.3389/fmicb.2013.00410