Motility and chemotaxis in alkaliphilic *Bacillus* species

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Abstract

Alkaliphilic *Bacillus* species grow at pH values up to approximately 11. Motile alkaliphilic *Bacillus* use electrochemical gradients of Na^+ (sodium-motive force) to power ion-coupled, flagella-mediated motility as opposed to the electrochemical gradients of H^+ (proton-motive force) used by most neutralophilic bacteria. Membrane-embedded stators of bacterial flagella contain ion channels through which either H^+ or Na^+ flow to energize flagellar rotation. Stators of the major H^+-coupled type, MotAB, are distinguishable from Na^+-coupled stators, PomAB of marine bacteria and MotPS of alkaliphilic *Bacillus*. Dual ion-coupling capacity is found in neutralophilic *Bacillus* strains with both MotAB and MotPS. There is also a MotAB variant that uses both coupling ions, switching as a function of pH. Chemotaxis of alkaliphilic *Bacillus* depends upon flagellar motility but also requires a distinct voltage-gated NaChBac-type channel. The two alkaliphile Na^+ channels provide new vistas on the diverse adaptations of sensory responses in bacteria.

Keywords

Extremophilic bacteria are of great utility because of the natural products they provide and the insights their adaptations provide into the plasticity of biological systems [1-3]. In

addition, studies of the adaptations to their specific environmental challenges often lead to discoveries that apply more broadly to nonextremophilic bacteria, including pathogenic strains. Studies of extremely alkaliphilic *Bacillus* species have led to insights into ion coupling to ATP synthesis and alkaline pH homeostasis that are now applied way beyond alkaliphiles [3-6], and recent studies of flagellar-based motility and chemotaxis in these extremophiles have identified participating components and raised questions that extend broadly [7-11]. Thus, the alkaliphile as a model system of bioenergetic work has shed light on swimming, which underpins chemotaxis, the ability to move away from detrimental conditions and toward favorable ones, and the ability to inhabit niches in animal hosts as well as environmental settings or perform complex behaviors [12,13].

Structure of bacterial flagella

Like most other motile bacteria, alkaliphilic *Bacillus* species use filamentous propeller-like flagella for swimming (Figure 1). Those alkaliphiles studied to date have peritrichous flagella, in other words, flagella that are distributed around the cell surface as opposed to being confined to one or both cell poles [9,11]. The flagellum is a complex rotary nanomachine that is composed of multiple copies of at least 25 different proteins (Figure 1) [14,15]. The structure is composed of a helical filament, a basal body that is embedded in the cytoplasmic membrane and a hook connecting the filament to the basal body. The flagellar motor in the basal body consists of a rotor and stator that function comparably to those of nonbiological motors. The rotor encompasses a switch complex that is composed of the proteins FliG, FliM and FliN, and is involved in the generation of the torque for flagellar rotation as well as in the control of the rotational direction, either counterclockwise or clockwise, and in the assembly of the flagella [16-25]. The membrane-embedded stators form a ring around the base of the flagella [26] and support conversion of the energy from transmembrane electrochemical ion gradients, in the form of a proton-motive force (PMF) or sodium-motive force (SMF), into mechanical energy [27]. MotA and MotB proteins were identified as the protein components of the stator of proton-driven motors of Gram-negative *Salmonella enterica* serovar Typhimurium and *Escherichia coli* in which the most extensive studies of bacterial motility and chemotaxis have been conducted [28-30]. MotA has four transmembrane helices (TM1–4). A large cytoplasmic domain between TM2 and TM3 is involved in generation of torque by its interaction with the C-terminal region of FliG [31]. MotB has one TM and a large periplasmic domain that binds to the peptidoglycan and acts as the anchor [32]. MotB also possesses a conserved and essential aspartate residue that is thought to play a critical role in proton movement [33,34]. The functional stoichiometry of each stator complex is thought to be 4A:2B [33,35]. The typical number of such complexes surrounding the basal motor appears to be at least 11 [26,36,37]. The torque, which is required for rotating the flagellar filament, is generated by the interaction between the rotor and the stator in the basal body.

Na$^+$-coupled stators

Initial studies of motility in Gram-positive bacteria showed that the rotational energy for the flagellar motor is provided by the electrochemical ion gradients across the cytoplasmic membrane, as had been established for Gram-negative bacteria [38]. In these studies, motility of *Streptococcus* spp. [39] and of *Bacillus subtilis* [40,41] was shown to be powered by the PMF as had been shown for *E. coli* and *S.* Typhimurium. Additional cations, such as sodium or potassium, were not required for motility (Figure 2).

As the energetic profile of aerobic alkaliphilic *Bacillus* strains was revealed through studies by several laboratories, motility was observed at alkaline pH values from 9 to 10.5 [42-44]. However, it was also clear that in this range of pH, the PMF available to energize proton-

coupled bioenergetic work such as motility decreases progressively [43,45]. Alkaliphilic *Bacillus* species were found to have sizeable 'reversed' chemical gradients of H^+, in other words, cytoplasmic pH values that are markedly lower than high external pH values during optimal growth [6,43,46-48]. In carefully pH-controlled studies in continuous cultures, alkaliphilic *Bacillus pseudofirmus* OF4 was shown to grow at pH values above 11. This alkaliphile sustains a cytoplasmic pH 2.3 units lower than the external pH of 10.5, resulting in the low bulk PMF of −44 mV, while growing logarithmically with a generation time of approximately 37 min on a semidefined, malate-containing medium and exhibiting a clear capacity for motility [43,47]. It was therefore anticipated that motility in such extremely alkaliphilic *Bacillus* strains would be energized by the larger SMF that exists under these conditions, as had earlier been found to be the case for ion-coupled solute uptake [49]. That Na^+ coupling was indeed used for motility was first demonstrated by Hirota *et al.*, who showed that the energy source for the rotation of flagellar motor of extremely alkaliphilic *Bacillus* species is the SMF and not the PMF [42,50]. However, the Na^+-dependent stator of the alkaliphilic *Bacillus* species was not the first SMF-driven Mot-type system to be identified. Rather, it was discovered that marine *Vibrio* species also use the SMF rather than PMF to power motility of their polar flagellum [51], and the Mot-type stator-force generator was designated PomA/PomB, a MotAB-like pair of proteins that required additional proteins, MotX and MotY, for torque generation in *Vibrio alginolyticus* [8,52-55]. Recently, Paulick *et al.* showed polar flagellar localization of both MotB and PomB in *Shewanella oneidensis* MR-1 and demonstrated the coexistence of the two sets of stator units in a single flagellar motor [56].

The MotPS that functions as the stator of the flagellar motor of alkaliphilic *Bacillus* species were first identified in *B. pseudofirmus* OF4 based on homology of the predicted products of a set of putative *mot* genes to MotAB [8]. The *motPmotS* genes are universally located downstream of the *ccpA* gene, which encodes a central regulator of carbon metabolism in *Bacillus* species and other Gram-positive organisms [57,58]. In extremely alkaliphilic *Bacillus halodurans* C-125 [59] and *B. pseudofirmus* OF4 [8], Na^+-dependent MotPS is the only stator and the flagellar motor is driven by SMF. By contrast, the more moderately alkaliphilic *Oceanobacillus iheyensis*, an alkaliphilic marine bacterium, has both sodium-coupled MotPS and proton-coupled MotAB (Figure 2) [60]. There are no reports of motility assays for this bacterium but studies described below for neutralophilic *B. subtilis*, which also has both MotAB and MotPS, suggest that both stators contribute to the torque required for flagellar rotation, with different relative contributions under different conditions.

Why is there so little motility of Na^+-dependent extremely alkaliphilic strains at near-neutral pH?

Bacillus pseudofirmus OF4 grows optimally up to pH 10.6 on semidefined malate-containing medium and the low end of its pH range under these conditions is 7.5 [43,61]. Sturr *et al.* reported that the motility of *B. pseudofirmus* OF4 declined at the low end of the pH range for growth and that no motility was observed at pH 7.5 [43]. This raised the possibility that in extreme alkaliphiles, which are adapted to growth at high pH, sodium-coupled processes are particularly sensitive to inhibition by protons. This has been hypothesized to be the case for sodium-coupled solute transporters of alkaliphilic *B. pseudofirmus* OF4 [62]. After swimming assays confirmed that motility of *B. pseudofirmus* OF4 is sodium dependent and the number of flagella per cell were shown to be comparable at pH 7.5 and 10.3, the possibility that competitive inhibition by protons was responsible for poor swimming at near-neutral pH was tested [63]. Indeed, motility was observed at pH values as low as 7 when the concentration of Na^+ was raised sufficiently and in the range of pH 8–10, swimming speed increased with increasing $[Na^+]$ up to 230 mM.

A lesson from the alkaliphile: MotPS is a second stator in neutralophilic *B. subtilis*

Once MotPS was recognized as the functional, sodium-dependent stator for motility of extreme alkaliphiles, it was of interest to investigate whether the homolog that had earlier been described in *B. subtilis*, but for which no function had been demonstrated [64], might be a second functional stator that responded to Na^+ and elevated pH in some neutralophiles [8]. Studies revealed that in *B. subtilis*, MotPS functioned as a Na^+-dependent stator that made a contribution to swimming of wild-type *B. subtilis*, especially at elevated pH, sodium and viscosity [8,9]. Hybrid forms were active and provided information about the contributions of the two stator components to different stator properties; this included the finding that the MotB and MotS proteins were the major determinants of ion specificity [9]. Up-motile variants of *B. subtilis* mutants lacking functional MotAB were described and found to have a mutation in the intergenic region between *ccpA* and *motPS* that resulted in more cotranscription of the latter two genes with the upstream *ccpA* [65]. This finding suggests that under appropriate selective pressure, MotPS could play a major role in motility of some *B. subtilis* strains. There have been increasing reports of dual flagellar systems in different bacterial species [66], with this one being the first example of two distinct stators that interact with a single rotor [8]. Other neutralophilic *Bacillus* strains also have both *motAB* and *motPS* loci, as indicated by genomic data (Figure 2). Recent studies have shown, using the two *B. subtilis* stators, that each of the stators with different single ion-coupling preferences can be modified by mutagenesis of the *motB* or *motS* gene to forms that can use both Na^+ and H^+, bringing it closer to the different mode of dual ion coupling that is described below for the single *Bacillus clausii* stator [11]. Additional studies will be required to determine how the ratio of MotPS to MotAB stators is determined in a given strain and to test the suggestion that a given flagellum is surrounded by a mixture of the two types. These are issues that are of general interest in the field [67].

One stator that couples to two different ions: flagellar stator & motility of *B. clausii* KSM-K16

Bacillus clausii strains are alkaliphilic bacteria that are noted for useful biological products such as alkaline proteases [68,69]. The genome sequence of *B. clausii* KSM-K16 was completed in 2005 and revealed only a single set of genes encoding a potential stator for this motile alkaliphile. These genes were predicted to encode a MotAB-like pair of proteins (GenBank accession no. AP006627) (Figure 2). The *B. clausii* stator proteins were closely related to the MotA and MotB that compose the proton-coupled stator in *B. subtilis* (identity: 52 and 45%; similarity: 73 and 64%, respectively), and were much less closely related to the MotP and MotS that compose the sodium-coupled stator in *B. subtilis* (identity: 32 and 28%; similarity: 57 and 55%, respectively). This raised the possibility that either *B. clausii* was only poorly motile in the alkaline edge of its pH range or that it had a novel type of MotAB stator that could couple to both sodium and protons. Studies of this question first showed that the organism was very motile over a range of pH that extended from pH 7.0 to 11 [11]. Therefore, the properties of the *B. clausii* MotAB (BCl-MotAB) were examined to test the hypothesis of a single, native stator with dual ion-coupling capacity. The BCl-MotAB was introduced into a stator-less (Δ*motAmotB*Δ*motPmotS*) mutant strain of *B. subtilis*, because *B. clausii* KSM-K16 is not yet genetically accessible. The heterologous BCl-MotAB functioned in the *B. subtilis* setting, as shown in Figure 3, making it possible to compare its properties with those of the two single stators of the host, MotAB and MotPS of *B. subtilis*, introduced into the statorless mutant in an identical fashion. The studies assessed ion dependence at different pH values as well as the pH-dependent effects of a sodium-channel inhibitor, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), an amiloride analog, and the

protonophore cyanide *m*-chlorophenyl hydra-zone (CCCP), which dissipates electrochemical proton gradients. The results strongly supported the hypothesis that BCl-MotAB is a bifunctional stator that uses protons at near-neutral pH and sodium at high pH and probably uses both at intermediate pH values.

It was further possible to introduce mutations into BCl-MotB that made the BCl-MotAB much more single-ion dependent, either for sodium or for protons. The strategy was developed from alignments of BCl-MotB with MotB and MotS. These alignments supported inferences with respect to which residues were of potential importance in ion specificity (Figure 4). A triple mutant of BCl-MotB with G42S, Q43S and Q46A mutations was constructed on this basis and behaved like the MotB subunit of a proton-coupled stator. A second triple mutant with V37L, A40S and G42S mutations behaved like the MotS subunit of the sodium-coupled stator. Further studies of single and double mutants showed that the Q43S mutation was critical for loss of sodium coupling and had to be introduced together with either the G42S or Q46A mutation to produce a proton-coupled form. The V37L mutation was critical for sodium selectivity and a combination of the V37L mutation and either the A40S or the G42S mutation was required for production of the BCl-MotB form that exhibits sodium coupling at low pH [11]. Thus, the novel bifunctional stator of *B. clausii* yielded insights into the determinants of ion-coupling specificity that will be useful in identifying other candidates in bacteria for dual function. When high-resolution structures of flagellar stators are achieved, such information will also play a role in modeling the cation sites.

Chemotaxis of *Bacillus* species

Motile bacteria can respond to stimulation by chemical and physical effectors in the external environment [12,27,70,71]. The temporal shift of temperature, pH and oxygen concentration can be effectors (attractants or repellents), as can diverse nutrient or inhibitory chemical compounds. The most intensively studied bacterial chemotaxis system is that of *E. coli*, which is thought to involve a less complex set of networks than that of *B. subtilis* chemotaxis [71-73], but still continues to provide novel information [74,75]. *B. subtilis* provides one of the other chemotaxis systems for which a great amount of information is available and is by far the most studied among *Bacillus* species, revealing commonalities with *E. coli* chemotaxis but also significant differences [71,72,76-78]. In both model systems, the bacteria swim in straight runs (with counterclockwise rotation of the flagellar bundle) with occasional tumbles (initiated by clockwise rotation) that increase the likelihood of a change in direction [71]. Therefore, movement toward attractants is favored by smooth runs in response to an attractant and movement away from repellents is favored by increased tumbling. Chemoreceptor proteins are usually transmembrane signaling proteins that bind external signaling molecules and/or respond to physical effectors in a manner that also initiates a change that is transmitted to the cytoplasmic domain. The chemoreceptors are called methylated chemotaxis proteins (MCPs) because of an adaptation feature of chemotaxis that involves a methylation/demethylation of sites on the MCPs. Clusters of MCPs have been found at the cell poles of rod-shaped bacteria [79-81]. The response of the MCPs is transmitted via the CheA histidine kinase that is complexed, together with adaptor protein CheW, on the cytoplasmic side. The resulting state of autophosphorylation of CheA determines the phosphorylation state of the response regulator CheY, which interacts with the switch domain of the rotor and determines the direction of rotation and, hence, the frequency of tumbling as opposed to runs. The response of the CheA kinase activity to binding of chemo-effectors to the MCPs is different in *E. coli* than in *B. subtilis*, and the *Bacillus* has distinct and more complex layers of adaptive capacity that correlate with the presence of chemotaxis proteins that are not found in *E. coli* (Table 1) [71,76]. *B. subtilis*

also has redundancies in several chemotaxis proteins, which is not observed in *E. coli* and is thought to increase robustness of *B. subtilis* chemotaxis (Table 1) [12,70,77].

Alkaliphilic *Bacillus* species show similar chemotaxis 'toolkits' to *B. subtilis* (Table 1) and are expected to share many of the same chemoeffectors as well as the general design principles of the signal transduction mechanism, since both of those show a high degree of conservation among even more distantly related bacteria [70-71]. There is as yet no information in alkaliphilic *Bacillus* species about the involvement of particular MCPs or other chemotaxis elements in responses to pH or PMF, as there are for *E. coli* [82-84]. However, evidence has emerged for a dependence of chemotaxis in alkaliphilic *B. pseudofirmus* OF4 on a voltage-gated sodium channel (see below). This incompletely understood layer of adaptation in alkaliphile chemotaxis may be distinct in these organisms, perhaps reflecting the challenge of alkaliphily and its dependence on a sodium cycle. Quite possibly, though, the involvement of a channel in alkaliphile chemotaxis is a particularly pronounced extremophile version of a role for an ion channel that will turn up in other bacteria and whose identification and study will be facilitated by the extremophile model.

Channel involvement in chemotaxis

In the aerobic, alkaliphilic *Bacillus* species, the respiratory chain pumps protons outward during electron transport and secondary sodium/proton antiporters (exchangers) convert the PMF produced by this proton pumping to a SMF and a 'reversed' pH gradient, in other words, they achieve the required acidification of the cytoplasm relative to the outside pH (Figure 5) [3,45]. Although alkaliphilic *Bacillus* species all have multiple sodium/proton antiporters encoded in their genomes, there is a specific antiporter, the multisubunit Mrp-type antiporter, which is essential for alkaliphily of aerobic *B. halodurans* C-125 and *B. pseudofirmus* OF4. It is the only bacterial antiporter that has been definitively shown to be irreplaceable by others of the organism's antiporter complement for alkaline pH homeostasis [85-88]. The same family of antiporters is found widely in bacterial pathogens [88]. The continuous antiporter-mediated efflux of sodium, which is coupled to cytoplasmic proton accumulation during respiration, necessitates continuous re-entry of sodium from the outside. It was expected that the MotPS sodium channel would be a major mediator of such sodium re-entry, especially under conditions in which the medium or environment was not rich in nutrients and other solutes that are taken up together with Na^+ [89]. Mutant studies showed that MotPS plays a modest role in sodium circulation in support of pH homeostasis under conditions in which both the concentrations of Na^+ and of solutes that enter with sodium are low. A distinct channel, the voltage-gated Na^+ channel called NaChBac in *B. halodurans* C-125 and $Na_V BP$ in *B. pseudofirmus* OF4, has a greater role in cytoplasmic pH homeostasis, as shown in mutants of the latter alkaliphile strain with defects in the *ncbA* gene encoding the channel [10,90].

The NaChBac–$Na_V BP$ type of channel is also found in other alkaliphilic *Bacillus* strains, as well as in other bacteria that face a spectrum of salt/alkali challenges [91]. The NaChBac family is of great general interest because of its homology with voltage-gated sodium channels of eukaryotes, which are profoundly important in various disease processes [90]. The smaller, bacterial version provides an excellent experimental model for structural studies [92,93]. It was also of great interest with respect to the physiology of bacterial motility and chemotaxis since the finding that *B. pseudofirmus* OF4 mutants lacking a functional $Na_V BP$ exhibited a striking chemotaxis abnormality, in other words, a 'tumbly' phenotype accompanied by 'inverse chemotaxis', wherein the bacteria moved away from nutrient attractants, such as malate, and toward repellent conditions, such as pH 10.5, in the absence of an energy source to support cell energetics [10]. The inverse chemotactic phenotype was not secondary to a problem with pH homeostasis because the inverted

response to nutrients was observed under conditions of pH and sodium in which pH homeostasis was normal. This suggests that the Na$_V$BP channel has a direct role of some kind in chemotaxis of the alkaliphilic *Bacillus*. Additional support for this suggestion was the observation that nifedipine, a sodium-channel blocker, caused inverse chemotaxis in the wild-type alkaliphile [10]. A role for ion channels in bacterial chemotaxis has been suggested multiple times, but had not previously been associated with a specific channel [94-96].

When properties of the Na$_V$BP channel shown in electrophysiological studies were compared with the conditions existing in its native setting, the results indicated that Na$_V$BP function may depend upon modulation by interaction with other proteins that provide 'additional triggers' for the channel [10]. Hypothetically, an interaction between the channel and the chemotaxis machinery might serve the dual purposes of activating the channel in some way that coordinates with chemotactic signaling and serves pH homeostasis simultaneously. This idea needs to be studied further, but thus far it has been shown that Na$_V$BP of *B. pseudofirmus* OF4 colocalizes at the cell poles of its native host with one of the MCPs, as indicated by its cross-reaction with antibodies against a *B. subtilis* MCP (Figure 5) [7].

Future perspective

Alkaliphilic bacteria are model organisms for study of sodium cycles that support motility, solute transport as well as pathogenicity in bacteria including *Pseudomonas aeruginosa* and *Vibrio cholerae* [97-99], and is implicated in many others [97]. The number of examples and distinct modes of sodium-dependent or dual ion-dependent motility are likely to continue to increase. This will be of general ecological and physiological interest and the findings are likely to include bacteria in which the motility system will be a useful target for inhibition of pathogenicity.

There also remain many basic questions that are yet to be answered, some of which have been mentioned. It will be important to unravel whether the stator rings around individual flagella contain multiple types of stators in organisms with single rotors and dual stator types. The controls that determine the ratio of the two types need to be resolved. It will also be of interest to determine the distribution of ratios in 'wild' rather than only of long-time laboratory-dwelling strains. Earlier studies suggested that the sodium-dependent MotPS channels have multiple Na$^+$-binding sites [9]; this merits further investigation into the nature and roles of such sites. A related issue is the relatively poor function of the alkaliphile MotPS stator at very high concentrations of Na$^+$ [9]. A similar phenomenon has been reported in *Vibrio alginolyticus* [100], and the possibility has been raised that there is a Na$^+$ site somewhere in the motor that acts as a brake.

The recent findings of diversity in the ion-coupling patterns of different bacteria should encourage those with interests in 'sodium energetics' to move beyond the coupling patterns of the ATP synthase as a major sentinel of whether a bacterium is primarily in the camp of sodium energetics as opposed to proton energetics (for example, see [101]). The findings reviewed here indicate that different bacteria display: proton-only coupling to motility, sodium-only coupling, and dual proton and sodium coupling motility using several different strategies, for example shared rotors and multiple stators; separate flagellar systems entirely; and a single stator that switches ions as a function of pH. The correlation of these different modes with degrees of overall use of sodium versus proton energetics would enrich the analyses greatly.

In the chemotaxis area, the basis for the effect of Na$_V$BP on alkaliphile chemotaxis needs to be elucidated. It may offer clues for identifying (perhaps more subtle) roles of channels in

chemotaxis of nonextremophilic bacteria. Conversely, the possibility of interactions between the channel and chemotaxis proteins at the cell poles requires further probing. Further study of Na$_V$BP or other NaChBac forms is expected to continue to contribute to mechanistic details about voltage-gated sodium channels.

Executive summary

Structure of bacterial flagella

- The bacterial flagellum is a complex structure that functions as a rigid propeller that turns as part of a rotary nanomachine with broad roles in physiology and pathogenicity.

- Stators of the flagellar motor encompass ion channels that transduce energy of the sodium- or proton-motive forces into mechanical energy.

Na$^+$-coupled stators

- A conserved pair of stator proteins, MotPS, that are homologs of MotAB mediate Na$^+$-coupled motility of extreme alkaliphiles.

- One of two stators interacting with a single motor in other *Bacillus* species.

Why is there so little motility of Na$^+$-dependent extremely alkaliphilic strains at near-neutral pH?

- Poor motility supported by alkaliphile MotPS at low external pH values results from competitive inhibition by protons that is reversible by high [Na$^+$].

- Use of a motor that is inhibited by protons is compatible with the hard-wired adaptation of extreme alkaliphiles to life at high pH.

A lesson from the alkaliphile: MotPS is a second stator in neutralophilic *Bacillus subtilis*

- Neutralophilic *Bacillus* strains, including *Bacillus subtilis*, exhibit dual ion-coupling capacity using MotPS and MotAB as stators for a single rotor.

One stator that couples to two different ions: flagellar stator & motility of *Bacillus clausii* KSM-K16

- A strain of the probiotic alkaliphile *B. clausii* has a novel form of dual ion coupling to motility in which a single MotAB stator uses both sodium and protons at different ranges of pH.

- Deductions from studies of alignments of the MotB and MotS subunits of sodium- and proton-coupled motility systems facilitated conversion of single ion-coupled stators to dual use and the *B. clausii* dual-ion stator to single-ion use (either sodium or protons).

- Additional examples of this type of bifunctional stator may now be recognized.

Chemotaxis of *Bacillus* species

- Alkaliphile genomes are predicted to encode an array of chemotaxis proteins that is comparable to the 'toolkit' of *B. subtilis*.

Channel involvement in chemotaxis

- A voltage-gated sodium channel with homology to the eukaryotic channels that have major roles in physiology and pathophysiology plays a role in alkaliphile pH homeostasis.

- The Na$_V$BP sodium channel of *Bacillus pseudofirmus* OF4 is required for normal chemotaxis of this alkaliphile and it colocalizes with chemoreceptors at cell poles.

- Mutational loss of the Na$_V$BP voltage-gated Na$^+$ channel of *B. pseudofirmus* OF4 results in 'inversed' chemotaxis.

Future perspective

- A sodium cycle is central to the lifestyle of alkaliphiles and many nonalkaliphilic and pathogenic bacteria.

- Further investigation of dual coupling systems will resolve the controls of the ratios of sodium- to proton-coupled stators in motility as well as the mechanism of the channel interaction with the chemotaxis system in alkaliphiles and, perhaps, beyond alkaliphiles.

- The multiple ion-coupling patterns found among different bacteria present a new sentinel for assessments of the engagement of different wild and clinical strains in sodium energetics versus proton energetics.

Acknowledgments

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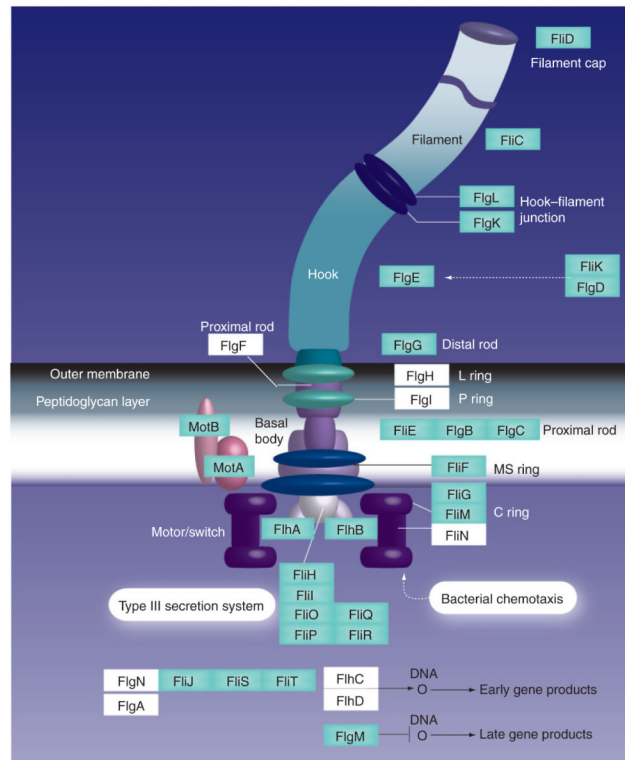
Figure 1. Flagellum in the Gram-negative bacteria *Escherichia coli*
Gram-positive species such as *Bacillus* lack the LP-ring assembly. Protein names, shown in green squares, are components that are conserved in many *Bacillus* species including alkaliphilic *Bacillus*. In *Bacillus* species, there is an FlhO protein that has been reported to substitute for FlgF, FlgI and FlgH [112]. An additional flagellar assembly factor, FliW, was reported in *Bacillus subtilis* and *Campylobacter jejuni* [113]. *B. subtilis* has the orthologs FliM and FliG, but instead of FliN this species has FliY, which is only homologous to FliN in its C-terminus [114]. *B. subtilis* has two kinds of flagellar stator complex, MotAB and MotPS. Some alkaliphilic *Bacillus* species only have a sodium-coupled, MotPS-type stator instead of a proton-coupled, MotAB-type one.
C: Cytoplasmic; L: Lipopolysaccharide; MS: Membrane and supramembranous; P: Peptidoglycan. Reproduced with permission from [201].
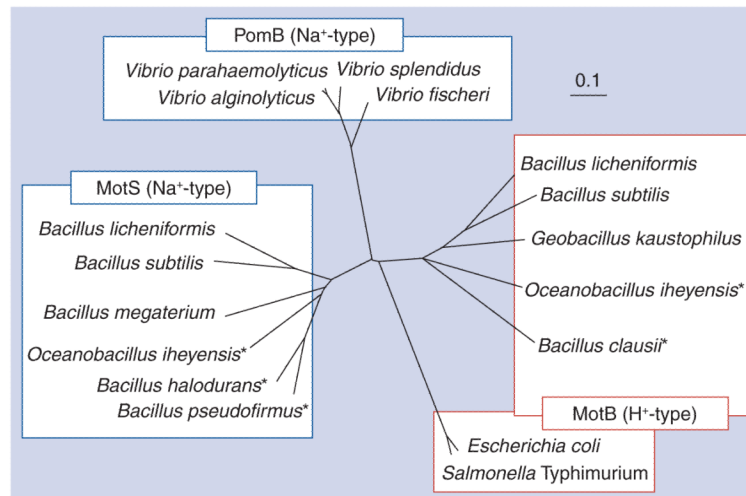
Figure 2. An unrooted tree shows relationships among the stator subunits MotB, MotS and PomB from *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus* and *Vibrio* species

Multiple sequence alignment by ClustalW [202] was used for calculating a multiple alignment and for generating an unrooted phylogenetic tree. Branch lengths are proportional to the sequence divergence and can be measured relative to the bar shown (bar = 0.1 substitutions per amino acid site).

*Proteins from alkaliphilic bacteria.
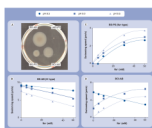
Figure 3. The motility of a stator-less *Bacillus subtilis* mutant (ΔABΔPS) expressing either *B. subtilis motAB*, *B. subtilis motPS* or *Bacillus clausii motAB*

(A) Motility in a soft agar plate assay. Fresh cells were inoculated into Luria-Bertani medium (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 Luria-Bertani medium (pH 7.0) and the swimming speed was measured at various concentrations of sodium in 1/20 Tryptone Yeast Extract (TY) medium. 1/20 TY medium contained 0–1 mM sodium. The pH values of 1/20 TY medium used in the swimming speed assays of *B. subtilis* strains are indicated by the key.

Bcl-AB: *B. clausii* motAB; BS-AB: *B. subtilis* motAB; BS-PS: *B. subtilis* motPS; ΔABΔPS: Deletion strain of the *motAB* and *motPS* genes.

Reproduced with permission from [11].

Figure 4. Alignment of the region containing the single transmembrane segment of MotB, MotS and PomB from *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus* and *Vibrio* species

The amino acid residues that are important for proton or sodium-ion selectivity are highlighted with red and blue, respectively. A conserved aspartic acid residue is critical for flagellar rotation and is highlighted with violet [33-34].

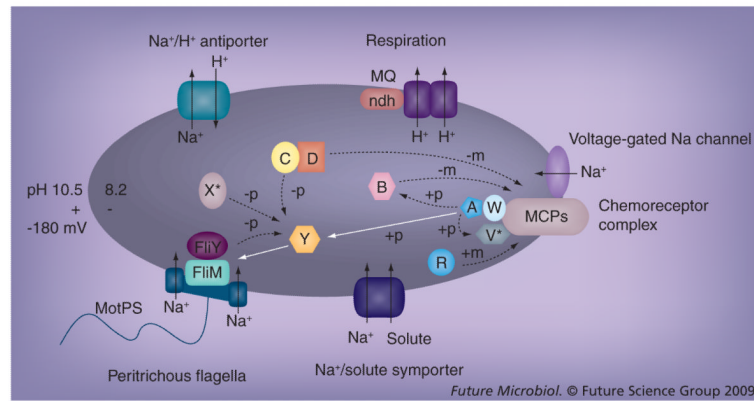Reproduced with permission from [11].

Figure 5. Relationship between Na$^+$-dependent motility, chemotaxis and the Na$^+$ cycle of extremely alkaliphilic *Bacillus* species

White arrows show signal transduction in support of chemotaxis. Dotted arrows show signal removal and adaptation systems. Solid black arrows show transport of ions and solutes. Alkaliphilic *Bacillus* species use a sodium-motive force for flagellar motility and solute symport [115]. Methyl-accepting chemotaxis proteins (chemoreceptors, MCPs), W, A, Y, B, R and FliM are chemotaxis components that are found in all bacteria that have flagellar motility [71]. V, C, D and FliY are not present in *Escherichia coli* but are found in many *Bacillus* species including alkaliphilic *Bacillus* (see Table 1). CheX is also present in several *Bacillus* species (e.g. *Bacillus halodurans* C-125). The asterisks show the proteins that are not conserved in alkaliphilic *Bacillus* species. Bacterial voltage-gated Na channels are found in alkaliphilic *Bacillus* species (e.g., *Bacillus halodurans*, *Bacillus pseudofirmus*, *Bacillus clausii* and *Oceanobacillus iheyensis*). The voltage-gated Na channel of *B. pseudofirmus* OF4 (Na$_V$BP) is required for normal chemotaxis of this alkaliphile and it colocalizes with putative chemoreceptors (McpX) at cell poles [7,10].

−m: Demethylation; +m: methylation; −p: Dephosphorylation; +p: Phosphorylation.
A: CheA; B: CheB; C: CheC; D: CheD; MCP: Methylated chemotaxis protein; MQ: Menaquinone; ndh: NADH dehydrogenase; R: CheR; V: CheV; W: CheW; Y: CheY.

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Table 1

Comparison of the chemotaxis protein homologs of alkaliphilic *Bacillus* species, *Bacillus subtilis* and *Escherichia coli*.

Protein	Functions	Escherichia coli	Bacillus subtilis	Bacillus halodurans	Bacillus clausii	Oceanobacillus iheyensis	Ref.
CheW	Adaptor protein	P0A964	P39802	Q9K8N6	Q5WEU4	Q8CXH0, Q8CX96	[102]
CheV	Adaptor protein	–	P37599	Q9KAM5, Q9KC62	Q5WG28	–	[77,102]
CheA	Histidine kinase	P07363	P29072	Q9K8N5	Q5WEU3	Q8ENE1 *	[103]
CheY	Response regulator	P0AE67	P24072	Q9KA46, Q9K8N7	Q5WFR9	Q8EQW9	[104]
CheR	Methyltransferase	P07364	P31105	Q9KCB8	Q5WGS9	Q8EQB5	[105,106]
CheB	Deamidase/methylesterase	P07330	Q05522	Q9KA55	Q5WFS3	Q8EQW0	[79,107]
CheC	Phosphatase	–	P40403	Q9KDX3	Q5WFS4	Q8EQV9	[77–78,108,109]
CheD	Deamidase/methylesterase	–	P40404	Q9KA57	Q5WFS5	Q8EQV8	[77,109]
CheX	Phosphatase	–	–	Q9KDX3	–	–	[109,110]
FliY	Phosphatase	–	P24073	Q9KA45	Q5WFR8	Q8EQX0	[78,109]
CheZ	Phosphatase	P0A9H9	–	–	–	–	[111]

The GenBank accession numbers are indicated.

*
Indicates the protein that was newly annotated in this research.