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Challenges and Adaptations of Life in Alkaline Habitats

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Contents

Abstract A vast array of organisms is known thriving in high pH environments. The biotechnological, medical, and environmental importance of this remarkable group of organisms has attracted a great deal of interest among researchers and industrialists. One of the most intriguing phenomena of alkaliphiles that engrossed researchers' attention is their adaptation to high pH and ability to thrive in the "extreme" condition which is often lethal to other organisms. Studies made in this line revealed that alkaliphiles deployed a range of adaptive strategies to overcome the various challenges of life in high pH environments. This chapter highlights some of the challenges and the most important structural and functional adaptations that alkaliphiles evolved to circumvent the hurdles and flourish in alkaline habitats. The fascinating alkaliphiles' pH homeostasis that effectively maintains a lower

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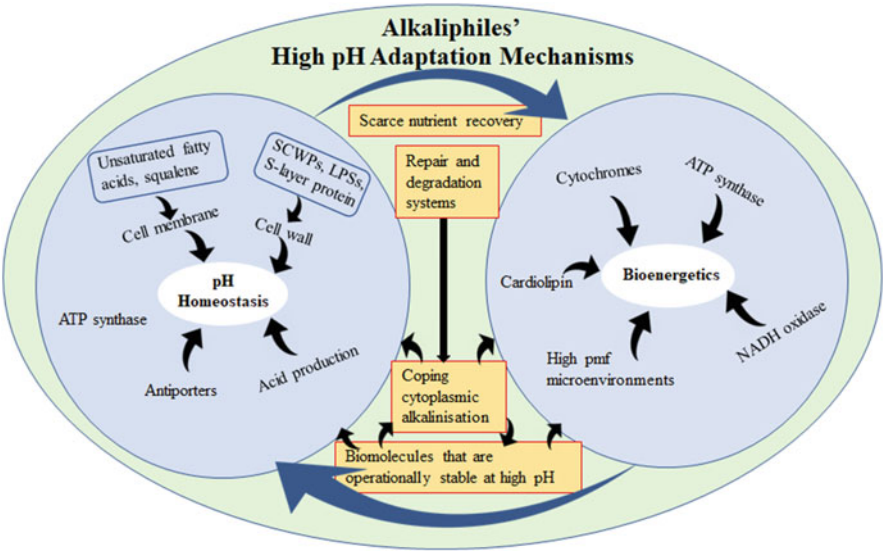
cytoplasmic pH than its extracellular environment and the remarkable bioenergetics that produce ATP much faster than non-alkaliphiles systems are reviewed in detail. Moreover, the adaptive mechanisms that alkaliphiles employ to keep the structural and functional integrity of their biomolecules at elevated pH are assessed.

It is undeniable that our understanding of alkaliphiles adaptation mechanisms to high pH is expanding with time. However, considering that little is known so far about the adaptation of life in alkaline milieu, it seems that this is just the beginning. Probably, there is a lot more waiting for discovery, and some of these issues are raised in the chapter, which not only summarizes the relevant literature but also forwards new insights regarding high pH adaptation. Moreover, an effort is made to include the largely neglected eukaryotic organisms' adaptation to high pH habitats.

Graphical Abstract



Keywords Alkaliphiles, Alkaliphiles adaptation, Antiporter, ATP synthase, Bioenergetic, Cardiolipin, Cytochrome, Eukaryotes, Extremophiles, pH homeostasis, Secondary cell wall, S-layer, Squalene, Unsaturated fatty acids

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMB	Bis(monoacylglycero) phosphate
CPA	Cation/proton antiporters

GH	Glycoside hydrolase
LPS	Lipopolysaccharide
Mrp	Multiple resistance and pH
MS	Mass spectrometry
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear magnetic resonance
OXPHOS	Oxidative phosphorylation
PCM	L-isoaspartyl protein carboxyl methyltransferase
PG	Peptidoglycan
P_i	Inorganic phosphate
pI	Isoelectric points
Pmf	Proton motive force
SCWP	Secondary cell wall polymers
S-layer	Cell surface layer
SLH	S-layer homology
SlpA	Surface-layer protein A
Smf	Sodium motive force
TCDB	Transporter classification database
TMH4	Trans-membrane helix-4
TMH5	Trans-membrane helix-5
TUA	Teichuronic acid
TUP	Teichuronopeptide
UT-A	Urea transporter A

1 Introduction

Organisms interact with their environment to ensure survival. They acquire resources such as nutrients from their environment and discharge waste and products out of their bodies. These fascinating complex processes are accomplished at astonishingly high rate of fidelity through numerous metabolic activities which give the identity of life. To sustain life, organisms should adjust to their environment. Thus, organisms evolve various mechanisms including structural and physiological features that allow them not only to survive but also thrive in their respective habitats. This process of evolving structural and functional mechanisms to thrive in an environment is known as adaptation.

The incredible evolution of organisms in response to environmental conditions gave rise to an impressive diversity of adaptive solutions to a range of habitats. This stretches life almost to every corner of the planet. Even places that once have been considered too hostile for life are found to be inhabited by various life forms. One of these "unusual" places is the different natural and man-made alkaline environments which are found inhabited by diverse groups of organisms known as alkaliphiles. Soda lakes, which are stable alkaline environments, are important habitats for

alkaliphiles from which many alkaliphiles have been isolated [1, 2]. Similarly, soda deserts, soda pans, solonchak soils, salt pans, oceans, etc. are known supporting this remarkable group of organisms [3–7]. Alkaliphiles have also been isolated from the steady-state alkaline environments that exist in the body of other organisms such as insect guts [8–10]. Serpentinization, low-temperature weathering of silicate containing calcium and magnesium minerals like olivine ($MgFeSiO_4$) and pyroxene ($MgCaFeSiO_3$), forms a highly alkaline Ca^{2+}-rich environment [11]. Alkaliphiles such as *Alkaliphilus hydrothermalis* [12] and *Serpentinicella alkaliphila* [13] have been isolated from such serpentine environments. Anthropogenic activities such as indigo dye production, potato peeling using KOH, cement/concrete production, electroplating, leather tanning, paper and board manufacture, mining, and herbicide manufacturing create alkaline environments [14–18]. Regardless of the difference in the genesis, chemistry, and stability, all known alkaline environments are inhabited by alkaliphiles, and this is discussed in detail by Kevbrin [19].

High pH environments are not easy to live in without special adaptations. For instance, maintaining structural integrity, bioenergetics, and intracellular pH homeostasis are barriers for non-alkaliphiles to survive and thrive in this extreme environment. On the other hand, alkaliphiles evolved adaptive solutions to circumvent these barriers and thrive lavishly in environments with pH values of up to over 13 [16]. However, adaptation always comes with price. Indeed, there is no single organism that flourishes in a pH range of 1–13. At least in this case, the rule of nature seems clear, when an organism evolves adaptations to thrive in specific pH condition (acidic, neutral, or alkaline), its fitness to live in a habitat of different pH condition is often compromised. Thus, the adaptation range of alkaliphiles determines their ability to survive in neutral conditions. As the optimum pH for growth varies among alkaliphiles, the ability to grow in neutral zone is also different. The growth of obligate alkaliphiles is compromised around neutral condition. On the other hand, facultative alkaliphiles can grow at neutral pH but not as lavishly as neutralophiles [20–22]. Similarly, at or above pH 10, the growth yield of facultative alkaliphiles is often lower than that of obligate alkaliphiles. This may indicate that alkaliphiles evolved to colonize high pH environments at a cost of losing growth potency around neutrality.

This chapter presents the grand challenges of life in high pH environments and tries to summarize the adaptive mechanisms deployed by alkaliphiles to circumvent the challenges and successfully colonize high pH habitats. Most of the studies made on high pH adaptations of organisms are related to alkaliphilic bacteria, and hence, the discussion in this chapter largely revolves around this group of organisms. On the other hand, there are several groups of unicellular and multicellular eukaryotes that are adapted to alkaline habitats. Studies on high pH adaptations of these eukaryotic organisms still remain scarce. Here, an effort is made to include the available information on adaptive mechanisms of multicellular organisms to high pH environment, fish.

2 The Grand Challenges to Thrive in Alkaline Habitats from a Neutralophilic Standpoint

A wide range of "bizarre" environments exist in the biosphere. Hot and frozen environments, sulfurous springs, solfataras, the deep-sea black smoker vents and cold seeps, acidic environments of anthropogenic and natural origin, and salt lakes are some among the many that fall in this category. These environments have their own challenges for life to thrive in. However, such sites are often found inhabited by organisms, which have specific adaptive solutions to the challenges of the respective extreme habitats. Likewise, alkaline environments have their own challenges, and some of the most important ones are discussed below.

The biochemical reactions of life are not spontaneous or self-driven; rather they are highly regulated and are mediated by specific enzymes which are operationally stable within a range of pH. The cytoplasmic pH of cells from various organisms is known to be within the neutral range [22], and the enzymes that catalyze the myriad biochemical reactions occurring inside cells are evolved to work optimally around this pH, neutrality. As the pH drifts away from the neutral range, the catalytic efficiency of the enzymes dwindles, and the cellular functional integrity drops. Like the functional integrity, the structural integrity of intracellular biomolecules is tuned to the cytoplasmic pH. The integrity of at least some of the important macromolecules such as proteins, lipids, and genetic materials can be labile at elevated pH, and the molecules become prone to precipitation or breakdown [23–25]. This structural and functional integrity impairment can be fatal and bring cellular demise. Thus, for biochemical reactions to proceed without a flaw and ensure survival, the intracellular pH should be maintained in the neutral range. However, when organisms are exposed to high pH condition, maintaining their cytoplasmic pH within the neutral range becomes difficult, and an upward drift in the cytoplasmic pH can occur. If this cytoplasmic pH rise remains unchecked, it ultimately kills the cell/organism. Thus, thriving in high pH environment requires an effective way of maintaining the intracellular pH close to neutrality and ability to withstand some degree of alkalinization. This process of maintaining pH within physiologically favorable range regardless of the extracellular environment is known as pH homeostasis.

In addition to pH homeostasis, at least non-photosynthetic aerobic prokaryotic life forms face another daunting task in high pH environment, bioenergetics. Living organisms require energy to perform the phenomena of life such as growth, reproduction, structure maintenance, movement, etc. Moreover, cells maintain order against chaos/randomness with expenditure of energy. If there is no energy that a cell uses to maintain order, chaos reign, and it loses viability. Thus, life-sustaining metabolic processes enable organisms to generate and store energy. In this regard, ATP is the most vital molecule which lays at the center of cellular bioenergetics. It is known as the energy currency of life which can store and shuttle chemical energy within cells. This energy-rich molecule can be produced by various cellular processes, most typically by F_1F_0-ATP synthase-mediated oxidative phosphorylation

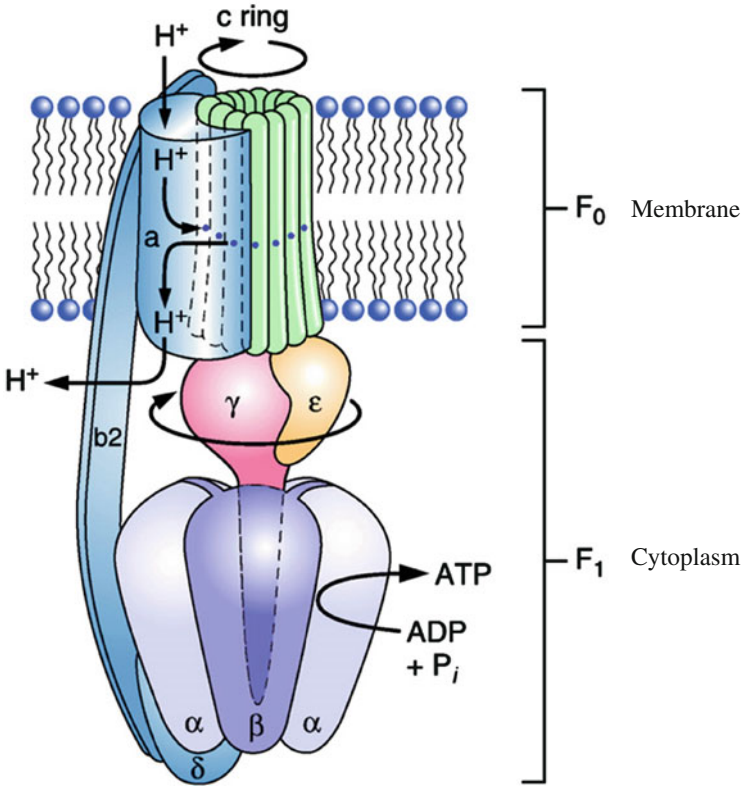


Fig. 1 A diagram representation of F_0F_1-ATP synthase with its subunits. Adopted from Hicks et al. [27] and reprinted with kind permission from Elsevier

(OXPHOS) or via photophosphorylation. However, some organisms in anoxic condition [26] and few aerobic cells such as matured red blood cells synthesize ATP through substrate level phosphorylation.

The F_1F_0-ATP synthase is a multi-subunit two-domain membrane-bound enzyme. The intracellular domain which is known as F_1 domain is hydrophilic, and the F_0 domain is hydrophobic, and most of it is embedded in the membrane. The F_0 domain has a functional center which captures protons from the bulk (extracellular) environment and channels them down to the cytoplasm (Fig. 1). This proton (H^+) translocation induces conformational changes in F_1 domain which in turn drives the synthesis of ATP from inorganic phosphate (P_i) and ADP. Neutralophiles and acidophiles have lower concentration of protons in their cytoplasm than their extracellular environment. Thus, the downhill movement of the ions across the membrane drives the ATP synthesis as explained by the chemiosmotic theory [28]. However, the efficiency of this proton motive force (pmf) diminishes in alkaline environments due to reversed proton gradient, which is higher in the cytoplasm than the extracellular environment. Thus, to flourish in alkaline

environments, it is necessary to evolve mechanisms that effectively generate energy carriers under this thermodynamically challenging condition.

High pH is known to degrade biological entities by disrupting molecular bonds between atoms in biomolecules. Thus, one of the challenges to thrive in alkaline environments is the maintenance of the cell structural integrity at high pH. Proteins, lipids, carbohydrates, and aromatic structures such as lignin which are the important structural components of different organisms are susceptible to alkaline conditions. For instance, keratin, a tough structural protein of hair, feather, horn, and nails decomposes at high pH [29]. This type of alkaline-mediated proteolysis of cellular proteins and peptides is sometimes referred to as liquefactive denaturation [23]. Studies on the degradation of plant biomass in aquatic environments have also revealed that the decomposition rate is linearly related to pH [30]. At high pH the plant cell wall cementing substance, lignin, decomposes, and the solubility of the hemicellulose fraction increases which results in degradation of plant biomass. Such susceptibility of structural biomolecules to high pH has led to the emergence of applications that use alkaline treatments to break down biological materials in various processes. Alkaline treatments are used in pulp and paper industry to break down the lignin and hemicellulose fractions of the plant biomass (Kraft pulping process), in molecular biology to digest bacterial cell wall during DNA (e.g., plasmid) extraction, in leather tanning to dehair skin and hides, in waste management to decompose keratin (e.g., feather) waste, etc. Lipids, which are important structural components of cells such as membranes, are also labile to high pH. The degradation process of lipids is known as saponification [23]. Degradation of structural components such as the cell membrane is lethal as it compromises the integrity of cells. In fact, alkaline solutions have long known for their disinfectant properties and are used as antimicrobial agents [31–34]. Alkaline solutions are also widely used as cleaning agents due to their ability of removing (by degrading and solubilizing) organic matter such as protein, lipid, and nucleic acids [32]. Thus, organisms that colonize alkaline habitats must evolve mechanisms which protect the cell integrity from the adverse effect of the extreme pH.

The challenges of life at high pH habitats are not restricted only to cell associated structures, but it also involves the structural and functional integrity of extracellular products. Cells produce and secrete various biomolecules to the extracellular environment to perform different tasks such as exopolysaccharides for protection, adhesion and biofilm formation, chemical signal molecules for cell-to-cell communication, enzymes for nutrient acquiring and recycling, bioactive compounds for defense and competition, etc. [35–40]. The efficiency of these biomolecules influences the success of the organism in colonizing a habitat. To fulfill the desired tasks, these products should be operationally stable in the habitat condition. Thus, the success of colonizing high pH habitats, at least partly, depends on the operational stability of the extracellular products. For instance, extracellular enzymes are very important to acquire nutrients by breaking down polymeric substrates to smaller pieces that can be transported to the cytoplasm across the cell envelope. But enzymes are optimally active and stable within a certain range of pH and can be denatured and cease to function outside this range. Since the extracellular biomolecules of

neutralophiles are evolved to function often around neutrality, the high pH of alkaline habitats can disrupt their activity and stability, which potentially starve the cell to death. Thus, one of the challenges in colonizing high pH habitats is to have extracellular products that are efficient and operationally stable at elevated pH.

Another important high pH environment challenge is related to nutrient availability. The bioavailability of some nutrients can be affected by the pH of the environment. Some nutrients become less available at high pH. However, since the genesis and chemistry of alkaline environments are different, the scarcity of nutrients could also vary from habitat to habitat. The scarcity problem in some soda lakes is mentioned here as an example. Nutrients such as P, Ca, Mg, Fe, etc. are less available in soda lakes [41, 42]. These substances either due to geological reasons or reactions with other constituents of the lakes form insoluble precipitates which severely diminish the bioavailability. However, these nutrients are important for normal metabolic process. For instance, several enzymes require metal ions such as Ca, Mg, and Fe for activity and/or stability [43]. Thus, the scarcity of these metals can severely impair the function of the enzymes and adversely affect the metabolic processes. The malfunctioning of metabolic processes can compromise cellular activities which can lead to survival deterioration. Thus, colonization of high pH habitats requires adaptive solutions to evade challenges related to poor nutrient availability.

What has been described in the above are some of the important challenges of life in alkaline environments, challenges that neutralophiles can face in high pH habitats. Probably, solving these challenges was the key adaptive evolution of alkaliphiles which allowed them to flourish in high pH environments. Below, the adaptive strategies deployed by alkaliphiles to circumvent these grand challenges of alkaline environments are discussed.

3 Adaptation of Alkaliphiles: Circumventing the Challenges of Alkalinity

Often, organisms colonizing extreme habitats have unique features that allow them not only to survive but also thrive in it. As discussed above, alkaline environments are bundled with tough challenges, and yet it is home for richly diverse group of organisms. The ability of lavishly growing at punishingly high pH in which neutralophiles cannot even survive for a while indicates the unique adaptive strategies deployed by alkaliphiles. Unraveling the secret of high pH adaptation of alkaliphiles has been the subject of several studies for decades. These studies have contributed to our knowledge of high pH adaptations of organisms. An effort is made here to summarize the important findings that depict the remarkable high pH adaptation mechanisms of alkaliphiles.

3.1 pH Homeostasis

The intracellular pH homeostasis is a vital adaptation shared by all alkaliphiles. Since Garland indicated that alkaliphiles maintain lower cytoplasmic pH than its external environment [44], the difference between the intracellular and extracellular pH values of alkaliphiles has been studied (Table 1). The results of these studies confirmed the established notion that alkaliphiles have an impressive capacity of maintaining low cytoplasmic pH while thriving in high pH environment. This pH homeostasis can uphold a difference of more than 2 pH units between the cytoplasm and extracellular environment [46–48, 50, 51]. Alkaliphiles use a variety of adaptive strategies to achieve this remarkable pH homeostasis (Fig. 2). Some of the most important adaptive mechanisms that play significant role in pH homeostasis such as acquiring H^+ from extracellular environment, reducing H^+ leakage from the cytoplasm, production of organic acid, and deterring the diffusion of OH^- from the extracellular environment are discussed below.

Table 1 The intracellular and extracellular pH values of some alkaliphiles

Organism	Extracellular pH	Intracellular pH	References
Exiguobacterium aurantiacum	9.4	8.4	McLaggan et al. [45]
Clostridium paradoxum	9.8	8.5	Cook et al. [46]
B. pseudofirmus OF4	10.5	8.3	Guffanti and Hicks [47]
B. pseudofirmus OF4	10.8	8.3	Sturr et al. [48]
B. pseudofirmus OF4	11.2	8.9	Sturr et al. [48]
B. pseudofirmus OF4	11.4	9.6	Sturr et al. [48]
B. halodurans C-125	9	7.9	Aono et al. [49]
B. halodurans C-125	9.5	8.1	Aono et al. [49]
B. halodurans C-125	10	8.2	Aono et al. [49]
B. halodurans C-125	10.5	8.4	Aono et al. [49]
B. alcalophilus	9	7.6	Guffanti and Hicks [47]
B. alcalophilus	10	8.6	Guffanti and Hicks [47]
B. alcalophilus	11	9.2	Guffanti and Hicks [47]

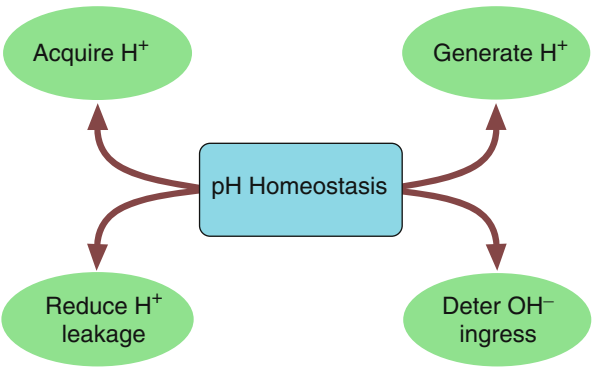


Fig. 2 The strategies alkaliphiles use to maintain low intracellular pH

3.1.1 High Level of Monovalent Cation/Proton Antiporters

Alkaliphiles tend to keep their cytoplasmic pH close to neutral range. To do this alkaliphilic cells maintain relatively high concentration of H^+ in their cytoplasm. One way of achieving this is by translocating H^+ from the extracellular environment into the cell and tightly controlling it. But there are two challenges to do this: the scarcity of H^+ in the extracellular environment and that the translocation and control are against concentration gradient. Alkaliphiles evolved mechanisms that solve these challenges. The monovalent cation/proton antiporters which exchange the intracellular cations such as Na^+ and Li^+ for the extracellular H^+ are believed to be the most important mechanism that alkaliphiles depend on for intracellular pH homeostasis [21, 52–59]. Based on the Transporter Classification Database (TCDB; http://www.tcdb.org), these antiporters are diverse and belong to two superfamilies. The cation/proton antiporters (CPA) superfamily which consists of five families including family CPA1 and CPA2 and the Na^+ transporting Mrp superfamily that comprises three families including family CPA3 which is among the most vital H^+ translocating antiporters of alkaliphiles [22, 60]. In addition to the families that belong to the two superfamilies, the Nha families, NhaA, NhaB, NhaC, and NhaD [61] are also involved in the homeostasis process [62].

Among the monovalent cation/proton antiporters, Na^+/H^+ antiporters which exchange cytoplasmic Na^+ for extracellular H^+ seem to be very crucial for pH homeostasis in alkaliphiles [21, 22, 54, 55]. Moreover, these antiporters are also used for Na^+ and volume homeostasis as well, like what it does in eukaryotic cells and their organelles [58, 63–66]. These antiporters avoid the accumulation of Na^+ to toxic level, while it maintains relatively higher H^+ concentration in the cytoplasm [21, 67]. The Na^+/H^+ antiporters are secondary active transporters which use the transmembrane electrical potential ($\Delta\psi$) generated by primary ion pumps such as the respiratory complexes [27] to efflux intracellular Na^+ [21, 54, 55, 68, 69]. In alkaliphiles, the monovalent cation/proton antiporter-mediated pH homeostasis is primarily specific for Na^+ but also accommodates Li^+ efflux. On the other hand, unlike alkaliphiles, neutralophiles use not only $Na^+(Li^+)/H^+$ antiporters but also K^+/H^+ antiporters [21, 22]. The specificity of the alkaliphiles monovalent cation/proton antiporters system to Na^+ is believed to avoid severe depletion of cytoplasmic K^+ that can potentially compromise some cytoplasmic processes [21] and enhances the cytotoxicity of Na^+ [21, 70, 71]. The other possibility might be that most of the studied alkaliphiles are adapted to habitats such as soda lakes with high level of Na^+; hence, it is ideal for such organisms to evolve a system that relies on the ample resource (Na^+).

Comparative analysis of genes encoding CPAs in genomes of alkaliphiles and neutralophiles revealed that there is no significant difference in the number of the genes between alkaliphiles and neutralophiles [54, 55]. However, the aggregate level of the Na^+/H^+ antiporter is much higher in alkaliphiles than in neutralophiles [21, 52, 53, 72]. This may be due to the greater burden of pH homeostasis at higher

extracellular pH values and the sole dependence of alkaliphiles on Na^+/H^+ antiporters unlike neutralophiles which also involve K^+/H^+ antiporters [21, 52].

Several studies have shown the vital importance of Na^+/H^+ antiporters in adapting high pH environments [73, 74]. This is clearly shown in the growth profile of neutralophiles with and without Na^+/H^+ antiporters. The growth of neutralophiles that lack functioning Na^+/H^+ antiporters is limited around neutrality, pH 6.3–7.7 [75, 76]. In these organisms, the rise in the environmental pH to alkaline range is accompanied by rapid alkalinization due to inefficient intracellular pH homeostasis, which hampers growth. But neutralophiles equipped with functional Na^+/H^+ antiporters can maintain their cytoplasmic pH around 7.5 and grow in environments with pH values of up to 8.5. Often, when the external pH value exceeds 8.5, neutralophiles start to grow slowly, and when the pH is over 9, their growth becomes severely impaired [21, 22]. At higher alkalinity, the neutralophiles fail to maintain their cytoplasmic pH below 8. Moreover, the physiology of these organisms is not adapted to function at high pH, and this results in a dramatic drop in growth rate as the external pH values increase. However, alkaliphiles which are endowed with high level of Na^+/H^+ antiporters can maintain their cytoplasmic pH at 7.5 even when growing in an environment of pH 9.5. Some of these alkaliphiles can grow at much higher pH, and in those conditions, the intracellular pH is expected to become way above the pH values at which neutralophiles can survive and/or grow.

Monovalent cation/proton antiporters can be products of a single gene or hetero-oligomers assembled from multiple gene products. The hetero-oligomer monovalent cation antiporters are known as Mrp [77]. Mrps are widely distributed among bacteria and archaea [77, 78] and involved in several physiological processes. In archaea, Mrps are used in the conversion of energy involved in metabolism and hydrogen production, while in bacteria, it is involved in nitrogen fixation, bile salt tolerance, arsenic oxidation, and pathogenesis [78]. In alkaliphiles it is believed that it plays a dominant role in pH homeostasis and sodium tolerance [21, 22, 60, 77, 79, 80]. The Mrp operon has six or seven genes which encode hydrophobic proteins required for optimal activity [79]. Structural analysis predicted that these antiporters have large surface which can facilitate the capturing of proton and funneling it into the antiporter [21, 22, 80].

The Na^+/H^+ antiporter systems exchange cytoplasmic Na^+ for extracellular H^+. However, the Na^+ leaving the cytoplasm must be replenished so that the antiporter-dependent pH homeostasis system works effectively; this is especially important when the extracellular Na^+ concentration is low [21, 22, 81–85]. Alkaliphiles use Na^+ solute symporters and Na^+-coupled motility channels known as MotPS for reentry of Na^+ to the cytoplasm [21, 78, 86, 87, 88]. Moreover, Na^+ uptake by alkaliphiles is accomplished through voltage-gated Na^+ channels known as NaChBac and NaVBP [86, 89–93]. The major Na^+ and H^+ entry and exit pathways are shown in Fig. 3.

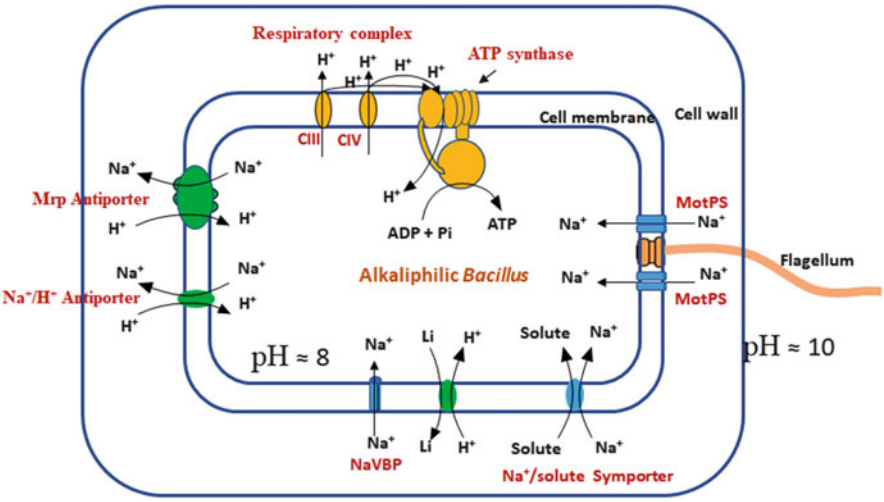


Fig. 3 A diagram of alkaliphilic *Bacillus* cell depicting some of the most important cellular entry and exit paths of H^+ and Na^+

3.1.2 Effective Proton Capturing by ATP Synthase and Inhibition of ATPase Activity

The capture and translocation of H^+ by F_1F_0-ATP synthases of cells adapted to neutral or acidic habitats are energetically favored as the H^+ concentration outside the cell is higher than that of the cytoplasm. On the other hand, alkaliphiles do not have the luxury of high H^+ concentration in their environment. H^+ is scarce in alkaline habitats, and hence, organisms adapted in such environments require an efficient system for capturing and translocating H^+. The analysis of the *atp* operon, the cluster of genes coding for F_1F_0-ATP synthase, of alkaliphilic *Bacillus* bacteria revealed a conserved lysine residue at position 180 (based on that of *B. pseudofirmus* OF4 numbering) of *a*-subunit [94] which exists only in alkaliphilic *Bacillus* gene sequences [95]. This conserved lysine in a thermoalkaliphilic strain, *Bacillus* sp. TA2.A1, was mutated to His, Arg, and Gly. Analyses of the ATP synthases carrying these mutations have shown that L180 is a specific adaptation of alkaliphiles that facilitate H^+ capture at high pH [94]. A broader mutational study on the ATP synthase of *a*-subunit of *B. pseudofirmus* OF4 indicated that the ATP synthase of alkaliphiles evolved to efficiently capture, translocate to the synthase core, and retain H^+ in the cytoplasm [95]. Therefore, ATP synthase is believed contributing to alkaliphiles pH homeostasis.

The ATP synthases of alkaliphiles have another remarkable contribution to the pH homeostasis. The F_1 domain of non-alkaliphiles ATP synthases is known not only synthesizing ATP but also hydrolyzing (ATPase activity) it. As shown in Fig. 4a, the hydrolysis of ATP drives the ATP synthase *c*-ring reverse rotation which pumps out proton to the extracellular environment. However, the ATP

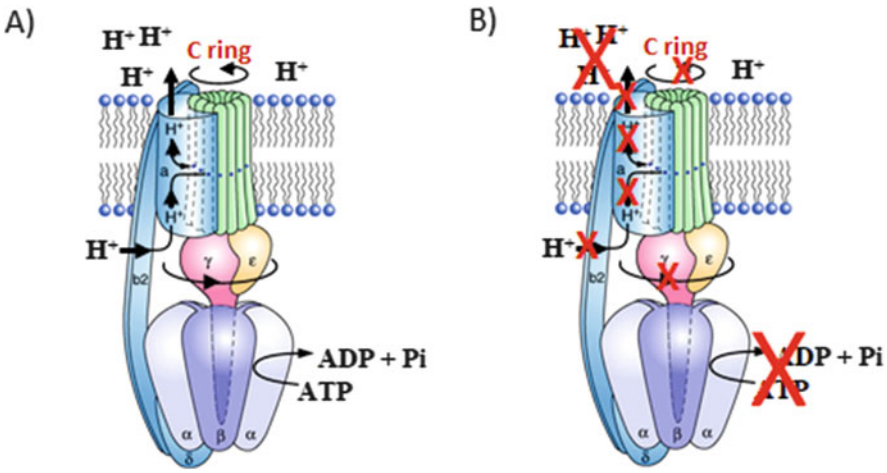


Fig. 4 The ATPase activity that pumps out H^+ in non-alkaliphiles (**a**) is absent in alkaliphiles, and cells retain H^+ (**b**)

synthase of aerobic alkaliphiles does not have the ATPase activity (Fig. 4b) and cannot translocate H^+ out of the cell [96–99], and this helps to retain the H^+ required for lowering the cytoplasmic pH. The importance of this adaptation is reflected on the *B. pseudofirmus* OF4 mutants K180H and K180G. These mutants exhibit high level of ATPase activity which compromised non-fermentative growth at pH 10.5 [95]. The adaptation features of the ATP synthase and the respiratory system that contributes to pH homeostasis in addition to ATP generation are discussed below in Sects. 3.2.1 and 3.2.2.

3.1.3 Acid Production

Extracellular pH is known to affect metabolic processes, and cells produce acids or alkali to offset the change in medium pH [100, 101]. For example, when *Escherichia coli* grows in high pH medium, it shifts its metabolism toward acid production [102]. The acid production process is facilitated by upregulating the deaminase, ATP synthase, and cytochrome *d* oxidoreductase activities. Like *E. coli*, many other organisms swing to acid production upon rise in the pH of the medium. Similarly, numerous alkaliphiles are known to produce acid that decreases the pH of the culture significantly [56, 103–105]. Alkaliphiles produce metabolic acid through sugar fermentation and amino acid deaminases. The acid production contributes to the pH homeostasis primarily by increasing the cytoplasmic H^+ concentration. Moreover, the acid production, in addition to preventing cytoplasmic alkalinization, can increase the availability of H^+ in the vicinity of the cell, and this can potentially contribute to alleviate the burden of capturing and translocating H^+ to cytoplasm.

3.1.4 Anion (OH^-) Deterring Cell Surface

The cell envelope, which comprises the cell membrane, cell wall, and associated cell surface depositions, is a barrier that prevents the cytoplasm from the direct effect of its environment. If cells must maintain a near-neutral cytoplasmic pH, the OH^- of the alkaline environment should not freely ingress into the cell. The cell surface of alkaliphiles contains acidic residues [106] that potentially repel the anions and prevent the rise in the cytoplasmic pH. Moreover, the anionic cell surface can capture H^+, especially H^+ pumped out of the cell such as by the respiratory complex. This ability of trapping H^+ might form a kind of H^+ reserve close to the membrane surface which hypothetically reduces the difficulty of capturing H^+ from the bulk alkaline environment. The H^+ trapped in the cell envelope can be retrieved such as by the Na^+/H^+ antiporters and ATP synthases and translocated to the cytoplasm, which contributes to the pH homeostasis. These trapped protons may also neutralize OH^- migrating to the cell membrane. Since the contribution of the cell envelope to high pH adaptation is significant, it is relevant to discuss it in detail.

3.2 Protective Cell Envelope

The cell envelope, which consists of cell membrane and cell wall (plus the outer membrane in Gram-negative bacteria), delineates the cell from its environment. This structure plays a vital role in the cell survival primarily by maintaining its content, while it allows controlled exchange of materials between the cell and its environment. Cells such as bacteria interact with their extracellular environment through their cell envelope. Thus, adaptations of such kind of organisms to their habitats often involve their cell envelope. There have been evidences that the cell envelopes of alkaliphiles are part of the high pH adaptation assemblies. Some of the most important studies that substantiate how cell envelope contributes to high pH adaptation of alkaliphiles are discussed below under the different components of the cell envelope.

3.2.1 Cell Wall: Secondary Cell Wall Polymers

Among alkaliphiles, members of the genus *Bacillus* are the most studied. Like other Gram-positive bacteria, the cell wall of *Bacillus* cells contains different polysaccharides. Most of these polysaccharides are covalently bonded to the peptidoglycan (PG), which is the prominent cell wall scaffolding structure. Based on structural properties, the cell wall polysaccharides of these organisms are categorized into three groups: (1) teichuronic acids [107, 108], (2) teichoic acids [109, 110], and (3) other polysaccharides which cannot be characteristically assigned to the other two groups [111, 112]. These polysaccharides have been considered to play

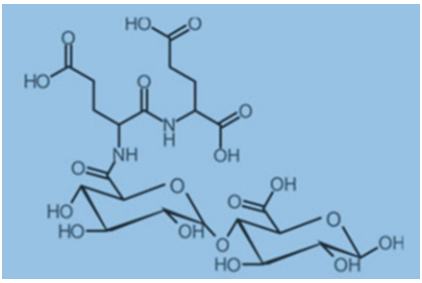


Fig. 5 A teichuronopeptide unit that forms the major polymeric cell wall component of alkaliphilic *Bacillus* strains

secondary role in cell wall function and hence referred to as "secondary" cell wall polymers (SCWPs). SCWP analysis of one of the most studied alkaliphiles, *B. halodurans* C-125, revealed that it is rich in negatively charged residues such as aspartic acid, galacturonic acid, glutamic acid, and phosphoric acid [113, 114]. The negatively charged residues glutamic and glucuronic acids form the major cell wall component of this alkaliphilic *Bacillus* strain, teichuronopeptide (TUP) (Fig. 5). This highly negatively charged cell wall structure interacts with cations such as H^+ [115]. The H^+ trapping by the alkaliphilic bacterial cell wall can delay the rapid loss of H^+ from the cell surface by equilibration effect of the alkaline bulk phase (the environment), and this significantly contributes to the pH homeostasis and bioenergetics of alkaliphiles. Moreover, the cell walls of alkaliphiles shield the cells from the detrimental effect of the high pH environment.

The negatively charged residues of SCWPs such as teichuronopeptide (TUP), teichuronic acid (TUA), and acidic amino acid chains in the cell envelope together with the trapped cations around the cell surface serve as barrier to OH^- [81, 83]. The anions of the SCWPs repel OH^-, while the trapped H^+ neutralizes OH^- escaping into the cell wall. Hence, SCWPs are expected to play a prominent cell protection role in high pH adaptation. Indeed, several studies confirmed its remarkable contribution to high pH adaptation. This includes (1) quantification of SCWPs revealed that cells produce more TUA and TUP when grown at alkaline than at neutral condition [106, 116], (2) removal of the cell wall of *B. halodurans* C-125 cells resulted in protoplasts that are not stable in alkaline medium [81, 83], and (3) *B. halodurans* C-125 mutants with disrupted TUP and TUA production poorly grow at pH 10.5 [81–84] and lose their alkaliphilicity [82, 84, 106, 117]. Moreover, electron microscopy analysis of an alkaliphilic *Bacillus* cell wall has shown that the thickness increases with increasing alkalinity of the growth medium [106]. The increase in the peptidoglycan and SCWPs was proportional [106], and hence, it can be speculated that at higher pH, the cells need a denser negative layer that ensures protection from the effect of high pH, and this is partly achieved by increasing the thickness of the cell wall.

3.2.2 Lipopolysaccharides

The nature of the Gram-negative and Gram-positive bacteria cell surfaces is different. Gram-negative cells lack SCWPs and do not seem to benefit from the high pH adaptation role of these structures. However, it seems that the outer membrane of Gram-negative bacteria plays more of the protection role. This membrane of Gram-negative bacteria contains lipopolysaccharides (LPS) which are exposed to the outer surface of the cells. Although, little is done on its involvement in high pH adaptation, it seems that it may function the same way as SCWPs of Gram-positive alkaliphiles. Indeed, structural analysis of the haloalkaliphilic strain, *Halomonas pantelleriensis* lipopolysaccharide *O*-chain revealed that it has a unique repeating unit, 4-*O*-((*S*)-1-carboxyethyl)-D-GlcA residue [118]. This repeating unit contains carboxyl groups which make the polymer highly negatively charged. Further, chemical, NMR, and MS study results show that the LPS of this haloalkaliphilic strain are very rich in carboxylate groups [118]. A similar observation of highly carboxylated LPS is reported from another Gram-negative bacteria, *H. magadiensis*. A protective buffering effect of this negatively charged LPS has been suggested [119], which is expected to be similar to that of SCWPs, repelling the OH^- by the anions of the LPS and neutralization by the trapped cations.

3.2.3 S-Layer Proteins

The cell envelopes of Gram-positive alkaliphiles are also known to have a special proteinaceous layer known as cell surface layer (S-layer) [120, 121]. S-layers are composed of identical (glyco) protein structures that form lattices on the bacterial cell surfaces. This layer is sometimes referred to as "nonclassical" SCWPs; however, based on compositional and structural analysis, Schäffer and Messner [122] suggested that it belongs to the third cell wall group of Araki and Ito [111]. Alkaliphilic cells express a range of S-layer proteins. For instance, 17 S-layer homology (SLH) domain-containing proteins, including S-layer protein A (SlpA), are identified in the genome sequence of *B. pseudofirmus* OF4 [123]. The contribution of S-layer to high pH adaptation has been assessed through mutational studies using *B. pseudofirmus* OF4 cells which produce SlpA both at neutral and alkaline conditions. Mutants that lack SlpA grow more slowly at pH 11 than the wild-type cells, especially when the Na^+ concentration was low [85]. On the other hand, the wild-type cells expressing SlpA grow slower at neutral condition than at high pH [85]. Although it seems that the expression of SlpA at neutral pH reduces growth efficiency, those facultative organisms expressing SlpA in the neutral range could benefit if sudden alkalinization happens. The results of the mutational studies indicate that the presence of SlpA on the cell surface has a high pH adaptive advantage. Like other cell wall proteins from alkaliphiles, SlpA has low (4.36) isoelectric point (pI) which is mainly due to its fewer arginine and lysine content. Like TUA and TUP, the relatively abundant negatively charged residues of SlpA favor H^+ accumulation and deter OH^- penetration [54, 55, 85, 124, 125].

Despite the fact that S-layer proteins exist in Gram-positive and Gram-negative bacteria [126], studies made so far on high pH adaptation role of S-layer proteins have been restricted to Gram-positive bacteria. Hence, relatively, little is known about the contribution of Gram-negative S-layer proteins to high pH adaptations. Scanning electron microscopy analysis of the surface of a Gram-negative bacterium, *Pseudomonas alcaliphila*, revealed that cells grown at pH 10 have rougher surface than those grown at pH 7 [127]. This might show the possibility that Gram-negative strains also make some surface depositions (S-layer proteins) to thrive in high pH environments. However, this needs specific experimental evidences. Further studies on other S-layer proteins of Gram-positive bacteria and probably other cell surface deposited proteins of alkaliphilic Gram-negative and Gram-positive bacteria may improve our understanding of these interesting proteins contribution to high pH adaptation.

3.2.4 Cell Membrane

The other component of the cell envelope that contributes to high pH adaptation is the cell membrane. The contribution of the outer membrane of Gram-negative bacteria is briefly discussed above in Sect. 3.2.1. In addition to serving as an anchor to negatively charged polymers, the cell membrane of alkaliphiles has shown a stunning difference in composition when compared to that of non-alkaliphiles. Even a difference is noted between the membrane of obligate and facultative alkaliphiles. For instance, a comparative analysis of the membrane fatty acid composition revealed that the unsaturated fatty acids account for 20% and up to 3% of the total phospholipid fatty acids of the obligate and facultative alkaliphilic *Bacillus* strains, respectively, when grown in alkaline condition [128]. Similarly, the membrane composition of an organism can vary with the pH of the growth medium. The membrane of facultative alkaliphiles grown in pH 7.5 medium was almost free of unsaturated fatty acids, while the unsaturated fatty acid content rises to about 3% when these cells were grown in pH 10.5 medium [128]. In another study, *Yersinia enterocolitica* cells were grown at pH 9 and pH 5, and the analysis of the fatty acid content of the cells revealed that the unsaturated fatty acid content was higher when it was grown at pH 9 and significantly decreased for cells grown at pH 5 [129]. A similar observation of high percentage of unsaturated membrane lipid has been reported for different alkaliphiles [130].

The rise in the content of unsaturated fatty acid seems correlated to the fatty acid desaturase (an enzyme that forms carbon double bonds in fatty acids) activity (Fig. 6). The membrane of obligate alkaliphiles has very high fatty acid desaturase activity, while the membranes of facultative strains do not have detectable activity [131]. As shown in Fig. 6, desaturase mediated reaction consumes oxygen. Aono et al. [132] have shown that the oxygen uptake rate of membrane vesicles of *Bacillus lentus* C-125 (which is later named *B. halodurans* C-125) grown at pH 9.9 is more than double than that of neutral grown. Part of this oxygen consumption may be related to the formation of unsaturated fatty acid bonds. However, this is yet to be experimentally proven. In general, very little is known about the role of desaturase in

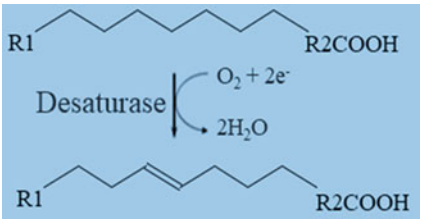


Fig. 6 Conversion of saturated fatty acid to unsaturated form by desaturase

high pH adaptation. Similarly, there are some substances that are known to be correlated to alkaliphiles or to growth at alkaline condition, but their high pH adaptation role is not clear. Bis(monoacylglycero) phosphate (BMB) could be an example. BMB exists in most alkaliphiles and known to be absent at least in many nuteralophiles [128, 133, 134]. But it is not clear if it contributes to high pH adaptation.

A difference is also observed in the membrane content of branched fatty acids. About 90% and 66–76% of the fatty acids in the phospholipids of obligate and facultative alkaliphiles, respectively, were found to be branched [128]. This branching may help in pH homeostasis by reducing H^+ leakage. It has been known that branched fatty acids are common in membranes maintaining H^+ gradient [135]. Moreover, studies revealed that the fatty acid chain length is tending to be shorter in facultative alkaliphiles cell membrane than those from obligate alkaliphiles. This may be related to inhibition of H^+ leakage. In the model proposed by Haines [136], it is indicated that branched fatty acids at the center of the bilayer are involved in preventing H^+ leakage. Thus, the longer the fatty acid chains, the better the chance it reaches at the center of the membrane bilayer. This probably suggests that the alkaliphilic membrane fatty acids which tend to be branched and longer plays a role in the pH homeostasis of these fascinating group of organisms.

Another interesting observation was made on the Gram-negative bacterium *Pseudomonas alcaliphila* fatty acid content which shows a difference in the amount of *cis*- and *trans*-unsaturated fatty acid with varying growth pH. When the bacterium is grown at high pH, the concentration of the *trans*-unsaturated fatty acid increases, while the amount of the *cis*-unsaturated fatty acid decreases proportionally [137]. But how this can contribute to the high pH adaptation is not clear. On the other hand, this phenomenon of high concentration of *trans*-unsaturated membrane fatty acids has been detected in bacteria exposed to environmental stresses including acidity [138–141]. Thus, one can speculate that its contribution to coping stress (including high pH) might be due to the better stability of the *trans* than the *cis* form.

Analysis of the content of alkaliphiles' membranes has also shown that it is rich in squalene and cardiolipin [128, 130, 142], which contain unsaturated bonds. In fact, one of the unique features of alkaliphiles membrane is the presence of high amount of isoprenes (including squalene) which accounts up to 40 mol% of the membrane lipids [128, 135]. The squalene and its derivatives account for about 10–11 mol% of the alkaliphilic *Bacillus* spp. total membrane lipid [128, 142]. Being apolar, this substance may occur inside the lipid bilayer and hence can serve as barrier and

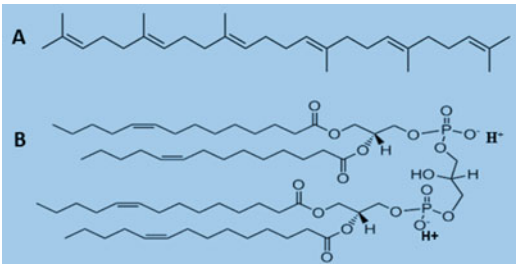


Fig. 7 Squalene (**a**) and cardiolipin (**b**), the two lipids abundant in the membrane of alkaliphiles

decrease membrane permeability for ions [128, 143]. It is interesting that these hydrocarbons are predominantly oriented parallel to the membrane plane [135], which probably enhances the barrier effect and minimizes H^+ leakage [136] and OH^- ingress. Thus, squalene seems be involved in the pH homeostasis of alkaliphiles. The other substance that exists at high concentration in alkaliphilic bacteria is cardiolipin [128], which is unsaturated anionic phospholipid. Cardiolipin has four unsaturated fatty acid chains an anionic structure which can trap H^+ [144]. Thus, like the other negatively charged residues of the cell envelope components such as SCWPs, it can trap cations and repel anions and hence play an important role in high pH adaptation. The structures of squalene and cardiolipin are shown in Fig. 7.

The composition, including its high content of unsaturated fatty acids, branched fatty acids, *trans*-unsaturated fatty acid, cardiolipin, and squalene, makes the membranes of alkaliphiles to function optimally at or above pH 9 [130, 131, 142, 143]. However, the membrane integrity of these alkaliphiles (especially that of obligate alkaliphiles) is compromised around neutral condition; it maintains low electrochemical ion gradient [145], becomes leaky, and tends to lyse [146]. This compromise can be one of the reasons why obligate alkaliphiles fail to grow at near-neutral pH while facultative alkaliphiles are able to grow well [147]. Thus, it is obvious that the cell membrane of alkaliphiles evolved adaptations for high pH environment. Among the membrane adaptations, the tendency of having more unsaturated fatty acid seems to be the most widely reported. However, so far, there is neither experimental nor theoretical explanation on how the unsaturated fatty acids contribute to high pH adaptation. Here, an attempt is made to propose an explanation how the unsaturated membrane lipid is involved in high pH adaptation.

The membranes of alkaliphiles are known to contain many proteins. Although there is no available information, at the time of writing, regarding the protein content difference among cells grown at neutral and alkaline conditions, one can speculate that there are more proteins bound to the membrane at high pH than at neutral condition. This is because the level of expression of proteins such as ATP synthase, cytochromes, antiporters, and other membrane proteins such as enzymes, etc. is high when alkaliphiles grow at elevated pH [21, 131, 148]. Moreover, the rate of denaturation due to the extreme pH condition is expected to be higher; hence, to compensate this, the synthesis of membrane-bound proteins could be enhanced at higher pH. The rise in the amount of proteins together with the enhanced level of

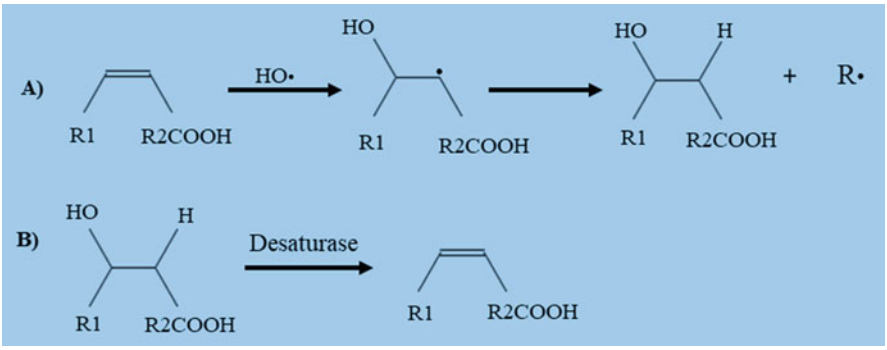


Fig. 8 The reaction of the hydroxy radical with C=C bond of the unsaturated fatty acid that results in saturation of the bond (**a**) and restoration of C=C bond by desaturase (**b**)

fatty acids and hydrocarbons can decrease the "free" volume within the alkaliphiles membrane. This molecular crowding can favor lipid-lipid interaction that may result in rigidity. Thus, it may be important to increase the membrane fluidity by increasing the unsaturated fatty acid content, and this is expected to alleviate the potential problems emanating due to membrane rigidity.

The other explanation for high amount of unsaturated fatty acids may be related to scavenging OH^-. The free radical OH^- is known to react with fatty acids or other hydrocarbon chains such as squalene in two alternative reaction routes. In one of the routes, H^+ is abstracted by OH^- from unsaturated bonds of lipids/hydrocarbons which are accompanied by the release of water. In the alternative route, the OH^- is added to the unsaturated bonds (Fig. 8). However, the addition of OH^- to the C=C is not only the dominant but also the fastest reaction route [149]. Thus, OH^- which somehow escapes through the outer barriers such as the cell wall and traversing the membrane will be captured by the double bonds of the unsaturated fatty acids (including cardiolipin's), squalene, and its derivatives in the same way antioxidants scavenge radicals. Thus, the presence of more unsaturated fatty acids in alkaliphiles cell membrane helps to capture efficiently the OH^- that traverses the membrane. The C=C readily reacts with OH^- and becomes saturated. However, one can speculate that the desaturase may act on the saturated fatty acid to unsaturated form, and the cycle continues (Fig. 8). However, this should be supported experimentally.

Thus, the double bonds between carbon atoms of unsaturated fatty acid can be involved in high pH adaptation through:

1. Improving membrane fluidity and facilitating material exchange.
2. The C=C bonds neutralize the OH^- traversing the membrane before it reaches the cytoplasm.

The phospholipid cardiolipin seems to have another important contribution to high pH adaptation, organization of membrane proteins, and facilitating ATP synthesis. Cardiolipin in mitochondria is known to facilitate the function of membrane-associated proteins, especially the formation of "supercomplex" proteins such as

those involved in shuttling of substances across the membrane and electron transport complexes [150–153]. As aforementioned, there is high presence of proteins in the membranes of alkaliphiles, and hence, one expects more cardiolipin at elevated pH to make these protein assortments assemble and function properly. Cardiolipin has a unique role in membranes involved in OXPHOS, aggregating the proteins involved in OXPHOS into a patch, and its headgroup serves as H^+ trap [144]. Since cardiolipin restricts pumped H^+ close to its headgroup domain, it possibly supplies H^+ to the ATP synthase [144]. Its close association to ATP synthase and respiratory complexes makes cardiolipin to play a unique role in the bioenergetics of alkaliphiles. As discussed below in Sect. 3.3.2, alkaliphiles pump out H^+ faster than non-alkaliphiles, and these protons need to be channeled to ATP synthase before it dissipates into the bulk phase. To this end, a microcircuit that facilitates the transfer of H^+ to ATP synthase has been proposed [27]. Based on its close association to cytochrome *c* oxidase and ATP synthase, and its unique role in patching these systems together, trapping H^+ and feeding it to ATP synthase, it seems that the microcircuit role is, at least partly, played by cardiolipin. Thus, it is not surprising that alkaliphiles have more cardiolipin in their membrane.

3.3 Bioenergetics

The pH homeostasis which effectively maintains a lower intracellular pH than that of the extracellular environment comes with bioenergetics challenge, difficulty of chemiosmotically driven ATP synthesis. Based on the chemiosmotic theory, cells generate ATP using pmf which is the sum of the transmembrane potential ($\Delta\Psi$) and the H^+ concentration gradient (ΔpH). In non-alkaliphiles, the relatively high concentration of H^+ in extracellular than in intracellular environment results in diffusion of H^+ to the cell, which is coupled to ATP synthesis by the ATP synthase. However, in alkaliphiles, this gradient is reversed, the intracellular H^+ concentration exceeds that of the extracellular, and hence, H^+ cannot diffuse to the cytoplasm. Although $\Delta\Psi$ increases at higher pH, it is not high enough to offset the chemiosmotically counterproductive pH gradient [20, 48, 50, 154]. Thus, it is obvious that the successful pH homeostasis raises problems concerning H^+-coupled OXPHOS-based ATP synthesis by prokaryotic alkaliphiles.

In photosynthetic alkaliphiles such as cyanobacteria, the ATP synthase is embedded in thylakoids which are suspended in the cytoplasm and hence not affected by the extracellular low H^+ concentration [155–157]. The pmf across the thylakoid membrane is higher than the pmf across the cytoplasmic membrane [158, 159]; thus, ATP can be produced chemiosmotically regardless of the high pH of their habitat. Probably, the same holds true for eukaryotic cells (organisms) that are adapted to high pH habitats and produce ATP using ATP synthase which is partly embedded in the inner membrane of mitochondria. However, there is no available information on how eukaryotic organisms thriving in alkaline environments generate ATP through OXPHOS.

Contrary to the thermodynamically unfavored condition, ATP synthesis by prokaryotic alkaliphiles is known to be more efficient at alkaline condition than in the near neutral range [47, 50, 51, 160]. Moreover, often aerobic alkaliphiles have higher growth rate and yield than neutralophiles [48, 161], which specifies that alkaliphiles are efficient in producing ATP. Studies have also shown that alkaliphiles, with few exceptions of anaerobes, depend on H^+-coupled ATP synthase to produce ATP and satisfy their energy requirement [20]. Thus, the fact that alkaliphiles do not exhibit energy shortage despite the thermodynamic hurdle of generating ATP in alkaline habitats marks that alkaliphiles have devised unique adaptive strategies to efficiently generate energy carriers at elevated pH. A number of experimentally supported and speculative adaptations have been forwarded to substantiate how prokaryotic alkaliphiles accomplish H^+-coupled ATP synthesis under the unfavorable low pmf. These adaptations which allow alkaliphiles to effectively generate ATP during high pH growth are discussed below.

3.3.1 ATP Synthase

Two types of ATP synthases are known in bacteria, those that are coupled to H^+ and those coupled to Na^+ [162]. Although it seems disadvantageous for alkaliphiles which are thriving in alkaline (low proton) environment to couple their energy carrier generating system to H^+, surprisingly non-fermentative aerobic alkaliphiles are entirely dependent on H^+-coupled ATP synthase [20, 98, 133, 163, 164]. Several studies have tried to decipher the reason behind why aerobic alkaliphiles couple their OXPHO-based ATP synthesis to H^+. Some of these studies have been focused on adaptation of alkaliphiles ATP synthase and able to identify certain unique features of the enzyme that seem to be correlated to high pH adaptation.

ATP synthase in non-alkaliphilic organisms is known to mediate both the synthesis and degradation of ATP. The ATPase activity that breaks down ATP to ADP and P_i is linked to pumping out H^+ from the cytoplasm. As discussed in Sect. 3.1.2, one of the phenomenal adaptations of this enzyme is inactivation of its ATPase activity. This inactivation, in addition to pH homeostasis, may contribute to energy saving. However, as revealed by several studies, ATP synthase mainly contributes to high pH adaptation through enhanced level of expression and specific adaptations of its subunits.

High Level Expression of ATP Synthase

One of the ATP synthase contributions to high pH adaptations seems to be related to the level of activity. Transcriptome and mutagenic studies revealed an increased expression and activity of ATP synthase at high pH. As pH increases, the energy demand to fuel cellular activities is also expected to rise [27], and hence an increase in the level of expression and activity of the synthase will compensate the high energy demand and contribute to minimize the low pmf effect on H^+-coupled ATP

synthesis. Moreover, it has been observed that ATP synthase expression increases when microbes such as *B. subtilis*, *Corynebacterium glutamicum*, *E. coli*, *Desulfovibrio vulgaris*, etc. are subjected to alkaline treatment [102, 148, 165–167]. ATP synthase synthesizes ATP when H^+ flows from cell surface to cytoplasm through it, and this contributes to the intracellular H^+ concentration. Hence, it is expected contributing to the pH homeostasis process. In fact, results of a mutational study reflect the pH homeostasis role played by ATP synthase. As aforementioned, Mrp antiporter is important in translocating H^+ from the extracellular environment to the cytoplasm and known to play a vital role in pH homeostasis. An Mrp antiporter deletion mutant of *B. subtilis* exhibited a rise in ATP synthase expression [168]. The rise in the level of synthase may compensate the loss in H^+ translocation due to the Mrp deletion. This also indicates that the bioenergetics and pH homeostasis processes are wired tightly.

Although high level ATP synthase expression is widely accepted and experimentally supported as means of high pH adaptation, it seems that the case is not universal. The transcriptome analysis of alkaline-stressed *Enterococcus faecalis* revealed that the ATP synthase was significantly downregulated when the cells were grown in pH 10 media [169]. The authors' findings also include a significant drop in the expression of the *nhaC* gene which encodes Na^+/H^+ antiporter. This also contradicts to the main stream notion that recognizes enhanced expression of the antiporter at high pH. However, the authors did not mention how these downregulations help the organism to survive the alkaline condition. However, it is tempting to speculate that *E. faecalis* is a lactic acid bacterium and in the presence of glucose, which the authors added in the medium they used, can produce acid, and this can possibly maintain low intracellular pH when grown in alkaline media. Thus, it is possible that in order to survive in the alkaline condition, the cells shift their metabolism to produce more acid and which may reduce the need for OXPHOS-based ATP synthase. However, this needs further studies. For instance, what will the transcriptome trend show if a non-fermentable medium is used?

Adaptations of the *a*-Subunit

ATP synthase is a multicomponent enzyme. One of these components believed to be involved in high pH adaptation is the *a*-subunit. Alignment studies on the *a*-subunit amino acid sequences of alkaliphilic and non-alkaliphilic *Bacillus* species revealed that the transmembrane helix-4 (TMH4) and transmembrane helix-5 (TMH5) are somehow distinct between these two groups of bacteria. The TMH4 of alkaliphiles has a conserved motif of 171MRxxxxVxxKxxxM, while TMH5 has two conserved residues, L205 and G212 [95, 170]. The fair conservation of these residues only among sequences of alkaliphiles suggests their possible role in high pH adaptation. Mutational studies on the conserved residues were done to elucidate their adaptive roles. Based on the analysis of the mutants, it seems that residues V177 and K180 are involved in H^+ uptake pathway [171]. Similarly, M171, M184, I185, and L205 are also believed to be relevant to the *a*-subunit proton pathway [95]. These authors also

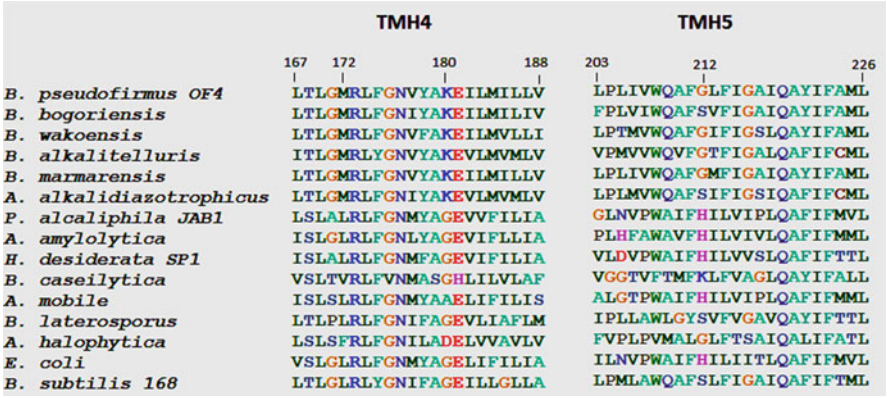


Fig. 9 Sequence alignment of the *a*-subunit of TMH4 and TMH5. The sequence GenBank accession numbers are given in parentheses. The alignment includes five Gram-positive alkaliphilic *Bacillus* strains – *B. pseudofirmus* OF4 (AAG48358), *B. bogoriensis* (WP_026672803.1), *B. wakoensis* (WP_034748151.1), *B. alkalitelluris* (WP_088077058.1), and *B. marmarensis* DSM 21297 (ERN51921.1). A sequence from a Gram-positive alkaliphilic anaerobe *Anaerobacillus alkalidiazotrophicus* (OIJ21792.1) is also included. Six sequences are from Gram-negative alkaliphiles – *Pseudomonas alcaliphila* JAB1 (APU32731.1), *Alkalimonas amylolytica* (WP_091342750.1), *Halomonas desiderata* SP1 (OUE41273.1), *Bogoriella caseilytica* (WP_123305435.1), *Alkalispirillum mobile* (WP_121443140.1), and *Brevibacillus laterosporus* (PCN45399.1). In addition, the alignment includes a sequence from alkaliphilic cyanobacteria *Aphanothece halophytica* (BAK19941.1) and two sequences of non-alkaliphilic strains, *B. subtilis* 168 (NP_391568.1) and *E. coli* (WP_078180285.1)

studied G212 mutant which exhibited H^+ leakiness. However, new sequence analysis (Fig. 9) shows that the conserved residues are not universal among alkaliphiles. These conserved residues are important only to alkaliphilic *Bacillus* strains and probably to related genera. If these residues are important as they are claimed to be, it would have been universal among alkaliphilic bacteria. But that does not seem to be the case. Even among alkaliphilic *Bacillus a*-subunit, some sequences lack conserved residues such as V177 or G212 [170]. Moreover, most of the mutational studies made so far did not pinpoint the exact high pH adaptive role of the conserved residues. However, the growing evidence suggests that the alkaliphilic *Bacillus* spp. ATP synthase *a*-subunit could be involved in preventing H^+ leakage. In addition, the results of the studies indicate a possibility that ATP synthase of alkaliphiles evolved an efficient system of capture and translocation of H^+ to cytoplasm. However, it needs further mutational and structural studies to establish the real high pH adaptation of these structures.

Adaptations of the *c*-Subunit

Amino acid sequence alignment studies on *c*-subunit of ATP synthase revealed the presence of two conserved motifs, 16AxAxAVA and 51PxxExxP, in alkaliphilic

Bacillus strains [170, 172, 173]. Neutralophilic *Bacillus* spp. have GxGxGNG motif in the TMH1. But, in alkaliphiles, this motif is substituted with 16AxAxAVA, which possibly indicates its potential importance in high pH adaptation. In the second conserved motif, the residue 51P is specific to alkaliphiles, and it is positioned close to the ion-binding residue E54. Mutational, structural, and sequence analysis of the *c*-subunit has shown features that may be recognized as high pH adaptations. Mutation of all the alanine of the 16AxAxAVA motif to glycine led to ATP synthesis activity loss by more than 80% [173]. P51 has been mutated to alanine and glycine. The mutant P51A exhibited a dramatic loss of ATP synthesis and non-fermentative growth at pH 10.5, whereas the P51G mutation did not affect the ATP synthesis capacity although it had growth problem at high pH and exhibits H^+ leakage [173]. Studies made on the mutant E54G revealed that it leaks H^+ and a 90% drop on non-fermentative growth. Although it needs further studies to expound how these identified motifs contribute to the high pH adaptation exactly, the results generated so far are valuable and indicate the involvement of the *c*-subunit in the adaptation of ATP synthesis at high pH. However, it is imperative to expand the study to other alkaliphilic genera as well to see the whole picture of the *c*-subunit high pH adaptation role.

3.3.2 Cytochrome

The adaptation of alkaliphiles respiratory system is believed compensating the pmf lost by the reversed transmembrane pH gradient [51]. To unravel this adaptive mechanism, different components of the respiratory system have been isolated and characterized. Analysis of the cytochrome content has shown that it increases with the cultivation pH [51, 161, 174]. This correlation possibly shows that the high pH adaptation of alkaliphiles involves cytochromes. In fact, characterization of isolated cytochrome *c* from alkaliphiles and neutralophiles revealed that the midpoint redox potential of alkaliphiles cytochrome *c* is much lower (<+100 mV) than that of neutralophiles (+220 mV) [161, 175]. However, the redox potential of cytochrome *c* oxidase, the terminal oxidase that accepts electron from cytochrome *c*, is similar between those of alkaliphiles and neutralophiles, +250 mV (cytochrome *a*) [161, 176]. The high midpoint redox potential difference between the terminal oxidase (cytochrome *a*) and cytochrome *c* drives the flow of H^+ and e^- faster across the membrane of alkaliphiles. This can create a H^+ gradient close to the membrane surface, especially the membrane part embedded with the respiratory system.

The H^+ gradient created by the respiratory complex activity is further enhanced and maintained by another unique feature of alkaliphiles cytochrome *c*, high electron retention capacity [177]. Studies made on soluble cytochrome *c*-552 of the Gram-negative facultative alkaliphilic *Pseudomonas alcaliphila* strain revealed that at alkaline pH, it has high electron retention ability and serves as an electron reservoir in the periplasmic space [174, 178]. A similar observation of electron retention is made for cytochrome *c*-550 of *Bacillus clarkii*, an obligate alkaliphile. The retention of electrons attracts H^+, and this contributes to the formation of high membrane

electrical potential ($\Delta\Psi$) for attracting H^+ from the outer surface membrane. This creates for each H^+ an enhanced ATP synthase driving force.

Faster pumping of H^+ by the respiratory complex may require an increased oxygen uptake and high level of electron donor (NADH). The respiratory and NADH activities of an alkaliphilic *Bacillus* have been studied [132]. The results indicate that the oxygen uptake and NADH oxidation activities increase with the rise in cultivation pH. The oxygen consumption studies revealed that the *Bacillus* grown at pH 7.2 and 9.8 has consumed 1.17 and 2.43 μmol oxygen atom/min/mg cell protein, respectively [132]. In addition to viable cells, the authors have also studied the activities of membrane vesicles prepared from the cell envelope of the alkaliphilic *Bacillus* cells. The trend was the same. When the cells were grown at pH 7–9 and 9.9, the oxygen uptake was 1.1–1.4 and 2.5 pmol oxygen atom/min/mg of the cell envelope protein used to make the membrane vesicles, respectively. Similarly, vesicles prepared from the *Bacillus* cells cultivated at pH 7–8.5 and 9.9 were able to oxidize 1.4–1.7 and 6.3 pmol NADH/min/mg cell envelope protein, respectively. On the other hand, an approximately 2.5 times lower oxygen consumption rate was reported for *B. clarkii* DSM 8720(T) cells at pH 10 than that of *B. subtilis* IAM 1026 cells at pH 7. This is despite the alkaliphilic *B. clarkii* 7.5 times higher rate of ATP synthesis than the neutralophilic *B. subtilis* [160]. Such a discrepancy regarding oxygen consumption is also reflected among different alkaliphiles [132], which suggests the high rate of oxygen uptake by NADH oxidation activities is not universal. In fact, it seems that at low level of aeration, the electron retention capacity of cytochrome *c* is more important in maintaining the transmembrane potential that drives the synthesis of ATP [177].

As it has been suggested, alkaliphiles generate pmf during the respiratory electron transport events [179]. At least theoretically, the rapid pumping of H^+ forms the high pmf before it gets equilibrated with bulk phase of the extracellular environment. However, the potential equilibration with the bulk phase should be minimized to tap the pmf for ATP synthesis. The high level of cytochrome *c* and cardiolipin that retains the H^+ close to the surface of the membrane seems to play a crucial role in preventing the dissipation of the pmf created by the respiratory complexes. Moreover, the retention of H^+ by the cytochromes and cardiolipin forms H^+ pool. If the cells have an effective means to shuttle the H^+ from the pool to the ATP synthase, it can generate ATP efficiently. This is expected to be more effective if the ATP synthases are located near to the respiratory complex, a task believed to be accomplished by cardiolipin. To this end, the presence of microcircuits that facilitate the transfer of H^+ to ATP synthase by connecting the surface of the H^+ pumping respiratory complexes and ATP synthases has been speculated [27]. The presence of specific interaction between cytochrome *c* oxidase and ATP synthase, which has been demonstrated in a reconstituted system [180], supports the speculation to some extent. The physical interaction between the respiratory and the ATP synthase complexes can efficiently sequester H^+ transfers during OXPHOS at high pH.

It seems that several factors contribute to enhance the efficiency of alkaliphiles' OXPHOS based ATP production. Among these factors, probably, the most important features include the presence of high amount of:

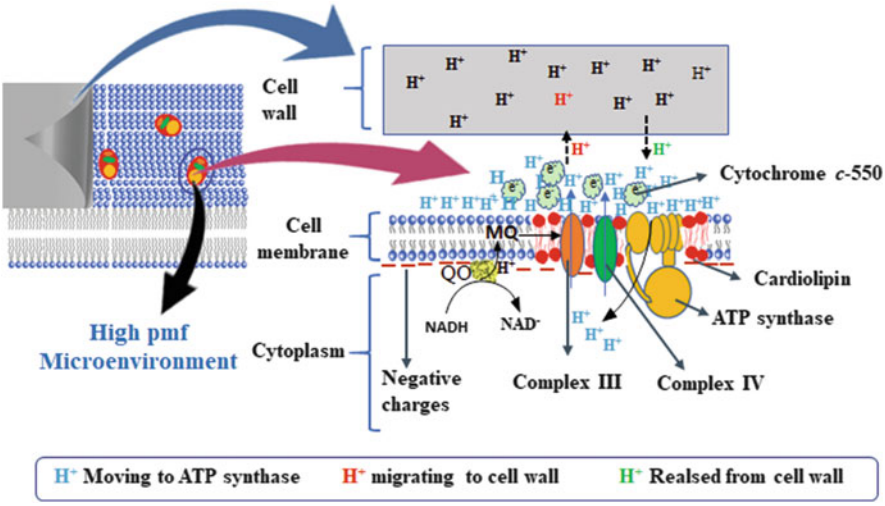


Fig. 10 An illustration of the high pmf microenvironment created by cardiolipin-induced aggregation of the respiratory complexes (complex III and IV). Quinone oxidoreductase (QO, NDH-2) transfers electrons to menaquinone (MQ) pool from which the electrons move to the menaquinol: cytochrome *c* (Complex III) and cytochrome *c* oxidase (complex IV). The rapid pumping of H^+ by the respiratory complexes and the retention of electrons by the cytochrome *c*-550 contribute to high pmf with more negative charges on the membrane facing the cytoplasm than the outer membrane

1. cardiolipin which aggregates the respiratory system together with the ATP synthase. It also restricts the H^+ coming from the respiratory complex close to ATP synthase and facilitate the H^+ transfer to the synthase [144],
2. cytochromes that pump out H^+ much faster than the normal pace of non-alkaliphilic organisms [161, 175] and cytochromes like cytochrome *c*-550 with high electron retention capacity [174, 177], and
3. ATP synthase which is efficient in translocating H^+ to its catalytic core (i.e. inhibition of H^+ leakage) [95, 170, 173].

The rapid pumping of H^+ by the respiratory complex and the restriction of these H^+ close to the surface of the aggregate create a microenvironment with high pmf which promotes the synthesis of ATP (Fig. 10). The H^+ translocated by the ATP synthase during ATP synthesis replenishes the H^+ pumped out by the respiratory complex, which contributes to maintain the low cytoplasmic pH. This microcircuit in the microenvironment produces ATP approximately seven times faster than that of neutralophiles [160, 177], and this may be one of the reasons why alkaliphiles grow faster and denser than neutralophiles.

3.4 Coping Intracellular Alkalinization

Although alkaliphiles are known to have an efficient pH homeostasis system that keeps the cytoplasmic pH well below that of the habitat, the intracellular pH can go above the neutral range. For instance, the intracellular pH of the facultative alkaliphile *B. psuedofirmus* has been reported reaching 9.6 when the cells were cultivated at pH 11.4 [48]. The maximum reported difference between the intracellular and extracellular pH of alkaliphiles is around 2.5 pH units [48]. As shown in Table 1, it seems that the cytoplasmic pH rises even higher as the cultivation pH for the organism reaches to its upper edge. Thus, although there is no available data, the cytoplasmic pH of alkaliphiles such as those growing at pH 13.5 [16] may exceed pH 10. In non-alkaliphiles, this high cytoplasmic pH can potentially impair cellular activities and integrities and ultimately kill the cell. But, as witnessed from their growth in extreme pH conditions, alkaliphiles evolved their intracellular system to remain active and stable at elevated pH. The possible strategies that alkaliphiles deploy to withstand cytoplasmic alkalinization may include (1) altering the expression/production profile of biomolecules, (2) evolving efficient intracellular repair system, and (3) production of biomolecules that are operationally stable at high pH. The production of biomolecules that are stable and functional at high pH is not restricted to intracellular products; it is an absolute necessity to extracellular products.

3.4.1 Altering the Expression/Production Profile of Biomolecules

Organisms endure stress by changing their gene expression profile and metabolic programming. Studies have shown that alkalinization is accompanied by up- and downregulation of several genes [102, 148, 169, 181]. Such changes bring the desired tolerance to the rising pH by (1) switching to alkali-tolerant variants, (2) increasing the level of biomolecules that mitigate the pH drift, (3) compensating the loss due to denaturation, and (4) activating the protein repair and degradation systems.

Switching Expression to Alkali-Tolerant Variants Some organisms have genes which encode variants of a product. These organisms, up on cytoplasmic pH rise, may switch to the expression of the alternative variant that encodes the protein which is operationally stable at alkaline condition [182]. Thus, inactivation of the alkali-sensitive proteins will not hamper the cellular process as the sensitive products are replaced by resistant variants. Alkaliphiles are known to produce biomolecules (such extracellular enzymes) that are active and stable at high pH. It is possible that these organisms use the same adaptation strategy to make alkali-resistant intracellular products. The adaptation mechanism for high pH operational stability of biomolecules is discussed in Sect. 3.5.

Producing More Biomolecules That Mitigate the pH Drift With rising intracellular pH, the level of some biomolecules such as ATP synthase, Na^+/H^+ antiporter, squalene, SCWPs, etc. increases [21, 102, 106, 128, 130, 148]. As aforementioned, these biomolecules play a significant role in the pH homeostasis of alkaliphiles, and hence, the upregulations of these biomolecules contribute to mitigate further increase in cytoplasmic pH. For instance, an increased level of ATP synthase expression results in pumping more H^+ to the cytoplasm which eases the cytoplasmic pH rise, while accumulation of squalene effectively limits H^+ leakage and OH^- ingress. In some organisms, cytoplasmic alkalinization is accompanied by metabolic acid production [102] which alleviates the cytoplasmic pH rise and protects the cell from the subsequent demise. In fact, many alkaliphiles are known producing organic acids and even reduce the culture pH significantly [56, 105].

Compensating Loss Due to Denaturation With the rise in cytoplasmic pH, the activity and integrity of intracellular biomolecules deteriorate. To compensate this loss, cells increase the production level of pH labile biomolecules. For example, as translation slows down and mRNAs are not stable at elevated pH, cells increase the level of mRNA to maintain the necessary level of protein synthesis [183, 184].

3.4.2 Activating Protein Damage Repair and Degradation Systems

The cells also use another strategy to maintain the necessary level of functional biomolecules, repairing the damage incurred by cytoplasmic alkalinization. Thus, it is expected that the cells activate their systems involved in repairing damages and/or recycling inactivated biomolecules. Among the damage repair systems, an increase in the level of chaperone and protein damage repair enzyme has been reported in relation to alkalinization [148, 185]. Many intracellular macromolecules that are vital for life are labile at high pH. The stability and activity of DNA, RNA, proteins, lipids, etc. can be severely affected by prolongated exposure to high pH. In general, it has been known that when cells are exposed to stress, the repair systems are often activated to mend problems suffered by the stress. Here, it may be relevant to mention the formation of isoaspartate and the associated repair system. Isoaspartate is an isomer of aspartic acid formed through the nucleophile attack of the γ-carbon in asparagine or aspartic acid residue side chains which forms a succinimide intermediate as illustrated in Fig. 11. The formation of isoaspartate affects the function and stability of many proteins [186–188]. In addition, the reaction can lead to deamidation of asparagine and formation of D-amino acids [189, 190]. If this damage remains uncorrected, the protein cannot properly perform its task. Thus, it is necessary that such protein damages must be repaired to maintain optimal cellular activities or the damaged protein should be degraded and removed. Cells produce L-isoaspartyl protein carboxyl methyltransferase (PCM), an enzyme which identifies and repairs such protein damages [191]. PCM encoding gene is widely distributed among unicellular and multicellular organisms [192], and in bacteria, it is linked to long-term stress survival [193]. High pH is known to aggravate isoaspartate

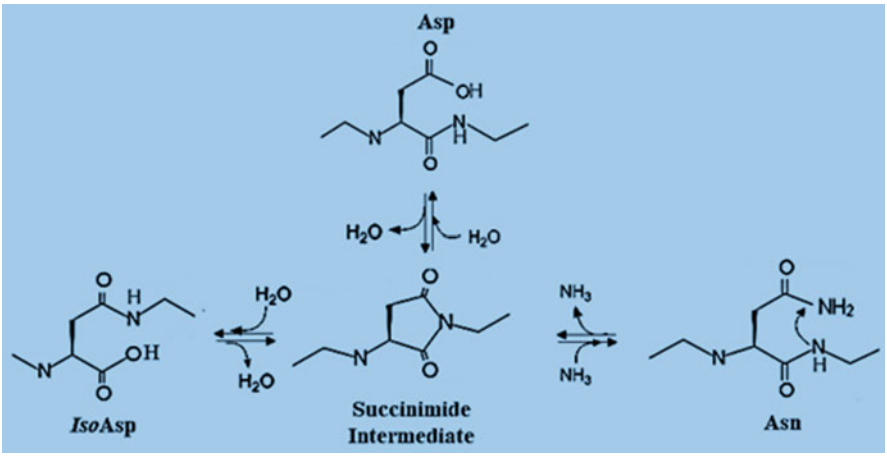


Fig. 11 Nonenzymatic conversion of Asp residue in peptide bonds to succinimide intermediate which converts to isoaspartate. The Asn residue converts to the intermediate through deamidation reaction

formation and deamidation [194–196]. Thus, the cytoplasmic protein damage is expected to increase with increasing alkalinization. Organisms that survive cytoplasmic alkalinization may have an efficient PMC that mends the damage caused by high cytoplasmic pH. Indeed, studies have indicated that protein repair mechanism of PCM is important to thrive in high pH conditions [197].

Another import repair system is the chaperon-mediated refolding of proteins. The rise in cytoplasmic pH can cause protein unfolding and aggregation. Chaperons are known to be involved in refolding proteins that are unfolded/aggregated by stresses. Studies have shown that acid stress in bacteria leads to chaperon production, which is used to adapt low pH environments [198–200]. If a parallel is drawn, alkaliphiles may also use the same strategy to alleviate high pH-induced protein unfolding/aggregation problems, especially related to sudden alkalinization. Although an increase in chaperone level has been reported in relation to alkalinization [148, 185, 201], little is known compared to its role in low pH tolerance.

Not all damages are reparable, and hence, it is possible that alkalinization may lead to accumulation of denatured biomolecules. However, for normal cellular activities, it is necessary to remove those biomolecules that are irreparably damaged. Thus, one of the relevant adaptations that alkaliphiles employ during cytoplasmic alkalinization may be enhancing the turnover rate of intracellular biomolecules. Inactivated biomolecules such as proteins should be degraded and replaced by newly synthesized active products to ensure normal physiology. An elevation in transcription of genes encoding proteases such as the ATP-dependent Clp, ATP-dependent La endopeptidase, and DnaK that are known in degrading nonfunctional proteins has been observed during alkalinization [148, 185]. However, there is no detailed study made so far on the actual involvement of these damage repair and recycling systems in high pH adaptation of alkaliphiles.

It is obvious that the data on high pH adaptations of alkaliphiles is still trickling in. However, it seems that far little is done in some areas. One of such cases is protein synthesis, which is one of the most crucial life processes vital for survival and growth. Several factors can influence this fascinating process, and pH is one of them. The optimum pH (pH 8.2–8.5) for cell-free protein translation systems of alkaliphilic origin have been reported to be only 0.5 pH units higher than that of neutralophiles [202]. However, the cytoplasmic pH of actively growing alkaliphiles can be much higher (>pH 10) such as when cells are grown close to pH 13, and hence, one expects lower rate of protein synthesis. On the other hand, alkaliphiles in general are known to grow faster than non-alkaliphiles [177], which suggest that alkaliphiles may have a very efficient protein synthesis at elevated pH. However, there is no available information how extreme alkaliphiles evolved their protein synthesis apparatus. Similarly, the adaptation of extreme alkaliphiles that shield their DNA and RNA from the effect of high pH is unknown. Since the *p*Ka of guanine (G) and thymine (T) is in the range of pH 9–10 [203], above pH 10, these residues get deprotonated and remain as negatively charged conjugate bases. This can break the hydrogen bonding between the two strands of the DNA helix and result in denaturation of DNA. This makes the DNA strand prone for damage and disrupts the replication and transcription processes. It has been reported that alkali stressed *E. coli* cells induce recA-independent DNA damage repair system [204], which suggests the possible involvement of the repair system in high pH adaptation of alkaliphiles. But the repair system is not enough by itself. There should be mechanism(s) that protect these vital macromolecules from high pH hostility.

3.5 Production of Extracellular Biomolecules Which Are Operationally Stable in Alkaline Milieu

Cells release products to their immediate environment to harvest nutrients, defense/competitional purposes, for communication, etc. At least theoretically, these products are evolved to work optimally in the host environment. Thus, products secreted by alkaliphiles are expected to be operationally stable in their high pH habitats. Among such products, enzymes have attracted a great deal of attention. Studies on alkaline active enzymes are done to understand the molecular mechanisms behind their structural and functional adaptation to high pH environment. Comparative sequence analysis and mutational studies revealed that alkaline-active enzymes exhibit reduced alkali susceptible residues and tend to increase alkali tolerant residues, especially in their exposed surfaces. The ionization state of residues such as Asp, Glu, His, Lys, and Arg side chains is determined by the pH of the environment. Thus, the distribution and frequency of these ionizable residues partly determine the pH adaptation of proteins. In line with this, Lys, Arg, Asn, His, Glu, and Asp residue content has been studied in relation to high pH adaptation [205–209]. These studies revealed the tendency of alkaline-active enzymes to have more Arg, His, and Gln in their structures. Since Arg has a higher *p*Ka than Lys,

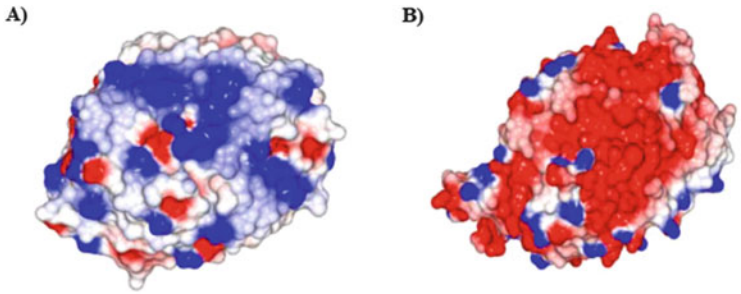


Fig. 12 The surface at the back of the catalytic cleft of an acid-active PDB 1B30 (**a**) and alkaline-active PDB 2UWF (**b**) xylanases. Negatively and positively charged surfaces are colored in red and blue, respectively

substitution of Lys by Arg may allow formation of hydrogen bonds at extremely high pH. His and Gln are largely neutral at alkaline condition, and this may be important to maintain the protein solubility in the alkaline condition. Asn is one of the most alkali susceptible residues [210, 211], and hence, its occurrence in alkaline-adapted proteins, especially on exposed surfaces, is relatively low [206]. This agrees to previous studies that involved mutational substitution of Asn with less susceptible amino acids and resulted in a better stability at high pH [212, 213].

Charged residues are known to play important roles in structural adaptation of biomolecules. Such residues are vital in high pH adaptations. Extracellular products of alkaliphiles tend to have more acidic residues on their surfaces than their non-alkaliphilic counterparts. For instance, deduced amino acid sequence analysis has shown that the externally exposed alkaliphile membrane protein loops have acidic residues, while the non-alkaliphile homologue loops have neutral/basic residues [214]. Similarly, as described above in Sect. 3.2, the surface exposed proteins and polysaccharides of alkaliphiles such as SplA, liposaccharides, and SCWPs are rich in negatively charged residues. Structural analysis of extracellular enzymes also reveals that their surface is more acidic than that of non-alkaline active counterparts. Figure 12 depicts the surface charge difference between xylanases that are optimally active at pH 5.6 [215] and 9–9.5 [216]. The alkaline active xylanase has more acidic surface than the acid-active enzyme. It seems that there is a consensus that the negatively charged surface of alkaliphiles extracellular products deters encountering negatively charged OH^- and protects the biomolecule from the aggressiveness of the high pH environment, a "Sword against sword" adaptation strategy.

When it comes to alkaline-active enzymes, it is not only their stability at high pH which is astonishing, but also their ability to optimally mediate reactions at elevated pH is intriguing. The interesting thing is that the catalytic residues and often their vicinity are highly conserved regardless of the origin of the enzyme. For instance, the endo-beta-1,4-xylanase from *Acidobacterium capsulatum* is optimally active at pH of 5 and loses its activity at or above pH 8 [217], while the xylanase from *B. halodurans* is optimally active around pH 9.5 and displayed nearly 20% of its optimal activity at pH 12 [216]. But these two enzymes belonging to the same family

(GH 10) share a similar catalytic pocket as well as identical catalytic residues (a pair of Glu). How these enzymes are able to ionize the catalytic residues in this wide range of pH and mediate the biocatalysis is fascinating.

The pH profile of enzymes such as glycosyl hydrolases is determined by the catalytic residues *pKa* values [209, 218, 219] which in turn are dependent on the microenvironment surrounding the catalytic residue. Thus, the nature of amino acids in the active site region plays a significant role in shaping the pH-activity profile of the enzymes. In general, amino acids with positive charges and hydrogen bonds lower *pKa* values, while carboxyl groups can increase or decrease the *pKa* values based on the electrostatic interaction between residues [220]. Thus, certain amino acids in the active site vicinity determine the *pKa* values by altering the active site electrostatic and dynamic aspects [221] through direct or indirect interaction with the catalytic residues. This kind of key residues, at least partially, determines the pH-dependent activities of enzymes [222], and mutational studies on such residues often shift the mutated enzyme pH-activity profile [223–225].

3.6 Adaptation to Low Nutrient Bioavailability

Nutrient bioavailability is a less studied challenge in high pH habitats. pH affects the availability of certain nutrients by determining its state (e.g., solubility), reaction with other substances, stability, etc. For instance, water in soda lakes is saturated with CO_2 that forms HCO_3^-/CO_3^{2-} which interacts with and precipitates divalent metal ions, making it less bioavailable. Thus, it is necessary for alkaliphiles to develop mechanisms that circumvent the problems related to the poor bioavailability of such nutrients. This can be achieved by deploying efficient retrieving systems for deficient nutrients or decreasing dependency on poorly available nutrients. In line with this, purification and characterization of some alkaline active extracellular enzymes revealed that the enzymes evolved some adaptive features including high affinity to metal cofactors [226] or became less dependent on it [227]. In fact, these properties are among the reasons why enzymes of alkaliphiles are desirable in detergent applications, resistant to the detergent chelator's effect.

Alkaliphiles are known to have efficient system of capturing and translocating scarce metal ions to the cytoplasm. One of the relatively well-studied scarce metals is iron. Iron is important in ATP production, and it is a crucial cofactor for enzymes involved in a variety of metabolic processes, and hence, it is essential for almost all organisms. Although it is one of the most abundant elements in nature, it is not readily available. Therefore, organisms employ different strategies to secure enough iron from their surroundings. As the solubility of iron decreases with increasing pH, it is vital for alkaliphiles to evolve a means to acquire iron. At alkaline conditions, iron exists in ferric state (Fe^{+3}) which reacts and forms the poorly soluble $Fe(OH)_3$. Thus, in alkaline environments, the bioavailability of iron is far below the requirement for living cells. At pH 10, the concentration of bioavailable iron is estimated to be approximately 10^{-23} M [228], which is much lower than the 10^{-18} M level at

pH 7 [229]. Considering the extreme scarcity of iron at high pH, one expects that alkaliphiles evolved a very efficient sequestering mechanism. Indeed, studies revealed that alkaliphiles produce very effective iron-binding chelators, siderophores. It is believed that siderophore-assisted iron acquisition is one of the critical adaptations of alkaliphiles in high pH habitats [229–231]. Studies made so far are limited to production (of siderophores). Detail biochemical characterization and structural analysis of these siderophores can be beneficial to advance our understanding on alkaliphiles adaptation and may also yield new siderophores of biotechnological importance. It is interesting that the first structural analysis proves the potential of alkaliphiles as sources of novel siderophores [229].

It is not only the metal ions' availability that is limited at highly alkaline conditions [232], other major nutrients such as nitrogen and phosphate could also be growth-limiting factors [16, 42, 233]. For example, NH_4^+ which serves as nitrogen source for a wide variety of organisms is mostly converted to volatile and toxic NH_3 and becomes unavailable in alkaline habitats of pH 10 and above. Studies have shown that the poor availability of nitrogen sources in some alkaline habitats makes the inhabitant alkaliphiles resort to utilization of certain unconventional resources such as cyanide and its derivatives as nitrogen source [230, 234].

In general, the limited work done so far indicates that there are challenges and associated adaptations regarding the bioavailability of certain nutrients in high pH habitats. It may be attractive for basic and applied areas to extend studies in this direction.

4 Adaptations of Eukaryotes to High pH Environments

There are numerous unicellular and multicellular eukaryotes such as ciliates, dinoflagellates, diatoms, fungi, green algae, invertebrates, fish, etc. that flourish in high pH habitats. Although there are very interesting studies on the diversity, taxonomy, population dynamics, ecological role, etc. of these eukaryotes [235, 236], there is very little information on how these organisms are adapted to their respective high pH habitats. Nearly all the studies regarding high pH adaptations of life have been focused on microorganisms (bacteria, archaea, and to some extent fungi). This may be due to several reasons such as their dominance/abundance, biotechnological interests, relatively easy handling, etc. On the other hand, studies on high pH adaptation of eukaryotes not only improve our understanding but also may enlighten us with new mechanisms. For instance, the cell membrane of most protozoans such as ciliates adapted to high pH environment may be exposed to the alkaline environment. It is of great interest to know how this membrane shields the cytoplasm effectively from the effect of the extreme pH. Moreover, the ciliates inner part of the cell membrane facing the cytoplasm is lined with proteinaceous structure known as pellicle. Although one expects that this pellicle may play an important role in the adaptation, there is no available information how this remarkable structure contributes in adapting alkaline habitats.

Alkaline environments, particularly the East African Rift Valley soda lakes are the most productive lakes on the planet and are supporting huge flocks of birds, especially flamingos (*Phoenicopterus roseus* and *Phoeniconaias minor*). These birds are associated with the soda lakes, wade and swim to feed on the cyanobacteria *Arthrospira* (previously known as *Spirulina*). However, these lakes are very alkaline and are hostile to practically all other forms of non-adapted life including humans. It is believed that the birds adapted to these hostile lakes have special tough skin and scales on their legs which prevent them from the alkali attack. However, detailed studies on how exactly this scale protects the birds' leg from the alkali effect are still lacking. The same holds true for other eukaryotes such as crustaceans, flagellates, insects, etc. that are thriving in high pH habitats. Relatively, fish adapted to alkaline lakes attracted attention which seems more due to economic importance than interest in basic understanding.

Although not all, some soda lakes are known for their fish. Fish such as the lake Magadi tilapia (*Alcolapia graham*) are adapted to thrive in hypersaline alkaline water that can kill other fish within minutes [237]. Studies made so far on high pH adaptation of fish revolved around two challenges, blood pH maintenance and ammonia excretion. When fish get transferred from neutral to alkaline water, the blood pH increased rapidly [238–241]. This is not mainly due to direct translocation of alkali to blood but driven by solubility of CO_2. Above pH 8.5, almost all CO_2 in water converts to bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), and this results in a CO_2 deficiency around the gill. This creates a faster diffusion of CO_2 from the blood of the fish [242]. The rapid loss of CO_2 from the blood leads to the rise in blood pH which is known as respiratory alkalosis [238–241]. Moreover, the abundant OH^- and HCO_3^- of the alkaline environment create an electrical gradient which facilitates the exchange of blood H^+ for environmental HCO_3^- [243] and this may potentially contribute to the blood pH rise. However, studies made on tilapia that live in a pH 10 soda lake revealed that it involved mechanisms that reduce gill permeability to HCO_3^- [244]. Moreover, these fish are adapted to handle high plasma pH [242, 245]. It has also been reported that fish adapted to high pH habitats lower the blood pH to physiological range by exchanging the blood Na^+ and HCO_3^-, respectively, for H^+ and Cl^- of the aquatic body [240, 241, 246].

The other potential challenge for fish to adapt high pH environment is accumulation of ammonia in the blood caused by its unfavorable passive diffusion across the gills [240]. Since protein catabolism continuously generates ammonia [247], if it is not removed effectively, it tends to accumulate in the body. High level of NH_3 is toxic as it binds to the brain *N*-methyl-D-aspartate (NMDA) receptors and cause an over-excitation and ultimate death [248, 249]. Terrestrial animals convert NH_3 to urea or uric acid at the expense of energy and then remove it from their bodies. Whereas fish, living in aquatic environment have become ammonotelic and directly discharge NH_3 through the surface of their gills without energy expenditure. However, such removal of NH_3 at gill surface is unfavorable in high pH environment. Fish adapted to high pH habitats are able to reduce NH_3 load by converting it to urea and discharge it through urea transporter A (UT-A) at the gills surface [246, 250]. This indicates that the fish adapted to high pH aquatic environments have the necessary biochemical machineries to process NH_3 into urea and

transporting it out of the blood. Most fish do have the genes necessary for this biochemical process; however, only those fish which are adapted to alkaline habitats are able to express these genes throughout their life [240].

The work done so far has improved our understanding how fish can adapt to high pH environment. However, there are still some unanswered questions such as adaptive mechanism of gills proteins/membranes which are directly exposed to the alkaline water. Moreover, due to the external fertilization of fish reproductive process, the gametes are deposited directly into the extreme habitats. How these gamete cells survive the high pH is yet to be discovered. Studies on other eukaryotic organisms' adaptation to the high pH environment will certainly add to the existing knowledge and should be encouraged.

5 Conclusion

It has been over four decades since researchers started unraveling the secretes of high pH adaptation. Over these years, very fascinating adaptive strategies of alkaliphiles have been described in numerous publications. To thrive in high pH environments, organisms evolved multilevel adaptations that are reflected in their unique functional and structural makeups. Adaptations related to pH homeostasis and bioenergetics of alkaliphilic prokaryotes have been widely and deeply studied. However, there are still issues that are waiting for proper scientific look. One of such issues that seem overlooked is the cytoplasmic alkalinization of extreme alkaliphiles like those growing around pH 13 and the associated physiological adaptations. At the extreme pH, though it is not experimentally proven, there is a possibility that the cytoplasmic pH can drift above pH 10, and this can, at least, theoretically affect the transcription and translation processes, DNA replication, the activities and stabilities of biomolecules including enzymes, DNA and RNA, etc. However, the fact that these unique organisms are growing in the extreme habitats indicate the cytoplasmic system is functional and hence must be adapted to high pH. On the other hand, although it is unlikely, there is a possibility that these organisms manage to keep the cytoplasmic pH below pH 10. If this happens, the intracellular and extracellular pH difference can reach over 3.5 pH units for alkaliphiles thriving at pH 13.5, and this obviously requires an extremely efficient pH homeostasis even by alkaliphiles standard. Thus, these extreme alkaliphiles to thrive in their habitats should evolve either an extraordinary pH homeostasis mechanism or unique adaptation that protects their cellular activities and biomolecules from the deleterious effect of high cytoplasmic pH (>pH 10). Which one of these alternatives nature has chosen remains to be seen?

Probably one of the most studied high pH adaptations is the bioenergetics. It is widely accepted that cell membranes of alkaliphiles have low proton motive force (pmf) which makes oxidative phosphorylation-based ATP production challenging. However, it seems that alkaliphiles solved this challenge primarily by evolving efficient respiratory complexes and ATP synthase that aggregates in a patch by the cardiolipin. The respiratory complexes pump H^+ faster, and the headgroup of the

cardiolipin restricts these H^+ within the microenvironment of the patch creating high pmf. The presence of cytochrome c such as cytochrome c-550 with high electron retention capacity significantly enhance the pmf. Moreover, due to the possible interaction of cardiolipin to the respiratory complexes and ATP synthase, it can to may continuously shuttle H^+ to the synthase, which may be one of the reasons why alkaliphiles ATP synthesis is more efficient and strictly H^+-coupled. Another contribution to high pH adaptation comes from unsaturated bonds of lipids. Double bonds are known to react with radicals such as OH^- faster than single bonds. Thus, the double bonds in squalene, squalene derivatives, and unsaturated fatty acids within the lipid bilayer scavenge OH^- traversing the membrane. Although it needs to be experimentally supported, desaturases probably re-establish the double bonds lost by reacting with ingressing OH^-.

It is very clear that our understanding of high pH adaptation is expanding due to the trickling information. However, the studies are still focused on prokaryotes. Even among prokaryotes, with very few exceptions, almost all the studies are directed to Gram-positive bacteria. On the other hand, there are largely diverse Gram-negative bacteria, archaea, and eukaryotes that are known thriving in high pH habitats. It could be interesting to include these groups of organisms in future studies.

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