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ADAPTATION IN HALOALKALIPHILES AND NATRONOPHILIC BACTERIA

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1. Introduction

Studies of the past decades revealed an impressive diversity of organisms that inhabit highly saline and alkaline environments (Duckworth et al., 1996; Oren, 2002, 2012; Grant and Sorokin, 2011). Most of the prokaryotes and even a few eukaryotic species inhabiting saline–alkaline systems are well adapted to high salinity/alkalinity and high pH conditions. In general, obligate or true halophiles are defined as organisms that require at least 0.2 M or 15 g/l of sodium salts (specifically NaCl) in their milieu, with optimal growth between 0.2 and 5.2 M of total Na⁺ (Kushner and Kamekura, 1988; Ventosa et al., 1998). Obligate (true) alkaliphilic organisms *sensu lato* are those living optimally at pH values of at least 9, mostly growing best between pH 9.5 and 10.5 (Krulwich, 2006). Based on their minimal salt or pH requirements, both categories of extremophiles could be further divided into slight, moderate, and extreme halophiles or alkaliphiles, respectively (Table 1) (Grant et al., 1998; Hamamoto and Horikoshi, 1992; Ventosa et al., 1998; Mesbah and Wiegel, 2008). Interestingly, while most of true halophiles are flourishing at neutral or slightly acidic pH (6–7), many true alkaliphiles are also halophiles in their requirements, being termed haloalkaliphiles and qualifying as polyextremophiles. On the opposite, non-halophilic alkaliphiles require high pH and low salt in their medium (Hamamoto and Horikoshi, 1992; Horikoshi, 1999). The term “natronophile” (“natron-loving” organisms or “chloride-independent sodaphile”) has been proposed for the alkaliphilic organisms that have an absolute requirement for Na₂CO₃/NaHCO₃ (Banciu, 2004; Sorokin et al., 2011a; Muyzer et al., 2011a). However, some natronophiles still require low concentrations of chloride ions for the optimal growth in soda brines. Life at haloalkaline conditions is energetically costly, and therefore, extreme haloalkaliphiles could hardly exhibit other extremophilic features (Oren, 2011). Moderate halophilic and thermoalkaliphilic anaerobic bacteria with validly published names belong to the orders *Halanaerobiales* (e.g., *Halonatronum saccharophilum*) and *Natranaerobiales*

Table 1. Main types of extremophilic microorganisms living at high salt and/or high pH conditions.

Growth characteristics	Range of pH (optimum pH)	Salt range (optimum salinity)	Representative bacterial species
Obligate (true) extreme halophile	5.2–8.5 (7–7.5)	12–30 % (15–25 %) (w/v) NaCl	<i>Salinibacter ruber</i>
Moderate halophile	5.0–8.5 (6.8–7.5)	3–15 % (7–12 %) (w/v) NaCl	<i>Halomonas halophila</i>
Facultative halophile	6.0–11 (6.8–7.5)	0–35 % (6–12 %) (w/v) NaCl	<i>Planococcus halophilus</i>
Halotolerant	5.0–8.5 (6.8–7.5)	0–15 % (1–5 %) (w/v) NaCl	<i>Salinivibrio costicola</i>
Obligate (true) alkaliphile	9.0–11.5 (9.5–10.5)	0.05–2 M (0.05–0.2 M) of total Na ⁺	<i>Bacillus alcalophilus</i> ATCC 27647
Facultative alkaliphile	7.5–11 (7.5–10)	0.05–0.1 M of total Na ⁺	<i>Bacillus pseudofirmus</i> OF4
Alkalitolerant	7.5–11 (7.5–10)	0.6–16 % (w/v) NaCl	Alkalitolerant <i>Bacillus</i> species (e.g., <i>Bacillus horikoshii</i>)
True extremely haloalkaliphile/ natronophile	8–10.7 (9.5–10.2)	10–30 % (12–25 %) (w/v) NaCl or 1.5–4.4 M (2–3.5 M) of total Na ⁺	<i>Thioalkalivibrio versutus</i>
True moderately haloalkaliphile/ natronophile	8–10.5 (9.0–10.0)	3–20 % (5–12 %) (w/v) NaCl or 0.5–2 M (1 M) of total Na ⁺	<i>Ectothiorhodospira haloalkaliphila</i>
Low-salt-tolerant natronophile	8–10.5 (9.5–10.0)	0.3–1.5 M (0.6 M) of total Na ⁺	<i>Thioalkalimicrobium aerophilum</i> AL3 ^T

(e.g., *Natronaerobius* spp. and *Natronovirga* spp.). Triple extremophiles are apparently exceptions to the above-mentioned statement, but they are not extreme in any of their optimal growth requirements (Zhilina et al., 2001; Mesbah et al., 2007; Mesbah and Wiegel, 2009). A more frequent situation is that of moderate haloalkaliphiles that are either thermotolerant (e.g., strains of the sulfur-oxidizing bacterium *Thioalkalivibrio versutus*, some actinomycetes isolated from saline soils) (Sorokin and Kuenen, 2005; Ningthoujam et al., 2009) or psychrotolerant (e.g., *Planococcus* spp. strain ZD22 isolated from a saline–alkaline soil in the vicinity of an oil field) (Li et al., 2006).

Besides the main metabolic types listed in Table 1, there are different degrees of mixed halophily and alkaliphily observed in various microbes. For example, strains of *Bacillus halodurans* are facultative alkaliphiles and moderately halotolerants, with optimal growth at pH 9–10 and a salt tolerance ranging from 0 up to 12 % NaCl (Nielsen et al., 1995). *Oceanobacillus iheyensis* HTE831 is facultatively alkaliphilic and extremely halotolerant and is closely related to *Bacillus* spp. It grows optimally at pH between 7.5 and 9.5, tolerating up to 21 % NaCl at pH 7.5. Isolated from deep-sea sediment, *O. iheyensis* can withstand pressures up to 30 MPa (Lu et al., 2001).

Often, saline–alkaline environments hosting haloalkaliphilic prokaryotes are exposed not only to relatively elevated temperatures but also to intense sunlight irradiation and, consequently, to a high rate of water evaporation during dry seasons (Grant and Sorokin, 2011). In these harsh conditions, survival of salt- and alkaline-loving microorganisms depends on a palette of adaptive mechanisms that aim to remain alive during prolonged periods of low water activity. Acute and long-term adaptation as a response to changing salinity and/or alkalinity include modulation of membrane fluidity, acquisition of osmoprotectants, adjustments of energy metabolism concomitant with fine-tuning of stress-sensing, chemotaxis, and nutrients transport at the membrane level. Model halophilic and alkaliphilic organisms have for a long time been used to test for phenotypic and genotypic response to variation of external salinity and pH, respectively. A few examples of detailed physiological investigations concerning the double-stress adaptation at high salinity and alkalinity are known, and they are mostly focused on natronophilic lithoautotrophic bacteria and haloalkaliphilic archaea. In this chapter, we endeavor to discuss up-to-date relevant findings in the haloalkaliphilic adaptative strategies as they were directly observed or as extrapolation of well-known alkaliphilic and halophilic settings. Accumulating information on genome sequences aids to reveal even more insights in the mosaic of various adaptive strategies to the haloalkaline environment.

2. Habitats and Diversity of Haloalkaliphilic and Natronophilic Bacteria

The majority of known obligate haloalkaliphilic and natronophilic bacteria and archaea are isolated from soda lakes and saline–alkaline soils. In the past, detailed investigations on the biota living in soda systems have been carried out mainly in

soda lakes from the East African Rift Valley and western USA. Currently, Central Asian (Chinese, Mongolian, and Siberian) soda lakes are under intense screening for microbial diversity.

2.1. SALINE–ALKALINE HABITATS

Saline–alkaline environments are characterized by increased salinity (>60 g/l total salinity) and high actual (soluble) alkalinity (up to molar concentrations) and pH (pH >9.5 – 11). Because in such environments salinity and alkalinity are simultaneously provided by a high concentration of sodium carbonate and sodium bicarbonate, they are also known as soda systems. Saline–alkaline (soda) lakes or soils are located mostly in arid and semiarid regions where particular climate, geochemical, hydrological, and topological factors interact. Differing from neutral saline lakes (pH 6–8, with low buffering capacity and high NaCl, Na_2SO_4 , K^+ , and Mg^{2+} concentration), soda lakes are strong natural buffering systems at pH >10 , containing high concentrations of Na_2CO_3 , NaHCO_3 , and, in some cases, a significant concentration of NaCl. One of the major chemical characteristics of soda lakes is the lack of solubilized divalent cations (Mg^{2+} , Ca^{2+}) that precipitate as carbonates under alkaline conditions. The early removal of divalent cation carbonates results in the accumulation of sodium or potassium carbonates, thus increasing the concentration of monovalent cations. By repeated cycles of concentration and evaporation, salts crystallize and brines are formed. In some parts of the world, the shallow lakes may end as a layer of solid rock termed trona (crystalline sodium sesquihydrate, $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3 \cdot 2\text{H}_2\text{O}$) by evaporation during dry seasons (Grant and Sorokin, 2011). A broad range of intermediate saline and/or alkaline lakes occurs by the mixing of the minerals in various ratios. Other important consequences of the soda brine chemistry, including the presence of high levels of soluble inorganic phosphorus, low toxicity of sulfide and nitrite, and high toxicity of ammonia, have important implications on the microbial system.

Salt lakes originating from sea are called thalassohaline, while those with land origin are termed athalassohaline (Oren, 2002). Most of neutral salt lakes and salterns (pH 7–8) are thalassohaline, while soda lakes are obviously of continental (athalassic) origin. Bodies of water with a significantly higher salinity (>80 g/l, w/v) than that of the seawater (35 g/l, w/v) are categorized as highly saline or hypersaline lakes (Poehls and Smith, 2009). Many soda lakes are also hypersaline, reaching a salinity of over 300 g/l. Well-known saline–alkaline lakes are located in eastern Africa (along the East African Rift Valley), the western and northern USA, the Middle East (Turkey, Armenia), and Central Asia (southern Siberia, northeastern and Inner Mongolia, China). Remote soda lakes are also located in other parts of the world, such as Australia, Central and South America (Chile, Venezuela), and southern Asia (India). Low-salt alkaline ponds and soils are described in Europe (Austria, Hungary, Serbia) (Tindall, 1988).

Saline–alkaline soils are either natural or man-made, formed upon intensive irrigation/evaporation cycles. Natural sodic (soda or soda solonchak) soils and deserts are formed in dry areas located in Europe (e.g., the Pannonian Basin), Asia (SW Siberia, NE Mongolia, Central China, India), Africa (Egypt), and North America. In comparison with soda lakes, the composition and diversity of microbial community living in soda soils is scarcely characterized (Lysak et al., 1994; Zenova et al., 2005; Sorokin et al., 2008e).

Saline–alkaline ecosystems have attracted the attention of investigators due to their unique chemical and biological features. Soda lakes are considered as models of ancient Martian or Archean terrestrial aquatic ecosystems (Kemp and Degens, 1985; Kemp and Kazmierczak, 1997; Zavarzin, 1993).

In spite of the apparently unfavorable physical and chemical conditions (high pH, high salinity, low water activity, paucity of divalent cations, high irradiation, elevated temperature, etc.), saline–alkaline environments display a full and balanced recycling of chemical elements (C, O, H, N, S, P, metals, etc.) and often a spectacularly high primary biomass production. In this category of extreme habitats, an almost complete trophic web assures the biological utilization and transfer of elements. Prokaryotes, represented by archaea and bacteria, as well as microscopic and macroscopic eukaryotes (protozoa, algae, brine shrimps, insect larvae) thrive in saline–alkaline lakes. Prokaryotes are the most diverse and numerous group of soda lake inhabitants. Recent advances in molecular biology have the potential to reveal complex metacommunities that play key roles in the ecological status of such habitats (Riesenfeld et al., 2004; Grant and Heaphy, 2010).

2.2. BIODIVERSITY OF SODA ENVIRONMENTS

As a general observation, in saline–alkaline environments, species diversity increases as the salinity and/or alkalinity decreases (Grant and Tindall, 1986). In diluted saline–alkaline lakes of the East African Rift, representatives of macrofauna, such as fishes (*Tilapia grahami*), are found. Brine shrimp (*Artemia* sp.), commonly found throughout the world's saline lakes, is documented in saline–alkaline Mono Lake (USA) (Dana and Lenz, 1986) as well as in hypersaline soda lakes in the Kulunda Steppe (Altai, Russia) (Sorokin et al., 2012d). In soda lakes with moderate salinity (<90 g/l) and pH <10, from East Africa and the western USA, zooplankton species including rotifers (e.g., *Brachionus plicatilis*), cladocerans (*Moina hutchinsoni*), and copepods (*Diaptomus sicilis*) were recorded (Grant and Tindall, 1986; Bozek, 1989). These microscopic invertebrates feed on phytoplankton such as microscopic algae and cyanobacteria. Haloalkaliphilic photoautotrophic cyanobacteria from the genera *Arthrospira* spp., *Cyanospira* spp., *Synechococcus* spp., and *Synechocystis* spp. consistently contribute to the high primary productivity in hyposaline and moderate soda lakes (Grant et al., 1990). Cyanobacteria are essential both for inorganic C fixation and O₂ production in the

ecosystem of saline–alkaline lakes. In addition to cyanobacterial populations, hypersaline soda lakes such as Lake Magadi in Kenya and Wadi Natrun haloalkaline lakes in Egypt, host significant communities of anoxygenic phototrophic purple bacteria belonging to *Ectothiorhodospira* and *Halorhodospira*, which also contribute to the primary biomass production (Imhoff et al., 1979; Grant and Tindall, 1986). Photolithoautotrophic haloalkaliphilic bacteria such as *Thiorhodospira sibirica* and *Thioalkalicoccus limnaeus* are the sole species of their genera that have been isolated from the low saline soda lakes from the Siberian steppe (Bryantseva et al., 1999a, 2000). Anoxygenic, photoheterotrophic heliobacteria such as alkaliphilic, low-salt-tolerant members of *Heliorestis* spp. were identified in low saline soda lakes from Siberia and Wadi Natrun (Egypt) (Bryantseva et al., 1999b; Asao et al., 2006). A relatively large nutritional and ecological spectrum of aerobic and anaerobic prokaryotes, bacteria and archaea, assures the natural cycling of the elements in the soda environments. Basically, all major metabolic groups are represented among the soda lakes inhabitants (Jones et al., 1998; Zavarzin et al., 1999; Grant and Sorokin, 2011).

Chemoheterotrophic aerobes thriving in soda lakes are well represented by archaea, as well as Gram-negative and Gram-positive bacteria. Haloalkaliphilic members of the family *Halomonadaceae* (e.g., *Halomonas magadiensis*, *H. kenyensis*, *H. mongoliensis*) have been isolated from soda lakes around the world, being an important part of the easily culturable aerobic chemoheterotrophic communities thriving in these ecosystems (Duckworth et al., 1996, 2000; Boltyanskaya et al., 2007; Shapovalova et al., 2008). Other haloalkalitolerant and haloalkaliphilic *Halomonas* spp. have been discovered in neutral saline lakes (*H. alkaliantarctica*), salt pools (*H. campaniensis*, *H. alkaliphila*), and saline soils (*H. campisalis*, *H. boliviensis*) (Poli et al., 2007; Romano et al., 2005a, 2006a; Mormile et al., 1999; Quillaguamán et al., 2004). Soda systems are the sources for many aerobic Gram-positive heterotrophs belonging to the phyla *Firmicutes* and *Actinobacteria*. Most *Bacillus* isolates from soda lakes or soils are alkaliphilic and moderately halophilic or halotolerant (*Bacillus alkalisediminis*, *B. aurantiacus*, *B. bogoriensis*, *B. daliensis*), and only a few of them have been categorized as true, although moderately, haloalkaliphiles, such as *B. localis* (Borsodi et al., 2008, 2011; Vargas et al., 2005a; Zhai et al., 2012; Márquez et al., 2011). A wealth of other *Bacillus*-related species with various degrees of mixed halo- and alkaliphily have been recently and continuously isolated from soda lakes: the halophilic and alkalitolerant *Salsuginibacillus kocurii* and *S. halophilus* (Carrasco et al., 2007; Cao et al., 2010), the moderately halophilic and alkalitolerant *Halolactibacillus alkaliphilus* (Cao et al., 2008), and the haloalkalitolerant *Amphibacillus jilinensis* (Wu et al., 2009).

The important question of polymer degradation at soda lake conditions has barely been touched. So far a possibility of anaerobic utilization of cellulose and pectin as growth substrates was demonstrated in pure cultures of fermentative haloalkaliphilic members of the *Clostridia* and the *Bacteroidetes* (Zhilina et al., 2005; Sorokin et al., 2011d, 2012c). Many fermentative and anaerobic respirers from soda lakes belong to the orders *Clostridiales*, *Halanaerobiales*,

and *Natronaerobiales*. From the *Halanaerobiales*, two natronophilic species of *Natroniella* have been described: the chemoorganotrophic homoacetogenic *Natroniella acetigena* (Zhilina et al., 1996) and the chemolithoautotrophic sulfidogenic *N. sulfidigena* (Sorokin et al., 2011b). Both have been isolated from anoxygenic sediments of soda lakes in Kenya and SW Siberia, respectively. *Natroniella sulfidigena* is capable of acetate-dependent sulfur respiration, being responsible of hydrogen sulfide formation at extremely haloalkaline conditions (pH >10; salinity >2 M Na⁺). Sulfate-reducing bacteria (SRB) thriving in the anoxic sediments of various soda lakes are represented by extremely or moderately natronophilic lithotrophs belonging to the order *Desulfovibrionales* (genera *Desulfonatronovibrio*, *Desulfonatronum*, and *Desulfonatronospira*) (Zhilina et al., 1997; Sorokin et al., 2008b, 2011c, 2012b; for a review, see Sorokin et al., 2011a). Moderately natronophilic heterotrophic SRB capable of utilizing propionate or volatile fatty acids as carbon and energy source have been recently assigned to the order *Desulfobacterales* and described as *Desulfonatronobacter acidivorans* and *Desulfobulbus alkaliphilus* (Sorokin et al., 2012a). The most recent example of an extraordinary sulfate reducer described as *Desulfohalophilus alkaliarsenatis* (Switzer Blum et al., 2012) was isolated from hypersaline–alkaline Searles Lake in California. This new member of the family *Desulfohalobiaceae* can grow by either sulfate or arsenate respiration at salt-saturating condition and high pH. The oxidative part of sulfur cycle in soda lakes is performed by natronophilic chemolithoautotrophic sulfur-oxidizing bacteria (SOB) belonging to four recognized genera: *Thioalkalimicrobium*, *Thioalkalivibrio*, *Thioalkalispira*, and *Thioalkalibacter*. Representatives of the former two genera are widely distributed in various soda lakes all over the world, while the latter two were only found occasionally (Sorokin and Kuenen, 2005; Banciu et al., 2008; Sorokin et al., 2011a). Haloalkaliphilic members of versatile fermentative spirochaetes (*Spirochaeta alkalica*, *S. africana*, *S. asiatica*, *S. americana*, and *S. dissipatitropha*) were isolated from brine sediments of Lake Magadi in Kenya, from sulfide-saturated mud of Lake Khatyn (Tuva, Siberia), and from sediments of the saline–alkaline Mono and Owens Lakes (California) (Zhilina et al., 1996; Hoover et al., 2003; Pikuta et al., 2009).

Examples of haloalkaliphilic and natronophilic Gram-positive anaerobes isolated from soda environments are the acetogenic *Natronoincola histidinovorans* (Zhilina et al., 1998) and members of the genus *Tindallia* (Pikuta et al., 2003; Alazard et al., 2007), halophilic and alkalithermophilic *Natronaerobius thermophilus*, *N. trueperi*, and *Natronovirga wadinatronensis* (Mesbah et al., 2007; Mesbah and Wiegel, 2009). Haloalkalitolerant and haloalkaliphilic methanotrophs such as *Methylomicrobium buryatense* (Kaluzhnaya et al., 2001), *M. alcaliphilum*, and *M. kenyense* (Kaluzhnaya et al., 2008; Sorokin et al., 2000) identified in the sediments of moderate or highly saline soda lakes are Gram-negative, aerobic bacteria responsible of methane oxidation and formation of CO₂ and various organic C₁ compounds. Methanol, formaldehyde, and formate formed as products of partial methane oxidation by aerobic methanotrophs, as well as methylamines produced by the breakdown of organic osmolytes, are used as carbon source by moderately

haloalkaliphilic aerobic methylobacteria such as *Methylophaga alcalica*, *M. natronica*, *M. lonarensis*, and *Methylnatronum kenyense* (Doronina et al., 2003a, b; Antony et al., 2012; Sorokin et al., 2007; for review, see Trotsenko et al., 2007).

In addition to bacterial populations, the prokaryotic communities of soda lakes comprise a variety of haloalkaliphilic archaea, many of them belonging to the *Halobacteriaceae* family. Haloalkaliphilic archaea isolated from soda lakes in East Africa, Central Asia, and the USA are found within the genera *Natrialba*, *Natronomonas*, *Natronococcus*, *Natronobacterium*, and *Natronorubrum* (Grant and Sorokin, 2011).

Other haloalkaliphilic bacteria possessing interesting metabolic characteristics or promising biotechnological applications have been isolated from saline-alkaline soils (e.g., the N_2 -fixing Gram-positive anaerobic *Natronobacillus azotifigens*) (Sorokin et al., 2008f), soda lakes sediments (*Nitriiliruptor alcaliphilus* capable of growing on nitriles) (Sorokin et al., 2009), soda lake water (arsenite-oxidizing *Alkalilimnicola ehrlichii*) (Hoeft et al., 2007), coastal lagoon mud (Gram-positive anaerobe fermentative bacterium *Halonatronum saccharophilum*) (Zhilina et al., 2001), etc.

3. Mechanisms of High-Salt and Alkaline Adaptation in Bacteria

3.1. ADAPTATION OF THE BACTERIAL CELL ENVELOPE TO HALOALKALINE CONDITIONS

Cell envelopes (cell wall and cytoplasmic membrane) are the first line of defense and, implicitly, of adaptation to changing of environmental factors. The main constituents of cell wall are polysaccharides and proteins, while membranes consist of mainly lipids and proteins. In bacteria, membrane lipids are typically represented by polar phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and diphosphatidylglycerol or cardiolipin (CL) and nonpolar lipids such as isoprenoids (e.g., quinones, squalene, pigments). Ester phospholipids are constituted from a glycerophosphate backbone and two fatty acids esterified at the C2 and C3 positions. Therefore, the bulk of cell-extracted fatty acids originates from the plasma membrane. In this regard, it is not surprising that a significant part of the halophilic and alkaliphilic adaptation kit in bacteria might include more or less subtle changes of the membrane fatty acid structure and composition. As can be seen from the following evidences, besides particular ways of adapting the lipid composition, there are some general rules that apply in most high-salt and high-pH adaptation strategies.

3.1.1. Phospholipid and Fatty Acid Composition of Halophilic, Alkaliphilic, and Haloalkaliphilic Membranes

Based on their hydrocarbon chain structure, fatty acids can be divided into two main classes: straight-chain and branched-chain fatty acids (BFAs). Alicyclic (cyclopropane) fatty acids (CFAs) contain a cyclic structure within their molecule

and are characteristic of Gram-negative bacteria (e.g., C19:0 cyclo ω 7c). In general, many Gram-positive bacteria have significant amounts of BFAs (Kaneda, 1977). The most common straight-chained fatty acids found in bacteria are the saturated C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C16:1 (palmitoleic acid), and the monounsaturated C18:1 (oleic acid). Branching of fatty acids generally occurs as iso and anteiso positioning of methyl residue at the second or at the third to the last carbon in the chain. Examples of iso-branched and anteiso-branched fatty acids that are widespread in Gram-positives are iso-C17:0 (15-methylpalmitic acid) and anteiso-C17:0 (14-methylpalmitic acid). Fatty acids are presently used as important chemotaxonomic markers in differentiation of bacterial species (Oren, 2012).

The major functional difference between the straight and branched chains, as well as between saturated and unsaturated fatty acids, resides in their contribution to the membrane fluidity (Kaneda, 1991). Variations of saturation, length, and branching in fatty acids are, therefore, one of the key factors changing the membrane fluidity as a short- and long-term response to environmental stress. Nevertheless, adjustments of the fatty acid composition are adding value to other adaptive changes in membrane lipids, such as modification of the zwitterionic to anionic lipid ratios (i.e., PE to PG and/or CL) or varying the production of isoprenoid derivatives.

The effects of varying salt concentration on membrane lipid composition are relatively well documented in halophilic bacteria with a broad salt tolerance such as *Vibrio* spp., *Halomonas* spp., and *Chromohalobacter* spp. (Adams et al., 1990; Adams and Russell, 1992; Russell, 1993; Vreeland et al., 1984; Arahall et al., 2001; Vargas et al., 2005b). As general conclusions drawn from experiments of salt stress effect on lipid composition in bacteria, one can affirm that the membrane fluidity is finely modulated by a complex interplay between environmental factors (temperature and salinity) on one side and cell response in terms of different phospholipids and fatty acids production on the other side. In Gram-negative halophilic bacteria (e.g., *Halomonas* spp.), growth at elevated salt concentration is correlated with high contents of straight chain, more unsaturated and lesser branched-chain fatty acids in the membranes (Monteoliva-Sanchez et al., 1988; Adams et al., 1990). At increasing salinity or at close to optimal growth salinity, the relative amount of long-chain acids (with C16 < C18) increases, whereas that of short-chain acids (C14 and/or C15) decreases (Imhoff and Thiemann, 1991; Valderrama et al., 1998). At high salt (as NaCl) concentration, the anionic phospholipids (PG) predominate over zwitterionic ones (PE). Additionally, CL and CFA increase at high salt concentration as observed in various moderately halophilic bacteria (for a review, see Ventosa et al., 1998). In the extremely halophilic bacterium *Salinibacter ruber*, CL is making up to 20 % of total polar lipids, a significantly higher proportion than that found in other halophilic bacteria (Lattanzio et al., 2009). The role of CL in the energetic membranes is well known. Cardiolipin is a major membrane lipid that binds *c*-type cytochromes and cytochrome *c* oxidase (Choi and Swanson, 1995; Robinson, 1993; Schägger, 2002)

maintaining their optimal activity. In archaeal membranes CL derivatives are associated with terminal oxidases and are considered essential for haloarchaea adaptation at osmotic stress (Corcelli, 2009). Significant amounts of CL are correlated with high cytochrome *c* contents and cytochrome *c* oxidase activity in the membranes of alkaliphilic *Bacillus* strains (Clejan et al., 1986; Hicks and Krulwich, 1995) as well as in the natronophilic SOB *Thioalkalivibrio* spp. (Banciu et al., 2005).

Adaptation to high pH is similarly reflected in the membrane composition. In the alkali-tolerant *Listeria monocytogenes*, the cells develop a higher proportion of BFAs of the anteiso form at pH 9. It has been suggested that the balance between anteiso- and iso-fatty acids may be more important than changes in the amounts and/or degrees of saturation of fatty acids in pH adaptation (Giotis et al., 2007). The facultatively alkaliphilic *Bacillus pseudofirmus* OF4 proved an excellent and intensively studied model organism to follow the effects of varying the external pH at the phenotype and genotype level. Like many *Bacillus* species, *B. pseudofirmus* OF4 possesses branched-chain fatty acids as major acids (90 % of total fatty acids). The obligate alkaliphilic *Bacillus* strains contain unusually high proportions of unsaturated fatty acids (20 % of the total fatty acids and most common being C16:1). In addition, obligate alkaliphilic strains of *Bacillus* have appreciable amounts of PG, CL, long-chain fatty acids, squalene, and C40 isoprenoids as compared to facultative strains (Clejan et al., 1986). Interestingly, the mixture of a high concentration of BFAs and a substantial fraction of unsaturated fatty acids (UFAs) may make the membrane leaky at a suboptimal (neutral) pH in the obligate alkaliphiles, as implied from the experiments performed by Dunkley et al. (1991).

In many Gram-positive moderately halophilic, alkaliphilic, or alkali-tolerant species related to genus *Bacillus* (e.g., *Alkalibacillus* spp., *Halalkalibacillus* spp., *Oceanobacillus* spp.), the prevailing cell fatty acids are the BFAs (see also Table 2). However, dominance of BFA in cell membrane seems to be a general feature of Gram-positives (Kaneda, 1991) suggesting that the distinctive haloalkaline adaptations might be accommodated within the cell wall structure. It is considered that the branched anteiso-C15 fatty acid has a comparable function to that of UFAs in bacteria with a straight-chain lipid type, mainly because it has the lowest phase transition temperature ($T_m = -16.5$ °C) of all BFAs (Kaneda, 1991). The biosynthesis of UFAs occurs either aerobically or anaerobically (Aguilar and de Mendoza, 2006). UFAs, mostly as C16:1 ω 7 and C18:1 ω 7, are relatively common among anaerobes such as members of the family *Clostridiaceae* (Zhu et al., 2009). Representatives of haloalkaliphilic anaerobic bacteria (e.g., *Tindallia* spp., *Natroniella* spp., *Natronincola* spp.) contain significant amounts of UFAs. UFAs have a lower transition phase temperatures than their saturated counterparts, and consequently, they may profoundly influence the membrane fluidity (Silvius, 1982; Aguilar and de Mendoza, 2006).

Cyclopropane fatty acids (CFAs) are synthesized by the transfer of a methylene group from *S*-adenosyl-L-methionine to a double bond of unsaturated

Table 2. Fatty acid (FA) composition of several representative species of Gram-positive and Gram-negative bacteria with various degrees of halophily and alkaliphily.

Species	Physiological type ^a	Optimal pH/salinity	Dominant FA ^b	References
Gram-positive bacteria				
<i>Bacillus locisalis</i> CGI ^T	Moderately haloalkaliphilic aerobe	10/10 % (w/v) NaCl	anteiso-C15:0, iso-C15:0, anteiso-C17:0	Márquez et al. (2011)
<i>Oceanobacillus thelyensis</i> KCTC 3954 ^T	Extremely halotolerant and facultatively alkaliphilic aerobe	7.5–9.5/3 % (w/v) NaCl	iso-C15:0, anteiso-C15:0, C16:0	Lee et al. (2006)
<i>Nitriinuptor alkaliphilus</i> ANL-iso2(^U)	Moderately halotolerant and obligately alkaliphilic aerobe	9.0–9.5/0.2–0.3 M of total Na ⁺	C16:0, iso-C14, C16:1, C14:0, iso-C16	Sorokin et al. (2009)
<i>Alkaliphilus halophilus</i> E2R ^T	Moderately haloalkaliphilic anaerobe	8.0/7.5 % (w/v) NaCl	iso-C15:0, iso-C15:1, iso-C13:0	Wu et al. (2010)
<i>Natronaerobius thermophilus</i> JW/NM-WN-LFT	Halophilic alkalithermophilic anaerobe	pH ^{55 °C} 9.5/~12 % (w/v) NaCl	iso-C15:0, iso-C17:0, C16 :0DMA	Mesbah et al. (2007)
<i>Tindallia magadiensis</i> Z-7934	Obligate natronophilic anaerobe	8.5/3–6 % (w/v) NaHCO ₃	C16:1, aldehyde-C16	Zhilina et al. (1998)
Gram-negative bacteria				
<i>Indibacter alkaliphilus</i> LW1 ^T and <i>Nitritalea halalkaliphila</i> LW7 ^T	Halotolerant and alkaliphilic aerobes	10/5 % (w/v) NaCl	iso-C15:0, iso-C17:0 3-OH	Anil Kumar et al. (2010a, b)
<i>Thioalkalimicrobium</i> <i>aerophilum</i> AL3 ^T	True natronophilic, low-salt-tolerant aerobe	9.8–10/0.6 M of total Na ⁺	C18:1, C16:1, C16:0	Banciu et al. (2005)
<i>Thioalkalivibrio versutus</i> ALJ15	True natronophilic, extremely salt-tolerant aerobe	10–10.2/0.6–1.2 M of (total Na ⁺)	C18:1, C16:0, eye-C19	Banciu et al. (2005)
<i>Halomonas campaniensis</i> 5AG ^T	Extremely halotolerant and facultatively alkaliphilic aerobe	9.0/10 % (w/v) NaCl	C18:1, C16:1, anteiso-C16:0, iso-C16:0	Romano et al. (2005a)

(continued)

Table 2. (continued)

Species	Physiological type ^a	Optimal pH/salinity	Dominant FA ^b	References
<i>Natronincola histidinovorans</i> Z-7940 ^T	Moderately haloalkaliphilic and natronophilic anaerobe	9.4/8–10 % (w/v) NaCl	C16:1, aldehyde-C16 C14:0; C16:0; C18:0	Zhilina et al. (1998)
<i>Natroniella acetigena</i> Z-7937 ^T	True haloalkaliphilic and natronophilic, anaerobe	9.7–10.0/12–15 % (w/v) NaCl	C14:0; C16:0, C18:0, C16:1, aldehyde-C16:1	Zhilina et al. (1996, 1998)
<i>Desulfonatronum thioautotrophicum</i> and <i>D. thiosulfatophilum</i>	Obligately moderate natronophilic anaerobes	9.5–10/0.4–0.6 M of total Na ⁺	iso-C17:1, iso-C15:0, C18:1, C16:1, C14:0	Sorokin et al. (2011c)

Taxa were chosen to reflect a broad variety of physiological types.

^aAll haloalkaliphilic species selected are mesophilic (optimal temperature 30–37 °C) with the exception of the halophilic alkalithermophile *Natronaerobius thermophilus*.

^bDominant fatty acids are listed in approximate order of decreasing concentration. In some situations, the summing of partial FA belonging to a structural type may exceed the dominant FA of other type.

fatty acid chains of membrane phospholipids. This conversion is catalyzed by cyclopropane fatty acid (CFA) synthase. CFA synthase is present in many bacteria and it is recognized to regulate the membrane lipid composition and fluidity, thus playing an essential part in the adaptation of bacteria in response to a drastic perturbation of the environment (To et al., 2011). The extremely salt-tolerant natronophilic *Thioalkalivibrio* strain ALJ15 has a very similar fatty acids composition to the closely related *Ectothiorhodospira* spp. and *Halorhodospira* spp. (Imhoff and Thiemann, 1991) and *Halomonas* spp. (Valderrama et al., 1998; Romano et al., 2001), in which C16:0, C18:1, and cyc-C19 are the dominant fatty acids. The concentration of monounsaturated C18:1 is, however, two times higher in haloalkaliphilic SOB than in the halophilic *Halomonas salina*. Moreover, the concentration of cyc-C19 is significantly (5 times) higher in the natronophilic *Thioalkalivibrio* spp. than that measured in *Ectothiorhodospira* and *Halomonas salina*. Increasing salt concentration induced the decrease of monounsaturated C18:1 and concomitant increase of cyc-C19 in *Thioalkalivibrio* spp. (Banciu et al., 2005), *Halomonas* spp. (*H. salina* and *H. halophila*) (Valderrama et al., 1998; Monteoliva-Sanchez et al., 1988), and *Lactobacillus* strains (Gilarova et al., 1994). This is consistent with the knowledge that cis-monounsaturated fatty acids like C18:1 can be converted into cyc-C19 and vice versa. This may suggest a salt-dependent activation of CFA synthase. The raised CFA content at the expense of unsaturated fatty acids would contribute to the increasing of membrane rigidity (Russell, 1993). A stimulation of CFA synthase activity by addition of organic compatible solutes (e.g., glycine betaine) was observed in the moderately halophilic bacterium *Pseudomonas halosaccarolytica* (Monteoliva-Sanchez et al., 1993). CFAs such as cyc-C19 are considered as biomarkers for Gram-negative bacteria in a similar way as the BFAs for the Gram-positives. They are common in the membrane of anaerobic SRB and have been associated with the low redox potential in soils (Dowling et al., 1986; Zelles, 1999).

The less common aldehydes and dimethylacetal (DMA) fatty acids are specifically obtained during extraction of cellular fatty acids from anaerobes, and they are often used to differentiate between species. Aldehydes and 1,1-dimethylacetals result from plasmalogens by chemical lysis during lipid analysis procedures (Mayberry and Lane, 1993). Bacterial plasmalogens are proven to have a substantial effect on membrane fluidity. In the plasmalogen-deficient mutants of *Megasphaera elsdenii*, Kaufman et al. (1990) have observed the prevalence of a more stable lamellar phase. Studies on the psychrophilic actinobacteria *Subtercola boreus* and *Subtercola frigoramans*, with high contents of BFAs and DMA, have revealed that lowering of the growth temperature favored the production of DMAs with shorter carbon chain lengths and the increase of anteiso-branched DMA at the expense of iso-branched congeners (Männistö et al., 2000). Generally it is believed that the presence of plasmalogens and their glycerol acetals could be responsible for substantial plasticity of bacterial membranes, especially for cells growing at a wide range of temperatures, salinity, and the presence of solutes such as hydrocarbons and solvents that have the

potential of perturbing the bilayer arrangement of the cell membrane. Another possible role of plasmalogens in anaerobic bacteria is to protect the cell against oxidative stress (Goldfine, 2010).

Polyunsaturated fatty acids (PUFAs) are typical for cyanobacterial membranes where they are believed to play important roles in growth, respiration, and photosynthesis. The γ -linolenic acid (GLA, γ C18:3) has an extremely low phase transition temperature (-60 °C) and abounds in membranes of halotolerant *Arthrospira* (*Spirulina*) spp. Its occurrence in *Arthrospira* may be important in protecting the photosynthetic machinery from photoinhibition at low temperatures in a similar fashion as shown in *Synechocystis* strains (Mühling et al., 2005; Tasaka et al., 1996).

Squalene (C30 isoprenoid) is a nonpolar lipid, likely to be located between the lipid monolayers, in the hydrophobic core of the membranes (Haus et al., 2002). Squalene can act as a barrier decreasing the membrane permeability for ions (Clejan and Krulwich, 1988). In the eukaryotic membranes, squalenes are precursors of cholesterol synthesis, a lipid that increases the membrane rigidity.

The alkaliphilic bacteria were shown to contain high concentrations of squalene and anionic phospholipids, especially cardiolipin (Clejan et al., 1986). Squalene and its synthesizing enzyme have been detected in natronophilic SOB *Thioalkalivibrio* spp., suggesting a reinforced structure of cell membrane (Banciu et al., 2005; Muyzer et al., 2011a).

Recently, a surprisingly high amount of the triterpene compound lanosterol has been detected in *Thioalkalivibrio paradoxus*, comprising 50 % from the total membrane lipids (D. Sorokin, unpublished data). This finding indicates a possible important structural function of sterols in the alkaliphily and warrants the necessity of closer attention. One of the possibilities is that these compounds may help the cell membrane to lower the membrane permeability to ions (i.e., protons, sodium ions) and also to cope with water availability in the surroundings. In this regard, the change of cell surface composition should also involve a change in hydrophilicity (and hydrophobicity) in order to control the water flux across cell envelopes. High salt is usually associated with low water activity and danger of desiccation, while lower salt than the optimal concentration correlates with higher water activity and excessive water inflow that may result in cell lysis. In this aspect it has been demonstrated that the cell surface of Gram-negative halophilic *Halomonas elongata* increased its hydrophilic nature with the elevation of external NaCl concentration (Hart and Vreeland, 1988).

3.1.2. The Acidic Cell Wall of Halo- and Alkaliphiles

In Gram-positive alkaliphilic and halophilic bacteria, the cell wall is clearly playing an important role in protecting the cell against the stress caused by high alkalinity and salinity.

The cell wall of facultatively alkaliphilic *Bacillus halodurans* C-125 is built from peptidoglycan and two acidic polymers: teichuronic acid (TUA) and teichuronopeptide (TUP). TUA consists of galacturonic acid, glucuronic acid,

and *N*-acetylglucosamine, while TUP is a complex of polyglucuronic acid and poly- γ -L-glutamic acid (Aono, 1990). Cell wall-defective mutants of *B. halodurans* C-125 lost their ability to grow at alkaline pH, indicating the essential role of the cell wall in survival at high pH (Aono and Ohtani, 1990). Although not obligatory, the presence of negatively charged polymers in the structure of the alkaliphilic cell wall may favor the attachment of cations (such as sodium and hydronium ions) and repel hydroxide ions (Aono, 1990; Horikoshi, 1999). The presence of TUP seems restricted to cell wall of a few alkaliphilic *Bacillus* species. Only recently, the comparative genome analysis of *Oceanobacillus iheyensis* has revealed the presence of a putative protein showing significant similarity to the *tupA* gene product involved in TUP biosynthesis in *B. halodurans* (Takami et al., 2002). Proteomic and genomic analyses in the extreme facultative alkaliphile *Bacillus pseudofirmus* OF4 have shown that TUP is lacking and is substituted by an S-layer of protein nature (e.g., SlpA) (Gilmour et al., 2000; Janto et al., 2011). Additionally, a capsule made of negatively charged poly- γ -D-glutamate is anchored to the outer surface of the S-layer. Poly- γ -D-glutamate (PGA) occurs in the cell envelope of several non-extremophilic (e.g., *Bacillus amyloliquefaciens*) and facultative halophilic bacteria such as *Planococcus halophilus* and the moderately halophilic *Halobacillus halophilus* (Kandler et al., 1983; Geng et al., 2011). An α -linked L-glutamate polypeptide is present as complex exopolymers in some representatives of the archaea, including the haloalkaliphilic archaeon *Natronococcus occultus* and the extreme halophilic *Natrialba aegyptiaca* (Niemetz et al., 1997; Hezayen et al., 2001).

3.2. OSMOADAPTATION IN HALOALKALIPHILIC AND NATRONOPHILIC BACTERIA

Presence of high sodium chloride and/or sodium (bi)carbonate concentration in the external milieu of living cells results in a high osmotic pressure and low water activity. This situation, often called osmotic stress, is also analogous to a desiccation stress. Cells that are not capable of response to such unusual physical and chemical environmental parameters will lose cytoplasmic water which tends to diffuse outward the cell. Non-halophilic organisms shrink and perish in highly saline conditions. On the other hand, halotolerants and halophiles developed several strategies to counterbalance the high external osmotic pressure. Extreme halophiles are narrowly specialized to live exclusively at high osmolarity and they are harmed and lysed in hypotonic conditions, the presence of too much water becoming a “water stress.” Besides the mechanical support of the cell wall (in Gram-positive bacteria) or a strengthened cell membrane (in archaea and Gram-negative bacteria), adaptation to high salinity/high osmotic pressure (or to variations of such parameters) is aiming to an isosmotic cytoplasm. Osmoadaptation of all living cells has same principle: synthesis and/or accumulation of osmotic compatible solutes (osmoprotectants or osmolytes). Based on their

chemical nature, two main categories of osmolytes are met in nature: inorganic and organic osmolytes. Inorganic osmolytes (i.e., KCl) are imported and accumulated in molar concentrations mainly by neutrophilic halophilic and natronophilic archaea as the so-called “salt-in” strategy of osmoadaptation. Some bacteria within the orders *Natranaerobiales* and *Halanaerobiales* and the renowned example of halophilic bacterium *Salinibacter ruber*, a member of the *Bacteroidetes*, also use KCl as prevailing osmolyte (Oren et al., 2002). Interestingly, short-term osmoadaptation in *Escherichia coli* and *Salmonella typhimurium* involves an initial quick import of potassium ions followed by synthesis of glutamate as counterion. This response is enough only to assure the survival of the bacterial cell. The long-term osmoadaptation in such halotolerants, however, is based on the accumulation and/or synthesis of organic compatible solutes (e.g., trehalose, proline, or glycine betaine) which are less harmful to the cytoplasmic constituents (Oren, 1998).

Almost all known haloalkaliphilic or natronophilic bacteria have adapted to withstand a high osmotic pressure either by taking up or by de novo synthesis of organic compatible solutes (“salt-out” strategy). Unlike inorganic osmolytes, the presence of organic compatible solutes in the cytoplasm is more “friendly” to cell components, especially lipids and proteins. The most preferred organic osmolytes of bacteria are ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) and glycine betaine (*N,N,N*-trimethylglycine), followed by certain amino acids (glutamate, proline) and disaccharides (sucrose, trehalose). Accumulation of osmolytes is part of salt stress adaptation in many known halotolerant nonpathogenic and pathogenic bacteria (e.g., strains of *Escherichia coli*, *Bacillus* spp., *Corynebacterium glutamicum*, *Listeria monocytogenes*). They also may act as thermoprotectants in thermophilic prokaryotes. Organic osmolytes have a wide occurrence in algae (e.g., glycerol in *Dunaliella* spp.) and invertebrates (e.g., trehalose in the brine shrimp *Artemia salina*) living in saline ecosystems.

3.2.1. The Universal Compatible Solute, Glycine Betaine

Glycine betaine, or simply betaine, is a trimethylated derivative of glycine: a highly polar, low molecular weight, and chemically inert molecule. Betaine is broadly used as osmoprotectant in all three domains of life: bacteria, archaea, and eukaryotes (Oren, 2008; Tuteja, 2007; Lim et al., 2007). In bacteria, glycine betaine is the main osmoticum of high-salt-tolerant cyanobacteria, of halophilic anoxygenic phototrophic bacteria, and of salt-tolerant non-halophilic and halophilic heterotrophic bacteria (Imhoff and Rodriguez-Valera, 1984; Oren, 2002).

In halophilic archaea, de novo biosynthesis of glycine betaine is a rarity; it was assigned to one representative of halophilic methanogens, *Methanohalophilus portucalensis* both by physiological and genetic evidences (Robertson et al., 1990; Lai and Lai, 2011). Additionally, osmoadaptation in *M. portucalensis* involves betaine import as well as accumulation of potassium ions up to 1.1 M concentration (Lai et al., 1991). The analysis of the haloarchaeal genomes known by 2011, however, has revealed that 9 out of 10 genomes encode members of the

betaine–choline–carnitine (BCC) transporter family (Anderson et al., 2011). Glycine betaine accumulation is a more preferred strategy in many halophilic bacteria and in some halophilic archaea (e.g., *Methanosarcina mazei*, *Halococcus hamelinensis*) (Mackay et al., 1984; Spanheimer and Müller, 2008; Burns et al., 2012). Several moderately halophilic/halotolerant and alkaliphilic/alkalitolerant Gram-positive bacteria such as *Alkalibacillus filiformis* and *Oceanobacillus oncorhynchi* accumulate glycine betaine and glutamate (Romano et al., 2005b, 2006b). In the halotolerant alkaliphilic *O. iheyensis*, a large number of solute transport proteins have been inferred from genome analysis. Several genes encode OpuD-like ABC transporters that facilitate betaine and choline uptake, as well as putative BCC transporters and Na⁺/proline or pantothenate symporters (OpuE transporters). It seems that the organism, which can grow between 0 and 18 % (3.6 M) NaCl, is employing an unusual high number of osmoprotectant transporters, especially when it must cope with a salinity of at least 3 M NaCl (Takami et al., 2002). Similar genes, although in a lesser number, are also present in genomes of the facultatively alkaliphilic and slightly halotolerant *Bacillus halodurans* C-125 and in the facultatively alkaliphilic *B. pseudofirmus* OF4 (Takami et al., 2000; Janto et al., 2011). Overall it seems that Gram-positive bacteria respond to osmotic stress by activating and using an important number of ABC and BCC transporters and proteins from the sodium/solute symporter (SSS) family. They possess the ability to import betaine when available in the growth medium or to import betaine precursors (i.e., choline) for cytoplasmic synthesis of this compatible solute.

De novo synthesis of betaine in halophiles is restricted to several autotrophic members of the cyanobacteria and the *Ectothiorhodospiraceae* (Oren, 2008). *Halorhodospira halochloris*, *Ectothiorhodospira marismortui*, and *Ect. haloalkaliphila* are well-known examples of glycine betaine accumulation in anoxygenic halophilic and haloalkaliphilic phototrophs (Galinski and Trüper, 1982; Imhoff, 1993). Unusually, in the haloalkalitolerant cyanobacterium *Aphanothece halophytica*, Laloknam et al. (2006) have detected a Na⁺/betaine transporter, BetT_{A. halophytica}. This transporter is a member of BCC transporter family and is related to OpuD in *B. subtilis* and ProU in *E. coli*. It has a high affinity for betaine and differs from Gram-positive OpuD by its low isoelectric point (4.58, in comparison with the basic value pI 9.54 of OpuD from *B. subtilis*). This is the first and only description of a betaine transport system in cyanobacteria to date. Computational analysis of the *Halorhodospira halophila* (strain DSM 244/SL1) genome has revealed the existence of putative BCC transporters (Hhal_2364, Hhal_1851, Hhal_1384) as well as of a glycine betaine/L-proline ABC transporter (Hhal_0233).

Obligately chemolithoautotrophic sulfur-oxidizing bacteria (SOB) of the genus *Thioalkalivibrio* are closely related to *Ectothiorhodospira* spp. and *Halorhodospira* spp., being the dominant sulfur-oxidizing bacteria in soda lakes. Most of the *Thioalkalivibrio* species are true natronophiles, with optimal growth at pH 10 and at 2 M of total Na⁺. Experimental evidence has shown that in

Thioalkalivibrio versutus strain ALJ15 grown in substrate-limited continuous culture at optimal salinity and pH, glycine betaine was the main organic compatible solute. When grown at different salt concentrations, strain ALJ15 accumulated betaine in a salt-dependent fashion: 1.5, 7.5, and 9 % of total dry weight at 0.6, 2, and 4 M Na⁺, respectively. Sucrose was produced as a minor secondary organic compatible solute (0.3–2.5 % of the total dry weight) in this organism, and its concentration was highest in cells grown at 2 M Na⁺. It was clear that *Thioalkalivibrio* spp. grown exclusively on organic-free medium is capable of de novo synthesis of glycine betaine as osmoadaptation strategy (Banciu et al., 2004a, b, 2005). Genome analysis in the *Thioalkalivibrio* spp. K90mix (phenotypically similar to strain ALJ15) and *Thioalkalivibrio sulfidophilus* HL-EbGr7 (phenotypically different from strain ALJ15) has indicated the presence of the genes for glycine sarcosine N-methyltransferase and sarcosine dimethylglycine methyltransferase. In addition, *Thioalkalivibrio* spp. genomes contain the gene for sucrose phosphate synthase needed for the production of sucrose as a minor compatible solute (Muyzer et al., 2011a, b). Biosynthesis of glycine betaine starting from glycine is a three-step methylation process: glycine sarcosine dimethylglycine betaine, and is an energetically expensive pathway impairing growth at extreme salinity conditions (Nyssölä et al., 2001; Oren, 1999). Other chemolithoautotrophic SOB that produce glycine betaine as main compatible solute include the extremely halophilic *Thiohalorhabdus denitrificans* and *Thiohalospira halophila* and the moderately halophilic and facultatively alkaliphilic *Thiohalospira alkaliphila*, all gammaproteobacteria. The first two species are among the most extreme halophilic lithoautotrophs known, with optimal salinity at 18 % w/v NaCl. They were isolated from surface sediments of inland hypersaline lakes in Russia, Crimea, and Central Asia and from a sea saltern at the shore of the Adriatic Sea. *Thiohalospira alkaliphila* was isolated from a hypersaline–alkaline lake in the Wadi Natrun and grows up to pH 10 and within a range of 0.5–4 M NaCl (optimum at 2 M NaCl). All these obligately chemolithoautotrophic SOB were grown on mineral media at 4 M and accumulated betaine up to one third of the total cell mass which was clearly much higher than in their natronophilic counterpart *Thioalkalivibrio* ALJ15 (Sorokin et al., 2008a, c). The reason for this is explained below.

Chemolithoautotrophic SOB such as *Thioalkalivibrio halophilus* HL17^T and *Thioalkalibacter halophilus* ALCO1^T grow well both at high NaCl and Na₂CO₃/NaHCO₃ concentration (up to 4 M of total Na⁺), equally at pH 7.5 and 10. Such versatile, facultatively alkaliphilic high-salt-tolerant halophilic and facultatively natronophilic strains are ideal candidates for a direct study of the physiological effects of chloride versus carbonate/bicarbonate anions at same (high) sodium concentration, as well as at of variable pH. The NaCl-grown biomass of *Tv. halophilus* contained 19.8 % (w/w) glycine betaine, while the soda-grown biomass contained 12.4 % glycine betaine at the same concentration (4 M) of total Na⁺ (Banciu et al., 2004b). A comparable trend has been observed in haloalkaliphilic *Thioalkalibacter halophilus*. Unlike *Tv. halophilus*, strain ALCO1 is producing

ectoine as the main compatible solute and betaine as a minor one. The specific concentration of osmolytes (ectoine and glycine betaine) measured in strain ALCO1 cells grown at 3 M NaCl (pH 7.5) was approximately two times higher than in cells grown at 3 M Na-soda, pH 10 (Banciu et al., 2008). Dissimilarities in the production of compatible solutes in NaCl- and soda-grown cells could be at least partially explained by physicochemical differences between NaCl and $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ solutions. A decisive parameter that may influence the accumulation of osmolytes in halophiles and natronophiles could be the osmotic pressure of the chloride- and soda-rich media which is determined primarily by the electrolytic features of two types of sodium salts (see Table 3 in Banciu et al., 2004b). The calculated osmotic pressure in the 4 M NaCl solution was 2.8 times higher than that of 2 M Na_2CO_3 (= 4 M Na^+). This result is reasonably close to the experimentally measured osmotic pressure in growth media with 4 M NaCl and 4 M Na^+ soda (as a mixture of $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$), at 30 °C, a value which is 1.8 times higher in chloride medium than in carbonate medium. This clearly indicates that apart from the many negative effects of high alkalinity pH, the life in soda brines also has some benefits as compared to the life in NaCl-rich environment. Such physiological experiments on model organisms, complemented by theoretical simulations, could add more hard evidence to our suggestion of using the term natronophily as a special, yet different, form of halophily. As sodium sulfate brines have essentially the same electrolytic properties as sodium carbonate, one may expect that, except for the high pH, the osmoadaptation pattern of natronophiles put into Na_2SO_4 brines might also be similar.

3.2.2. Ectoine Is a Multivalent Compatible Solute

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a derivative of aspartate. It was discovered in the phototrophic bacterium *Halorhodospira halochloris* (Galinski et al., 1985). In a similar manner as betaine, ectoine is accumulated by import and/or synthesis in salt-stressed cells. Unlike glycine betaine which is ubiquitous among bacteria, ectoine is the preferred compatible solute in many aerobic chemoheterotrophic eubacteria. The hydroxylated derivative of ectoine, β - (or 5)-hydroxyectoine, is more frequently found among halophilic and halotolerant Gram-positive bacteria (Severin et al., 1992; Detkova and Boltyanskaya, 2007). Ectoine alone is a minor compatible solute in the anoxygenic phototrophic *Halorhodospira* spp., while together with hydroxyectoine, ectoine is the dominant osmolyte in *Halomonas* spp., *Salinivibrio* spp. among the *Proteobacteria*, and in *Marinococcus* spp. and *Virgibacillus* spp., members of the *Firmicutes* (Severin et al., 1992; Grant, 2004).

By using ^{13}C -NMR spectroscopy and HPLC analysis, Kuhlmann and Bremer (2002) have proven de novo synthesis of ectoine in a variety of *Bacillus* and *Bacillus*-related species grown under salt stress conditions. The obligate alkaliphilic *B. alcalophilus* synthesized ectoine, the halophilic and alkalitolerant *Virgibacillus salexigens* produced both ectoine and hydroxyectoine, whereas *Virgibacillus pantothenicus* synthesized both ectoine and proline. The

ability to synthesize ectoine from L-aspartate-semialdehyde in a three-step pathway is widespread within the genus *Bacillus* and closely related taxa. In these Gram-positive microbes, ectoine biosynthetic genes, *ectABC*, encode diaminobutyric acid (DABA) acetyltransferase (EctA), DABA aminotransferase (EctB), and ectoine synthase (EctC). Expression of *ect* genes is controlled by salinity or osmotic pressure outside the cell (Louis and Galinski, 1997). The *ectABC* genes and the proteins for ectoine synthesis are highly conserved among the *Proteobacteria* and the *Actinobacteria* that synthesize ectoine (Lo et al., 2009).

In certain species (e.g., *Halomonas elongata*), stress conditions such as elevated temperature trigger hydroxylation of some ectoine to form hydroxyectoine. In species capable of hydroxyectoine formation, two different genes, *ectD* and *ectE*, coding for ectoine hydroxylase have been identified (*ectD* in *Virgibacillus salexigens* and *ectDlectE* in the halotolerant gammaproteobacterium *Chromohalobacter salexigens*) (Bursy et al., 2007; García-Estepa et al., 2006).

Most ectoine synthesizing species have also developed ectoine import systems. In *V. pantothenicus*, another gene, *ectT*, encodes a protein (EctT) that is a member of the BCC carriers. The EctT transporter specifically mediates the import of ectoine and hydroxyectoine but also possesses minor uptake activities for proline and glycine betaine (Kuhlmann et al., 2011).

In the extremely halotolerant *Halomonas elongata* DSM 2581^T, Grammann et al. (2002) discovered a novel solute transporter, termed TeaABC, that belongs to the TRAP (tripartite ATP-independent periplasmic) transporters family. The osmoregulated transporter TeaABC is encoded by three genes (*teaABC*), and it mediates the uptake of ectoine and hydroxyectoine. Its assumed physiological role in *H. elongata* would be the recovery of ectoine that is excreted or lost in the surroundings of the cell by a yet unknown mechanism. Active recovery of ectoine (and/or other organic osmolytes) after solute excretion upon osmotic downshock might be an energy-saving strategy for salt tolerance in organisms that must cope with shifts of salinity in their environment (Oren, 1999). The amino acid sequences of the small, TeaB (YP_003899344.1) and large, TeaC (YP_003899345.1) transmembrane components of TeaABC carrier from *H. elongata* are significantly similar (40 and 56 %, respectively) to those of the small (NP_244258.1) and large (NP_244257.1) subunits of TRAP transporter deduced from the *B. halodurans* genome.

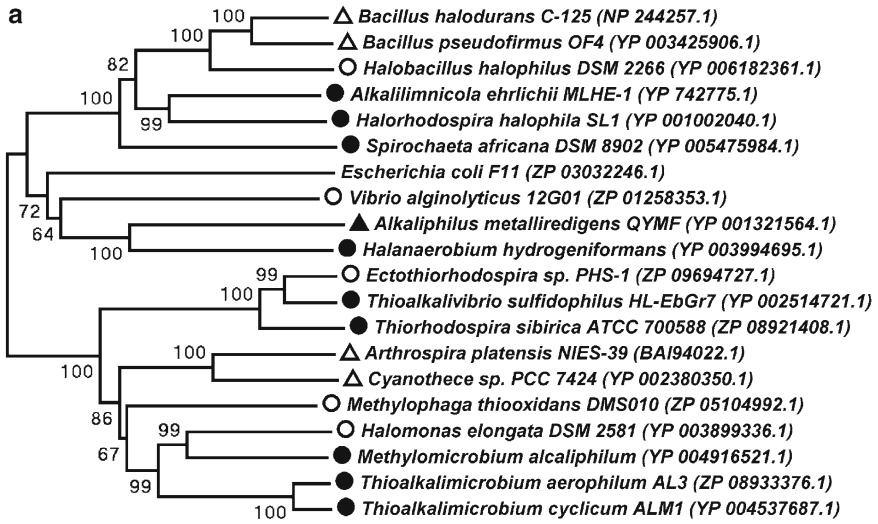
In aerobic halophilic and halotolerant methylobacteria from soda lakes, the main compatible solute is ectoine, followed by glutamate and sucrose. The total pool of compatible solutes is enlarged by increasing NaCl concentration in *Methylobaculum alcaliphilum*, *Methylophaga natronica*, *M. alcalica*, and *M. lonarensis* (Trotsenko and Khmelena, 2002; Doronina et al., 2003a, b; Trotsenko et al., 2007; Antony et al., 2012). The gene cluster *ectABC* has been identified in *Methylobacter marinus* and the natronophilic *Methylobaculum kenyense* AMO1^T. In other C₁-utilizing bacteria (e.g., the haloalkaliphilic *Methylobaculum alcaliphilum* ML1 and *Methylophaga alcalica* M8, and the

moderate halophilic *Methylophaga thalassica* 33146^T and *Methylarcula marina* h1^T) the *ectABC-ask* operon is additionally present, encoding aspartate kinase (Ask). Upstream the gene cluster *ectABC-ask*, another gene, *ectR*, encodes the MarR-like transcriptional regulator named EctR. In methano/methylotrophic bacteria hydroxyectoine is lacking, a fact proven by the absence of *ectD*-like genes (Reshetnikov et al., 2011).

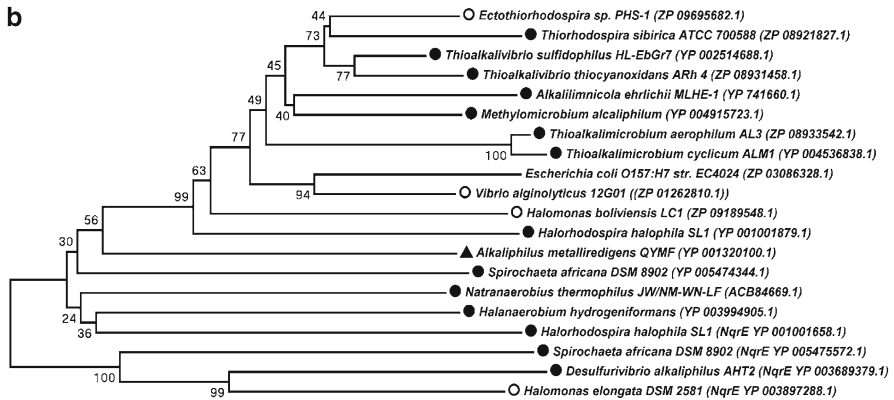
Chemolithoautotrophic natronophilic and haloalkaliphilic SOB from the genera *Thioalkalimicrobium* and *Thioalkalibacter* also produce ectoine as the prevailing compatible solute (Banciu et al., 2005, 2008). The production of ectoine in *Thioalkalimicrobium aerophilum* AL3^T is clearly stimulated by increasing the total Na⁺ concentration from 0.2 to 1.2 M in the growth medium. At the highest salinity tolerated by the organism (1.2 M of total Na⁺), ectoine accounted for 8.7 % of the total dry weight. Another organic osmolyte detected in strain AL3^T in minor amounts was glutamate. The glutamate production decreased with increasing salt concentration (Banciu et al., 2005). Apparently, species of *Thioalkalimicrobium* are also capable of ectoine uptake by the TRAP system. A BLASTP search for the 388 amino acid sequence of a putative extracellular solute-binding protein, family 7, from *Thioalkalimicrobium aerophilum* AL3^T (Accession number ZP_08933362.1) has retrieved 71 and 57 % identity with the TRAP dicarboxylate transporter – DctP subunit from *Thiomicrospira crunogena* XCL-2 (YP_392275.1) and *Marinomonas* sp. MWYL1 (YP_001343266.1), respectively, and 50 % identity score with the TRAP transporter substrate-binding protein from *Halomonas elongata* DSM 2581 (YP_003899494.1). The phylogenetic relationships of the DctM subunit of TRAP-like proteins found in several alkaliphilic, haloalkaliphilic, and halophilic bacteria are presented in Fig. 1a. Moreover, genome sequences of *Thioalkalimicrobium* spp. have also indicated the possible presence of BCC transporters in these SOB.

Although ectoine and betaine require comparable energy investments from the cell metabolism, there are a number of advantages of using ectoine over glycine betaine. Ectoine and hydroxyectoine are not only involved in osmoprotection of salt-stressed cells, but they are required for heat- and cold-shock adaptation of bacterial cells (Bursy et al., 2008; Kuhlmann et al., 2011). Hydroxyectoine has protective and stabilizing effects on proteins, including enzymes, when cells are exposed to thermal stress or desiccation, apparently playing same role as mannosylglycerate compounds in archaea (Lippert and Galinski, 1992; Borges et al., 2002). Ectoines might enhance the fluidity of the cell membrane through increasing the hydration of the surface and the mobility of the lipid head groups (Harishchandra et al., 2010).

Besides its prevalent osmoprotective role, ectoine can act as a nitrogen storage compound, as carbon and/or energy source (Galinski and Herzog, 1990; Khmelenina et al., 1999; Vargas et al., 2006). It can be degraded and reused by the cells. Ectoine degradation proceeds via hydrolysis to N α -acetyl-L-2,4-diaminobutyric acid, followed by deacetylation to diaminobutyric acid. In *Halomonas elongata*, diaminobutyric acid can either generate aspartate or

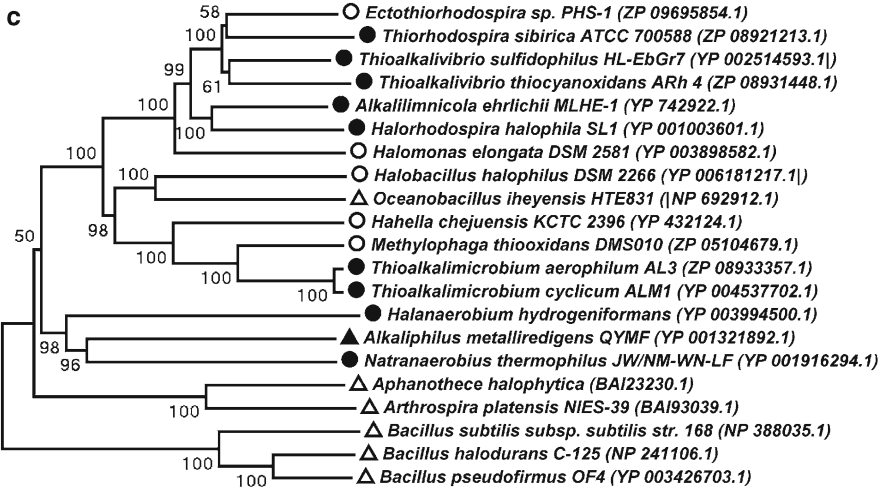


DctM subunit of TRAP transporter (C4-dicarboxylate/H⁺ symporter)

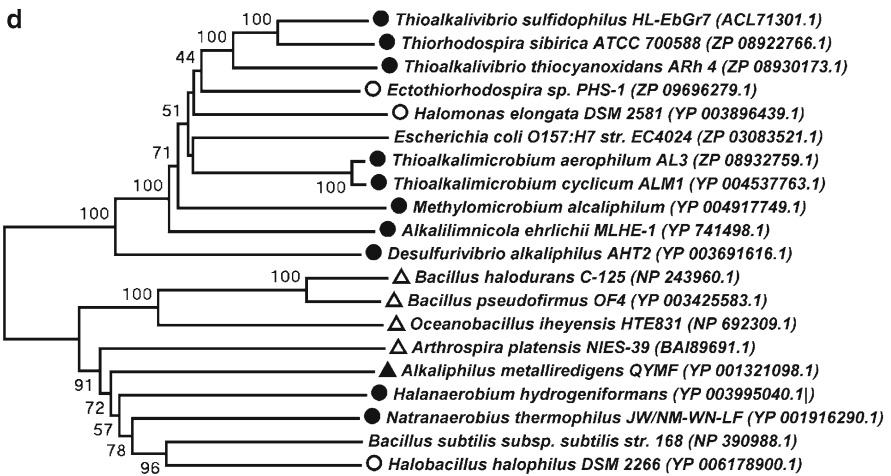


RnfA/NqrE (Na⁺-translocating NADH dehydrogenase)

Figure 1. Phylogeny of four transporters essential for haloalkaliphilic adaptation: DctM (a), RnfA/NqrE (b), MrpA/MnhA (c), and TrkH/TrkH-like proteins (d) from different haloalkaliphilic (filled circles), halophilic or halotolerant (open circles), and alkaliphilic/alkalitolerant bacteria (filled triangles/open triangles). Proteins of non-extremophilic bacterial species were used as reference sequences. Gene products or proteins were selected after BLASTP analysis. Subsequently, the selected protein sequences were aligned by ClustalW. Evolutionary analyses were conducted in MEGA5 by using the neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The bar indicates 10 % sequence difference. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in units of the number of amino acid differences per site. All ambiguous positions in amino acid sequences were removed for each sequence pair.



MrpA/MnhA subunit of Mrp or Mrp-like complex (Na⁺/H⁺ antiporter, CPA3 family)



TrkH/TrkH-like subunit of Trk system (K⁺/H⁺ antiporter)

Figure 1. (continued)

reenter the ectoine synthesis pathway, forming a cycle of ectoine synthesis and degradation. However, a comparison of the available bacterial genomes indicated that the ectoine degradation pathway exists predominantly in non-halophilic bacteria unable to synthesize ectoine. Energetic considerations indicated that ectoine turnover might be fast and it could be finely tuned by the cell's necessity to respond promptly to changing conditions (Schwibbert et al., 2011). On the other hand, occurrence of ectoine as the main compatible solute in halotolerant and halophilic alkaliphilic aerobic methane-oxidizing bacteria as well as in low-salt-tolerant natronophilic *Thioalkalimicrobium* spp. is correlated with the limitation of their tolerance to high salt (<15 % w/v NaCl). At the same time, most of haloalkaliphilic phototrophic SOB (e.g., *Halorhodospira* spp.), natronophilic SOB (e.g., *Thioalkalivibrio* spp.) and SRB (e.g., *Desulfonatronospira* spp.), and extremely halophilic SOB (i.e., *Thiohalorhabdus* spp., *Thiohalospira* spp.) thrive under a broad range of salinity up to extreme salinity (>20 % w/v NaCl), and this ability is often associated with the use of betaine as the main compatible solute (Oren, 1999; Banciu et al., 2005; Sorokin et al., 2008b; Muyzer et al., 2011a, b). These observations allow us to conclude that the glycine betaine is a more efficient osmolyte at extreme salt concentration.

3.2.3. Glutamate as an Additional Anionic Osmolyte

Glutamate is an acidic compatible solute usually associated with low-salt response in halotolerant non-halophilic marine bacteria, moderately halophilic bacteria from the *Firmicutes* and the *Gammaproteobacteria*, and methanogenic archaea. In most cases, glutamate is accompanied by various other compatible solutes (potassium ions, glycine betaine, glutamine, ectoine, proline). As already stated, some aerobic methylotrophic and chemolithoautotrophic SOB bacteria from soda lakes are capable of salt-dependent glutamate production.

In the moderately haloalkaliphilic *O. iheyensis* and *B. halodurans*, glutamate synthesis from the branched-chain amino acids (i.e., leucine, isoleucine, and valine) is thought to play a notable role in alkaline adaptation. The putative ABC transporters for branched-chain amino acids identified in their genomes could facilitate the uptake of glutamate precursors. Since L-glutamic acid is negatively charged at pH values above its pKa (3.9 or 4.3), the converted L-glutamic acid and its accompanying proton could contribute to the cytoplasmic pH which is about two units lower than the external pH of around 10.5 (Krulwich et al., 2007; Takami, 2011).

3.2.4. Sucrose and Trehalose: Minor Osmolytes with Stabilizing Roles

Nonreducing disaccharides such as sucrose and trehalose are energy costly compounds involved in osmotic stress adaptation of many freshwater and low-salt-tolerant cyanobacteria (e.g., *Synechococcus* spp., *Phormidium* spp.) and non-halophilic and slightly halophilic bacteria when they need to adapt to elevated salt concentrations (Mackay et al., 1984; Oren, 1999). Unlike polyols and amino acid derivatives used as osmolytes, sucrose and trehalose have a lesser stabilizing

effect on enzymes at high salt and have lower water solubility. Disaccharides and their derivatives are the most energetically expensive compatible solutes, and therefore, their synthesis should be carefully managed in salt-stressed cells. As main compatible solute (5 % w/w, at 2.5 M Na⁺), sucrose is present in the moderately halophilic SRB *Desulfonatronovibrio*, together with the rare osmolyte compound *N*-acetylglutaminylglutamine amide (N-AGGN) found at an approximate concentration of 2 % w/w (Sorokin et al., 2011c). Sucrose, in a mixture with the amino acid glutamate, is used as a secondary compatible solute in aerobic methylotrophs from soda lakes, where sucrose increased significantly at the higher growth limit of salinity (Doronina et al., 2003a, b). As a minor osmolyte, sucrose is also present in chemolithoautotrophic SOB of the genus *Thioalkalivibrio*, where it reaches a maximum concentration (2.5 % of cell dry weight) at the optimal total sodium concentration (2 M) (Banciu et al., 2005).

Trehalose has multiple uses in osmoprotection, as thermolyte and as anti-drying agent in low-salt-tolerant cyanobacteria, halophilic actinomycetes, moderately halophiles, and thermophilic bacteria (Mackay et al., 1984; Alarico et al., 2005; Roberts, 2000). In the extremely halophilic actinomycete *Actinopolyspora halophila* grown at 24 % NaCl, Nyssölä and Leisola (2001) showed that main compatible solute was betaine (33 % of the cellular dry weight), followed by a significant amount of trehalose (9.7 % w/w). In the moderately halophilic sulfate-reducer *Desulfovibrio halophilus* grown at 15 % NaCl, trehalose is accumulated as principal osmolyte in the absence of exogenous betaine (Welsh et al., 1996). In combination with ectoine, trehalose is implicated in the thermal tolerance of halophilic *Chromohalobacter salexigens* (Reina-Bueno et al., 2012). Recent progress on deciphering prokaryotic genomes has allowed identification of genes for trehalose synthesis in halotolerant cyanobacteria and in halophilic and haloalkaliphilic bacteria and archaea. The widely distributed pathway of trehalose synthesis that involves trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP) could be inferred from genomes of *Synechococcus* spp., *Synechocystis* spp., *Halalkalicoccus jeotgali*, *Haladaptatus paucihalophilus*, *Haloterrigena turkmenica*, *Natronobacterium gregoryi*, *Natrialba magadii*, etc., as well as from those of the halophilic bacterium *Salinibacter ruber* and the haloalkaliphilic “Halanaerobium hydrogeniformans.” Another pathway converts maltodextrins (maltooligosaccharides, glycogen, and starch) to trehalose in two enzymatic steps catalyzed by maltooligosyl trehalose synthase (TreY) and maltooligosyl trehalose trehalohydrolase (TreZ), respectively (Elbein et al., 2003). Trehalose synthesis by TreY and TreZ has been observed in archaea belonging to *Sulfolobus* but can also be assumed from the genome sequence of the haloalkaliphilic methanotrophic bacterium *Methylomicrobium alcaliphilum* (Avonce et al., 2006; personal BLASTP search). It is worth mentioning that exogenous trehalose can be taken up by specialized import systems (e.g., phosphotransferase system – PTS – trehalose-specific enzyme II, and trehalose/maltose binding protein, TMBP) found in a large number of heterotrophic bacteria and archaea, as concluded from a BLASTP analysis of available amino acid sequences.

Following its uptake, trehalose is phosphorylated to trehalose-6-phosphate which can be further degraded by trehalose-6-phosphate hydrolase (TreA in *B. subtilis* and TreC in *E. coli*) to provide the cells with glucose (Helfert et al., 1995; Rimmele and Boos, 1994). TreA-like proteins are present in halophilic and alkaliphilic *Bacillus* species (*Bacillus halodurans*, *B. clausii*, *B. selenitireducens*, and *B. pseudofirmus* OF4), as well as in *Oceanobacillus iheyensis* and *Halobacillus halophilus*.

3.2.5. K^+ Is the Main Osmolyte in Anaerobic Extreme Haloalkaliphiles

The natronophilic acetogenic *Natroniella acetigena* from the order *Halanaerobiales* as well as halophilic thermoalkaliphiles belonging to the *Natranaerobiales* (*Natranaerobius* spp. and *Natronovirga* spp.) have adopted the salt-in strategy to withstand high salinity conditions. In *N. acetigena*, the measured intracellular concentrations of K^+ (ca. 0.9 M) were almost two orders of magnitude higher than the K^+ level in the medium. At the same time, intracellular Na^+ and Cl^- concentrations were close to the extracellular values (Detkova and Boltyanskaya, 2007). Intracellular K^+ concentration in representatives of the *Natranaerobiales* is reliably kept at constant values of 0.2–0.3 M over a wide range of external K^+ (8–400 mM), at optimal or suboptimal pH and salinity. Apparently, in halophilic alkalithermophiles of the order *Natranaerobiales*, potassium homeostasis is tightly regulated. Accumulation of K^+ is accompanied by that of Cl^- ions, which reach molar concentration. However, the sum of both ions does not equilibrate the cytoplasm osmolarity with that of the outer environment. The genome analysis of *Natranaerobius thermophilus* has revealed the ability of this organism to import and even to synthesize glycine betaine. Apparently, this finding was confirmed experimentally. Intracellular concentrations of glycine betaine and glutamate have been detected in molar concentrations in *Natranaerobius thermophilus*. Such an observation, if confirmed by a detailed experimental study, would constitute a first known case of a fermentative extreme halophile capable of producing organic compatible solutes and use the “salt-in” strategy concomitantly (Mesbah and Wiegel, 2012).

3.3. ADAPTATION OF THE OXIDATIVE PHOSPHORYLATION (OXPHOS) MACHINERY TO OVERCOME LOW *PMF*

In highly saline and alkaline environments, the burden due to elevated osmotic pressure is doubled by an extremely low level of H^+ concentration. The bulk medium where haloalkaliphiles and natronophiles live is weakly supporting a favorable proton-motive force (*pmf*) to drive ATP synthesis by oxidative phosphorylation pathway as postulated by Peter Mitchell. Despite the apparently adverse pH gradient, alkaliphilic and, implicitly, the haloalkaliphilic prokaryotes flourish at alkaline conditions. Moreover, haloalkaliphiles are splendidly facing the challenge of expensive life in a hyperosmotic medium by synthesizing energetically costly compatible solutes. To respond to the multiple extreme factors, which additionally include high irradiation and, sometimes, relatively high temperatures

(45–60 °C), true haloalkaliphilic and natronophilic microbes must have an efficient energy metabolism. The adaptative modifications are met not only in the core of the ATP synthesis process but are also found in the respiratory chain components and in the overall organization of the cytoplasmic enzyme machinery. In the following we will briefly discuss the alkaliphilic adaptations of the respiratory chain components as it is best known from several model organisms.

3.3.1. Features of Alkaliphilic F-Type ATP Synthase

Three major ways of generating the ATP are known in various haloalkaliphiles and natronophiles: (i) membrane-bound oxidative phosphorylation in aerobic chemotrophs, (ii) photophosphorylation linked to photosynthetic inner membranes of aerobic and anaerobic phototrophs, and (iii) substrate-level phosphorylation of anaerobic (or fermentative) bacteria. Mixed sources of ATP could be found in non-obligately metabolic situations such as facultatively aerobes and in anaerobic phototrophs that rely on either (i) and (iii) or (ii) and (iii). One of the most intriguing types of energy metabolism occurs in obligately aerobic chemolithoautotrophic alkaliphilic bacteria, where anabolic maintenance and active transport processes rely almost exclusively on oxidative phosphorylation which is directly affected by the apparently suboptimal proton electrochemical gradient.

Oxidative phosphorylation (OXPHOS) is a membrane-localized metabolic pathway that couples the generation of an electrochemical gradient with ADP phosphorylation to form ATP. In prokaryotes the transmembrane electrochemical gradient, usually based on protons, is the result of the respiration process. Respiratory chain components perform simultaneous electron transfer from an internal or external donor to an oxygen molecule as the final electron acceptor, coupled with proton pumping across the membrane toward the outer or positive (P) side of the cell. The free Gibbs energy stored in the electrochemical gradient is transferred to chemical bonds within the ATP molecule by the ATP synthase activity of F-type primary pump. Universally found in all three domains of life, F-ATP synthase is a reversible enzyme that catalyzes both ATP synthesis and hydrolysis. The hydrolytic activity of the enzyme is mainly observed in anaerobes, while in many other bacteria, this function is low or latent. The equilibrium between the hydrolytic and the synthetic function of F-type ATP synthase is carefully controlled to maintain appropriate cytoplasmic ATP and ADP concentrations. The catalytic domain of the ATP synthase complex (also termed F_1) is located in the cytoplasm. The F_1 portion of ATP synthase consists of $\alpha_3\beta_3\gamma\delta\epsilon$ subunits and is relatively well conserved among all organisms. The transporting part of the F-ATP synthase (F_0) is a transmembrane complex made of a variable number of subunits (ab_2c_{10-15} in bacteria) facing the external milieu of the cell, as well as the hydrophobic membrane. The F_0 factor of ATP synthase is highly variable among different prokaryotic species. The critical issue that functioning of ATP synthase toward ATP synthesis must address is the apparently low electrochemical gradient. Therefore, it is expected that specific structural adaptations of this key enzyme to be found in the *a*-, *b*-, and *c*-subunits of the F_0 part.

The alkaliphilic adaptations of the F-ATP synthase and OXPHOS both at the physiological and the genetic levels have been documented in-depth in the aerobic, facultatively alkaliphilic bacterium *Bacillus pseudofirmus* OF4 (for review, see Hicks et al., 2010). As previously mentioned in this review, the facultative alkaliphilic and/or halophilic organisms are appropriate models to study the changes and adaptations at the genotype and phenotype levels.

There are certain key features of the alkaliphilic F_1F_0 -ATP synthase from *B. pseudofirmus* OF4 that allow ATP synthesis under conditions of low *pmf*, and they are summarized below together with general and particular considerations regarding other alkaliphiles.

1. *The functioning of the alkaliphilic F-ATP synthase toward ATP synthesis is H⁺-coupled.* So far, all F-type ATP synthases with a high rate of ATP synthesis from true and facultative alkaliphiles are proton-coupled. The recent examples of proton-driven F₁F_o-ATP synthase deduced from genome analysis are the aerobic lithoautotrophic extreme natronophilic *Thioalkalivibrio* spp. that demands high sodium concentration for optimal respiratory activity and growth (Banciu et al., 2004a; Muyzer et al., 2011a, b) and the anaerobic natronophiles with respiratory metabolism *Desulfurivibrio alkaliphilus* and *Desulfonatrosospira thiodismutans* (Sorokin et al., 2008b, d; Hicks et al., 2010). In anaerobic alkaliphilic, alkalitolerant, or haloalkaliphilic bacteria, ATP is almost entirely formed by substrate-level phosphorylation. However, Na⁺-driven F-type ATP synthase is present in several species with various degrees of alkaliphily (e.g., the alkalithermophilic *Clostridium paradoxum* and the halophilic thermoalkaliphilic *Natranaerobius thermophilus*) (Ferguson et al., 2006; Mesbah and Wiegel, 2011). The only cyanobacterium with Na⁺-translocating ATPase activity reported so far is the alkaliphilic and halotolerant *Aphanothece halophytica* (Soontharapirakkul and Incharoensakdi, 2010). In these bacteria, a membrane-bound V-type ATPase works at the expense of ATP to translocate Na⁺ outwardly, thus contributing to sodium homeostasis in cytoplasm but not to the energy generation. Same type of the sodium-coupled ATPase has been detected in the genomes of a natronophilic clostridium *Dethiobacter alkaliphilus* (Sorokin et al., 2008d; Hicks et al., 2010) and a fermentative low G+C firmicutes *Amphibacillus* (Kaieda et al., 1998; Satoh and Koyama, 2005).
2. *The intimate construction of alkaliphilic F-ATP synthase favors the catalysis of ATP synthesis and blocks the hydrolytic activity.* The regulation of bacterial F-ATP synthase activity is achieved by two major mechanisms. Tight but reversible binding of ADP as magnesium salt is an effective inhibitory process that slows the ATPase activity in all known F-ATP synthases (Minkov et al., 1979; Feldman and Boyer, 1985; Konno et al., 2011). In bacterial and chloroplast F-ATP synthase, the ϵ subunit is an “endogenous inhibitor.” Fine-tuning of conformational changes in the ϵ subunit is controlled by the *pmf* and the ATP/ADP ratio (Kato-Yamada et al., 1999; Suzuki et al., 2003). It is very likely that haloalkaliphilic ATP synthase is not exceptional in this regard.

For the Na^+ -translocating ATP synthase from *Natranaerobius thermophilus*, with a high hydrolytic activity and a low capacity for ATP synthesis, Mesbah and Wiegel (2011) have proposed a regulatory mechanism mediated by the ϵ subunit.

3. Most of adaptive modifications (as compared with the neutralophilic ATP synthase) have been identified in the F_0 factor.

In the alkaliphilic *Bacillus* or *Bacillus*-related species (i.e., *B. alcalophilus*, *B. halodurans*, *B. pseudofirmus* OF4, *B. selenitireducens*, and *Caldalkalibacillus thermarum* TA2.A1), the c -ring is made up of 13 subunits, a slightly higher number than that of decameric c -ring of most neutrophilic cells. Accordingly, the H^+ to ATP ratio in these alkaliphiles would be 4.3, higher than the more usual 3.3 proton equivalents for one mol of synthesized ATP as calculated for neutrophilic ATP synthase (Hicks et al., 2010). Comparative analysis of genomic sequences revealed alkaliphilic-specific sequences in the genes coding for a - and c -subunits as well as a different pattern of alkaliphilic motifs in the Gram-positive alkaliphiles as compared with Gram-negative ones. Moreover, different levels of alkaliphily are accordingly reflected in these sequences (Hicks et al., 2010). Two specific amino acid sequences related to alkaliphily in *Bacillus* species were observed in the primary structure of the c -subunit: AxAxAXA – or at least three alanine residues, and PxxExxP – two proline residues flanking the glutamic acid with a critical carboxylate residue. The first motif is apparently contributing to spatial ordination of the c -ring, while the second is responsible for ion binding (Wang et al., 2004; Liu et al., 2009).

Another peculiarity of the primary structure of alkaliphilic c -subunits is the presence of a threonine residue (Thr³³) near the loop edge of the hairpin-like structure, which may influence the dynamics of the rotor part of the ATP synthase edifice. Recently, a high resolution crystal structure of the rotor ring in *B. pseudofirmus* OF4 unveiled a rather “tulip beer glass” shape of the c -subunit ring, different from the “hour-glass” shape of the c -rotors in other bacteria. At the same time, the alkaliphilic motifs in c -subunits promote tight proton binding in the ion-binding site in the form of a tetra-coordinated hydronium ion (H_3O^+) (Preiss et al., 2010).

A brief comparative analysis by multiple alignments of available sequences for ATP synthase c -subunits from several haloalkaliphilic species (the aerobic Gram-negative *Thioalkalivibrio* spp., *Thioalkalimicrobium* spp., and *Methylo-microbium alcaliphilum*; anaerobic Gram-negative *Thiorhodospira sibirica* and *Ectothiorhodospira* spp.; anaerobic Gram-positive *Alkaliphilus* spp.) indicated the presence of “non-alkaliphilic” motif GxA/GxGxG, the same motif as found in c -subunit of *Escherichia coli* or *Oceanobacillus iheyensis* (see also Hicks et al., 2010). When searching for the second motif of c -subunit, analogous to alkaliphilic PxxExxP, same observation applied: non-alkaliphilic GxxD/ExxP/T motif has been detected in all surveyed haloalkaliphiles. Thus, it seems that minute changes in the last amino acid of the N-terminus motif would influence the

kinetics of ion binding at the nearby glutamate/aspartate residue. Thr³³ was not found, but all analyzed extremophiles contain the nonpolar Ala³³ residue instead.

In the stator part of F₁F₀-ATP synthase, the *a*-subunit of true alkaliphilic *Bacillus* spp. contains at least two crucial amino acids, Lys¹⁸⁰ and Gly²¹², that are not found in the neutrophilic counterpart. Situated at the interface between the *a*- and the *c*-ring, Lys¹⁸⁰ and Gly²¹² are essential for ATP synthesis at elevated pH, being probably implicated in proton capturing and transferring to the rotor ring of ATP synthase (Fujisawa et al., 2010). Our brief search within the sequence databases has indicated the substitution of Lys¹⁸⁰ and Gly²¹² in the haloalkaliphilic *Thioalkalivibrio* spp., *Thioalkalimicrobium* spp., *Methylomicrobium alcaliphilum*, and *Thiorhodospira sibirica* in an *E. coli*-like pattern (Lys¹⁸⁰/Gly²¹² being replaced with Gly/His or Lys). These substitutions in places that face each other in trans-membrane helices 4 and 5, respectively, could ensure similar microenvironment for transferring the protons as it does in the *a*-subunit of alkaliphilic *Bacillus* spp. Additionally, the external surface of the *a*-subunit in alkaliphilic *Bacillus* spp. is somewhat more polar than the analogous region of non-alkaliphilic *Bacillus* species or *E. coli*. This region may have an enhanced capacity of retaining H⁺ on the outer surface of the membrane (Wang et al., 2004).

The molecular adaptations of ATP synthase from alkaliphilic, aerobic, and Gram-positive species only partially explain the ability of such organism to withstand a broad range of pH and/or extreme alkalinity. In particular, the phosphorylation potential (ΔG_p), the parameter that reflects the energy required to sustain the [ATP]/[ADP][Pi] ratio, is unexpectedly high under conditions in which the *pmf* is relatively low. As discussed by Hicks et al. (2010), in *B. pseudofirmus* OF4 grown at pH 10.5, the measured *pmf* is -50 mV, while ΔG_p is 478 mV. Similarly, in the thermophilic *Caldalkalibacillus thermarum* grown at pH 10, the observed *pmf* was -78 mV, while ΔG_p was around 430 mV. Close values (*pmf* = -58 to -89 mV, and ΔG_p = 477–491 mV) were measured in a sucrose-energized cell suspension of *Natranaerobius thermophilus* (Mesbah et al., 2009). In all situations, the observed ΔG_p is close to that measured in the neutrophiles. However, in the obligately anaerobic fermentative haloalkaliphile, the measured ΔG_p does not necessarily reflect the capacity of ATP synthesis by OXPHOS. Based on the relation $[H^+]/[ATP] = \Delta G_p / pmf$ and assuming the value of 3.3 for $[H^+]/[ATP]$, the theoretical *pmf* value that supports the observed ΔG_p would be around two times higher. A slightly increased $[H^+]$ to $[ATP]$ ratio of 4.3, corresponding to tridecameric *c*-rings of *B. pseudofirmus* and *C. thermarum*, does not suffice to obtain such a phosphorylation potential (Hicks et al., 2010). In this light, the hypotheses of localized proton gradients or local proton microcircuits that boost the efficiency of proton transfer, sequestration, and transport from respiratory chain components to the ATP synthase complex have been now revived since Williams proposed the proton circuits theory in 1961 (Williams, 1988; Guffanti and Krulwich, 1992; Hicks et al., 2010). In support of this theory, some evidence has risen. Localized microcircuits of protons and fast proton diffusion alongside the membrane surface have been directly measured in liposomes

by using a fluorescence correlation spectroscopy (FCS)-based approach (Brändén et al., 2006). Several other reports exist on the possibility of faster diffusion of protons along the lipid and/or protein surface of membranes than the expelling of protons out into the bulk phase (Heberle et al., 1994). Protons extruded during respiration were apparently retained close to the outer surface of the cell membrane in alkaliphilic *B. clarkii* K24-1U, but this observation needs further confirmation (Yoshimune et al., 2010).

3.3.2. Adaptation of Respiratory Chain Components at Alkaline pH

The aerobic facultatively alkaliphilic *Bacillus pseudofirmus* OF4 as well as *B. halodurans* C-125 and *Oceanobacillus iheyensis* have a primary H⁺-extrusion respiratory chain with *caa3*- and *bd*-type terminal oxidases (Krulwich et al., 1998; Takami et al., 2000, 2002). Additionally, cytochrome *bo3* oxidase is inferred from the *O. iheyensis* and *B. halodurans* genomes (Takami et al., 2000, 2002). Cytochrome *caa3* oxidase is induced during growth at alkaline pH. Liu et al. (2007) observed close physical interaction between *caa3* oxidase and F₁F₀-ATP synthase that might increase the efficiency of proton transfer to the F-ATP synthase. Cytochrome *bd* is a non-proton-pumping quinol oxidase that is upregulated during the alkaline pH adaptation in *E. coli* K-12 concomitantly with a decrease in expression of proton-extruding complexes (cytochrome *o* and types I and II NADH dehydrogenases) of the respiratory chain. Such a concerted response would probably diminish the export of protons from the cytoplasm during generation of *pmf* (Maurer et al., 2005).

The midpoint redox potential of cytochrome *c*, one of the main components of the respiratory chain, is considerably lower (< +100 mV) in alkaliphilic than in neutrophilic bacteria (approx. +220 mV). At the same time, the cytochrome *caa3* oxidase (complex IV) that accepts electrons from cytochrome *c* has an apparently normal redox potential (approx. +250 mV) (Muntyan and Bloch, 2008). Moreover, cytochrome *c* is highly abundant in alkaliphilic *Bacillus* when grown at alkaline pH (for reviews, see Hicks and Krulwich, 1995; Krulwich et al., 1998). It is suggested that the overall construction of the respiratory chain permits a quicker and more efficient H⁺ translocation and electron flow along the membrane in alkaliphiles than in neutralophiles (Goto et al., 2005).

In the aerobic natronophilic SOB, dominant cytochromes are both of the *c*- and *b*-type, in *Thioalkalivibrio* spp., and *c*-type only in *Thioalkalimicrobium* spp. Terminal oxidases have been identified as *caa3*-, *ccb3*- and *o*-type in the first genus, and cytochrome *ccb3* type in the latter. Presence of cytochrome *ccb3* oxidase, a low efficient proton pump (H⁺/e⁻ = 0.2–0.75) with high oxygen affinity ($K_m \sim 7$ nM) and a relatively low midpoint potential of its active site heme ($E_{m,7} \sim -60$ mV), is indicative of microaerobic adaptation (Pitcher et al., 2002; Rauhamäki et al., 2009). Microoxic conditions are likely in the saline environment where oxygen solubility is affected by presence of salts. In *E. coli* cytochrome *o* oxidase is produced under oxygen-rich conditions, does not extrude protons, and has a relatively low oxygen affinity ($K_m = 1.4$ – 2.9 μ M) (Tseng et al., 1996). Cytochrome *o* oxidase oxidizes ubiquinol but not cytochrome *c*, representing an

alternative route for electron transfer. Its presence in *Thioalkalivibrio* spp. with two altogether different terminal oxidases and low-potential cytochrome *b* may suggest a highly controlled, multiple-way electron flow and versatility of growing under variable oxic/microoxic conditions.

The high sodium concentration in the external medium of haloalkaliphilic bacteria would have a dramatic effect on their internal milieu unless they possess extruding systems to adjust their internal sodium level. One of such regulators of Na^+ -homeostasis could be the Na^+ -dependent NADH:quinone oxidoreductase (Nqr or type III NAD dehydrogenase). Nqr is a respiration-dependent primary sodium pump that couples sodium extrusion with electron transfer from NADH to ubiquinone to generate *smf*. Nqr is present in many Gram-negative marine bacteria such as *Vibrio* spp. and *Shewanella* spp. where it is related to the absolute sodium requirement for growth (Oh et al., 1991). Coexistence of proton-translocating NADH dehydrogenase (NDH-1) and Nqr is predicted from the genomes of pathogens (e.g., *Pseudomonas* spp., *Neisseria* spp., *Yersinia* spp.), obligate chemolithoautotrophs (*Nitrosomonas europaea*), and euryarchaeota (*Methanosarcina acetivorans*) (Melo et al., 2004). Homologous *nqr* genes (*rnfABCDE*) that code for the Rnf complex were recently identified in the genome of the natronophilic *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp., as well as in other soda-loving bacteria (Fig. 1b). In *Thioalkalivibrio* spp., the *rnf* operon coexists with the *nuo* operon (*nuoABCDEFGHIJKLMN*) encoding type I proton-pumping NADH dehydrogenase (NDH-1). Rnf are bacterial redox-driven ion pumps with flavin-binding subunits (D and G) homologous to Na^+ -Nqr B and C subunits (Steuber, 2001; Backiel et al., 2008). Genes coding for Nqr as well as for NDH-1 (*nuo* genes) were also discovered in the genome of haloalkaliphilic SRB *Desulfurivibrio alkaliphilus* (Pereira et al., 2011). Coexistence of both types of NADH dehydrogenases suggests a finely tuned redox metabolism in association with sodium extrusion from the cytoplasm.

Type II NADH dehydrogenase (energy-uncoupled NADH dehydrogenase or NDH-2) capable of sodium export as well as of Na^+/H^+ antiport (NapA) activity was described in the moderate halophile *Halobacillus dabanensis* D-8 (Yang et al., 2006). Genes for NDH-2 exist in the Gram-positive alkaliphilic *B. halodurans* C-125, *B. clausii*, and *B. pseudofirmus* OF4. Their predicted products are two types of NHA-2. NHA-2A found to have high sequence similarity (>70 %) among alkaliphilic *Bacillus*, while the gene for NHA-2B of *Bacillus pseudofirmus* OF is 56 % similar to that of putative “ Na^+ -NADH dehydrogenase (NapA)” from *Halobacillus dabanensis*. None of the NHA-2 expressed proteins has Na^+/H^+ antiport activity in *B. pseudofirmus* OF4, and they are supposed to play a gating role for electron entry in the respiratory chain (Liu et al., 2008). Interestingly, the facultatively alkaliphilic and halotolerant marine bacterium *Oceanobacillus iheyensis* seems to rely only on NDH-2, lacking other types of respiratory NADH dehydrogenases (Melo et al., 2004).

Isoprenoid (respiratory) quinones function as electron and proton carriers in respiratory and photosynthetic chains. Quinones are also known as antioxidants.

Ubiquinones (high-potential quinones, $E_m = +70$ to $+112$ mV) are found in eukaryotes and are widespread in aerobic alpha-, beta-, and gammaproteobacteria. Menaquinones (low-potential quinones, with $E_{m7.5}$ between -110 and -70 mV) are typical for prokaryotes and are often related to anaerobic metabolism (Nowicka and Kruk, 2010). In many haloalkaliphilic, alkalitolerant halophilic, and moderately halophilic Gram-positive bacteria, the major respiratory quinone is menaquinone (MK)-7 (e.g., in *Bacillus locisalis*, *B. qingdaonensis*, *B. chagannorensis*, *Salsuginibacillus kocurii*) (Márquez et al., 2011; Wang et al., 2007; Carrasco et al., 2007). MK-7 is also dominant in the membranes of alkali-tolerant halophilic, moderately halophilic, and alkaliphilic halotolerant Gram-negative bacteria belonging to *Virgibacillus* spp., *Mongoliococcus* spp., and *Mongoliitalea* spp. (Chen et al., 2009; Liu et al., 2012; Yang et al., 2012). Haloalkaliphilic species of *Halomonas* (*H. campaniensis*, *H. desiderata*, *H. campisalis*) contain MK-9 and MK-8 as dominant quinones. In aerobic natronophilic *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp. that belong to the *Gammaproteobacteria*, the dominant respiratory quinone is the high midpoint potential ubiquinone-8 (UQ-8) (Sorokin and Kuenen, 2005). A combination of ubiquinones and menaquinones is characteristic of phototrophic sulfur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira*. MK-7 and UQ-7 are major respiratory quinones in *Ect. magna*, *Ect. shaposhnikovii*, and *Ect. vacuolata* (Bryantseva et al., 2010). The closely related haloalkaliphilic *Halorhodospira halophila* distinctively utilizes two menaquinones, MK-8 and MK-7, for the photosynthetic electron transfer reaction and UQ-8 for the respiratory reaction (Schoepp-Cothenet et al., 2009).

3.4. Na^+ -DEPENDENT FLAGELLAR MOVEMENT

Naturally, the concentration of sodium ions is higher in the surrounding medium than within the cell. When speaking of alkaliphilic, halophilic, and haloalkaliphilic bacteria, the favorable (inwardly oriented) sodium gradient in the form of an electrochemical sodium gradient or sodium-motive force (*smf*) is exploited in several ways. Flagellar movement driven by *smf* has been well documented in haloalkalitolerant *Vibrio* spp. and in alkaliphilic *Bacillus* spp. and haloalkaliphilic bacteria (Atsumi et al., 1992; Fujinami et al., 2009; Krulwich et al., 2007). A Na^+ -dependent flagellar motor, similar to PomAB of *Vibrio* spp. and MotPS of *Bacillus* spp., was predicted from the genomes of *Thioalkalivibrio sulfidophilus* HL-EbGr7 and *Thioalkalivibrio* K90mix. These motor proteins are also closely related to a similar protein of *Halorhodospira* spp. Remarkably, the sodium-dependent movement coexists with proton-driven MotB-like flagellar motors in *Thioalkalivibrio* spp., suggesting that the chemotaxis of these bacteria is finely regulated through a complex relationship between osmo- and pH-sensing (Muyzer et al., 2011b). In low-salt-tolerant natronophilic *Thioalkalimicrobium* spp. and *Methylomicrobium alcaliphilum*, the genome

sequences indicated the presence of H⁺-dependent flagellar motility, both putative OmpA/MotB complex and MotA-like flagellar stator protein being present. In *M. alcaliphilum*, however, MotA (YP_004918404.1) and PomA (YP_004917195.1) gene products coexist.

3.5. pH AND ION HOMEOSTASIS IN THE CYTOPLASM OF HALOALKALIPHILES

The most important strategy of regulating cytoplasmic pH in bacteria depends on the activity of primary and secondary proton transporters. Primary H⁺-pumps comprise proton-extruding complexes of the respiratory chain and H⁺-driven F-ATP synthase, while secondary proton transporters are antiporters that exchange monovalent cations for protons (Padan et al., 2005; Krulwich et al., 2011). The key antiport system of halo- and alkaliphiles introduces H⁺ while extruding Na⁺ that inherently accumulates within the cell living at high salinity and/or alkalinity. The alkaliphilic and/or haloalkaliphilic Na⁺/H⁺ antiporters are part of a constellation of many types of transporting proteins that seems to have minute particular adaptations in almost every species. The concerted result of membrane transport activity is the accomplishment of ionic (i.e., H⁺, Na⁺) and volume homeostasis.

3.5.1. Na⁺/H⁺ Antiporters

Presently, Na⁺/H⁺ antiporters are classified into five families (NhaA to NhaE) in the monovalent cation–proton antiporter (CPA) superfamily (<http://www.tcdb.org/>). Nha's differ in their structure, topology, substrate affinity, stoichiometry, and kinetics of ion exchange.

Members of the Na⁺/H⁺ antiporter NhaA family are commonly found among all living cells and make an essential contribution to the Na⁺ and pH homeostasis during alkaline adaptation of non-extremophilic and extremophilic organisms (Padan et al., 2001). The activity of electrogenic NhaA is regulated by the cytoplasmic pH, and it catalyzes the inward translocation of 2H⁺ in exchange with 1Na⁺ (or Li⁺) that is extruded, resulting in a net positive charge accumulation inside the cytoplasm. The secondary transport achieved by NhaA is energized by the electric potential ($\Delta\psi$) component of *pmf*. The prototype member of this family is NhaA from *E. coli* (Ec-NhaA). Ec-NhaA has a cytoplasmic pH-sensing domain in loop VIII-IX. It has a high affinity for sodium with apparent $K_m = 0.2$ mM Na⁺ at pH 8 (Gerchman et al., 1993; Tzuberly et al., 2004). Expression of the *nhaA* gene is modulated by Na⁺ at the transcriptional level, while the pH-dependent activity is an intrinsic function of the translated protein. The growth of *E. coli* at alkaline pH supported by the combined action of two transporters, NhaA and a multidrug transporter MdfA. Apparently, NhaA is aiding non-alkaliphilic cells to withstand alkalinity up to pH 9, while MdfA, which has Na⁺/H⁺ as well as K⁺/H⁺ antiport activity in addition to efflux of multiple drug substrates

in exchange for H^+ , takes over at higher pH (Padan et al., 2005). Ec-NhaA has maximal activity at pH 8.5, and growth of *E. coli* cells at pH >9 would raise the cytoplasmic pH at inhibitory value for NhaA activity. Ec-NhaA has many homologs and paralogs in bacteria and archaea (for review, see Padan, 2008). A survey of the database (BLASTP) for available protein sequences or gene products closely related to NhaA has revealed few homologs in halophilic *Halomonas* spp. and *Chromohalobacter salexigens*.

Two members of the NhaC family of Na^+/H^+ antiporters have been originally uncovered in *Bacillus pseudofirmus* OF4 and *Bacillus subtilis*, respectively. One member (also designed as Bp-NhaC) is a relatively high-affinity, electrogenic Na^+/H^+ antiporter with a minor role in alkaliphily (Ito et al., 1997); the other is a paralog described as mediating an electroneutral malate- H^+/Na^+ -lactate antiport important in malate uptake at low *pmf* (Wei et al., 2000). Bp-NhaC of *B. pseudofirmus* remains the only member of the NhaC family that is functionally characterized. Genes coding for NhaCs exist in both Gram-negative bacteria and Gram-positive bacteria as well as archaea (i.e., *Haloferax* spp.). An NhaC homolog is predicted from the genome of the halophilic thermoalkaliphilic *Natranaerobius thermophilus* JW/NM-WN-LF (Zhao et al., 2011). The *nhaC* gene product of *N. thermophilus* showed high amino acid identity (42–44 %) with similar products in the genomes of the alkaliphilic *Bacillus clausii* KSM-K16, *B. halodurans* C-125, the obligately anaerobic, non-halophilic and moderately alkaliphilic *Alkaliphilus metalliredigens* QYMF and *A. oremlandii* OhILAs, as well as with a partial protein sequence from *Halomonas boliviensis* LC1 (acc. no. ZP_09188066).

Since the first reports on Na^+/H^+ antiport activity in the moderately halophilic *Salinivibrio costicola* and the slightly halophilic *V. parahaemolyticus* (Hamaide et al., 1983; MacLeod et al., 1988), evidence arose for the simultaneous presence of NhaA-, NhaB-, and NhaD-type antiporters in halophilic bacteria of *Vibrio* spp. Genes coding for NhaA and NhaB from *V. parahaemolyticus* complemented mutants unable to grow at neutral pH and high salinity (0.5 M LiCl) or at alkaline pH (8.5) in the presence of low salt concentration (0.05 M LiCl). Expression of *nhaA* and *nhaB* was proposed to be pH-regulated (Kuroda et al., 2005). Genes for the latter antiporter, NhaD (or Vp-NhaD), identified for the first time in *V. parahaemolyticus* by Nozaki et al. (1998), were also isolated and their product functionally characterized in *Vibrio cholerae* (Vc-NhaD) and the haloalkaliphilic *Alkalimonas amylolytica* (Aa-NhaD) (Dzioba et al., 2002; Ostroumov et al., 2002; Liu et al., 2005).

NhaD proteins catalyze $Na^+(Li^+)/H^+$ antiport at a yet unknown stoichiometry. Homologs of *nhaD* gene products are found among many proteobacteria, including truly halophilic *Halomonas* spp. (Kurz et al., 2006). Activity of NhaD is optimal at alkaline pH ($pH_{opt} = 8$ for Vc-NhaD; 8.5–9 for Vp-NhaD, and 9.5 for Aa-NhaD). The activity of NhaD from *Alkalimonas amylolytica* has a relatively low Na^+ affinity ($K_m = 0.5$ mM Na^+) and it is optimal at a concentration of 600 mM Na^+ (Liu et al., 2005). These characteristics of haloalkaliphilic NhaD (Aa-NhaD) seem in accordance with the supposed chemical parameters

of the cytoplasm during growth at saline and alkaline conditions. However, pH homeostasis in *Alkalimonas amylolytica*, capable of growing optimally at pH 10–10.5 and 2–3 % (w/v) NaCl, is achieved upon combined activities of at least three other ion transporters: the K^+/H^+ antiporter (Aa-NhaP), the K^+ efflux pump (Aa-KefB, homolog of glutathione-regulated K^+ efflux protein B, KefB from *E. coli*), and, to a lesser extent, the calcium/cation antiporter (Aa-CaxA) (Wei et al., 2007).

3.5.2. Mrp and Mrp-Like Antiporters

In aerobic alkaliphilic *Bacillus* strains, growth at alkaline pH is strictly dependent on the presence of external Na^+ . Therefore, in these bacteria, pH homeostasis is mainly accomplished by a variety of Na^+/H^+ antiporters (Krulwich et al., 2011). A large hetero-oligomeric Mrp antiporter that extrudes Na^+ (K^+ or Li^+) in exchange for H^+ has a crucial role for survival at high pH (Ito et al., 2001; Morino et al., 2008, 2010). Unlike Ec-NhaA that is a single-gene product, the multidrug resistance efflux pump Mrp from *Bacillus* spp. is a seven-component system (MrpA–MrpG) encoded by an operon containing seven genes. Originally described in *Bacillus halodurans* C-125 by Hamamoto et al. (1994), it turned out that Mrp genes are virtually spread in many Gram-negative and Gram-positive bacteria and archaea, with vital contribution to cytoplasmic pH regulation and general physiology of microorganisms (Swartz et al., 2005). Mrp-like clusters from cyanobacteria have been demonstrated to be involved in salt stress tolerance and CO_2 deficiency-induced expression (Blanco-Rivero et al., 2005). A full Mrp-like complex (Ap–MrpA–G) with Na^+/H^+ and Li^+/H^+ antiport activity was functionally described in the halophilic and alkalitolerant cyanobacterium *Aphanothece halophytica* (Fukaya et al., 2009). An interesting tripartite clustering of the genes *bicA*, *napA1-2*, and an *mrp*-like gene was described in *A. halophytica*. The first gene codes for Na^+ -dependent HCO_3^- uptake, the second for a pH-regulated Na^+/H^+ antiporter, active at alkaline pH (Price, 2011; Wutipraditkul et al., 2005). A model of cooperation between the three transporters was proposed in *A. halophytica*. At alkaline and saline conditions, when CO_2 becomes limiting, the Na^+ extruded by Na^+/H^+ antiport catalyzed by NapA1-2 and the Mrp-like complex drives the inward flux of HCO_3^- . The pH of the cytoplasm is maintained at a tolerable level by simultaneous proton import (Fukaya et al., 2009). Genes of the *mrp*-like operon (*mhA-G*), together with those for other cation/proton antiporters (*nhaP*), have been reported in the natronophilic *Thioalkalivibrio* spp. (Muyzer et al., 2011a, b) as well as in the genomes of the arsenite-oxidizing *Alkalilimnicola ehrlichii* and *Halorhodospira halophila* (see also Fig. 1c).

3.5.3. K^+ Transport and Intracellular Homeostasis

It is expected that the alkaliphiles retain an outwardly directed K^+ gradient. Therefore, K^+/H^+ antiporters might be energized by the electrochemical potassium gradient to drive protons inside the cell. However, K^+/H^+ antiporters are not expected to play central roles in cytoplasmic acidification in alkaliphiles as the

removal of intracellular K^+ is not desirable. Accumulation of (monovalent) cations in the halo/alkaliphilic cytoplasm and particularly of K^+ is a strategy either to neutralize the charge of anionic osmolytes (i.e., glutamate, chloride ions) or to compensate for the risk of Na^+ toxicity at high intracellular pH (Padan and Krulwich, 2000).

Potassium transporters participate in the overall pH, K^+ , and volume homeostasis in non-halophilic or slightly halophilic bacteria (e.g., *E. coli*, *Vibrio alginolyticus*) (Radchenko et al., 2006; for a review, see Epstein, 2003), as well as in osmoregulation of *Halanaerobiales* and haloarchaea (Oren, 2002). K^+ transporters that contribute to pH and K^+ homeostasis of halo- and/or alkaliphiles are NhaPs belonging to the monovalent cation/proton antiporter-1 (CPA1) family and members of the K^+ transporter (Trk) family. The Aa-NhaP – $K^+(NH_4^+)/H^+$ antiporter was characterized in *Alkalimonas amylolytica* (see above) (Wei et al., 2007). The Ap-NhaP – $Na^+(Ca^{2+})/H^+$ antiporter has been functionally described in the halotolerant cyanobacterium *Aphanothece halophytica*. It exhibits Na^+/H^+ antiporter activity over a wide pH range (5–9) and shows high Ca^{2+}/H^+ antiporter activity at alkaline pH (Waditee et al., 2001).

The K^+ uptake protein TrkH could be inferred from the genome sequences of natronophilic and haloalkaliphilic *Thioalkalimicrobium* spp., *Thioalkalivibrio* spp., *Methylomicrobium alcaliphilum*, and *Desulfovibrio alkaliphilus* (Fig. 1d). It was also characterized in *Alkalimonas amylolytica* (Guo et al., 2009). Kinetics experiments revealed that the Aa-TrkAH complex has an optimal activity at pH >8.5 and it is salt tolerant. The TrkAH complex has a channel-like shape and a large acidic extracellular region. It catalyzes NAD^+ -regulated K^+ uptake possibly coupled with H^+ import (Domene and Furini, 2012).

3.5.4. Sodium Import Closes the Sodium Cycle in Salt- and Alkaline-Adapted Cells

Two major routes for Na^+ entry in prokaryotic cells are known: Na^+ /solute symport and Na^+ diffusion mediated by channels (including the flagellar Na^+ channel).

Na^+ -dependent solute uptake is essential in providing sodium for antiporter activity as well as nutrients (i.e., sugars, amino acids) or precursors (such as choline) for the purpose of general metabolism including osmoadaptation. Na^+ /solute symporters are ubiquitously spread in many bacteria and archaea as well as in eukaryotes. They belong to the sodium/solute symporter (SSS) family according to the Transporter Classification system (Saier et al., 2009). Activity of Na^+ /solute symporters is energized by the electrochemical Na^+ gradient (*smf*) (Jung, 2001). Na^+ -mediated import of L-proline by homologs of the PutP symporter from *E. coli* and *Vibrio parahaemolyticus*, respectively, is largely found in pathogens, slightly halophilic marine bacteria, extreme halophilic bacteria (*Salinibacter ruber*, *Halomonas elongata*), and archaea (*Halobacterium* spp., *Haloferax* spp.) (Jung et al., 2012).

Na^+ -coupled import of a nonmetabolizable solute (α -aminoisobutyric acid) has been reported in alkaliphilic *Bacillus* as early as 1985 by Krulwich

et al. Glutamate and sucrose uptake are dependent on the sodium gradient in *Caldalkalibacillus thermarum* TA2.A1. Apparently the electrical component of the *smf* is the driving force for solute uptake in this thermoalkaliphile (Peddie et al., 1999, 2000). Among the anaerobic halophilic and thermoalkaliphilic bacteria, Na^+ -coupled solute symport was detected in *Natranaerobius thermophilus* (Zhao et al., 2011). In comparative physiological experiments with anaerobic alkaliphilic bacteria from soda lakes, Pitriuk et al. (2004) concluded that metabolism and substrate-level energy formation during growth at alkaline pH in the saccharolytic fermenters *Spirochaeta alkalica*, *Amphibacillus tropicus*, and the *Halanaerobiales* representative *Halonatronum saccharophilum* heavily depend on transmembrane H^+ and Na^+ gradients.

The bacterial Na^+ -selective channels Na_vBac belong to the voltage-gated ion channel (VIC) superfamily. The prototype protein of Na_vBac is the NaChBac channel discovered in the alkaliphilic *Bacillus halodurans* C-125 (Ren et al., 2001). The sodium-selective NaChBac is a channel protein with a high specificity for Na^+ , although its activation and inactivation kinetics are slower than that of Na_vs (Ren et al., 2001; for a review, see Charalambous and Wallace, 2011). NaChBac homologues are encountered in diverse marine bacteria and/or bacteria living in alkaline niches (Koishi et al., 2004; Ito et al., 2004). A recent BLASTP search reported by Irie et al. (2010) yielded 26 homologs of NaChBAC, including those from *Oceanobacillus iheyensis*, the anaerobic *Alkalilimnicola ehrlichii*, and the haloalkaliphilic *Halorhodospira halophila*. However, our updated BLASTP evaluation of NaChBAC homologs indicated their presence in *Halomonas* spp. (37 % amino acid identity), *Ectothiorhodospira* spp. (39 %), and *Thioalkalivibrio* spp. (40 %).

A Na^+ -channel potentiated at elevated pH has been described as playing roles in pH homeostasis and pH-sensing in the facultatively alkaliphilic *Bacillus pseudofirmus* OF4. Deletion of the gene (*ncbA*) coding for the voltage-gated Na^+ channel Na_vBP impaired growth at alkaline pH, indicating its significant contribution to the cytoplasmic ion homeostasis. Moreover, Na_vBP plays an active role in the motility and chemotaxis only during growth at pH 10 (Ito et al., 2004; Fujinami et al., 2007).

3.5.5. Bicarbonate Transport in Lithotrophic Haloalkaliphiles and Natronophiles

Uptake of carbon dioxide (CO_2) and/or bicarbonate (HCO_3^-) by obligately autotrophic organisms is essential for providing the inorganic carbon needed in assimilation. Soda lakes are virtually unlimited sources for carbonate (CO_3^{2-}), but, since it is not transported, the main source of inorganic carbon in soda lakes seems to be HCO_3^- which becomes less accessible at very high pH (>10). Therefore, the soda lake autotrophs have to rely on active transport of bicarbonate ion. However, hypersaline soda lakes host a high abundance and diversity of photo- and chemolithoautotrophic SOB from the family *Ectothiorhodospiraceae*

(*Thioalkalivibrio* spp., *Halorhodospira* spp.) (Sorokin and Kuenen, 2005; Kovaleva et al., 2011). Low to moderately saline soda lakes, instead, have an impressive seasonal biomass production by oxygenic phototrophic cyanobacteria.

In cyanobacteria, the CO₂-concentrating mechanism (CCM) involving the uptake of CO₂ and HCO₃⁻ is driven by light energy (Kaplan et al., 2008). To date, at least two CO₂ uptake systems bound to thylakoids, and three HCO₃⁻ transporters localized in plasma membrane covering the entire range of inorganic carbon availability are known in cyanobacteria. By this CCM, inorganic carbon is captured in the cytoplasm and delivered as HCO₃⁻ through thylakoid membranes to the RuBisCO active site (Price, 2011). Two membrane transporters for bicarbonate are sodium-dependent: the inducible, high-affinity HCO₃⁻/SO₄²⁻ transporter, SbtA (Shibata et al., 2002), and the low-affinity, high-flux HCO₃⁻ transporter, BicA, identified in marine cyanobacteria (Price, 2011). The HCO₃⁻/Na⁺ symporter SbtA is a multi-pass membrane protein that is induced at low dissolved CO₂. SbtA, identified in *Synechocystis* PCC 6803, has a high affinity for bicarbonate (K_{0.5} < 5 μM HCO₃⁻), and its function is dependent on the *smf* generated by the activity of the Na⁺/H⁺ antiporter (Shibata et al., 2002). SbtA is particularly interesting as it works at low bicarbonate and high sodium concentration. Genes related to the *sbtA* gene from *Synechocystis* PCC 6803 are present in the genome sequences of the natronophilic SOB *Thioalkalivibrio* K90mix, *Thioalkalivibrio thiocyanoxidans* ARh4, *Thioalkalimicrobium aerophilum* AL3, and *Thioalkalimicrobium cyclicum* ALM1, but not in *Thioalkalivibrio sulfidophilus* HL-EbGr7. In all natronophilic chemolithoautotrophic SOB, however, a gene for low-affinity high-flux HCO₃⁻ transporter, SulP, is present (Muyzer et al., 2011a,b; personal BLASTP search). A comparative analysis of gene products also revealed homologous sequences of *sbtA* in haloalkalitolerant cyanobacteria (*Synechococcus* spp., *Anabaena* spp., and *Cyanothece* spp.) (Muyzer et al., 2011b). Apparently, SbtA bicarbonate transporters are not restricted to autotrophic bacteria. From a survey of the protein sequence database, homologous genes for SbtA are identified in alkaliphilic *Bacillus* and *Bacillus*-related species (*B. pseudofirmus* OF4 – acc. nr. YP_003425900.1, *B. halodurans* C-125 – acc. nr. NP_244736.1, and *Caldalkalibacillus thermarum* – acc. nr. ZP_08531624.1). In a complementation experiment, a gene fragment that encodes a homolog to the SulP transporter family member, BicA, from the haloalkaliphilic, strictly heterotrophic *Alkalimonas amylolytica* N10 allowed an alkali-sensitive *E. coli* mutant to grow at high pH (Wei et al., 2007). Interestingly, the closest homolog of the BicA protein from *Alkalimonas amylolytica* (acc. nr. ABG37985.1) is an uncharacterized anion transporter from haloalkaliphilic methanotroph *Methylomicrobium alcaliphilum* (acc. nr. YP_004916439.1) with 61 % amino acid identity. It is tempting to speculate that the active uptake of HCO₃⁻ in heterotrophic haloalkaliphilic bacteria could be an indicative of inorganic C fixation by anaplerotic pathways that alternatively replenish the Krebs cycle during starvation periods (Ashworth and Kornberg, 1966).

3.6. ADAPTATION TO HIGH-LIGHT AND OXIDATIVE STRESS BY SPECIFIC MEMBRANE PIGMENTS

Apart from their specific roles in photosynthesis (i.e., bacteriochlorophylls and carotenoids) and light-driven transport processes (rhodopsins), pigments produced by several halophilic and haloalkaliphilic prokaryotes may intervene in high-light/UV protection and prevent oxidative-stress damages as antioxidants. Such biological functions may be attributed to carotenoids from archaeal membranes and halophilic bacteria (e.g., bacterioruberin from *Halobacterium salinarum*, salinixanthin of *Salinibacter ruber*) (Oren, 2002). Natronochrome and chloronatronochrome, two yellow pigments composed of a C15-polyene chain attached to a phenyl group, were chemically characterized from the membranes of non-photosynthetic natronophile *Thioalkalivibrio versutus* ALJ15 (Takaichi et al., 2004). Yellow-membrane-bound pigments have been extracted from the chemolithoautotrophic SOB *Thiohalospira* spp. (Sorokin et al., 2008a). No functional characterization has been done on these yellow polyene pigments, but their possible involvement in light- and oxidative-stress protection was suggested, as well as a function as an additional membrane barrier to H⁺ and Na⁺ leakage in a similar manner as squalene (Haines, 2001; Hauss et al., 2002). Xanthomonadin, a structurally related yellow pigment isolated from the outer membrane of the phytopathogenic gammaproteobacterium *Xanthomonas* spp., was demonstrated to improve the survival rate of cells under high light conditions and to protect lipids against peroxidation in vitro (Poplawsky et al., 2000; Rajagopal et al., 1997).

4. Conclusions

Most moderate and extreme haloalkaliphilic and natronophilic prokaryotes are mainly distributed within the groups of aerobic Gram-negative bacteria, anaerobic, low G+C, Gram-positive bacteria, and haloarchaea. Haloalkaliphiles and natronophiles take up and/or produce compatible solutes in a similar manner as halotolerant and halophilic prokaryotes. The unique feature, however, is met in natronophiles that demand high soda medium and must apparently face a lower osmotic pressure at the same sodium concentration than the true halophiles. This situation has presumably important consequences to the bioenergetics of natronophiles. One such consequence is the energy saved out of synthesizing and/or importing high amounts of organic compatible solutes, energy that could be further invested in a multitude of low-cost/high-benefit strategies to overcome the low *pmf*. H⁺-dependent ATP synthesis is universally found among aerobic alkaliphilic, haloalkaliphilic, and natronophilic bacteria. High production of low-potential cytochromes of the *c*-type, point mutations in the H⁺-translocating subunits of F-ATP synthase are complemented by a bifurcated electron transfer chain. Such adaptations build a highly efficient OXPHOS machinery under the conditions of an unfavorable electrochemical proton gradient. Last but not least,

a broad variety of Na⁺- and pH-regulated transporters are interconnected and differently expressed to maximize the exploitation of the favorable electrochemical sodium gradient (*smf*). *Smf* is the driving force for flagellar movement and, indirectly, for chemotaxis, as well as for acidification of cytoplasm, a critical process to resist the alkaline surrounding. Other adaptations that have not been discussed in this chapter are met in the structures and kinetics of intracellular and extracellular enzymes of haloalkaliphiles. Overall, the physiology and genetic evidences indicate a conserved, general pattern of salt and alkaline adaptation over a broad range of alkaline pH (9–11) and salinity (up to saturation) in haloalkaliphiles/natronophiles. Genomes of salt- and alkaline-tolerant strains of *Bacillus* spp. and *E. coli*, the most common experimental models, apparently encode the basic survival kits to respond the moderate osmotic and alkali shocks. However, those kits are not sufficient to cross to the regions where true haloalkaliphiles are flourishing. Further in-depth analyses of alkaliphilic and haloalkaliphilic genomes completed by integration with other “omics” and physiological evidences would definitively enlighten us on the bases as well as on the peculiarities of haloalkaline adaptation in prokaryotes.

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