Chapter 14
Halophiles Exposed Concomitantly to Multiple Stressors: Adaptive Mechanisms of Halophilic Alkalithermophiles

Noha M. Mesbah and Juergen Wiegel

14.1 Introduction

The world of halophilic microorganisms is highly diverse. Microorganisms adapted to life at high salt concentrations are found in all three domains of life: Archaea, *Bacteria* and *Eucarya*. In many ecosystems, halophiles are present with such high numbers that their presence is immediately recognized; the bright red color of marine saltern and crystallizer ponds found world-wide is due to populations of halophilic Archaea (order *Halobacteriales*), *Bacteria* (*Salinibacter*) and *Eucarya* (*Dunaliella salina*).

Most environments explored for the presence of halophiles are thalassohaline environments, i.e., that originated from the evaporation of seawater. Their chemical and ionic compositions reflect that of seawater and have a neutral or slightly alkaline pH. There also exist deep-sea brines, found at the bottom of the Red Sea, the Mediterranean Sea and the Gulf of Mexico. These brines are often anoxic and have very high salinity (approaching saturation) accompanied by elevated temperature and metal ion concentration. A number of interesting extremophiles have been isolated from deep-sea brines, including *Flexistipes sinusarabici* (an anaerobic heterotroph growing optimally at 2.7 M NaCl, pH 7 and 45–50°C (Fiala et al. 1990)), *Salinisphaera shabaensis* (an anaerobic heterotroph growing optimally at 1.5 M NaCl, 37°C and pH 7.5 (Antunes et al. 2003)), and *Halorhabdus tiamatea* (an anaerobic archaeon with optimal growth at 4 M NaCl, pH 7.0 and 45°C (Antunes et al. 2008)).

In many athalassohaline environments, i.e., hypersaline environments not originating from sea water, life at extreme salinity is combined with the need to

N.M. Mesbah (✉)
Department of Biochemistry, Suez Canal University, Ismailia 41522, Egypt
e-mail: noha_mesbah@pharm.suez.edu.eg

J. Wiegel
Department of Microbiology, University of Georgia, Athens, GA 30602, USA
e-mail: jwiegel@uga.edu

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survive at alkaline pH and in some cases, extreme temperature. These environments are fed by surface or river water and hypersalinity arises as a result of evaporative concentration driven by sunlight. Most of these environments are solar-heated, though a few also contain hot springs, such as saline Mono Lake, California or some of the South African Rift lakes. The ionic composition of athalassohaline environments is different from that of seawater, being dominated by potassium, magnesium, sodium and carbonate ions. Examples of athalassohaline environments include the alkaline soda lakes of Egypt (Wadi An Natrun), the Dead Sea, the East African soda lakes of the Kenyan–Tanzanian Rift Valley, the soda lakes of northern China and Inner Mongolia, salt flats of Nevada and Oregon, and Mono Lake, California.

As many athalassohaline environments are characterized by alkaline pH and/or elevated temperature in addition to high salinity and intense solar radiation, microorganisms inhabiting these environments must be adapted for survival and growth under this combination of extreme conditions. These microorganisms, termed the halophilic alkalithermophiles, not only survive, but grow optimally under conditions of high salt concentrations, alkaline pH and elevated temperature (Mesbah and Wiegel 2008; Bowers et al. 2009). The mechanisms enabling this tripartite lifestyle are essential for understanding how microorganisms grow under inhospitable conditions and will be explored in this review article.

14.2 Halophilic Alkalithermophiles

14.2.1 Growth Conditions of Halophilic Alkalithermophiles

Halophilic alkalithermophiles are a unique group of extremophiles which grow optimally at the combined extreme conditions of elevated salt concentration (>1.5 M), alkaline pH (greater than 8.5 measured at optimal temperature for growth, see Mesbah and Wiegel (2006) and Wiegel (1998) for details) and elevated temperature (>50°C). Table 14.1 contains definitions for different categories of halophiles, alkaliphiles and thermophiles.

In describing the optimal and marginal growth conditions for halophilic alkalithermophiles, care must be taken as the value of one extreme growth condition is affected by the other. For example, the measured pH value of a medium is dependent on temperature because of changing p*K*a values of different medium components at different temperatures (Wiegel 1998). Thus, the pH of the medium when measured at room temperature will be different from its pH when it is measured at the elevated growth temperature using temperature-calibrated electrodes and pH meters. For neutral pH, the difference in pH is small, usually less than 0.3 pH units. However, at acidic or alkaline pH values, the difference can be larger than 1 pH unit. Thus, to facilitate comparison of published data, it is important to know the conditions under which the pH was determined. It has been

Table 14.1 Definitions of different extremophiles

Growth characteristic	Minimum	Optimum	Maximum
Thermotolerant	T_{min} –	$T_{opt} < 50°C$	$T_{max} < 60°C$
Thermophile	T_{min} –	$T_{opt} \geq 50°C$	$T_{max} \geq 60°C$
Extreme thermophile	$T_{min} \geq 35°C$	$T_{opt} \geq 65°C$	$T_{max} < 85°C$
Hyperthermophile	$T_{min} \geq 60°C$	$T_{opt} \geq 80°C$	$T_{max} \geq 85°C$
Alkalitolerant	$pH_{min} \geq 6.0$	$pH_{opt} < 8.5$	$pH_{max} > 9.0$
Alkaliphile[a]			
Facultative	$pH_{min} < 7.5$	$pH_{opt} \geq 8.5$	$pH_{max} \geq 10.0$
Obligate	$pH_{min} \geq 7.5$	$pH_{opt} \geq 8.5$	$pH_{max} \geq 10.0$
Halotolerant	$NaCl_{min}$ –	$NaCl_{opt}$ 0.25–1.5 M[b]	$NaCl_{max} \leq 2.5$ M
Halophile	$NaCl_{min}$ 1 M	$NaCl_{opt} \geq 1.5$ M	$NaCl_{max}$ –
Extreme halophile	$NaCl_{min} \geq 1.5$ M	$NaCl_{opt} \geq 2.5$ M	$NaCl_{max}$ –

[a]For thermophiles and psychrophiles, the pH must be measured at the growth temperature for the microorganism

[b]1 M NaCl = 5.84% NaCl wt/vol

proposed (Mesbah and Wiegel 2008; Wiegel 1998) that the temperature at which the pH is measured and the pH meter calibrated be indicated with a superscript, e.g., $pH^{55°C}$.

Another interaction observed is the effect elevated salt concentration has on pH measurements. In solutions with high Na^+ concentrations, Na^+ can be read as H^+ by the pH electrode, and this is particularly pronounced at high pH values (>10). This phenomenon, known as the "Na^+ error," can be overcome to some extent with the use of a specific glass combination electrode. The glass membrane in this electrode is specifically constructed to reduce the Na^+ error at high-pH values, and it may also allow use of the electrode over a wider range of temperatures.

The first anaerobic, moderately halophilic alkalithermophilic eubacterium to be described was *Halonatronum saccharophilum*. It is a spore forming bacterium that belongs to the order *Halanaerobiales* (Zhilina et al. 2004). The optima for growth are 1.1–1.5 M NaCl, pH^{RT} 8.5 and 36–55°C, which represents a very broad temperature optimum. *H. saccharophilum* has a fermentative metabolism and grows on mono- and disaccharides, starch and glycogen. *H. saccharophilum* is the first alkaliphilic representative of the order *Halanaerobiales*, which is dominated by extremely halophilic anaerobes.

With a larger Na^+ ion requirement and more alkaline pH optimum, *Natranaerobius thermophilus* represents the first true anaerobic halophilic alkalithermophilic bacterium described (Mesbah et al. 2007b), and is the first representative of the novel order *Natranaerobiales* within the phylum *Firmicutes*. *N. thermophilus* grows (at $pH^{55°C}$ 9.5) between 35 and 56°C, with an optimum at 53°C. The $pH^{55°C}$ range for growth is 8.5–10.6, with an optimum at $pH^{55°C}$ 9.5 and no detectable growth at $\leq pH^{55°C}$ 8.2 or $\geq pH^{55°C}$ 10.8 (Fig. 14.1a). At the optimum pH and temperature, *N. thermophilus* grows in the Na^+ range of 3.1–4.9 M (1.5–3.3 M of added NaCl, the remainder from added Na_2CO_3 and $NaHCO_3$) and optimally between 3.3 and 3.9 M Na^+ (1.7–2.3 M added NaCl).

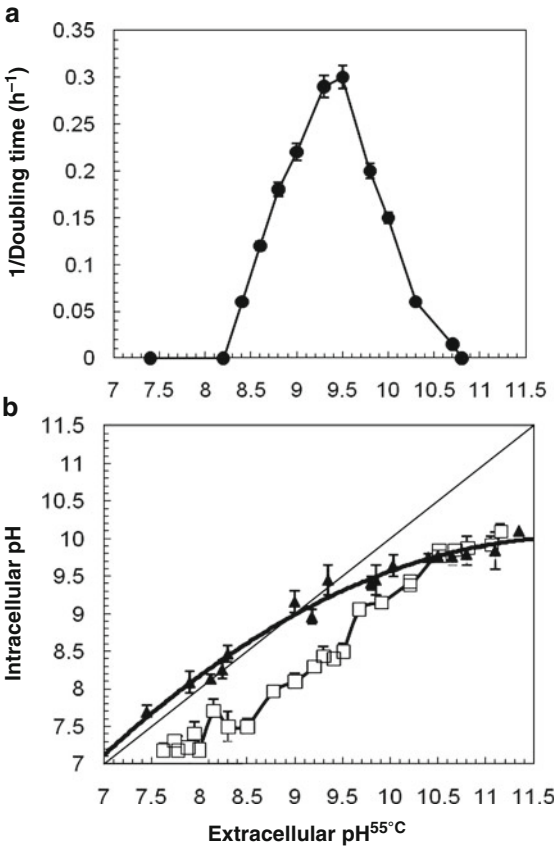

Fig. 14.1 Bioenergetic parameters in *Natranaerobius thermophilus*. (**a**) Effect of external $pH^{55°C}$ on growth of *N. thermophilus* in batch culture. (**b**) Effect of external pH on intracellular pH in sucrose-energized cell suspensions (*open square*) and non-energized cell suspensions (*filled triangle*). The *diagonal line* represents absence of a ΔpH

Two other anaerobic halophilic alkalithermophiles have been described recently. *Natranaerobius trueperi* grows (at $pH^{55°C}$ 9.5) between 26 and 55°C, with an optimum at 52°C. The $pH^{55°C}$ range for growth is 8.0–10.8, with an optimum at $pH^{55°C}$ 9.5 and no detectable growth at $\leq pH^{55°C}$ 7.8 or $\geq pH^{55°C}$ 11.0. At the optimum pH and temperature, *N. trueperi* grows in the Na^+ range of 3.1–5.4 M (1.5–3.8 M of added NaCl, the remainder from added Na_2CO_3 and $NaHCO_3$), and optimally at 3.8 M Na^+ (2.2 M added NaCl) (Mesbah and Wiegel 2009).

Natronovirga wadinatrunensis (the type species for the second genus described within the novel order *Natranaerobiales*) grows (at $pH^{55°C}$ 9.9) between 26 and 56°C, with an optimum at 51°C. The $pH^{55°C}$ range for growth is 8.5–11.5, with an optimum at $pH^{55°C}$ 9.9 and no detectable growth at $\leq pH^{55°C}$ 8.3 or $\geq pH^{55°C}$ 11.7. At the optimum pH and temperature, *Nv. wadinatrunensis* grows in the Na^+ range of 3.3–5.3 M (1.7–3.7 M of added NaCl, the remainder from added Na_2CO_3 and $NaHCO_3$), and optimally at 3.9 M Na^+ (2.3 M added NaCl) (Mesbah and Wiegel 2009).

A number of other aerobic and facultatively anaerobic halotolerant alkalithermophiles have been isolated, but their names have not been validly published and

in some instances they were not characterized beyond the genus level. "Caloramator halophilus" is an obligately proteolytic facultatively aerobic member of the *Firmicutes* isolated in our laboratory from salt flats located in northern Nevada. It is thermophilic, growing optimally at 64°C (temperature range 42–75°C), and alkaliphilic, growing optimally at $pH^{60°C}$ 9.2, and not growing below $pH^{60°C}$ 8.1 or above 10.8. It is unusual in having a large NaCl range for growth: it grows between 0 and 3 M NaCl, with an optimum at 1.5 M (NM Mesbah, P Maurizio and J Wiegel, unpublished).

"Bacillus thermoalcaliphilus" is a chemoorganotrophic, facultatively anaerobic bacterium isolated from mound soil infested with the termite *Odontotermes obesus* (Sarkar 1991). The optima for growth are 1.5 M NaCl, pH^{RT} 8.5–9.0 and 60°C. *Bacillus* STS1, isolated from the same soil termite mounds as "B. thermoalcaliphilus," grows optimally at 60°C, pH 9.0 and 1 M NaCl (Sarkar 1991). Another example is *Bacillus* sp. BG-11, isolated from an alkaline thermal environment in India. It is a facultatively aerobic, non-motile, spore-forming rod that is capable of growth at temperatures greater than 55°C, NaCl concentrations greater than 1.5 M and pH values 7.5–9.0 (Kevbrin et al. 2004).

Among the Archaea, *Natronolimnobius aegyptiacus* grows (at $pH^{55°C}$ 9.5) between 29 and 65°C, with an optimum at 54–57°C. The $pH^{55°C}$ range for growth is 7.3–10.8, with an optimum at $pH^{55°C}$ 9.5 and no detectable growth at $\leq pH^{55°C}$ 7.1 or $\geq pH^{55°C}$ 10.9. At the optimum pH and temperature, *Nl. aegyptiacus* grows in the Na^+ range of 2.9–5.5 M (2.8–5.3 M of added NaCl, the remainder from added Na_2CO_3 and $NaHCO_3$), and optimally at 4.5 M Na^+ (4.3 M added NaCl) (Bowers et al. in press). To our knowledge, *Nl. aegyptiacus* represents most extreme halophilic alkalithermophile among the Archaea.

14.2.2 Habitats of Halophilic Alkalithermophiles

The halophilic alkalithermophiles isolated and characterized thus far, *N. thermophilus, N. trueperi, Nv wadinatrunensis, Nl aegyptiacus* and *H. saccharophilum*, as well as other undescribed species "*N. grantii*" and "*N. jonesii*" (Bowers et al. 2009; KJ Bowers, NM Mesbah and J Wiegel, unpublished) were isolated from sediments of soda lakes in the Wadi An Natrun, Egypt and Lake Magadi in the Kenyan–Tanzanian Rift Valley (Zhilina et al. 2004; Mesbah et al. 2007b; Mesbah and Wiegel 2009; Bowers et al. in press). As will become apparent below, the ability of the halophilic alkalithermophiles to survive and grow optimally in the presence of multiple stressors is an adaptation to life in their natural environments.

14.2.2.1 Wadi An Natrun, Egypt

The Wadi An Natrun is a depression in the Sahara desert located 90 km northwest of Cairo. The bottom of the valley is 23 m below sea level and 38 m below the Rosette branch of the river Nile. Along the valley stretches a chain of seven large

alkaline, hypersaline lakes in addition to a number of ephemeral pools. Water is supplied by underground seepage from the river Nile and occasional winter precipitation. The depth of the lakes ranges between 0.5 and 2 m, and is regulated by seasonal changes in influx seepage and evaporation. High evaporation rates and arid climatic conditions during the summer months cause the salinity to rise above 5 M (Mesbah et al. 2007a).

The Wadi An Natrun lakes are extreme in several aspects; total dissolved salt concentrations are between 91.0 and 394 g L^{-1} in all seven of the lakes and the water in all lakes has pH values between 9 and 11. The lakes are solar heated. Salinity and temperature remain the same throughout the water column. Temperatures between ambient and 60°C were measured in the water below the salt crusts formed on the surface (NM Mesbah and J Wiegel, unpublished). Salinity in most of the lakes approaches saturation, with measured values between 2.5 and 5 M. The water in the most hypersaline lakes is anoxic, with dissolved oxygen concentrations below 0.2 ppm.

The Wadi An Natrun lakes are populated by dense communities of halophilic alkaliphilic microorganisms. The water displays different shades of red, purple and green according to their content of halophilic Archaea, photosynthetic purple bacteria and *Cyanobacteria*. Microbial mats occur along the lake floors and margins. The mats grow under a thin (1–2 mm) layer of sand. They consist of thin pink layers of purple photosynthetic bacteria and red halophilic Archaea followed by thick black layers containing decayed organic matter and sulfide minerals. Molecular analysis of the prokaryotic community of three large lakes of the Wadi An Natrun revealed a diverse range of prokaryotes, a high proportion of which had less than 90% 16S rRNA sequence identity to any sequences deposited in GenBank. The majority of the sequences retrieved were affiliated with the *Firmicutes*, *Alphaproteobacteria*, *Bacteroidetes*, and *Halobacteriales* (Mesbah et al. 2007a).

14.2.2.2 The Kenyan–Tanzanian Rift Valley

The Kenyan–Tanzanian rift valley harbors a series of six highly alkaline lakes. The salinities of these lakes vary from 1 M in the northerly lakes to 5 M in the lakes in the south, with roughly equal proportions of sodium carbonate and chloride as the major salts (Grant et al. 1999). The Rift valley is a volcanically active region and many of the soda lakes are fed by hot springs at temperatures of 45–96°C. The microbial community of these soda lakes contains alkaliphilic representatives of all major trophic groups of *Bacteria* and Archaea. It is hypothesized that there is active cycling of carbon, sulfur and nitrogen between these groups under both aerobic and anaerobic conditions. The aerobic environment of these soda lakes is dominated by *Cyanobacteria* and proteobacteria of the genera *Ectothiorhodospira* and *Halorhodospira*, contributing to photosynthetic primary production in the lakes (Jones et al. 1998; Rees et al. 2004) and haloalkaliphilic archaea (Grant et al. 1999). The soda lake anaerobic environment is dominated by chemoorganotrophic and

sulfate-reducing members of the *Firmicutes* (Jones et al. 1998). In addition to *Halonatronum saccharophilum*, a fermentative Gram-negative microorganism belonging to the *Thermotogales* was isolated. It is capable of growth at temperatures up to 78°C and pH values above 10.5, but cannot tolerate large sodium chloride concentrations (Jones et al. 1998). It has not been characterized beyond the genus stage.

14.3 Adaptive Mechanisms of Anaerobic Halophilic Alkalithermophiles

Life at high salt concentrations, alkaline pH values, high temperatures in addition to intense UV radiation undoubtedly requires special adaptive physiological mechanisms. Each extreme growth condition, whether high salt concentration, alkaline pH or high temperature, poses a number of physiological and bioenergetic problems.

14.3.1 Adaptation of Anaerobic Halophilic Alkalithermophiles to High Salinity

Halophilic microorganisms must maintain their cytoplasm at least isoosmotic with their surroundings in order to prevent loss of water to the environment. Maintenance of a turgor pressure requires a hyperosmotic cytoplasm. All halophilic microorganisms tested thus far, with the possible exception of the halophilic Archaea of the family *Halobacteriaceae*, maintain a turgor pressure (Oren 1999).

There are two mechanisms that enable halophilic microorganisms to live in high salt concentrations, the "high-salt-in" strategy involves accumulation of equimolar concentrations of inorganic ions (predominantly potassium) in the cytoplasm. This method necessitates that all intracellular proteins be stable and active in the presence of molar concentrations of potassium and other salts (Oren 2002). This method has been observed in *Salinibacter* spp., *Halanaerobiales* and Archaea of the *Halobacteriaceae*. The "low-salt-organic-solutes-in" strategy is based on the accumulation and/or de novo synthesis of water soluble organic solutes which do not interfere with the activity or stability of normal enzymes (Oren 2002). This mode has been observed in moderate aerobic halophiles tolerating Na^+ concentrations up to 1.5 M (Oren 2008; Ventosa et al. 1998). In rare cases, both strategies can be combined (see below).

Analysis of intracellular ions in energized cell suspensions of the anaerobic, halophilic alkalithermophilic *N. thermophilus* showed that at its optimal growth conditions of 53°C, $pH^{55°C}$ 9.5 and 3.5 M Na^+, intracellular K^+ concentration was 250 mM. The intracellular K^+ concentration did not vary with changes in the

K^+ concentration of the culture medium. This concentration remained constant below extracellular $pH^{55°C}$ 9.5, but increased sharply at more alkaline extracellular pH values reaching 540 mM at $pH^{55°C}$ 10.6, the maximum growth pH for *N. thermophilus*. These values were consistent with those measured in exponentially growing cells under the same growth conditions. The intracellular Na^+ concentration in both exponentially growing and energized cell suspensions was 8 mM at extracellular $pH^{55°C}$ 9.5, and increased to 33 mM at $pH^{55°C}$ 10.5 (Mesbah et al. 2009). Concentrations of other ions (Mg^{2+}, Mn^{2+}, Li^+) were in the nanomolar range. These data indicate that *N. thermophilus* does not rely solely on the "high-salt-in" strategy for osmotic adaptation.

Analysis of the complete genome sequence of *N. thermophilus* showed that it contains genes for the biosynthesis and uptake of the osmotic solute glycine betaine. Physiological assays showed that, when grown at 52°C and $pH^{55°C}$ 9.5, intracellular concentration of glycine betaine increased more than twofold, from 410 mM at 3.3 Na^+ to 1.07 M at 4.5 M Na^+ (Table 14.2; B Zhao and J Wiegel, unpublished). Intracellular concentration of the amino acid glutamate (the only amino acid present in millimolar amounts in the cells) also increased greatly, from 19 mM in the presence of 3.3 M Na^+ to 207 mM at 4.5 M Na^+. The intracellular concentration of glycine betaine was only affected by changes in the extracellular Na^+ concentration but was not significantly affected by alterations in either temperature or extracellular pH (Table 14.2). On the other hand, intracellular concentration of glutamate increased almost threefold in response to an increase in temperature (Table 14.2). These data indicate that the accumulation of the amino acid plays roles in the adaptation to both high salinity and temperature.

The data above imply that the anaerobic, halophilic, alkalithermophilic *N. thermophilus* utilizes a combination of both the "high salt-in" and the "low-salt-organic-solutes-in" strategies for osmotic adaptation. The finding of glycine

Table 14.2 Effect of salinity, $pH^{55°C}$ and temperature on accumulation of glycine betaine and glutamate in cells of *Natranaerobius thermophilus* (data from B Zhao and J Wiegel)

Growth condition	Glycine betaine Intracellular concentration (mM)	Glutamate Intracellular concentration (mM)
Effect of salinity $pH^{55°C}$ 9.5, 52°C		
3.3 M Na^+	410	19
3.9 M Na^+	570	150
4.5 M Na^+	1,070	207
Effect of temperature $pH^{55°C}$ 9.5, 3.9 M Na^+		
37°C	500	61
54°C	630	178
Effect of $pH^{55°C}$ 52°C, 3.9 M Na^+		
$pH^{55°C}$ 8.5	630	148
$pH^{55°C}$ 10.5	500	121

betaine in molar concentration inside cells of *N. thermophilus* is significant, as it has been previously thought that anaerobic halophiles usually adapt to high salinity using the "high-salt-in" strategy alone, and do not contain osmotic solutes (Oren 2008). The majority of anaerobic halophiles isolated thus far belong to the order *Halanaerobiales*, and, with the exception of *Halonatronum saccharophilum*, are mesophilic/thermotolerant and tolerate neutral to slightly alkaline pH. The question therefore arises that the "high-salt-in" strategy could pose bioenergetic problems during growth at high salinity in combination with alkaline pH and high temperature. Indeed, as described below, *N. thermophilus* uses a dual mechanism for cytoplasm acidification, one involving cytoplasmic buffering mediated by an acidic proteome (p*I* between 4 and 5 based on amino acid analysis) and possibly by accumulation of acidic amino acids (glutamate) and unusual polyamines. The presence of molar concentrations of cations in the cytoplasm, as would occur during the "high-salt-in" strategy, would prematurely neutralize the interior of the cell, thereby compromising cytoplasm acidification towards the alkaline end of the pH growth profile for *Natranaerobius thermophilus*. It is also plausible that a high intracellular cation concentration will destabilize intracellular enzymes already adapted to be structurally stable and function at an intracellular pH that is alkaline compared to non-halophilic alkaliphiles and alkalithermophiles (Cook et al. 1996; Olsson et al. 2003).

N. thermophilus has also been shown to have a requirement of chloride in its growth medium (KJ Bowers and J Wiegel, unpublished results). *N. thermophilus* required 1.2 M of chloride ions for growth in the presence of 3.3 M Na^+, and showed no growth at or below 1 M chloride. A requirement for chloride has been previously described for several microorganisms, such as *Halobacillus halophilus*, *Salinibacter ruber* and *Halanaerobium praevalens* (Müller and Oren 2003; Roeßler and Müller 1998). Microorganisms of the *Halanaerobiales* such as *H. praevalens* use chloride and other inorganic ions to maintain the cytoplasm iso-osmotic with their environments i.e., "high-salt-in" strategy. *H. praevalens* possesses an intracellular chloride concentration of 2.2 M, roughly equivalent to that of its extracellular environment (Oren 2002). Similar results were reported for *S. ruber* (Oren et al. 2002). In *Halobacillus halophilus*, flagellin biosynthesis is strongly dependent upon the presence of chloride, as is organic compatible solute transport and synthesis (Roeßler and Müller 2001, 2002). Chloride has also been identified as a signal molecule for organic compatible solute modification in environments with constantly changing salt concentrations (Saum and Müller 2008; Müller and Saum 2005).

Since *N. thermophilus* uses a combination of both the "high-salt-in" and "low-salt-organic-solutes-in" strategies for osmotic adaptation, it is difficult to predict the role that intracellular chloride ions play during growth at high salinity, alkaline pH and elevated salt concentration. It is possible that intracellular chloride ions play a role in stabilization of intracellular proteins, as has been reported for other negatively charged compatible solutes (Martins et al. 1997).

14.3.2 Adaptation of Halophilic Alkalithermophiles to Alkaline pH

Halophilic alkalithermophiles not only cope with high salinity but also with alkaline pH. They are faced with all the bioenergetic problems faced by alkaliphiles, primarily an inverted ΔpH, suboptimal proton motive force and the need to constantly acidify the cytoplasm whilst growing in a dearth of protons. The extent to which the two stressors i.e., high salinity and alkaline pH interact and produce syntropic actions is not yet understood. Therefore, the adaptations of halophilic alkalithermophiles to alkaline pH are discussed from the viewpoint of alkaliphiles.

Generally, microorganisms must maintain a cytoplasmic pH that is compatible with optimal functional and structural integrity of the cytoplasmic proteins that support growth. Many non-extremophilic microorganisms grow over a broad range of external pH values, from 5.5 to 9.0, and maintain a cytoplasmic pH that lies within the narrow range of pH 7.4–7.8 (Padan et al. 2005). The consequences of not being able to do so are profound. The anaerobic *Caloramator fervidus* (basonym *Clostridium fervidum*), (Collins et al. 1994) has bioenergetic processes that are entirely Na^+-coupled and lacks active H^+ extrusion or uptake systems that can support pH homeostasis. *C. fervidus* is unable to generate a pH gradient or regulate cytoplasmic pH. As a result, it grows only within the narrow pH range of 6.3–7.7 (Speelmans et al. 1993).

The key bioenergetic difficulty faced by all alkaliphiles is that, in contrast to neutralophiles and acidophiles, they have a reversed ΔpH ($pH_{in} - pH_{out}$), i.e., their cytoplasmic pH is more acidic than the extracellular pH. Thus, alkaliphiles must have mechanisms for cytoplasmic acidification and/or homeostasis. A number of adaptive strategies are used for intracellular pH homeostasis. These strategies include (a) increased expression and activity of monovalent cation/proton antiporters, (b) increased ATP synthase activity that couples H^+ entry to ATP generation, (c) changes in cell surface properties, and (d) increased metabolic acid production through amino acid deaminases and sugar fermentation. Among these strategies, monovalent cation/proton antiporters play an essential and dominant role in cytoplasmic pH regulation and also have a role in Na^+ and volume homeostasis (Krulwich et al. 1998; Padan et al. 2001, 2005; Slonczewski et al. 2009).

14.3.2.1 Cytoplasm Acidification and ΔpH in *Natranaerobius thermophilus*

Bioenergetic analyses on energized cell suspensions of the anaerobic, halophilic, alkalithermophilic *N. thermophilus* showed that it is capable of cytoplasm acidification. *N. thermophilus* maintains a ΔpH of approximately 1 unit ($acid_{in}$) over an extracellular $pH^{55^\circ C}$ range of 7.5–11.5 (Fig. 14.1b). This ΔpH of ~1 unit was due to active cytoplasm acidification as indicated by the collapse of the gradient (measured at optimal extracellular conditions) in the presence of inhibitors that

dissipate the ΔpH (e.g., 3,3′,4′,5-tetrachlorosalicylanilide, nigericin). Furthermore, the ΔpH was absent in non-energized suspensions of *N. thermophilus* (Fig. 14.1b) (Mesbah et al. 2009). As the external $pH^{55°C}$ was increased from 8.0 to 11.0, the intracellular $pH^{55°C}$ increased from 7.2 to 9.9 (Fig. 14.1b). The ΔpH across the cell membrane (1 pH unit, $acid_{in}$) did not collapse, even at the upper and lower $pH^{55°C}$ boundaries for growth. This indicates that cessation of growth at more alkaline pH values is not due to a decrease in ΔpH as has been reported for non-halophilic alkaliphiles and alkalithermophiles, but rather due to excessive alkalinization of the cytoplasm (Sturr et al. 1994; Cook et al. 1996; Olsson et al. 2003). The intracellular pH of *N. thermophilus* increases to 9.9 at the maximum extracellular pH allowing growth ($pH^{55°C}$ 10.6). To our knowledge, this is one of the highest intracellular pH values measured in an alkaliphile.

Analysis of the intracellular pH in non-energized cell suspensions of *N. thermophilus* (under aerobic conditions) showed a ΔpH close to zero at external $pH^{55°C}$ less than 9.5, but appearance of a ΔpH of approximately 1 unit at external $pH^{55°C}$ values greater than 9.5. This ΔpH was partially collapsed by protonophores and ionophores. These data suggest that at the alkaline range of the pH growth profile of *N. thermophilus*, cytoplasm acidification is achieved by an energy-independent mechanism (cytoplasmic buffering, discussed below).

14.3.2.2 Active Cytoplasm Acidification and Cation/Proton Antiporters in *Natranaerobius thermophilus*

Cation-proton antiporters catalyze the active efflux of Na^+ and/or K^+ in exchange for H^+ from outside the cell. $Na^+(K^+)/H^+$ antiporters are secondary active transporters and use the energy of the electrochemical gradient of ions across the cell membrane to drive antiport. Cation-proton antiporters support cytoplasmic pH homeostasis and allow tolerance to alkali (Slonczewski et al. 2009).

Bacterial genomes typically contain 11–14 gene loci predicted to encode $Na^+(K^+)/H^+$ antiporters. The completed genome of *N. thermophilus* was found to contain 17 predicted genes for monovalent cation-proton antiporters (Mesbah et al. 2009; Krulwich et al. 2009). Cloning and heterologous expression of 12 of these genes identified in the draft genome of *N. thermophilus* showed that 8 of them were capable of complementing Na^+ and alkali-sensitivities of triple-antiporter deficient *Escherichia coli* KNabc. The remaining four complemented the K^+ uptake deficiency in K^+-uptake deficient *E. coli* TK2420 (Mesbah et al. 2009). The predicted antiporters belonged to different antiporter families, including the cation-proton antiporter (CPA)-1, CPA-2 and NhaC-families. In addition, there was one antiporter, Nt-Nha that according to sequence analyses was a stand-alone Mrp-type protein (Mesbah et al. 2009). All these protein families contain transporters that play roles in cytoplasmic pH regulation, extrusion of intracellular Na^+ and cell volume regulation (Radchenko et al. 2006; Wei et al. 2007; Slonczewski et al. 2009).

All eight of the putative antiporter proteins displayed electrogenic antiport when heterologously expressed in membranes of triple-antiporter deficient *E. coli* KNabc,

indicating that they catalyze antiport at the expense of the electrochemical membrane potential (−174 mV, relatively negative inside the cell) present across the cell membrane of *N. thermophilus*. Electrogenicity is an important property of cation/proton antiporters that support alkali resistance (Padan et al. 2005; Slonczewski et al. 2009). All eight putative antiporters showed Na^+ and K^+-dependent consumption of the membrane potential.

The kinetic and biochemical properties of the eight antiporter proteins were well suited to the intracellular conditions of *N. thermophilus*. Seven antiporter proteins exhibited strong $Na^+(K^+)/H^+$ antiport activity, and one showed only K^+/H^+ antiport activity. Together, the eight antiporters functioned over a range of Na^+ and K^+ concentrations, consistent with the ability of *N. thermophilus* to grow over a range of salinities. Antiporters of *N. thermophilus* have alkaline pH optima for activity, ranging between $pH^{37°C}$ 8.5 and 8.8, consistent with the intracellular pH measured in cells of *N. thermophilus* (Fig. 14.2). One antiporter had a more alkaline profile (pH 8.5–10) with an optimum at pH 9.5. This could potentially allow the bacterium to survive and grow at the alkaline intracellular pH reached in *N. thermophilus* when exposed to alkaline stress. The pH profiles for the different antiporters were overlapping, indicating that they play concomitant roles in intracellular pH and/or salt tolerance of *N. thermophilus*. Concomitant roles were further confirmed when it

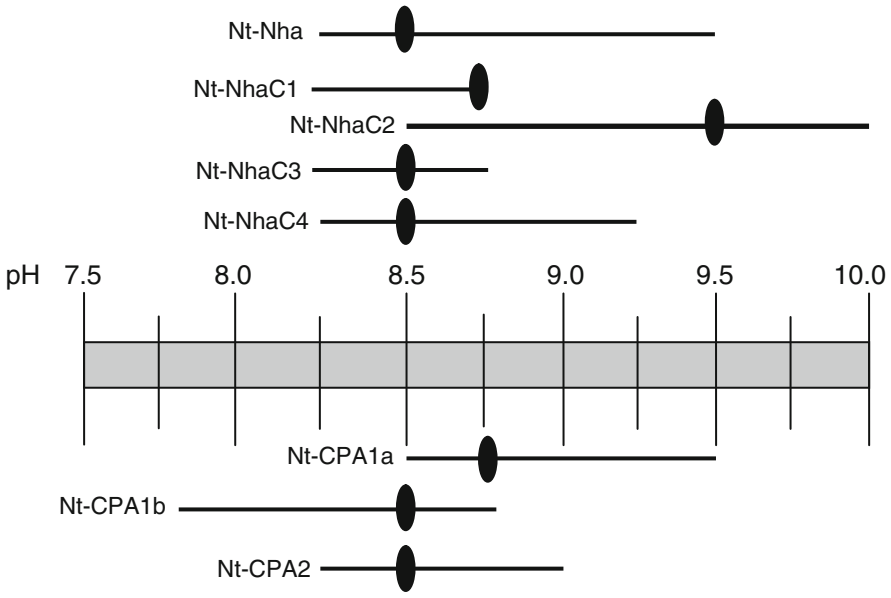

Fig. 14.2 pH activity profiles of cation/proton antiporters of *Natranaerobius thermophilus*. Optimal pH is indicated with a *filled circle*

was shown that they are constitutively expressed when *N. thermophilus* was exposed to both acid- and alkaline-stress (Mesbah et al. 2009).

A number of notable features exist about antiporter-mediated cytoplasm acidification of *N. thermophilus*. The unusually large number of predicted antiporter gene loci in the genome (the most observed in an alkaliphile thus far (Krulwich et al. 2009)) suggests that the total number of antiporters in the antiporter-complement of a bacterium is influenced by the number of environmental challenges faced by the bacterium and not simply the intensity of a single environmental challenge. *N. thermophilus* has 17 predicted antiporters; the extremely alkaliphilic, non-halophilic and mesophilic *Bacillus halodurans* C-125 has only five predicted antiporters (Takami et al. 2000). It is possible that extremophiles facing only one extreme require only a limited number of antiporters which are specifically adapted to face the bioenergetic difficulties posed by that particular extreme. On the other hand, poly-extremophiles such as *N. thermophilus* need a large complement of antiporters, their overlapping roles and properties will serve as defense mechanisms against the large array of bioenergetic problems posed by multiple extreme conditions. This assumption is further supported by the constitutive expression of antiporter genes under both acid and alkaline stress, indicating that they are important for growth under different environmental conditions. Genetic studies (deletion/mutation) are needed to determine whether individual antiporters play specific roles under certain growth conditions. However, no genetic systems exist for halophilic, alkalithermophilic bacteria.

Another notable feature of *N. thermophilus* antiporters is their ability to transport both Na^+ and K^+ during cytoplasm acidification, and the presence of a specific K^+/H^+ antiporter. This is in contrast to the aerobic alkaliphiles studied thus far, which use Na^+ exclusively as a substrate for cytoplasm acidification (Padan et al. 2005). The ability to use K^+/H^+ antiport in addition to Na^+/H^+ antiport for cytoplasm acidification is beneficial for an anaerobic halophilic alkalithermophile. Aerobic alkaliphiles typically use H^+-coupled bioenergetics; there is no competition for the intracellular Na^+ substrate. On the other hand, anaerobic, non-halophilic alkalithermophiles have Na^+-coupled mechanisms for pH homeostasis and solute transport (Speelmans et al. 1993; Prowe et al. 1996; Ferguson et al. 2006). *N. thermophilus* has been shown to have a Na^+-coupled F_1F_0-type ATPase which, when tested in vitro, functions primarily in the ATP hydrolysis direction, i.e., it expels Na^+ from the cell at the expense of ATP (NM Mesbah, J Wiegel, manuscript in review). This Na^+-coupled ATPase will compete with the Na^+/H^+ antiporters for the intracellular Na^+ substrate. In this case, K^+/H^+ antiporters can continue to acidify the cytoplasm after the Na^+/H^+ antiporters and the Na^+-coupled ATPase sufficiently reduce the intracellular Na^+ concentration. *N. thermophilus* accumulates approximately 250 mM of K^+ inside its cytoplasm when grown at optimal conditions (extracellular K^+ was 8.4–500 mM), this concentration more than doubles to 540 mM when grown under alkaline stress (extracellular $pH^{55°C}$ 10.2). Accumulation of intracellular K^+ can fuel K^+-dependent antiport. Four of the 12 predicted antiporter genes did not show cation/proton activity but did support K^+ uptake in a K^+-uptake deficient strain of *E. coli*. It is possible that

these transporters couple K^+ uptake with exchange of organic molecules, as has been reported for the *Bacillus subtilis* YqkI malic/Na^+-lactate antiporter (Wei et al. 2000).

Another notable feature of *N. thermophilus* antiporters is the significant contribution of NhaC antiporters to cytoplasm acidification and alkali resistance. Four of the eight putative *N. thermophilus* antiporters showing cation/proton antiport activity when heterologously expressed in antiporter-deficient *E. coli* were homologous to other antiporters of the NhaC family (Mesbah et al. 2009). This is in contrast to what has been reported in extremely alkaliphilic aerobes. In these microorganisms, NhaC antiporters play minor roles in alkali-resistance; deletion of the NhaC antiporter in extremely alkaliphilic, aerobic *Bacillus pseudofirmus* OF4 did not significantly affect growth and cytoplasm acidification at pH 10.5 (Ito et al. 1997). Its major role was Na^+ extrusion from the cell at lower pH values. *Bacillus subtilis* NhaC does not have a prominent role in pH homeostasis (Padan et al. 2005). Among the cyanobacteria, the only cation/proton antiporter reported to contribute to alkaline pH resistance of the aerobic, alkaliphilic cyanobacterium *Synechococcus elongatus* belonged to the CPA-2 family (Billini et al. 2008). In the aerobic, alkaliphilic and moderately halophilic *Alkalimonas amylolytica*, the three antiporters shown to participate in alkali resistance belonged to the calcium/cation transporter, CPA-2 and CPA-1 families (Wei et al. 2007). It follows that NhaC antiporters play larger roles in alkali resistance in anaerobes than in aerobes. Indeed, the gene encoding the Na^+/H^+ antiporter NhaC-2 in the anaerobic, non-halophilic *Desulfovibrio vulgaris* was up-regulated during alkaline stress. An *nhaC-2* deletion mutant showed increased susceptibility to alkaline pH compared to the wild-type, showing that this NhaC antiporter plays an important role in adaptation to alkaline stress (Stolyar et al. 2007).

14.3.2.3 Cytoplasmic Buffering in *Natranaerobius thermophilus*

As discussed above, comparison of the intracellular pH in energized and non-energized cells of *N. thermophilus* showed that it utilizes a dual-mechanism for cytoplasm acidification; at extracellular pH at and below the optimal growth $pH^{55°C}$ of 9.5, the cytoplasm is acidified via the action of at least eight electrogenic cation/proton antiporters. As the extracellular pH increases beyond the optimum, antiport-dependent cytoplasm acidification stops, but the cytoplasm remains approximately 1 pH unit more acidic than the pH outside the cell. Under these conditions, cytoplasmic buffering capacity contributes to cytoplasm ΔpH homeostasis. Analysis of the proteome of *N. thermophilus* showed that, consistent with being a halophile, the isoelectric point of proteins is predominantly acidic, ranging between 4 and 5. Thus, at the alkaline intracellular pH of *N. thermophilus*, the majority of proteins will be negatively charged. As the internal pH increases, the charge on the proteome will be become more negative. In order to reach overall neutrality inside the cell, cations, such as H^+, K^+ and Na^+ will enter the cell, leading to a more acidic interior (Mesbah et al. 2009). Thereby, it is hypothesized that cessation of growth of

N. thermophilus at the alkaline pH range is due to cytoplasm alkalinization due to saturation of the cytoplasmic buffering capacity, and not due to ΔpH collapse as was reported for the non-halophilic, anaerobic alkalithermophile, *Clostridium paradoxum* (Cook et al. 1996).

Cytoplasmic buffering has been reported in aerobic alkaliphiles where it works concomitantly with antiporters to acidify the cytoplasm (Slonczewski et al. 2009). Cytoplasmic buffering does not replace the role of antiporters in cytoplasm acidification. The fact that antiporter-based homeostasis comes to a stop when approaching the pH_{max} of *N. thermophilus* and is replaced by cytoplasmic buffering suggests that the buffering capacity of cytoplasm in anaerobic halophilic alkalithermophiles is stronger than that of aerobic, non-halophilic, mesophilic aerobes. This could be due to differences in the isoelectric point and ionization of the cells proteome, or due to differences in the intracellular content of the cells.

14.3.3 Adaptation of Halophilic Alkalithermophiles to Elevated Temperature

The third stressor faced by halophilic alkalithermophiles is that of elevated temperature. Generally, thermophiles are faced with the challenge of controlling cytoplasmic membrane permeability at high temperatures. Increased motion of lipid molecules at elevated temperatures results in increased permeability to protons. Due to this motion, water molecules become trapped in the lipid core of the membranes allowing protons to hop from one molecule to the other. Other ions, unlike protons, can diffuse through the membrane. Diffusion is a temperature dependent process hence membrane permeability to ions will increase as well (Konings et al. 2002).

Bacterial cell membranes contain lipids composed of two fatty acyl chains esterified to glycerol. The third hydroxyl group of glycerol is linked to hydrophilic phosphor- or glyco-containing headgroups. These lipids are organized in a lipid bilayer such that the polar headgroups are exposed to the water phases and the acyl chains are directed towards the hydrophobic interior of the membrane. To keep the cytoplasmic membrane in a liquid crystalline state, thermophilic bacteria have been shown to increase the chain length of the lipid acyl chain, the ratio of iso/anteiso branching and/or the degree of saturation of the acyl chain (Albers et al. 2006).

Analysis of the phospholipid fatty acid (PLFA) profile for *N. thermophilus* showed that the polar and neutral fatty acid compositions were similar, with a predominance of iso- and, to a lesser extent, anteiso-branched 15:0 fatty acids (Table 14.3). Unexpectedly, the PLFA profile did not change significantly in response to either acid and alkaline stress or hyposalinity and hypersalinity (Table 14.3). The PLFA profile of *N. thermophilus* does not show the hallmarks of a thermophilic bacterium, there were no fatty acids longer than 18 carbons and some branched and unsaturated fatty acids (Table 14.3). This could possibly explain

Table 14.3 Effect of $pH^{55°C}$ and salinity on the membrane lipid composition of *Natranaerobius thermophilus*

Growth condition[a]	Na^+ 3.9 M $pH^{55°C}$ 9.5	Na^+ 3.1 M $pH^{55°C}$ 8.5	Na^+ 4.9 M $pH^{55°C}$ 8.5	Na^+ 3.1 M $pH^{55°C}$ 10.5	Na^+ 4.9 M $pH^{55°C}$ 10.5
Polar lipids					
Terminally branched saturated fatty acids					
i15:0	77.2[b]	65.1	74.0	78.4	74.9
a15:0	11.3	7.6	10.8	10.2	12.2
i17:0	1.8	2.8	2.5	2.6	2.6
a17:0	0.9	0.8	0.8	0.8	1.0
Monoenoic fatty acids					
16:1 ω 7c	0.7	4.4	1.1	0.9	0.8
Branched monoenoic fatty acids					
i17:1 ω 7c	1.2	0.9	1.5	1.3	1.2
Normal saturated fatty acids					
14:0	2.4	3.6	3.2	1.9	2.9
16:0	2.1	7.0	3.3	1.9	2.4
Polyenoic fatty acids					
18:2 ω 6	0.0	1.4	0.0	0.0	0.0
Neutral lipids					
Terminally branched saturated fatty acids					
i15:0	54.4	42.4	57.8	61.5	53.1
a15:0	11.5	6.4	10.3	9.2	10.8
i17:0	5.9	11.3	9.6	10.3	13.9
a17:0	2.4	1.7	2.2	2.3	3.0
Monoenoic fatty acids					
16:1 ω 7c	2.7	3.9	2.6	1.7	0.0
18:1 ω 9c	3.0	8.5	0.0	0.0	0.0
Branched monoenoic fatty acids					
i17:1 ω 7c	2.4	2.0	0.0	3.2	3.9
Normal saturated fatty acids					
14:0	2.2	2.3	3.0	1.7	2.3
16:0	7.3	12.3	9.9	5.6	9.1
18:0	4.8	4.0	3.2	1.9	2.5
Polyenoic fatty acids					
18:2w6	0.0	1.9	0.0	0.0	0.0

[a]All cultures were grown at 52°C
[b]Numbers denote the percentage of total polar and neutral lipids, respectively

why *N. thermophilus* is a moderate thermophile, showing no growth at temperatures greater than 56°C. While it was not possible to examine the effect of temperature on the PLFA profile of *N. thermophilus* due to poor biomass yield at the extremes of the temperature profile (NM Mesbah and J Wiegel, unpublished), it is not expected that there will be variation in the PLFA profile. *N. thermophilus* grows optimally at a temperature of 52°C, at temperatures greater than 54°C the growth rate plummets and growth ceases at 56°C (Mesbah et al. 2007b). Such a small difference between the optimal growth temperature and the maximum growth temperature is not sufficient to induce massive changes in the PLFA profile. The maintenance of

a ΔpH even at alkaline extracellular pH values indicates that the cell membrane of *N. thermophilus* remains relatively impermeable to protons, i.e., protons are not lost from the cytoplasm during alkaline stress.

Polyamines are low-molecular weight, aliphatic polycations found in the cells of all living organisms. The most common polyamines in microorganisms are putrescine (1,4-diaminobutane), spermine and spermidine. Due to their negative charge, polyamines bind to macromolecules such as DNA, RNA and proteins. Polyamines are involved in diverse processes, and have been reported to play critical roles in the stabilization of proteins and nucleic acids during exposure to extremes of temperature (either hot or cold) (Tabor and Tabor 1985). Polyamines have been shown to stabilize DNA and RNA in thermophilic cells (Terui et al. 2005). The presence of the polyamines tetrakis(3-aminopropyl)ammonium and spermidine were shown to be critical for thermophile protein biosynthesis in *Thermus thermophilus* near the optimal growth temperature of 65°C (Uzawa et al. 1993).

Analysis of the polyamine content of *N. thermophilus* showed the presence of spermine, spermidine and putrescine, in addition to two unidentified polyamines (Table 14.4). One of the unidentified polyamines had an elution time that coincides with that of tetrakis(3-aminopropyl)ammonium, a branched polyamine found in hyperthermophiles (Terui et al. 2005). These polyamines could play a role in stabilization of nucleic acids and proteins in the thermophilic *N. thermophilus*. Analysis of the changes in intracellular concentrations of polyamines in response to changing temperature and genetic analyses are needed to determine the precise role each polyamine plays in the adaption of *N. thermophilus* to high temperature in the presence of high salinity and alkaline pH.

It is interesting that, in addition to increasing in response to high salinity, the intracellular concentration of the amino acid glutamate increases from 61 to 178 mM in response to an increase in growth temperature from 37 to 54°C (Table 14.2). Amino acids typically serve as organic compatible solutes in halophilic bacteria (Roberts 2005). A number of studies have revealed that the role of organic compatible solutes extends beyond protection against osmotic stress to protection against high temperature, freezing and desiccation (Santos and da Costa 2002; Welsh 2000). In many thermophiles studied, the level of compatible solutes increases when cells are grown at supraoptimal temperatures, indicating that compatible solutes play a role in protection of cellular components from the effects of heat (Silva et al. 1999; Gonçalves et al. 2003). Compatible solutes of thermophiles

Table 14.4 Polyamines of *Natranaerobius thermophilus*

Polyamine	Intracellular concentration (nmol/mg dry weight)
Putrescine	22.8
Spermidine	45.5
Spermine	59.0
N^1-Aminopropylagmatine[a]	22.8
Unknown	7.9

[a]Further structural analyses needed for confirmation

are generally negatively charged, where as mesophiles accumulate neutral solutes (Martins et al. 1997). The increase in intracellular content of the negatively charged amino acid glutamate therefore indicates that it plays a role in adaption of the anaerobic halophilic alkalithermophile *Natranaerobius thermophilus* to heat.

14.3.4 Resistance to UV Radiation and Other Adaptive Mechanisms of Anaerobic Halophilic Alkalithermophiles

Since anaerobic halophilic alkalithermophiles are exposed to intense solar radiation and periods of desiccation in their natural environments, it is expected that they have the ability to survive desiccation and γ-radiation. However, cells of *Natranaerobius thermophilus* did not show desiccation resistance for periods greater than 12 h (KJ Bowers, M.Sc. thesis). *N. thermophilus* also did not show resistance to ionizing radiation; cells exposed to 1 kGy of γ-radiation had a survival rate of 0.01%, cells exposed to 3 kGy of γ-radiation lost viability. The resistance of *N. thermophilus* to γ-radiation was only slightly higher than that of *E. coli* (KJ Bowers, M.Sc. thesis).

Both type species of the genera of the family *Natranaerobiaceae*, *N. thermophilus* and *Natronovirga wadinatrunensis* showed remarkable resistance when exposed to the three wavelengths of UV A, B, and C radiation (385, 312 and 254 nm, respectively). *N. thermophilus* displayed greater resistance, as evidenced by a greater number of viable cells (KJ Bowers, M.Sc. thesis) (Table 14.5). Such high adaptation to UV exposure is most probably an adaptation to the intense solar irradiation commonly encountered in hypersaline habitats.

Microorganisms have developed multiple strategies for surviving UV radiation. These include cell cycle arrest and activation of various pathways for repair of UV-damaged DNA. Tolerance mechanisms also exist, such as DNA recombination and lesion by-pass which allow cells to survive unrepaired lesions in their DNA (Boubriak et al. 2008). Bacterial nucleotide excision repair has also been shown to be required for repair of UV damage in the extremely halophilic archaeon *Halobacterium* NRC-1 (Crowley et al. 2006). Analysis of the genome sequence of *Natranaerobius thermophilus* showed that it possesses homologs for the *recFOR* genes, the *radA* and *radC* genes, in addition to homologs for DNA mismatch repair proteins MutS and MutN. The RecFOR pathway is a pathway of homologous recombination that repairs DNA; it can repair single- and double-stranded gaps

Table 14.5 Survival of *Natranaerobius thermophilus* and *Natronovirga wadinatrunensis* after UV radiation exposure (J Blamey, unpublished results)

	N. thermophilus	*N. wadinatrunensis*	*E. coli*
385 nm (34 J cm^{-2})	75% at 30 h	60% at 30 h	0% at 2 h
312 nm (46 J cm^{-2})	80% at 28 h	70% at 28 h	0% at 2 h
254 nm (60 J cm^{-2})	50% at 28 h	45% at 28 h	0% at 2 h

(Handa et al. 2009; Hiom 2009). RecO plays a vital role in radioresistance of *Deinococcus radiodurans* (Xu et al. 2008). A *recO* null mutant of *Deinococcus radiodurans* was extremely sensitive to irradiation with gamma rays and UV light. MutS and MutN play roles in DNA mismatch repair, which recognizes and repairs erroneous incorporation of bases that can arise during DNA damage (Kunkel and Erie 2005). RadA and RadC are archaeal proteins and play roles in DNA repair in the extremely halophilic archaea of the *Halobacteriales* (Boubriak et al. 2008). The presence of several putative DNA repair systems in the genome of *N. thermophilus* could explain its resistance to UV radiation. Functional genetic analyses are needed to confirm the role of each gene in the UV resistance of *N. thermophilus*.

Susceptibility to desiccation is interesting. It is possible that desiccation or a decrease in water activity results in irreversible destabilization of and denaturation of intracellular proteins. The proteome of *N. thermophilus* is acidic, with an isoelectric point between 4 and 5. It follows that intracellular proteins are stabilized primarily by polar and ionic interactions. Absence of water can compromise the polar bonds necessary for protein stabilization.

14.4 Conclusions

Anaerobic halophilic alkalithermophiles are an unusual group of extremophiles capable of robust growth under the combined extremes of high salinity, alkaline pH and elevated temperature. *Natranaerobius thermophilus* has been used as a prototype for investigation of the adaptive mechanisms allowing this unique lifestyle. *N. thermophilus* grows well when confronted with the combined challenges of high salinity (3.3 M Na^+), $pH^{55°C}$ and a temperature of 53°C. Several mechanisms appear to contribute to this extraordinary feat. First, *N. thermophilus* appears to use a combination of both the "high-salt-in" and "low-salt-organic-solutes-in" strategies for osmotic adaptation. *N. thermophilus* accumulates approximately 250 mM of K^+ (extracellular concentration 8.4 mM) and 1.1 M of the compatible solute glycine betaine when grown at 3.3 M Na^+, making it the first anaerobe known to accumulate compatible solutes. It has been assumed till now that anaerobic halophiles rely solely on the "high-salt-in" strategy for osmotic adaptation.

N. thermophilus displays an unusual pattern of cytoplasm pH homeostasis. It maintained a ΔpH of 1 unit throughout the entire pH range of growth, and the ΔpH did not collapse, even beyond the upper and lower ends of the pH growth profile. *N. thermophilus* tolerates a high cytoplasmic pH (when compared to anaerobic alkaliphiles and alkalithermophiles), and the growth rate drops significantly as the intracellular pH rises, indicating that growth cessation is due to excessive cytoplasm acidification and not due to collapse of the ΔpH as described for anaerobic, non-halophilic alkalithermophiles.

N. thermophilus possesses an unusually large number of cation/proton antiporters, and all of them contribute to cytoplasm acidification and pH homeostasis

at the optimal growth pH for *N. thermophilus*. Kinetic properties of these antiporters showed that they have similar and overlapping roles, and expression analyses indicated genes encoding these antiporters are constitutively expressed under acid- and alkaline-stress. It appears that extremophiles growing under multiple extremes need a large complement of antiporters, their combined and overlapping roles necessary to cope with the multiple bioenergetic problems posed by multiple extreme conditions. Cation/proton antiporters of *N. thermophilus* are capable of using both Na^+ and K^+ for cytoplasm acidification. *N. thermophilus* couples different bioenergetic processes such as solute transport and ATP hydrolysis with Na^+-pumping. Therefore, the ability to use multiple cations for antiport allows them to continue to acidify the cytoplasm in the event of a decrease in cytoplasmic Na^+ content. Kinetic analyses of the individual antiporter genes also showed a new role for NhaC antiporters in cytoplasm acidification. Previously, antiporters of the NhaC family have been thought to function mainly in Na^+ extrusion from the cell and do not play major roles in alkali resistance in aerobic alkaliphiles. It appears that they play different roles in anaerobic halophilic alkalithermophiles than they do in aerobic alkaliphiles.

N. thermophilus utilizes a dual-mechanism for cytoplasm acidification. At extracellular pH values at and below the optimum, cytoplasm acidification is achieved by a large number of cation/proton antiporters. When the extracellular pH rises beyond the optimum however, antiport-mediated cytoplasm acidification comes to a stop, and the high cytoplasmic buffering capacity (mediated by an acidic proteome) contributes to cytoplasmic acidification and maintains the ΔpH at more alkaline pH values. Aerobic alkaliphiles have also been shown to have high cytoplasmic buffering capacity, but it works concomitantly with antiporters for cytoplasm acidification and does not replace antiporters in alkali resistance.

N. thermophilus does not appear to alter its cell membrane lipid composition in response to salt- and pH-stress. It also does not show an unusual phospholipid fatty acid profile, indicating that the combined extremes of high salt, alkaline pH and high temperature do not influence the membrane lipid composition. *N. thermophilus* is a moderate thermophile, it grows optimally at 53°C and the growth rate plummets at temperatures greater than 55°C. It is possible that the membrane lipid composition plays a role in limiting the upper temperature for growth of this poly-extremophile.

Similar to other thermophiles, *N. thermophilus* accumulates polyamines and the negatively charged amino acid glutamate in its cells, with intracellular glutamate concentrations more than doubling in response to elevated temperature. It follows that in anaerobic halophilic alkalithermophiles, polyamines and compatible solutes not only protect against osmotic stress, but also contribute to protection against temperature stress.

A model showing the bioenergetic processes of *N. thermophilus* is shown in Fig. 14.3. *N. thermophilus* is a fermentative bacterium and generates the bulk of its ATP via substrate level phosphorylation. The membrane bound F-type ATPase of *N. thermophilus* is Na^+-coupled and is geared primarily in the hydrolysis direction, fuelled by cytoplasmic ATP and expelling Na^+ from the cytoplasm. Na^+-pumping

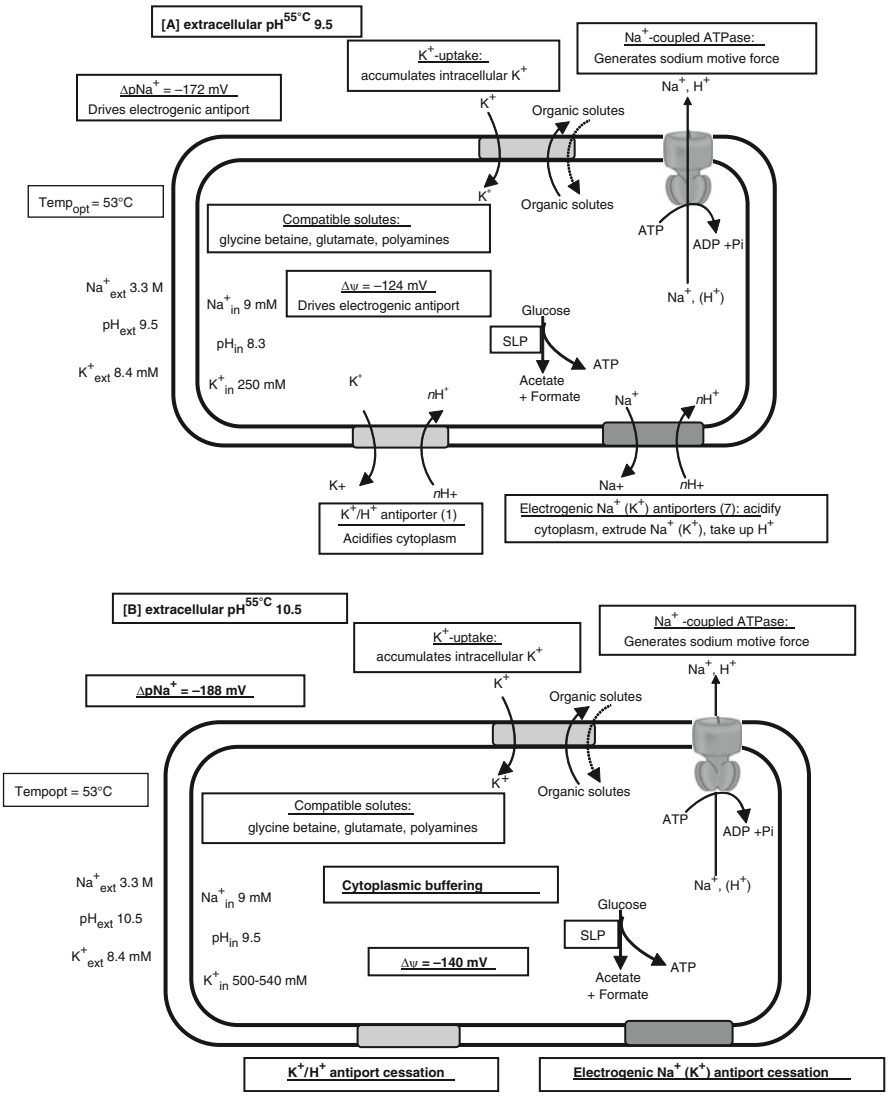

Fig. 14.3 Schematic diagram of bioenergetic processes of *Natranaerobius thermophilus* under optimal conditions (**a**) and alkaline stress (**b**)

contributes to the generation of an electrochemical gradient across the cell membrane, which is critical for driving electrogenic cation/proton antiport. *N. thermophilus* utilizes two distinct methods for cytoplasm acidification under conditions of high salt concentration, alkaline pH and elevated temperature. At extracellular $pH^{55°C}$ values at and below the optimum, acidification of the cytoplasm is achieved via a large cohort of electrogenic cation/proton antiporters that are able to translocate Na^+ and K^+ ions in exchange for protons. As the

extracellular $pH^{55^{\circ}C}$ increases, energy-dependent antiport activity stops, and acidification is probably achieved by physiochemical forces such as cytoplasmic buffering. *N. thermophilus* also uses two mechanisms for adaption to high salinity, accumulating both the inorganic cation K^+ and the organic compatible solute glycine betaine in its cytoplasm. The PLFA profile of *N. thermophilus* is not influenced by its combined growth extremes; however it appears that during growth at high temperature in the presence of alkaline pH and high salinity, both organic compatible solutes and polyamines play roles in adaptation to high temperature. All the above strategies allow *N. thermophilus* to adapt to combined extreme growth conditions, and also enable it to adapt to fluctuations in extracellular pH, Na^+ or temperature.

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