ANAEROBIC HALOPHILIC ALKALITHERMOPHILES: DIVERSITY AND PHYSIOLOGICAL ADAPTATIONS TO MULTIPLE EXTREME CONDITIONS

by

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(Under the Direction of Juergen Wiegel)

ABSTRACT

Halophilic alkalithermophiles are poly-extremophiles adapted to grow at high salt concentrations, alkaline pH values and temperatures greater than 50ºC. Halophilic alkalithermophiles are of interest from physiological perspectives as they combine unique adaptive mechanisms and cellular features that enable them to grow under extreme conditions. The alkaline, hypersaline lakes of the Wadi An Natrun, Egypt were chosen as sources for isolation of novel halophilic alkalithermophiles. These lakes are characterized by saturating concentrations of NaCl (5.6 M), alkaline pH (8.5-11) and temperatures of 50ºC due to intense solar irradiation. The prokaryotic communities of three large lakes of the Wadi An Natrun were assessed using 16S rRNA clone libraries. The Wadi An Natrun lakes are dominated by three phylogenetic groups of *Bacteria* (*Firmicute*s, *Bacteroidetes*, α- and γ-proteobacteria) and two groups of *Archaea* (*Halobacteriales* and *Methanosarcinales*). Extensive diversity exists within each phylogenetic group; half of the clones analyzed did not have close cultured or uncultured relatives. Three novel halophilic alkalithermophiles were isolated from the Wadi An Natrun. A novel order, *Natranaerobiales*, was proposed to encompass these novel isolates. *Natranaerobius thermophilus* was chosen as a model for more detailed physiological studies. Analysis of the

bioenergetic characteristics of *N.thermophilus* revealed the absence of cytoplasmic pH homeostasis. Rather, *N.thermophilus* has the novel feature of maintaining the cytoplasmic pH at a constant 1 unit below that of the extracellular pH, the cytoplasmic pH continuously changed with the extracellular pH. To investigate the mechanism of this dynamic cytoplasmic pH regulation, genes encoding putative cation/proton antiporter proteins were functionally characterized. The physiological characteristics of these cation/proton antiporters were well suited to the intracellular conditions of *N.thermophilus*. Collectively, antiporter proteins function over the whole pH range for growth of *N.thermophilus* and display antiport activity over a wide range of Na^+ ion concentrations. Thus, they are capable of providing active cytoplasmic acidification even if the intracellular environment of *N.thermophilus* changes. Most of the antiporter proteins belong to the NhaC family of cation/proton antiporters; a group that is primarily involved in expulsion of intracellular Na^+. These results indicate that these antiporters provide *N.thermophilus* tolerance to both alkaline pH and high salt concentrations.

INDEX WORDS: Halophile, Alkaliphile, Thermophile, Extremophile, Alkalithermophile, *Natranaerobiales, Natranaerobius thermophilus, Natronovirga*, Wadi An Natrun, Microbial diversity, Antiporter, Intracellular pH, Proton motive force, Membrane potential, Phosphorylation potential

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW*

The following introduction describes in general terms extremophiles and poly-extremophiles and the reasons to study them from chemotaxonomic, phylogenetic, and physiological perspectives. The first section outlines functional definitions of extremophiles and focuses on description of the two particular classes of poly-extremophiles studied in this thesis, the anaerobic alkalithermophiles and anaerobic halophilic alkalithermophiles. The next section describes hypersaline environments as biotopes for prokaryotic life, the diversity of microorganisms encountered in them, and how they are ideal locations for isolation of extremophiles. The following sections discuss the physiological aspects of survival and growth under extreme conditions with a focus on high salt, temperature and alkaline pH extremes. The mechanisms developed by different groups of prokaryotes to cope with the requirements of life under these extreme conditions are discussed in detail. Interactions between adaptive mechanisms to the different extreme conditions are presented and implications for poly-extremophiles are described. Finally, the last section outlines the main objectives of this work.

* Adapted from:

- Mesbah, N.M. and J. Wiegel. 2008. Life at extreme limits: The anaerobic halophilic alkalithermophiles. p 44-57. *In* J. Wiegel, M. Adams and R. Maier (eds.), The Incredible Anaerobes: From Physiology to Genomics to Fuels". Annals of the New York Academy of Sciences, New York.

- Mesbah, N.M. and J. Wiegel**.** 2005. Halophilic thermophiles: Life at the extremes, p. 91-118. *In* T. Satyanarayana and B.N. Johri (eds.), Microbial diversity: Current perspectives and potential applications. I.K. International Publishing House Pvt. Ltd., New Delhi.

EXTREMOPHILES: MICROORGANISMS LOVING THE EXTREMES 'WHEN EXTREME IS NORMAL'

We usually associate the extremes with properties outside of the ordinary. This leads to the question 'What is biologically normal?' From an anthrocentric point of view, 'normal' is what is comfortable for us as human beings. Thus, what we regard as 'normal' are ambient temperatures (~24ºC), neutral or near-neutral pH values between 6 and 8, atmospheric pressure and an atmosphere containing 20% vol/vol oxygen. Everything outside this comfort zone, such as temperatures above 40ºC, acidic or alkaline pH values, low atmospheric pressures (high altitudes) or high pressure (deep ocean) are regarded as 'extreme'. However, these 'normal' conditions are extreme conditions for the extremophiles; microorganisms that not only survive, but grow and thrive under harsh environmental conditions such as elevated (≥ 50ºC) or low (≤10ºC) temperatures, acidic (≤ 5) or alkaline (≥ 8.5) pH values, and very high pressure such as deep ocean sediments. In fact, many extremophiles cannot grow, divide or even survive under environmental conditions that we humans call 'normal'. For example, the aerobic, thermophilic *Sulfolobus* spp. can only grow at temperatures greater than 70ºC and pH values less than 3.0. At temperatures less than 30ºC, the microorganisms cannot grow as they are no longer able to maintain the internal pH (Huber & Prangishvili, 2006).

For this work, 'normal' conditions are defined as those where the microorganism will a) grow optimally at the shortest measured doubling time, b) attain reasonable cell densities (OD_{600} 0.3-1.0), c) remain viable indefinitely in a vegetative state, and d) form a major component of the prokaryotic community composition in its natural 'extreme' habitat. An excellent example is *Thermoplasma*, which lives in burning and smoldering coal piles, and can only grow in acidic (pH 0.5-3.0) environments at elevated temperatures (optimally around 60^{o}C). It cannot survive at

neutral pH values or ambient temperatures (Segerer *et al.*, 1988). There also exist some facultative extremophiles, which are microorganisms that can survive and grow under extreme conditions, but grow and multiply faster under human-defined 'normal' conditions.

Diversity of Extremophiles

The term 'extremophile' is collective for a great variety of *Bacteria* and *Archaea* that grow optimally under 'extreme' or 'abnormal' conditions; such as acid or alkaline pH, high or low temperature, and extremes of atmospheric pressure, salt and organic ion concentrations. Extremophiles are best characterized according to their growth profiles, using marginal data for certain culture or environmental conditions such as for NaCl range ($NaCl_{opt.}$, $NaCl_{min}$, $NaCl_{max}$) or temperature profile (T_{opt}, T_{min}, T_{max}). Examples of extremophiles include, but are not limited to: thermophiles (high temperature), psychrophiles (low temperature), acidophiles (low pH), alkaliphiles (high pH), piezophiles (high pressure, formerly called barophiles), halophiles (high salt concentration), osmophiles (high concentration of organic solutes), oligotrophs (low concentration of solutes and/or nutrients) and xerophiles (very dry environment). The term 'extremophile' also includes microorganisms able to grow in the presence of high metal concentrations or microorganisms that grow with unusually short doubling times e.g. *Vibrio natriegeans* grows with a doubling time of 5-6 minutes in a continuous culture (F. Canganella and J. Wiegel, unpublished). Table 1.1 contains definitions for different extremophiles. It is important to note that the criteria defining an extremophile are different for prokaryotes and eukaryotes. In general, a eukaryote is considered to be extreme at far less harsh conditions than prokaryotes.

Oxygen as an 'Extreme'

It is hypothesized that life evolved under anoxic conditions, i.e., absence of molecular oxygen (Baross, 1998, Wiegel, 1998). It thus follows that the first forms of life on Earth were anaerobic, and gained energy by an anaerobic metabolism. There are many anoxic niches in the environment and even inside the human body where obligate anaerobes predominate. In this article, we will refer to microorganisms that can grow both in the presence and absence of oxygen as facultative aerobes to stress the anaerobic origin of life and that the ability to use oxygen as an electron acceptor is an adaptation that happened at a later time from an evolutionary standpoint. We also stress that most anaerobes are able to survive in nearly all environmental conditions found on Earth.

Poly-Extremophiles

Whereas extremophiles are usually defined by one extreme, many natural environments pose two or more extremes such as acidic hot springs, alkaline hypersaline lakes and dry sandy deserts. These environments harbor acidothermophiles, halophilic alkaliphiles and UV/radiation resistant oligotrophs, respectively. Acidothermophiles are widely distributed because of the frequent occurrence of hot springs with acidic pH values (≤ 3.0). Acidic hot springs arise due to the presence of sulfuric acid formed by microbial and chemical oxidation of sulfur compounds. Many halophiles are also alkaliphiles as salt lakes, whether they are of marine origin (thalassic), or terrestrial origin (athalassic), are alkaline due to accumulation of carbonate salts.

When dealing with poly-extremophiles, problems arise when describing their optimal and marginal growth data as the value of one of the extreme growth conditions could be affected by the other. For example, the measured pH value of a medium is dependent on temperature due to

changing pKa values of different medium components at different temperatures (Wiegel, 1998). Thus, the pH of the medium when measured at room temperature will be different than when it is measured at the elevated growth temperature using temperature-calibrated electrodes and pH meters calibrated at the same growth temperature. 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 (especially in complex media) 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. Wiegel proposed (1998) that the temperature at which the pH measurement was taken and the pH meter calibrated be indicated as a superscript, e.g. $pH^{55^{o}C}$.

Similarly, for halophilic alkaliphiles, the elevated salt concentration has an effect on measured pH values. 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 by 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 the use of the electrode over a wider range of temperatures. For example, the ThermoScientific Orion ROSS ™ Ultra ® pH electrode used in our laboratory reduces the 'Na^+ error' to neglible amounts at pH values less than 12.

Extremophiles adapted to more than two extremes are much less common than those adapted to two extremes, with the exception of the aerobic thermacidophilic archaea. This most likely reflects lack of exploration of these novel groups of extremophiles and/or the improper selection of media and culture conditions. Two examples of 'poly-extremophiles', the anaerobic alkalithermophiles and anaerobic halophilic alkalithermophiles, have been isolated, cultivated and characterized in our laboratory, and are discussed below.

Anaerobic Alkalithermophiles

The anaerobic alkalithermophiles isolated thus far are neither the most alkaliphilic nor most thermophilic of the extremophiles. From the presently characterized anaerobic alkalithermophiles, it appears that there is a correlation between pH and temperature optima of the extremophiles; the larger the temperature optimum the lower the pH optimum and vice versa (Figure 1.1). This is not only true for species among different genera, but also for different strains of the same species (Kevbrin *et al.*, 2004). The scarcity of alkalithermophiles with pH optima $\geq$ 9.5 and temperature optima $\geq$ 65ºC could be due to physiological reasons. Growth under both of these extreme conditions requires specific adaptations of the cell wall and membrane compositions to minimize permeability to protons and cations. Alkalithermophiles are also faced with the burden of acidifying cytoplasmic pH whilst growing in a dearth of protons, and various other bioenergetic problems such as suboptimal proton motive force and phosphorylation potential. However, the existence of such isolates cannot be ruled out, as there have been isolates reported at such temperatures on the acidic side of the pH scale. Obligately aerobic archaeal *Picrophilus* species were isolated from solfataric areas in Japan. These isolates grow at pH values 0-4, and temperatures 55-65ºC (Schleper *et al.*, 1995). The genome of *Picrophilus torridus* contained genes encoding for a putative proton-pumping NADH dehydrogenase (complex I) and for quinol and cytochrome oxidation (Futterer *et al.*, 2004). Genes encoding for an A_0A_1-ATPase were also detected. This effective respiratory chain allows rapid extrusion of protons from the cell hence preventing cytoplasmic acidification. In addition, genome analysis showed evidence of various pathways for organic acid degradation. Organic acids are lethal for acidophiles as they behave as uncouplers of oxidative phosphorylation.

Interestingly, the genes that enhance the ability to *P. torridus* to cope with extremely acidic conditions were acquired by horizontal gene transfer (Futterer *et al.*, 2004).

Thus, it follows that the lack of 'hyper-alkalithermophiles' could be due to lack of exploration of this group and/or improper selection of medium and culture conditions. The fact that the genes required for surviving extreme acidic conditions were acquired by horizontal gene transfer is intriguing, as this implies that the 'hyper-alkalithermophiles' could possibly have evolved by obtaining the necessary genes from extreme alkaliphiles and extreme thermophiles.

The Anaerobic Halophilic Alkalithermophiles

The anaerobic halophilic alkalithermophiles require, in addition to high temperature and alkaline pH, an elevated salt concentration (≥ 1.5M NaCl) for growth. Definitions for halophiles are shown in Table 1.1. The first anaerobic, moderately halophilic alkalithermophilic bacterium 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 36-55ºC, which represents a very broad temperature optimum. *H. saccharophilum* has a fermentative metabolism and grows on mono- and di-saccharides, 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*, described in **Chapter 3**, represents the first true anaerobic, extremely halophilic alkalithermophilic bacterium described thus far (Mesbah *et al.*, 2007a). *N. thermophilus* grows (at $pH^{55ºC}$ 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 ≤ $pH^{55ºC}$ 8.2 or ≥ $pH^{55ºC}$ 10.8.

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). We have also isolated an additional species of the genus *Natranaerobius* and a novel genus, *Natronovirga* (described in **Chapter 4**). They are both extremely halophilic, growing optimally between 1.5-2.0 M NaCl, and obligately alkaliphilic, growing optimally between $pH^{55ºC}$ 9.5-9.9 and not below $pH^{55ºC}$ 8.2. They are also moderately thermophilic, growing optimally between 51 and 53ºC.

A number of other aerobic and facultatively aerobic halotolerant alkalithermophiles have been isolated, but not validly published and in some instances not characterized beyond the genus level. '*Caloramator halophilus*' is an obligately proteolytic, facultatively aerobic *Firmicute* isolated in our laboratory from salt flats located in northern Nevada. It is thermophilic, growing optimally at 64ºC (T_{range} 42-75ºC), and alkaliphilic, growing optimally at $pH^{60ºC}$ 9.2, and not growing below $pH^{60ºC}$ 8.1 or above 10.8. It is unusual in having a large NaCl range for growth, it grows between 0-3.0 M NaCl, with an optimum at 1.5 M (N.M. Mesbah, P. Maurizio and J.Wiegel, unpublished).

'*Bacillus thermoalcaliphilus'* is a chemoorganotrophic, facultatively aerobic 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 '*Bacillus 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).

SALINE AND HYPERSALINE ENVIRONMENTS

The majority of the earth's surface is saline. Waters of the oceans and seas that cover 71% of the Earth contain about 35 grams of dissolved salts per liter. Higher salt concentrations are often found in environments such as salt marshes, where hypersalinity arises due to evaporative concentration driven by sunlight. Even higher concentrations of salt, up to saturation, exist in naturally occurring inland salt lakes such as the Great Salt Lake, Utah, the Dead Sea, Jordan, and the hypersaline lakes of the Wadi An Natrun, Egypt and the Kenyan-Tanzanian Rift. Gradients of increasing salt concentrations are found in man-made evaporation ponds and multi-pond solar salterns located near sea shores worldwide, and less frequently in inland lakes. Additional hypersaline environments include salted food products as salted fish and subterranean brines often associated with oil fields. Deep hypersaline anoxic basins are also found in the Eastern Mediterranean Sea (van der Wielen *et al.*, 2005). All these environments, from seawater salinity to NaCl-saturated brines, have been shown to be habitats for prokaryotic life (Jones *et al.*, 1998, Mesbah *et al.*, 2007b, van der Wielen *et al.*, 2005).

There are two types of hypersaline environments, thalassohaline and athalassohaline. The ionic composition of water in these different habitats can differ greatly. Thalassohaline environments arise due to evaporation of seawater, and the ionic composition of water in these environments reflects that of seawater during the first stages of evaporation. Sodium is the predominant cation, Cl^- the main anion, followed by SO_4^{2-}, and the pH is neutral to slightly alkaline. The ionic composition of the water changes as it is concentrated by evaporation. When evaporation reaches the solubility limit of $CaSO_4$, it precipitates at a total salt concentration between 100 and 120 grams per liter (Oren, 2006b). Thus, brines that enter saltern-crysallizer ponds are depleted in calcium. During the subsequent precipitation of NaCl, the ionic

composition once again changes, and the relative concentration of K^+ increases. The water of Great Salt Lake, Utah still reflects the ionic composition of the seawater that it arose from even though it has been detached from the sea for thousands of years. Thus its waters are still classified as thalassohaline. Thalassohaline brines are usually at neutral or near-neutral pH (7.0-8.5).

Athalassohaline lakes arise from non-seawater sources and are fed either by underground water seepage or surface water. The size and salinity of these lakes is controlled by a balance between evaporation and freshwater drainage to their basins. The ionic composition of athalassohaline lakes differ markedly from that of seawater. Alkaline soda lakes are depleted in divalent cations Mg^{2+} and Ca^{2+}, contain high concentrations of carbonate/bicarbonate, and reach pH values of 10-11 and higher. Chloride and bromide are the dominant anions, and sulfate is very low (Oren, 2006b). The Dead Sea is an exception; divalent cations, Mg^{2+} and Ca^{2+}, are more abundant than monovalent cations Na^+, K^+. The pH of the Dead Sea water is slightly acidic, around 5.8-6.0. Alkaline athalassohaline lakes are present in diverse geographic locations such as in the Wadi An Natrun, Egypt, East Africa (Lake Magadi and other lakes in Kenya and Tanzania), California, Nevada, Tibet, China, Inner Mongolia and India.

Hypersaline environments are prime habitats for isolation of extremophiles

Most hypersaline environments are characterized by, in addition to salt concentrations approaching saturation, intense ultra-violet and solar radiation , temperatures reaching 50ºC, alkaline pH values ranging between 9 and 11.5, and scarcity of molecular oxygen in sediments and brines. Despite these inhospitable conditions, numerous culture dependent and -independent studies have shown vast prokaryotic communities within hypersaline waters and the salt crusts

and sediments beneath. The unique combination of high salt concentrations, alkaline pH and elevated temperature make these environments ideal for isolation of halophilic alkalithermophiles that can not only survive, but grow under these harsh conditions.

PHYLOGENY OF PROKARYOTES LIVING AT HIGH SALT CONCENTRATIONS

Halophilic behavior is found all over the phylogenetic tree of prokaryotes, within both the *Bacteria* and *Archaea* (Figure 1.2). Within the archaeal domain exist the most salt-requiring microorganisms in the order *Halobacteriales*, represented by a single family, the *Halobacteriaceae*. *Halobacterium* and its relatives require at least 1.5-2.5 M NaCl for survival, growth and structural stability (Grant, 2001). Halotolerant microorganisms are also encountered within the class *Methanococci,* order *Methanosarcinales* (genera *Methanohalophilus, Methanohalobium, Methanosalsum*). All these belong to the phylum *Euryarchaeota*, no halophilic representatives have been identified within the *Crenarchaeota*. Halophilic microorganisms are widespread in the bacterial domain; halophiles have been identified within the phyla *Firmicutes, Proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria and Spirochaetes* (Figure 1.2).

Phylogenetically coherent groups of halophiles: The *Halobacteriales, Halomonadaceae* and *Halanaerobiales*

There exist within the small subunit rRNA gene sequence-tree of life three phylogenetically and physiologically coherent groups that are comprised exclusively of halophiles. Within the *Euryarchaeota* there is the order *Halobacteriales*, comprised of a single family, the *Halobacteriaceae* (Oren, 2006d). Among the *Bacteria*, the family *Halomonadaceae*

(order *Oceanospirllales*, γ-proteobacteria) is predominantly composed of halophiles (Arahal & Ventosa, 2006). The third phylogenetically coherent group contains the anaerobic fermentative bacteria of the order *Halanaerobiales* (phylum *Firmicutes*, families *Halanaerobiaceae* and *Halobacteroidaceae*) (Oren, 2006c).

The archaeal order *Halobacteriales.* Members of the order *Halobacteriales* are aerobic heterotrophs that gain energy primarily by oxidation of carbon compounds via the tricarboxylic acid (TCA) cycle (Grant, 2001, Oren, 2006d). In some cases, the TCA cycle is used in combination with the glyoxylate cycle and respiratory electron transport using a chain of cytochromes (Oren, 2006d). Halophilic archaea are capable of utilizing a wide range of substrates such as amino acids, sugars, organic acids and polymeric substances such as starch, gelatin, casein and Tween 80. Several species of the *Halobacteriales* have been shown to produce exoenzymes such as proteases, lipases and amylases (Bhatnagar *et al.*, 2005, Gimenez *et al.*, 2000, Hutcheon *et al.*, 2005). In addition, unusual substrates such as aliphatic and aromatic hydrocarbons are used by certain members of the *Halobacteriales* (Bertrand *et al.*, 1990). Many halophilic archaea are also able to grow anaerobically, which could be an adaptation to the low solubility of oxygen in salt-saturated brines. Modes of anaerobic growth documented within the *Halobacteriales* include the use of alternative electron acceptors such as nitrate, dimethylsulfoxide, trimethylamine, fumarate, fermentation of arginine and light-driven anaerobic growth facilitated by bacteriorhodopsin (Oren, 2008).

The order *Halobacteriales* is known to contain the most halophilic microorganisms that will suffer irreversible damage and even lyse when suspended in solutions containing less than 1.5-2.5 M NaCl (Grant, 2001). However, culture-dependent and independent studies have shown

that members of the *Halobacteriales* are not restricted to hypersaline ecosystems such as salt lakes or solar salterns, but can also inhabit lower-salinity environments where the NaCl concentration is less than 1 M (Elshahed *et al.*, 2004, Purdy *et al.*, 2004, Savage *et al.*, 2007). It is hypothesized that localized areas of high NaCl concentrations are sufficient to prevent their lysis.

Other halophilic and halotolerant *Archaea*

All of the archaea in this group are anaerobic. The most halophilic methylotroph is found in the genus *Methanohalobium* (order *Methanosarcinales*), represented by a single species, *Methanohalobium evestigatum*. *M. evestigatum* is extremely halophilic, growing optimally at 4.3 M NaCl, with a range of 2.6-5.1 M. It is neutrophilic, growing optimally at pH values 7.0-7.5. It is also moderately thermophilic with a wide temperature optimum, 40-55ºC, with no growth above 60ºC or below 35ºC. *M. evestigatum* is strictly methylotrophic; it can use trimethylamine, dimethylamine and methylamine substrates for growth and methanogenesis, but cannot utilize acetate, formate or H_2/CO_2. *Methanohalobium* like species are present in anoxic sediments of hypersaline environments (Zhilina, 2001).

The three known members of the genus *Methanohalophilus* (order *Methanosarcinales*) are moderately halophilic (optimal growth with 1.0-2.5 M NaCl), mesophilic and neutrophilic. They also grow on C_1- substrates, producing methane and carbon dioxide. As with *Methanohalobium*, they are unable to catabolize acetate, formate, H_2 and secondary alcohols. Species of the genus *Methanohalophilus* are also found within anoxic sediments of saline systems (Boone, 2001).

Methanosalsum zhilinae, isolated from anoxic sediments in both the Wadi An Natrun, Egypt and the Kenyan-Tanzanian Rift, is differentiated from *Methanohalobium* and *Methanohalophilus* by being alkaliphilic, it grows optimally between pH 8.7 and 9.5 (pH range 8-10), and bicarbonate is required for growth. It is moderately halophilic, growing optimally between 0.4 and 0.7 M Na^+ (Na^+ range 0.2-2.1 M) and slightly thermotolerant, growing most rapidly between 35 and 45ºC. As with *Methanohalobium* and *Methanohalophilus*, energy metabolism is by formation of methane from methyl amines, methanol or dimethyl sulfide; acetate, formate, H_2/CO_2 and alcohols other than methanol are not used (Mathrani *et al.*, 1988).

The bacterial family *Halomonadaceae.* The family *Halomonadaceae* is a group of metabolically versatile, moderately halophilic, facultatively aerobic, heterotrophic microorganisms. Members of the family *Halomonadaceae* have been isolated from a variety of environments including thalassohaline salt lakes and brines, saline soils, athalassohaline, alkaline habitats and salted fish, meat and other foods (Ventosa *et al.*, 1998). The halomonads are generally aerobic, but can grow anaerobically in the presence of nitrate as an electron acceptor (Arahal & Ventosa, 2006). Salt requirement and tolerance are variable among the different species, and often vary according to the type of nutrients available. Complex media stimulate growth at high salt concentrations. *Halomonas elongata* can grow in complex medium at NaCl concentrations between 0.5 and 3.4 M. In defined medium where only glucose and alanine served as organic nutrients, NaCl tolerance was decreased and growth occurred within a narrower salt range (Ventosa *et al.*, 1998). In the presence of nutrients such as glycine betaine, the halomonad *Salinivbirio costicola* was able to grow at 4 M NaCl, growth at 3 M NaCl did not require glycine betaine. The largest NaCl range for growth of *S. costicola* was found in peptone

and tryptone medium (up to 4.0 M NaCl). In a defined medium, *S. costicola* could not grow at NaCl concentrations greater than 2.3 M (Ventosa *et al.*, 1998).

The bacterial order *Halanaerobiales*. All members of the order *Halanaerobiales* are strict anaerobes, oxidase and catalase negative, and do not contain cytochromes (Rainey *et al.*, 1995). Most species grow fermentatively on sugars (in some cases amino acids), and produce acetate, ethanol, lactate and propionate as major fermentation products (Oren, 2006c). Two species, *Acetohalobium arabaticum* and *Natroniella acetigena*, have a homoacetogenic metabolism and produce acetate as the main end product of their metabolism (Kevbrin *et al.*, 1995, Zhilina *et al.*, 1996). Several species of the *Halanaerobiales* can use oxidized sulfur compounds as electron acceptors. *Halanaerobium congolense*, *Halanaerobium saccharolyticum*, *Halanaerobacter lacunarum* and *Halobacteroides elegans* can all reduce elemental sulfur to sulfide (Oren, 2006c). Nitrosubstituted aromatic compounds can also serve as electron acceptors; nitrophenols, nitrobenzene and 2,4-dintiroaniline can be reduced by *Halanaerobium praevalens* and *Orenia marismortui* (Oren, 2006c).

All known members of the *Halanaerobiales* are moderately halophilic, growing optimally at NaCl concentrations between 1.2 and 2.5 M. A minimal NaCl concentration of 0.3-1.2 M is required for survival depending on the species. Certain members are also thermophilic; *Halothermothrix orenii* grows optimally at 60ºC; and alkaliphilic; *Natroniella acetigena* grows optimally at pH 9.7-10.0 (Cayol *et al.*, 1994, Zhilina *et al.*, 1996).

Other halophilic and halotolerant *Bacteria*

In addition to the *Halomonadaceae* and *Halanaerobiales*, halophiles are spread all over the phyla and orders of the bacterial domain. Halophilic *Bacteria* vary widely in their metabolic properties and include aerobic and anaerobic chemoheterotrophs, photoautotrophs, photoheterotrophs and chemolithotrophs (Oren, 2008).

Within the α-proteobacteria, the orders *Rhodospirillales*, *Rhodobacterales* and *Rhizobiales* all contain halophilic representatives. Among the *Rhodospirillales*; *Rhodovibrio salinarum*, and *R. sodomensis* grow optimally at 2 M NaCl and can tolerate NaCl concentrations as high as 3 M. They are slightly thermotolerant (optimal growth between 40 and 42ºC), neutrophilic and preferentially grow photoheterotrophically under anoxic conditions in the light (Garrity *et al.*, 2005). Among the *Rhodobacterales*, *Rhodothalassium salexigens* grows optimally between 1 and 1.3 M of NaCl, and can tolerate NaCl concentrations as high as 3.3 M, making it one of the most halotolerant species of the purple nonsulfur bacteria (Drews, 1981). *R. salexigens* is also neutrophilic and thermotolerant (pH_{opt} 7.0, T_{opt} 40ºC), and grows under anoxic conditions photoheterotrophically. The thermophilic *Dichotomicrobium thermohalophilum* (order *Rhizobiales*) grows optimally at 1.8 M NaCl, 50ºC and pH 8.5 (Hirsch & Hoffman, 1989). It is an obligate aerobe, chemoorganotroph and is characterized by a unique tetrahedral cell morphology with 1-4 hyphae.

Anoxygenic photosynthesis in hypersaline environments is also carried out by species of the genera *Ectothiorhodospira* and *Halorhodospira* of the γ-proteobacteria (order *Chromatiales*). *Ectothiorhodospira* and *Halorhodospira* species are characterized by their obligate requirement for both high salt concentrations and alkaline pH (Imhoff, 2001). *Ectothiorhodospira* spp. have total salt optima ranging between 0.3 and 1.2 M, with tolerances as high as 2.5 M by

Ectothiorhodospira haloalkaliphila and as low as 0 M by *Ectothiorhodospira shaposhnikovii.* (Imhoff, 2005a). Thus, *Ectothiorhodospira* spp. are considered to be marine bacteria with elevated salt tolerance. *Halorhodospira* spp. are much more halophilic, all known species require at least 1.5 M total salt concentrations for growth (Imhoff, 2005b). *Halorhodospira* spp. are well adapted to elevated temperatures, alkaline pH and high light intensity commonly found in hypersaline systems, and have not been isolated from marine habitats. *Halorhodospira* spp. bloom in concentrated brines of alkaline soda lakes and have been reported to impart a bright red color in the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (Imhoff *et al.*, 1979). *Halorhodospira halophila* is the most commonly encountered of this extremely halophilic genus. It shows optimal growth at 1.8-5.3 M NaCl, pH 8.5-9.0 and 42ºC. Cells of *Halorhodospira halophila* are obligately phototrophic, strictly anaerobic, and lyse at NaCl concentrations less than 0.5 M (Imhoff, 2005b). Additional halophilic purple bacteria of the genus *Halochromatium* and *Thiohalocapsa* have been isolated from reduced sediments of coastal salterns that are exposed to light. Species within these two genera have NaCl optima between 1.3 and 1.8 M NaCl, and grow photolithoautotrophically under anoxic conditions in the light with sulfide or S^0 as electron donors (Imhoff & Caumette, 2005a, 2005b).

The δ -proteobacteria harbors the most salt-tolerant sulfate reducers. *Desulfohalobium retbaense*, isolated from the hypersaline Lake Retba in Senegal, can grow at NaCl concentrations up to 4 M (optimal growth occurs at 1.5 M NaCl)(Ollivier *et al.*, 1991). Other halophilic isolates such as *Desulfovibrio halophilus, Desulfovibrio oxyclinae* and *Desulfonatronum lacustre* tolerate NaCl concentrations up to 3 M (Caumette *et al.*, 1991, Krekeler *et al.*, 1997). *Desulfonatronum lacustre* is also extremely alkaliphilic, growing optimally at pH 9.5, with no growth at pH 8.0 or below. The maximum pH for growth is 10.0 (Pikuta *et al.*, 1998).

The phylum *Firmicutes* harbors several moderately halophilic aerobic microorganisms within the order *Bacillales* and include the genera *Halobacillus*, *Bacillus*, *Gracilibacillus, Salibacillus* and *Salinicoccus* (Oren, 2002). A number of anaerobic halophiles within the order *Clostridiales* have been isolated, including *Clostridium halophilum*, growing optimally at 1 M NaCl, the sulfate-reducing *Desulfotomaculum halophilum*, growing optimally at 1 M NaCl, the extremely alkaliphilic *Natronincola histidinovorans* and members of the genus *Tindallia*, all growing optimally between 0.8 and 1.5 M NaCl (Fendrich *et al.*, 1990, Kevbrin *et al.*, 1999, Pikuta *et al.*, 2003, Tardy-Jacquenod *et al.*, 1998, Zhilina *et al.*, 1998).

Cyanobacterial mats are abundantly present in hypersaline lakes and saltern evaporation ponds. Oxygenic photosynthesis by cyanobacteria can occur at NaCl concentrations up to saturation. Even though the major planktonic primary producers in hypersaline environments are eukaryotic algae of the genus *Dunaliella* (Oren, 2002); cyanobacteria such as *Aphanothece halophytica*, *Halospirulina tapeticola*, and *Spirulina* spp. are abundant in benthic microbial mats that cover shallow sediments of salt lakes and saltern ponds (Nubel *et al.*, 2000, Oren, 2008). The taxonomy of the halophilic cyanobacteria is still poorly developed.

Finally, the most halophilic bacterium to be isolated and described thus far is *Salinibacter ruber*, first isolated from saltern crystallizer ponds in Spain (Anton *et al.*, 2000). Similar to the extreme halophilic archaea of the order *Halobacteriales, Salinibacter ruber* grows optimally between 3.3 and 5 M NaCl, and does not grow at NaCl concentrations below 2.5 M (Anton *et al.*, 2002). However, cells of *S. ruber* retain their morphology and do not lyse under hypoosmotic conditions (Oren, 2006a). *Salinibacter* isolates are pigmented, obligate aerobes that prefer low nutrient concentrations (yeast extract, amino acids and some sugars) for growth. While it was previously assumed that aerobic *Salinibacter* spp. do not play a significant role in the microbial

community inhabiting salt-saturated environments, fluorescence in situ hybridization experiments have shown that *Salinibacter* spp. can form 5-25% of the total prokaryotic community in hypsersaline environments (Anton *et al.*, 1999). In addition, pigment analysis revealed that the salinixanthin pigment of *S. ruber* contributes up to 5% of the total prokaryotic carotenoids that colored the crystallizer ponds in Alicante, Spain red-pink (Oren & Rodriguez-Valera, 2001). Thus, this genus is widespread in hypersaline environments with salinities between 2.5 M and saturation.

ALKALIPHILIC AND THERMOPHILIC HALOPHILES

Many halophilic prokaryotes are also adapted to other forms of environmental stress. Thus, alkaliphilic, thermophilic and psychrophilic halophiles are also known. No acidophilic halophiles have been described thus far. The Dead Sea is the most acidic hypersaline habitat known (pH 6.0), and is the most promising source of moderately acidophilic halophiles.

Many hypersaline environments such as the Wadi An Natrun, Egypt, Lake Magadi, Kenya, and soda lakes in China, are characterized by high concentrations of carbonates in addition to high salinity. The pH values in these soda lake environments range between 9.5 and 11.5. Several aerobic haloalkaliphilic archaea and bacteria have been isolated and described (Oren, 2002, 2008). Anaerobic, haloalkaliphilic bacteria also occur in these environments. Lake Magadi was shown to harbor a diverse anaerobic community including cellulolytic, proteolytic, and homoacetogenic bacteria, most of which have not been identified beyond the genus level (Jones *et al.*, 1998). The homoacetogen *Natroniella acetigena* was isolated form Lake Magadi. Its pH optimum is 9.8-10.0, and can grow at pH values as high as 10.7 (Zhilina *et al.*, 1996).

Growth at high temperatures most likely represents an adaptation to the intense solar irradiation and relatively high temperatures often encountered in salt lakes in both desert and tropical areas. The low specific heat of brines allows them to quickly reach high temperatures when heated by sunlight. Many aerobic halophilic archaea of the order *Halobacteriales* have high temperature optima. *Halorhabdus utahensis, Haloterrigena thermotolerans, Haloarcula quadrata, Halobacterium salinarum, Halorubrum saccharovorum, Halorubrum coriense, Haloferax volcanii* and *Haloferax mediterranei* all have temperature optima between 47 and 54ºC (Grant, 2001). Among the anaerobic *Bacteria* of the order *Halanaerobiales*, moderately thermophilic representatives have been identified. *Halothermothrix orenii* was the first true thermophilic, halophilic microorganism to be described. It was isolated from a warm saline lake in Tunisia (Cayol *et al.*, 1994). It grows optimally at 60ºC, and can grow at temperatures as high as 68ºC in the presence of 3.3 M NaCl. *Acetohalobium arabaticum* strain Z-7492 has a temperature optimum of 55ºC at 3.4-4.0 M NaCl (Kevbrin *et al.*, 1995). *Thermohalobacter berrensis* (order *Clostridiales*), is moderately halophilic and thermophilic, growing optimally at 0.8 M NaCl and 65ºC (Cayol *et al.*, 2000).

ADAPTIVE MECHANISMS OF ANAEROBIC ALKALITHERMOPHILES AND HALOPHILIC ALKALITHERMOPHILES

Life at high salt concentrations, alkaline pH values and high temperatures 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 outlined below.

Adaptive mechanisms of halophiles

Halophilic microorganisms must maintain their cytoplasm; in terms of ionic concentration, 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, with the possible exception of the halophilic archaea of the family *Halobacteriaceae,* maintain a turgor pressure (Oren, 1999).

Two different strategies enable halophilic and halotolerant prokaryotes to withstand the high osmotic stress associated with their hypersaline habitats: (i) cells accumulate and maintain high concentrations of inorganic ions (other than Na^+) in the cytoplasm (the 'salt-in' strategy). In this case, all the intracellular enzymatic machinery is adapted to the presence of high salt concentrations. (ii) cells maintain low cytoplasmic salt concentrations but accumulate high concentrations of low molecular weight compatible solutes (the 'low salt in' or 'compatible solute' strategy). No adaptation of intracellular machinery is required.

The salt-in strategy is used by three phylogenetically unrelated groups; the extremely halophilic aerobic archaea of the family *Halobacteriaceae*, the anaerobic bacteria of the order *Halanaerobiales,* and the extremely halophilic *Salinibacter ruber* of the *Bacteroidetes* (Oren, 2006b, Oren *et al.*, 2002). No organic osmotic solutes have been found in the members of these groups. The cytoplasm in these microorganisms is characterized by the presence of molar concentrations of K^+ cations; Cl^- is the predominant anion. Microorganisms using the salt-in strategy are obligate halophiles, high concentrations of inorganic ions both intra- and extra-cellularly is necessary for proper maintenance of cell shape, membrane structure and enzyme activity.

The compatible-solute strategy is used by most moderately halophilic bacteria with exception of the above mentioned members of the order *Halanaerobiales* and *Salinibacter ruber*. The use of organic compatible solutes allows a great deal of flexibility and adaptability to a wide range of NaCl concentrations (Oren, 2008).

a. The 'Salt-in' Strategy. Analysis of intracellular ionic concentrations in the halothermophile *Halobacterium salinarum* showed that it contained an extremely high intracellular salt concentration and that the ionic composition of the intracellular milieu was different from that of the outside medium. For *H. salinarum* cells in early exponential growth phase, the intracellular concentration of K^+ was 4.57 M whilst the medium concentration was 0.03 M. The intracellular concentration of Na^+ was 1.37 M, but the medium concentration was 4 M (Oren, 2006b). High intracellular concentrations of Na^+, K^+ and Cl^- were also measured inside cells of mesophilic members of the order *Halanaerobiales*, with K^+ being the predominant cation. The concentrations were high enough to be isotonic with the medium (Oren *et al.*, 1997). Apparent intracellular concentrations of K^+ measured in *Salinibacter ruber* grown in medium with 3.3 M NaCl were 3 M. Insignificant amounts of compatible solutes were detected in *S. ruber*, so it appears that it also utilizes the salt-in strategy (Oren *et al.*, 2002).

For microorganisms using the salt-in strategy for osmotic adaptation, all structural cell components and enzymes must be adapted to the presence of molar concentrations of inorganic salts. Molar concentrations of salts inside the cell are devastating to proteins and other macromolecules; they result in collapse of protein structure due to increased hydrophobic interactions within and between protein molecules. In addition, salt ion hydration reduces the

availability of free water below the level required to maintain essential biological processes (Oren, 2006b).

Evolutionary modifications required to engineer proteins so that they become halophilic have included the incorporation of a large number of acidic amino acids (glutamate and aspartate) into the surface of proteins. The pI values of enzymes from extreme halophiles are typically around 4. The carboxylic groups of the acidic amino acids sequester and organize a tight network of water and hydrated K^+ ions around the protein and form internal salt bridges with basic amino acid residues to provide internal structural rigidity to the protein (Dennis & Shimmin, 1997). Another characteristic feature of halophilic proteins is their low content of hydrophobic amino acid residues that is offset by a high content of borderline hydrophobic amino acids, serine and threonine. Without a high salt concentration, very weak hydrophobic interactions are not sufficient to maintain structural stability. Salt ion hydration reinforces these weak hydrophobic interactions and results in protein molecules assuming a more tightly folded conformation (Oren, 2006b).

These adaptations make cells strictly dependent on the continuous presence of high salt concentrations for maintenance of structural stability. When suspended in hypoosmotic solutions, repulsion of the negatively charged acidic side groups on the surface of the protein results in structural collapse. Most enzymes and proteins of the *Halobacteriales* denature when resuspended in solutions containing less than 1-2 M salt. Similarly, intracellular enzymes from the anaerobic fermentative *Halanaerobiales* function better in the presence of molar concentrations of salt than in salt free medium. Many enzymes are more active in the presence of KCl than NaCl, which correlates with the fact that K^+ is the predominant intracellular cation (Ebel *et al.*, 1999).

b. The 'Salt-out' (Compatible Solute) Strategy. In most other halophilic and halotolerant bacteria and the halophilic methanogenic archaea, osmotic balance is provided by small organic molecules that are either synthesized by the cell or taken up from the medium when available. The compatible solute strategy does not require specifically adapted proteins. Compatible solutes are polar, highly soluble molecules that are uncharged or zwitterionic at physiological pH. Compatible solutes include amino acids and derivatives, quaternary amines such as glycine betaine, sugars or sugar derivatives as sucrose, trehalose and 2-sulfotrehalose and polyols as glycerol and arabitol (Roberts, 2005).

Intracellular concentrations of compatible solutes are regulated according to the salinity of the medium; allowing this strategy to provide a high degree of adaptability of cells to changes in the salt concentration of the environment. However, this strategy is only highly effective in environments with up to 1.5 M salt. Above this concentration it is less effective and energetically unfavorable (Oren, 1999). However, compatible solutes have been observed in the extremely halophilic black yeast *Hortaea werneckii*., In *H. werneckii*, glycerol accumulates intracellularly when growing at salinities up to 1.5 M, but then remains unchanged at NaCl concentrations between 1.5 and 3 M. This indicates that there is possibly another compatible solute(s) in this halophilic black yeast (Plemenitas & Gunde-Cimerman, 2005). The intracellular cation concentration of *H. werneckii* was far below the extracellular cation concentration and hence unlikely to be involved in osmotic balance (Plemenitas & Gunde-Cimerman, 2005). In the case of halophilic alkalithermophiles with broad NaCl ranges between 1.5 and 4 M, it would be expected that they either combine both strategies to cope with high salt concentrations or use the salt-in strategy alone.

Maintenance of a turgor pressure by a halophile, whether by the salt-in or salt-out strategy, requires a highly impermeable cell wall and cell membrane in order to maintain the large extracellular sodium gradient and to prevent diffusion of the intracellular K^+ and/or accumulated compatible solute(s). However, at high temperatures (≥ 45ºC), the rate of Na^+ diffusion through the membrane increases as it is a temperature dependent process (Konings *et al.*, 2002). In addition, a study by Vossenberg *et al.* (1999b) showed an increase in the Na^+ permeability of liposomes prepared from haloarchaeal cell membranes as the NaCl concentration increased from 0.5 to 4 M. Nevertheless, numerous halophilic thermophiles have been brought into culture, thus it follows that the halophilic thermophiles must have developed unique mechanisms, particularly novel membrane lipid compositions, which remain less permeable to ions at both elevated salt concentrations and temperatures.

Adaptive mechanisms of alkaliphiles

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 7.4-7.8 (Padan *et al.*, 2005). The consequences of not being able to do so are profound. The anaerobic *Caloramator fervidus*, previously named *Clostridium fervidus* (Collins *et al.*, 1994) (Patel *et al.*, 1987), has bioenergetic processes that are entirely Na^+-coupled and lacks active H^+ extrusion or uptake systems that can support pH homeostasis. As a result, it grows only within the narrow pH range of 6.3-7.7 that corresponds to an optimal cytoplasmic pH (Speelmans *et al.*, 1993).

The key bioenergetic difficulty faced by all alkaliphiles is that of cytoplasmic acidification and/or homeostasis. A number of adaptive strategies are used for intracellular pH homeostasis. These strategies include: (i) increased ATP synthase activity that couples H^+ entry to ATP generation, (ii) increased expression and activity of monovalent cation/proton antiporters, (iii) changes in cell surface properties, and (iv) 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 intracellular Na^+ and volume homeostasis (Padan *et al.*, 2005).

Bacteria have multiple monovalent cation/proton antiporters: there are at least four in the facultative aerobe *Escherichia co*li (Padan *et al.*, 2001), five in aerobic *Bacillus subtilis* (Fujisawa *et al.*, 2005), and four in alkaliphilic *Bacillus* strains (Ito *et al.*, 1997, Takami *et al.*, 2000). Detailed information on individual Na^+/ H^+ and $Na^+(K^+)/ H^+$ antiporters is beginning to provide insights into the basis for the role that specific antiporters have in cytoplasmic pH homeostasis of individual bacterial strains.

Although microorganisms have multiple monovalent cation/proton antiporters forming complex H^+ and Na^+ cycles within the cell, these different antiporters do not contribute equally to cytoplasmic pH regulation and homeostasis. Properties of an antiporter that control its impact on cytoplasmic pH acidification include the stoichiometry and kinetics of the exchange, the affinity (K_D) of substrate binding, positive and negative effectors of activity, and reaction mechanism. Very little work has been done in this respect with anaerobic alkaliphiles, alkalithermophiles, and even more so for halophilic alkalithermophiles.

Importance of electrogenicity for Na^+/ H^+ antiport-dependent pH acidification. If Na^+/ H^+ antiporters are to be effective at cytoplasmic acidification, transport must be electrogenic rather than electroneutral (Padan *et al.*, 2005). An electroneutral exchange of cytoplasmic Na^+ for extracellular H^+ is a 1:1 exchange where no net flux of charge is involved. Thus, the membrane potential ($\Delta\psi$) component of the proton motive force does not contribute to energizing the exchange. Microorganisms extrude Na^+ from their cytoplasm by diverse expulsion mechanisms because it is cytotoxic (Padan & Krulwich, 2000). As a result, there is no outwardly directed Na^+ gradient to support electroneutral Na^+/ H^+ antiport. Electroneutral Na^+/ H^+ antiport can only equilibrate the ΔpH component of the proton motive force, but cannot drive the accumulation of H^+ necessary for cytoplasmic acidification. Electrogenic Na^+/ H^+ antiporters exchange a larger number of H^+ for each extruded Na^+ during a turnover, so that a net positive charge moves inward. Thus the $\Delta\psi$ component of the proton motive force, which is negative inside relative to outside the cell, is able to drive the inward H^+ proton movement and cytoplasmic acidification can be achieved. The coupling stoichiometry of the Na^+/ H^+ antiporter NhaA of *E.coli* was determined to be 2 H^+/1 Na^+ (Taglicht *et al.*, 1993). For other Na^+/ H^+ and $Na^+(K^+)/ H^+$ antiporters that function in cytoplasmic acidification, precise coupling stoichiometries have not yet been determined. No work has been done on identifying and characterizing antiporters in anaerobic alkaliphiles, alkalithermophiles, haloalkaliphiles or halophilic alkalithermophiles.

Substrate and cation specificity of antiporters. While studies on antiporter mediated cytoplasmic acidification have been focused so far on the $Na^+(Li^+)/ H^+$ specific antiporters, other types of antiporters have been described. For example, the electroneutral Bs-MleN antiporter

catalyzes the 2 H^+ -Malate^{2-} /Na^+ -Lactate^{1-} exchange in *Bacillus subtilis* (Wei *et al.*, 2000). Driven by inwardly directed malate and outwardly directed lactate gradients, the external dicarboxylic acid malate enters with H^+ and cytoplasmic lactate exits with Na^+, hence supporting cytoplasmic accumulation of H^+. Theoretically, an electroneutral K^+/ H^+ antiporter can support H^+ uptake as alkaliphiles usually maintain an outwardly directed K^+ gradient. Involvement of K^+/ H^+ antiport activity in cytoplasmic acidification has been reported in the neutrophilic *E. coli, Enterococcus hirae, Vibrio alginolyticus* and most recently the alkaliphilic *Alkalimonas amylolytica* (Kakinuma & Igarashi, 1995, Nakamura *et al.*, 1992, Plack Jr & Rosen, 1980, Wei *et al.*, 2007). NhaK, a *Bacillus subtilis* transporter belonging to the cation-proton antiporter family-1, also catalyzes K^+/ H^+ antiport at alkaline pH (Fujisawa *et al.*, 2005). However, it is not anticipated that K^+/H^+ antiporters play dominant roles in cytoplasmic acidification in alkaliphiles, as they result in depletion of cytoplasmic K^+. A high cytoplasmic concentration of K^+ is physiologically important for alkaliphiles, as it ameliorates the increased cytotoxicity of Na^+ at high pH (Padan & Krulwich, 2000).

Antiporters and the alkaliphile Na^+ cycle. In line with the essential role of Na^+/ H^+ antiporters in alkaliphily, alkaliphile pH homeostasis is strictly dependent on the presence of Na^+ (Cook *et al.*, 1996, Padan *et al.*, 2005). Alkaliphiles must use Na^+-specific antiporters in order to avoid the risk of lowering cytoplasmic K^+ concentration. However, for optimal cytoplasmic acidification, re-entry routes for Na^+ are crucial to provide substrate for continuous antiporter activity. One such route is Na^+ -coupled transport of solutes and metabolites into the cell. Such a route has been postulated for the anaerobic alkalithermophiles *Clostridium paradoxum* and *Anaerobranca gottschalkii* (Ferguson *et al.*, 2006, Prowe *et al.*, 1996). Initial data also showed

that solute uptake in the anaerobic halophilic alkalithermophile *Natranaerobius thermophilus* is Na^+-coupled (**Appendix B**). An investigation by Pitryuk *et al*. (2004) on the effects of metabolic inhibitors on energy generating processes in the anaerobic haloalkalithermophile *Halonatronum saccharophilum* showed that its metabolism depends on both Na^+ and H^+ gradients.

Another route for Na^+ re-entry to the cell is via a Na^+-translocating ATPase. This ATPase will catalyze the synthesis of ATP in conjunction with pumping of Na^+ ions into the cell. However, cloning and characterization of the ATPase in the anaerobic alkalithermophile *Clostridium paradoxum* showed that it is Na^+-coupled and incapable of ATP synthesis. It is used solely to pump Na^+ out of the cell (Ferguson *et al.*, 2006). ATPase activity in inverted membrane vesicles prepared from cells of *Anaerobranca gottschalkii* was also found to be Na^+-dependent (Prowe *et al.*, 1996), but its ability to synthesize ATP has not been determined.

An additional re-entry route for Na^+ is through specific Na^+ channels that are activated at alkaline cytoplasmic pH. The alkaliphilic, aerobic *Bacillus pseudofirmus* OF4 possesses a voltage-gated Na^+ selective channel termed Na_VBP. Deletion of the gene encoding this protein showed that it plays an important role in cytoplasmic pH homeostasis. It also has roles in motility and chemotaxis, two characteristics that are observed only when this facultative alkaliphile is grown at high pH (Ito *et al.*, 2004). Consistently, the activity of Na_VBP is potentiated by high pH, but the precise interplay between Na_VBP signaling and pH homeostasis has not been determined.

Adaptive strategies of alkaliphiles apart from the Na^+ cycle. Adaptive mechanisms of anaerobic alkaliphiles and alkalithermophiles have yet to be investigated in detail. Studies on aerobic alkaliphilic *Bacillus* species revealed the presence of specific secondary cell wall

polymers associated with the peptidoglycan that are crucial for survival at pH 10.5 but not at 7.5 (Padan *et al.*, 2005). The alkaliphilic *Bacillus halodurans* has negatively charged teichuronic acid and teichuronopeptides as major secondary cell wall polymers. A mutant strain that does not produce either teichuronic acid or teichuronopeptides showed poor growth at pH 10.5 (Aono *et al.*, 1999). *Bacillus pseudofirmus* OF4 possesses an acidic S-layer closely associated with the peptidoglycan cell wall. Deletion of the gene encoding this S-layer resulted in growth defects at pH 11.0, and enhanced growth at pH 7.5 (Padan *et al.*, 2005). In both *Bacillus halodurans* and *Bacillus pseudofirmus* however, secondary cell wall polymers are neither sufficient nor necessary substitutes for active antiporter-based cytoplasmic acidification. It is presumed that the positive effect of these polymers is due to their ability to bind cations and trap them near the cell surface, hence enhance the availability of H^+ and Na^+ ions for pH homeostasis and other bioenergetic processes.

Alkaliphilic *Bacillus* species also have large proportions of cardiolipin and squalene in the cell membrane. It is hypothesized that cardiolipin traps protons at the membrane surface and squalene lowers the proton permeability of the cell membrane (Padan *et al.*, 2005), but these proposed roles have not been experimentally proven. The phospholipid fatty acid profile of *Natranaerobius thermophilus* when grown at its optimal growth conditions of $pH^{55ºC}$ 9.5, 1.5 M NaCl and 53ºC revealed a unique pattern of branched chain dimethyl acetals (Mesbah *et al.*, 2007a) (**Chapter 3**). It is unclear at this stage what role these unique fatty acids are playing in cellular bioenergetics.

Finally, a transporter-based alkali adaptation different from Na^+/H^+ antiporters has been reported in *Bacillus pseudofirmus* OF4, and plays a critical role when *B. pseudofirmus* is grown in an amine rich medium. AmhT (ammonium homeostasis transporter) facilitates ammonium

efflux from cells (Wei *et al.*, 2003). At alkaline pH, this prevents accumulation of ammonium inside the relatively acidic cytoplasm, and hence prevents ammonium accumulation from subverting cytoplasmic acidification processes. Instead, expulsion of NH_4^+ from the cytoplasm results in formation of NH_3 in the alkaline external environment. The NH_3 gas volatilizes, and leaves a proton in the medium, which in turn can be used for cytoplasmic acidification by other antiporters in the cell membrane.

Adaptive mechanisms of thermophiles

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). Thermophiles must also have mechanisms to preserve protein structure at elevated temperatures.

Studies on permeabilities of bacterial and archaeal cytoplasmic membranes showed that *Bacteria* and *Archaea* adjust the permeability of the cytoplasmic membrane to the growth temperature of the microorganism (Vossenberg *et al.*, 1995). In psychrophilic and mesophilic bacteria and archaea, the proton permeability of the cytoplasmic membrane is kept constant by increasing the lipid acyl chain length, degree of saturation and ratio of iso- and anteiso-branched fatty acids as temperature increases (Vossenberg *et al.*, 1999a). However, thermophilic bacteria and archaea growing at temperatures greater than 50^oC encounter increased membrane proton permeability because they are no longer able to compensate by adjusting the lipid composition.

The membrane proton permeability of the facultatively aerobic bacterium *Geobacillus stearothermophilus,* anaerobic *Thermotoga maritima* and the aerobic archaeon *Sulfolobus acidocaldarius* increases exponentially with temperature. The sodium ion permeability is several orders of magnitude lower than the proton permeability, thus many thermophiles have Na^+ coupled bioenergetics (Konings *et al.*, 2002).

The mechanisms underlying thermal stability of proteins are yet to be fully explained. Several hypotheses have been proposed, including greater hydrophobicity of amino acid content, deletion/shortening of surface loops, increased packing of the protein core, larger subunit interfaces, and increased interaction across subunit interfaces allowing stabilization via 'fortified' oligomerization. All these theories have been proposed based on sequence analyses of homologs of thermophilic and mesophilic proteins (Kumar *et al.*, 2007).

Structural surveys have shown that thermophilic proteins have more non-polar cores than mesophilic homologues, and contain greater frequencies of well packed residues. This adaptation minimizes surface energy and the hydration of apolar surface groups while burying hydrophobic residues and maximizing packing of the core. The majority of proteins in the genome of the anaerobic thermophile *Thermotoga maritima* (optimal growth at 80ºC) have higher contact order than their mesophilic homologs (Robinson-Rechavi & Godzic, 2005). Genome analyses have also revealed that reduction in surface loop content of thermophilic proteins and increased proline content of the surface loops are involved in reduced protein flexibility and increased thermostability (Kumar *et al.*, 2007).

An increase in hydrophobic residues seems to interfere with the mechanism of protein adaptation to high salt concentrations, since the borderline hydrophobic residues serine and threonine are replaced with the more hydrophobic alanine/proline and valine/isoleucine

respectively (Kumar *et al.*, 2007). This could possibly explain why all the obligate extreme anaerobic halophiles are moderate thermophiles, with T_{max} not exceeding 60^{o}C. Above this temperature minimizing hydration and polar groups at the surface of the protein are necessary in order to maintain stability. The presence of high concentrations of intracellular ions would interfere with protein folding and decrease stability. However, the anaerobic *Halothermothrix orenii* grows optimally at 60ºC and can grow at temperatures as high as 68ºC (Cayol *et al.*, 1994). *H. orenii* belongs to the order *Halanaerobiales*, which is known to utilize the salt-in strategy for osmotic adaptation. Thus it follows that this microorganism must have developed novel adaptive mechanisms to be able to survive and grow in the presence of both of these extreme conditions.

INTERACTION BETWEEN SALT, ALKALINE AND TEMPERATURE STRESS: IMPLICATIONS FOR THE ANAEROBIC HALOPHILIC ALKALITHERMOPHILES

Alkaline, salt and temperature stressors have significant interplay with one another. First, there is an overlap between alkaline stress and salt stress. Halophiles commonly have higher intracellular Na^+ contents than their non-halophilic counterparts. As pH rises, however, Na^+ cytotoxicity greatly increases (Padan & Krulwich, 2000). The toxicity of Na^+ is also dependent upon the status of cytoplasmic K^+ concentration; the larger the K^+ concentration in the cytoplasm, the more tolerant the cell becomes to intracellular Na^+. Second, temperature stress overlaps with that of alkaline stress; high temperatures increase cell membrane permeability to H^+ (Konings *et al.*, 2002) which can be detrimental to alkaliphiles that must keep their cytoplasmic pH 1-2.5 units lower than the external pH. Third, temperature stress also overlaps with salt stress; high temperature alters membrane permeability to Na^+, albeit to a much lesser extent than membrane permeability to H^+. This will counteract the efficacy of systems functioning to keep cytoplasmic

Na^+ concentrations low (and these are essential for survival as intracellular Na^+ is cytotoxic), and might also compromise the efficiency of cation-proton antiporters as some of them have roles in regulating osmolarity (Padan *et al.*, 2005). Fourth, alkaline pH, high salt concentrations and temperatures all interplay with cell membrane composition. While increased temperatures cause an increase in membrane fluidity and permeability, alkaline pH and high salt concentrations cause an increase in the degree of unsaturation of the fatty acids of the cytoplasmic membrane with a concomitant increase in rigidity. An additional stress intersection includes adaptation of intracellular proteins to high temperature, which involves increasing hydrophobic packing, and adaptation to high intracellular ionic concentrations which involves increasing the protein content of hydrophilic amino acids.

Studies on the adaptive mechanisms of poly-extremophiles are scarce and many do not take into account the effect of elevated salt concentration. Studies on the alkalithermophilic anaerobes *Clostridium paradoxum* and *Anaerobranca gottschalkii* showed that, like mesophilic alkaliphiles, the magnitude of the proton motive force is suboptimal for H^+-coupled processes (Cook *et al.*, 1996, Prowe *et al.*, 1996). *Clostridium paradoxum* is capable of cytoplasm acidification; it increases its ΔpH across the cell membrane ($pH_{out} - pH_{in}$) by as much of 1.3 units (Cook *et al.*, 1996). At pH values greater than 10.0, near its maximum pH range, the ΔpH and $\Delta\psi$ gradually declined, and the intracellular pH significantly increased till it was almost equal that of the extracellular medium. *Clostridium paradoxum* has an absolute requirement for 50-200 mM of Na^+ for growth, and growth is inhibited by the sodium ionophore monensin, and amiloride, an inhibitor of Na^+/ H^+ antiporters. This indicates the importance of the Na^+ cycle in this anaerobic alkalithermophile. *Clostridium paradoxum* is also sensitive to the F-type ATPase inhibitor N,N'-dicyclohexylcarbodiimide, indicating the presence of an F-type ATPase in this

anaerobe (Ferguson *et al.*, 2006). The F_1F_0-ATPase of *Clostridium paradoxum* is a Na^+ -translocating ATPase that is used to generate an electrochemical gradient of Na^+ that could be used to drive other membrane related bioenergetic processes such as solute transport. Cloning of the *atp* operon revealed absence of the C-terminal region of the ε subunit that is essential for ATP synthesis (Cipriano & Dunn, 2006). Thus, the F-type ATPase of *Clostridium paradoxum* is used solely as a pump for generation of a Na^+ gradient, and is not involved in ATP synthesis.

It is unclear how the additional stress of elevated NaCl concentration will interplay with the adaptive mechanisms discussed above to cope with elevated pH and temperature. The presence of a Na^+ -translocating ATPase would play a role in extrusion of excess Na^+ from the cytoplasm. However, continuous extrusion of Na^+ and possibly K^+ from the cytoplasm would compromise osmolarity, which is necessary for maintenance of a turgor pressure at elevated salt concentrations.

In addition, the challenge of acidifying the cytoplasm whilst growing in alkaline conditions, a dearth of protons, remains to be investigated. While a high H^+/Na^+ stoichiometry makes it possible for an antiporter to use the $\Delta\psi$ to acidify the cytoplasm at alkaline pH, it does not solve the problem of H^+ capture by the antiporter when the external concentration of H^+ is low. The efficiency of a Na^+/ H^+ antiporter is also compromised by the increased membrane permeability to both H^+ and Na^+ posed by elevated temperatures and salt concentrations respectively. No cellular features in poly-extremophiles have yet been described that could trap protons on the surface of the cell, similar to those suggested for membrane-embedded electron transport or light-driven proton pumps.

Finally, one of the major challenges encountered by the anaerobic alkalithermophiles and halophilic alkalithermophiles concerns their anaerobic and hence fermentative metabolism. The

anaerobic alkalithermophile *Clostridium paradoxum* and the anaerobic halophilic alkalithermophiles *Natranaerobius thermophilus, Natranaerobius trueperi, Natronovirga wadinatrunensis* and *Halonatronum saccharophilum* are all fermentative chemoorganotrophs that produce their energy (ATP) in the cytoplasm by substrate level phosphorylation. Thus, in comparison with the aerobic extremophiles which produce ATP via respiratory electron transport, ATP production in the fermentative anaerobes is significantly less. Many of the key adaptive mechanisms for the halophilic alkalithermophiles and alkalithermophiles, such as maintenance of the crucial Na^+ cycle, solute uptake, compatible solute synthesis and synthesis of impermeable cell wall and cell membrane constituents, are ATP requiring processes. As the anaerobic extremophiles mentioned above are capable of rapid growth under extreme conditions (doubling times 16 min to 3.5 hours), it follows that the anaerobic extremophiles must have developed highly efficient methods for energy production and turnover that allow them to, not only survive, but grow rapidly under multiple extreme conditions. Figure 1.3 outlines the bioenergetic problems facing anaerobic halophilic alkalithermophiles, and shows a diagrammatic summary of ion extrusion mechanisms, the Na^+ cycle, and salt adaptation mechanisms with hypothesized roles in anaerobic halophilic alkalithermophiles.

OBJECTIVES OF THE DISSERTATION

Prior to this thesis, only two halophilic alkalithermophiles, *Halonatronum saccharophilum* and '*Bacillus thermoalcaliphilus'*, had been isolated. Given the ambiguities outlined above surrounding the physiological and adaptive mechanisms employed by this unique group of poly-extremophiles, an understanding of their basic physiology and biochemistry is necessary for understanding the mechanism of their survival and their evolutionary history on

Earth. Hypersaline environments are promising environments for isolation of halophilic extremophiles. Work in this dissertation was primarily focused on the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. Building on culture-dependent studies by others on this environment, this work employed both culture-dependent and independent approaches to identify the total prokaryotic community of this environment, and to isolate novel halophilic alkalithermophiles that can be used as model microorganisms for subsequent physiological studies.

Specific objectives are:

1. Elucidation of the bacterial and archaeal community compositions within water and sediments of three of the largest lakes of the Wadi An Natrun via 16S rRNA clone libraries and comparison of prokaryotic diversity with that of other hypersaline environments.
2. Isolation and characterization (phenotypic and phylogenetic) of novel anaerobic halophilic alkalithermophiles from water and sediments of lakes in the Wadi An Natrun valley.
3. Investigation of the mechanisms of cytoplasmic pH acidification and regulation within the novel halophilic alkalithermophile, *Natranaerobius thermophilus*.

Results from these studies are presented in the following five chapters (2-6) as published/ publishable manuscripts. The final chapter (Chapter 7) summarizes the results of this work. The ensuing appendices detail methods employed during the progress of this dissertation that have not been described in detail in the preceding chapters.

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Table 1.1: Definitions of different extremophiles. Definitions for thermophiles and alkaliphiles adapted from Wiegel (1998), definitions for halophiles are adapted from Oren (2002)

Growth Characteristic	**Minimum**	**Optimum**	**Maximum**
Thermotolerant	T_{min} --	$T_{opt} < 50^oC$	$T_{max} < 60^oC$
Thermophile	T_{min} --	$T_{opt} \geq 50^oC$	$T_{max} \geq 60^oC$
Extreme Thermophile	$T_{min} \geq 35^oC$	$T_{opt} \geq 65^oC$	$T_{max} < 85^oC$
Hyperthermophile	$T_{min} \geq 60^oC$	$T_{opt} \geq 80^oC$	$T_{max} \geq 85^oC$
Alkalitolerant	$pH_{min} \geq 6.0$	$pH_{opt} < 8.5$	$pH_{max} \geq 9.0$
Alkaliphile[1]			
Facultative	$pH_{min} < 7.5$	$pH_{opt} \geq 8.5$	$pH_{max} \geq 10.0$
Obligate	$pH_{min} \geq 7.5$	$pH_{opt} \geq 8.5$	$pH_{max} \geq 10.0$
Non-halophile	$NaCl_{min}$ --	$NaCl_{opt} \leq 0.5$ M[2]	$NaCl_{max} \leq 1$ M
Halotolerant	$NaCl_{min}$ --	$NaCl_{opt}$ 0.25 – 1.5 M	$NaCl_{max} \leq 2.5$ 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	$NaCl_{max}$ --

[1] For thermophiles and psychrophiles, the pH must be measured at the growth temperature for the microorganism (Wiegel, 1998)

[2] 1M NaCl ~ 6% NaCl wt/vol

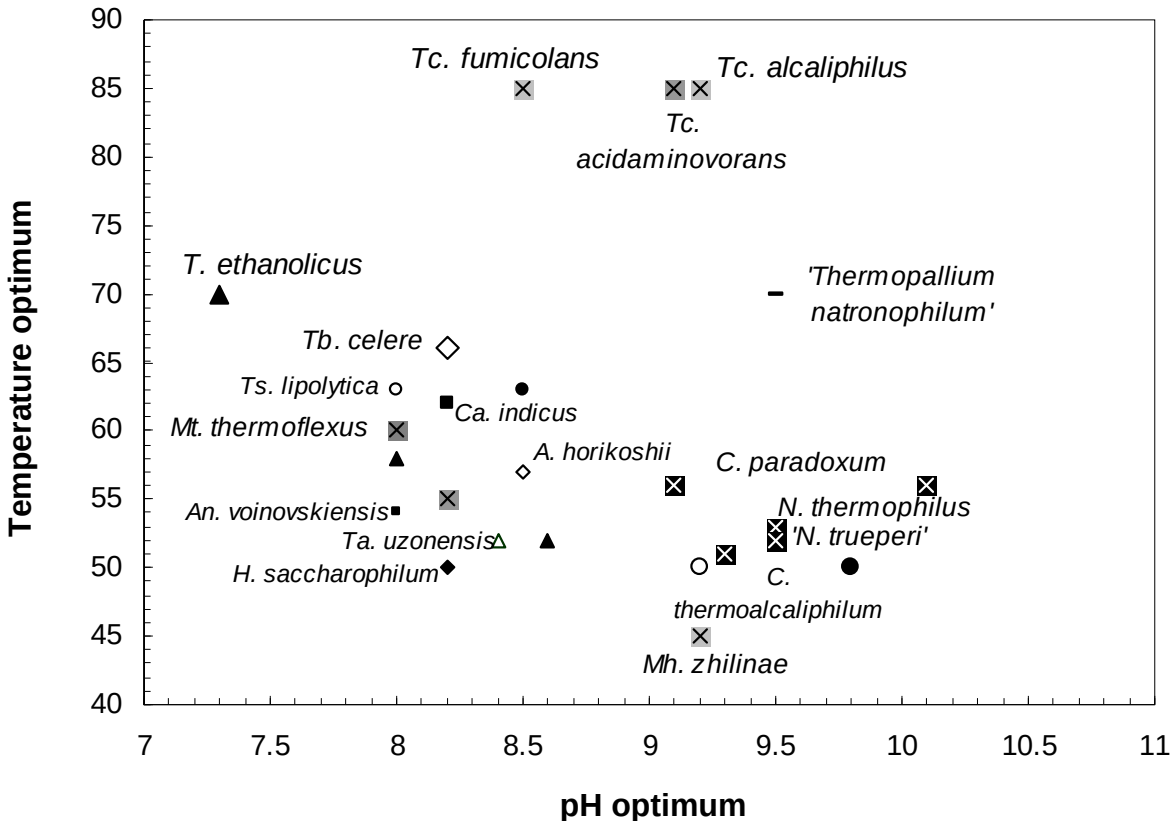

Figure 1.1. Anaerobic alkalithermophiles and halophilic alkalithermophiles graphed according to their temperature and pH optima. Solid symbols represent pH values measured at room temperature, open symbols represent pH values measured at the corresponding growth temperature. Genera have been abbreviated for legibility: A: *Anaerobranca*, An: *Anoxybacillus*, C: *Clostridium*, Ca: *Caloramator*, H: *Halonatronum*, Mh: *Methanosalsum*, Mt: *Methanothermobacter*, N: *Natranaerobius*, T: *Thermoanaerobacter*, Ta: *Thermalkalibacillus*, Tc: *Thermococcus*, Ts: *Thermosyntropha*

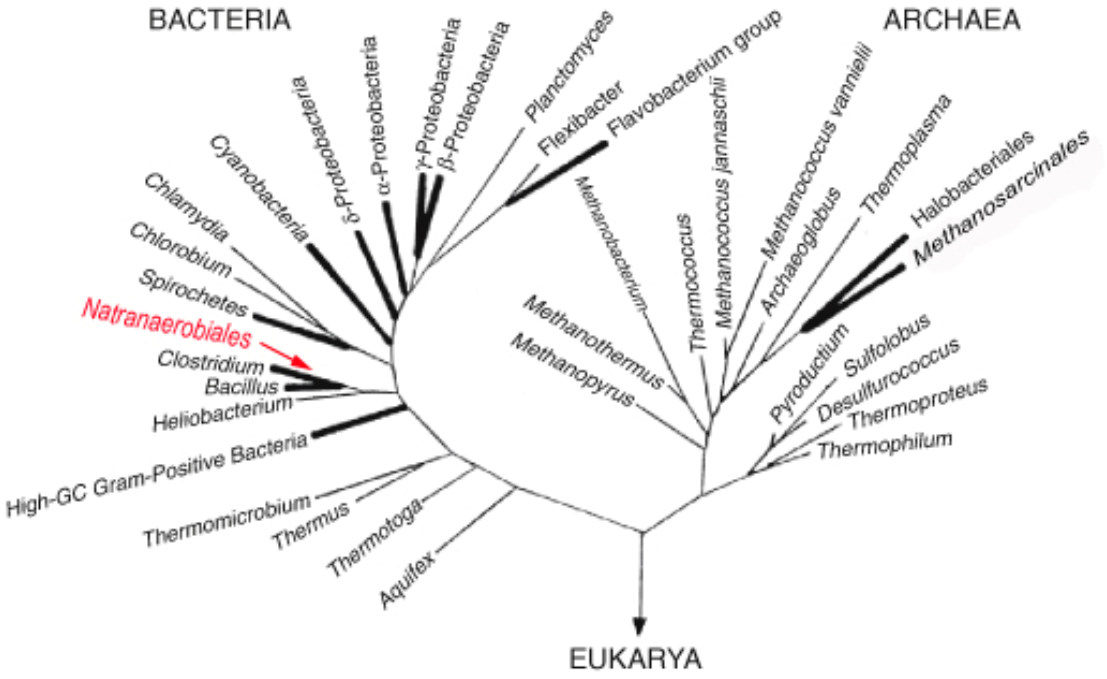

Figure 1.2. Phylogenetic tree of the *Bacteria* and *Archaea* based on 16S rRNA sequence comparisons. Bold lines indicate branches containing microorganisms able to grow optimally at NaCl concentrations greater than 10% wt/vol. Red arrow indicates the position of the order *Natranaerobiales* within the phylogenetic tree. Adapted from Oren,2006b.

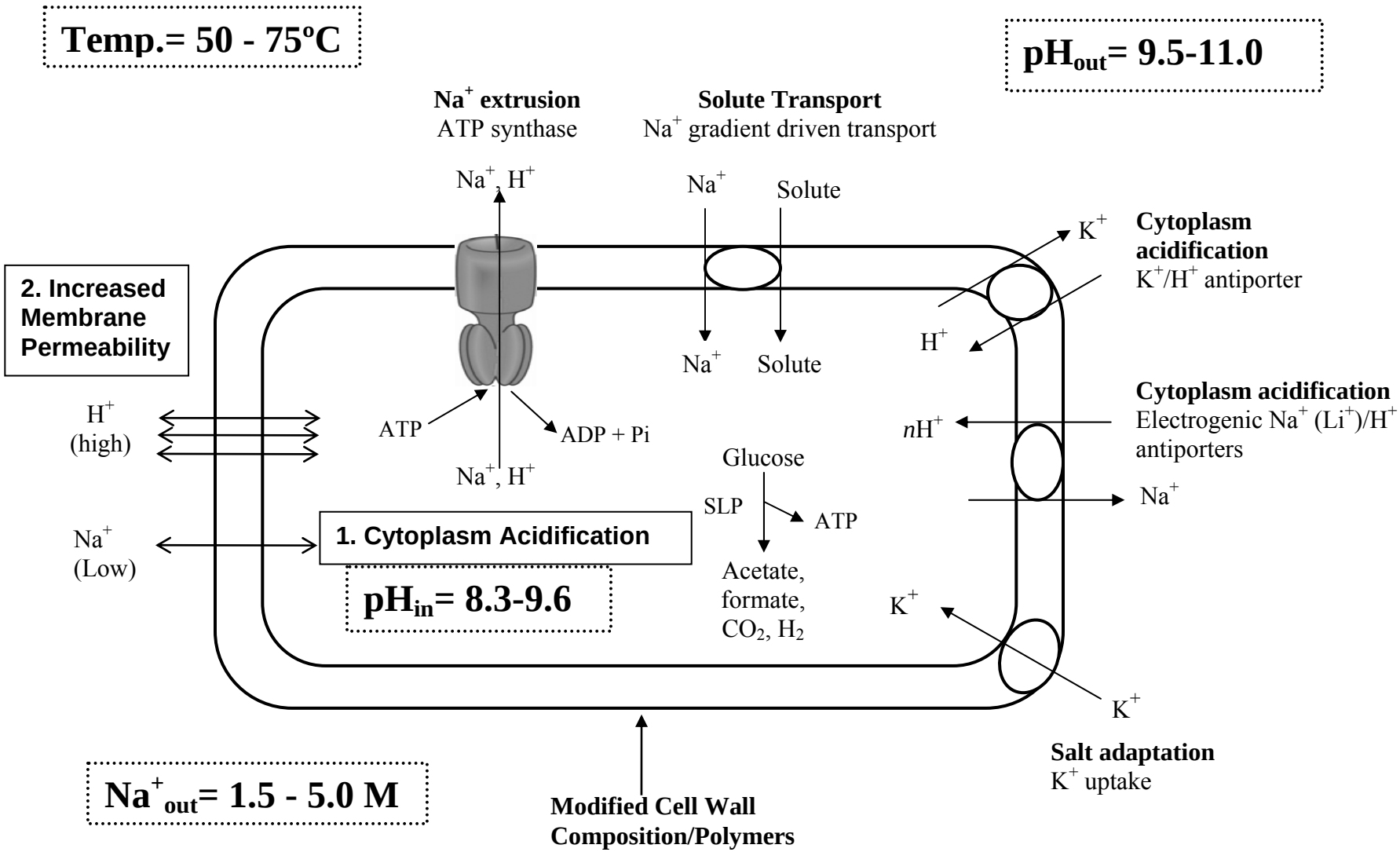

Figure 1.3. Diagrammatic summary of bioenergetic problems, the Na^+ cycle, and potential adaptive mechanisms hypothesized to be employed by anaerobic halophilic alkalithermophiles. SLP= substrate level phosphorylation.

CHAPTER 2

NOVEL AND UNEXPECTED PROKARYOTIC DIVERSITY IN WATER AND SEDIMENTS OF THE ALKALINE, HYPERSALINE LAKES OF THE WADI AN NATRUN, EGYPT[*]

[*] Mesbah, N.M., S.H. Abou-El-Ela, and J. Wiegel. *Microbial Ecology*. 54: 598-617. Reproduced with permission of publisher.

Abstract

The phylogenetic diversity of the bacterial and archaeal communities in the water and sediments of three large lakes of the Wadi An Natrun were investigated using 16S rRNA clone libraries. The bacterial community was diverse, 769 clones formed 345 OTUs defined at 99% 16S rRNA sequence identity. The bacterial community in both the water and sediments of the lakes was dominated by clones affiliated with the low G+C Gram-type positive group, α-proteobacteria and *Bacteroidetes*, (11-39, 11-30, and 10-37% of OTUs observed respectively), patterns that have been observed in previously described alkaline, athalassohaline systems. However, a relatively high proportion of *Firmicutes* related clones in the water of the lakes and α-proteobacteria in the sediments was observed. The bacterial community composition of the water and sediment of the same lake and of different lakes was significantly different ($P < 0.05$). OTUs related to the γ-proteobacteria were more abundant in the sediment of Lake Fazda, whereas the sediment of Lake UmRisha was dominated by members of the δ-proteobacteria. The proportion of γ-proteobacterial and *Bacteroidetes* affiliated OTUs were predominant in water of Lake UmRisha and differed significantly from other lake waters (Chi-squared analysis, $p \leq 0.01$). The more oxygenated and dilute nature of Lake Hamra was reflected in its microbial community composition, with the abundance of *Bacillales* sequences in the water, absence of *Halanaerobiales, Clostridiales*, and *Archaea* in the water, and presence of representatives of more phyla such as the *Actinobacteria, Spirochaetes* and *Verrucomicrobia*. The archaeal community composition appeared less diverse, 589 clones resulted in 198 OTUs defined at 99% 16S rRNA sequence identity and all sequences fell into the phylum *Euryarchaeota*. Phylogenetic analysis showed that many of the sequences were distantly related (83-90% 16S rRNA sequence identity) to cultured and uncultured archaea, with many clones forming clusters that branch

deeply within the *Euryarchaeota*. Forty two and 53% of the bacterial and archaeal clones had less than 90% 16S rRNA sequence identity to previously described sequences. This indicates that the water and sediments of the Wadi An Natrun harbor a unique and novel prokaryotic diversity that is different from what has been described among other alkaline, athalassohaline lakes.

Introduction

The Wadi An Natrun[†] is a depression in the Sahara desert located in Egypt 80 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 (Abd-el-Malek & Rizk, 1963). 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 0.5-2 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 30% (wt/vol).

Leecheate in seepage water infiltrating the valley is the source of salts in the lakes. Geochemical properties of the lakes were first reported more than 100 years ago (Schweinfurth & Lewin, 1898), and then by Imhoff *et al*. (1979) and Taher (1999). The Wadi An Natrun lakes are extreme in more than one aspect; total dissolved salt concentrations between 91.0 and 393.9 g/L have been reported for all seven of the lakes and all lakes have pH values between 8.5 and 11 (Taher, 1999). Salinity and temperature are the same throughout the water column. The lakes are eutrophic ecosystems (Imhoff *et al.*, 1979). Reported levels of inorganic and organic nutrients are high, with measured phosphate concentrations between 116 and 6,830 μM, nitrate between

[†] While different from previously published spellings, this spelling of the Wadi An Natrun is the one that phonetically agrees the most with the pronunciation of the name in the Arabic language. It is also consistent with spelling used by Abdel-Malek and Rizk (1963)

53 and 237 μM , ammonia between 2 and 461 μM , and dissolved organic carbon in the range 136-1,552 mg/L (Imhoff *et al.*, 1979).

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. Abdel-Malek and Rizk (1963) documented the importance of bacterial sulfate reduction in the increase in alkalinity of the area and also stated that the deposition of natron ($Na_2CO_3.xH_2O$) is due to the activity of sulfate reducing bacteria. However, they were unable to detect sulfate reducing bacteria in bottom sediments of most of the hypersaline lakes. Extensive sulfide oxidation is also expected to be carried out by dense communities of photosynthetic sulfur bacteria (*Halorhodospira* and *Ectothiorhodospira* spp.) and chemoautotrophic sulfur-oxidizing bacteria. Enrichment cultures demonstrated the presence of aerobic sulfide-oxidizers and autotrophic ammonia oxidizers (Imhoff *et al.*, 1979).

The Wadi An Natrun lakes have yielded a substantial number of novel prokaryotic species, *Archaea* as well as *Bacteria* (Fritze, 1996, Imhoff & Truper, 1977, Mathrani *et al.*, 1988, Soliman & Truper, 1982, Sorokin *et al.*, 2002, Sorokin *et al.*, 2003). These microorganisms participate in aerobic and anaerobic cycling of carbon, nitrogen and sulfur, hence suggesting active cycling of these elements in the ecosystem. However, the extent to which these species form a dominant component of the microbial community of the lakes has not been determined. Molecular ecological studies based on identification of 16S rRNA sequences isolated directly

from DNA extracted from biomass have not been performed. The only molecular analysis of microbial diversity of the Wadi An Natrun performed thus far has been of cellulolytic enrichment cultures, and focused on the identification of cellulase genes (Grant *et al.*, 2004).

Both cultivation and molecular based studies have been employed to reveal the diversity of bacterial and archaeal communities in hypersaline environments. Various hypersaline lakes from around the world have been studied, including Mono Lake, CA (Humayoun *et al.*, 2003, Scholten *et al.*, 2005), soda lakes in the Kenyan-Tanzanian Rift Valley (Rees *et al.*, 2004), soda lakes in Mongolia (Sorokin *et al.*, 2004) and Inner Mongolia in China (Ma *et al.*, 2004), athalassohaline lakes of the Atacama desert, Chile (Demergasso *et al.*, 2004), saline, meromictic lake Kaiike in Japan (Koizumi *et al.*, 2004), saline Qinghai Lake, China (Dong *et al.*, 2006) and athalassohaline Lake Chaka, China (Jiang *et al.*, 2006). Despite these recent studies, however, understanding of microbial diversity in hypersaline environments is still limited in comparison with diversity of marine and freshwater environments. In addition, the number of investigations related to hypersaline lake sediments is low. Many of the recent studies have been concerned primarily with the water column of the lakes.

The primary objective of this study was to supplement previous culture-dependent studies on the Wadi An Natrun by analysis of the molecular phylogenetic composition of the microbial communities present in water and sediments of three of the largest lakes in the valley and to compare with similar environments. Furthermore, the potential importance of the physiochemical properties of the lakes in structuring the microbial communities is evaluated. The compositions of communities were compared by using phylogenetic statistics to assess similarity of sequence collections.

Methods

Site description and sample collection. Samples for DNA extraction were collected from three soda lakes of the Wadi An Natrun in September of 2003. Lake Fazda, a hypersaline lake situated at 30°19'43.50" N, 30°24'29.68' E, Lake UmRisha, a saturated hypersaline lake (30°20'48.70" N, 30°23'08.14" E) and Lake Hamra, a more dilute lake (30°23'48.28" N, 30°19'13.39" E), were sampled. Water samples approximately 30 cm below the surface were collected into sterile Pyrex® 100 mL bottles (Corning Inc.). Sediment samples were collected at 20 cm below the bottom of the lake. All samples were immediately stored at 4°C and then frozen at -20°C upon arrival to the Suez Canal University. The samples were shipped at 4°C to the US. Upon arrival, they were immediately frozen at -80°C for DNA extraction.

Physiochemical properties of the lakes were measured in May of 2005. In situ sodium chloride concentrations were measured with a refractive index spectrophotometer calibrated for NaCl (Leitz, Germany). pH and temperature were measured with a Solomat WP4007 water quality monitoring system (Solomat Neotronics, Norwalk, CT) and selective electrodes. Sulfide concentration was measured with an ion-selective electrode (Cole-Palmer® EW-27502-41). Water samples, 5 mL, were added to 20 mL vials containing 5.0 mL of sulfide antioxidant buffer. Standards ranging from 10-1000 ppm were prepared using deionized water. The electrode was immersed in the sample or standard, and the millivolt signal was recorded after stabilization. Sulfide concentrations in samples were calculated from regression of data for standards. Dissolved oxygen, ammonium and phosphate were measured using field colorimetric saltwater Hach kits.

Sediments from lakes Fazda and UmRisha were similar; they were dark black with a strong odor of hydrogen sulfide. The dark black core was covered in both lakes by a thin red

layer and then a thin brown layer of sand. Water of Lake Fazda was brown red and turbid. Water of Lake UmRisha was clear and bright pink. There was a thick layer of white precipitated salt on the lake bottom. Sediment from Lake Hamra was dark brown. There was no sulfide odor nor could we detect any white precipitate on the shore or in the lake. Lake Hamra was the most dilute of the sampled lakes. There was a freshwater spring present in the lake approximately 10 m from the shore. More details on the sites can be found in previously published papers (Imhoff *et al.*, 1979, Taher, 1999).

DNA extraction from sediment and water samples. Community genomic DNA was extracted from frozen sediment and water samples (-80°C). Sediment, 10 g wet weight, from each of the three lakes, was placed in 50 mL centrifuge tubes. Approximately 15 mL of lake water was placed into 50 mL centrifuge tubes. The water samples contained a large amount of dissolved matter which quickly blocked 0.2 μm filters. As a result, centrifugation was the method of choice for isolation of cell matter. All water samples gave a visible pellet upon centrifugation. After 3 successive washings in ice cold phosphate buffered saline, 10 mL of lysis buffer (2.5% wt/vol SDS, 50 mM Tris.Cl, 100 mM EDTA) was added to the tubes. The tubes were then subjected to three freeze-thaw cycles consisting of freezing at -80^{o}C for 30 minutes followed by thawing at 70^{o}C for 30 minutes. Freeze-thaw was the chosen method of cell-lysis as it avoids excessive shearing of DNA that can happen during bead-beating or sonication. The tubes were then centrifuged, and the supernatant transferred to new tubes. To the remaining pellet, 10 mL of lysis buffer was added, and the tubes were heated at 70^{o}C till the pellet dissolved. The tubes were then centrifuged and the supernatant combined with the other one. Then, 0.05 g of potassium acetate was added per mL of supernatant to remove humic materials and other soil impurities and the tubes were incubated at 4^{o}C overnight. The tubes were then centrifuged for 30 minutes, and the

supernatants were decanted into new tubes. An equal volume of phenol:chloroform:isoamyl alcohol was added to each sample, vortexed briefly, and centrifuged for 20 minutes. The aqueous phase was transferred to a new tube and extracted again with chloroform:isoamyl alcohol (24:1). DNA was precipitated out of the aqueous phase by the addition of 100% ethanol. After precipitation, the tubes were centrifuged for 20 minutes. A dark brown pellet was visible in each tube. The pellets were air dried and then resuspended in 500 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The DNA preparations were finally purified by passage through the Wizard ® DNA Clean-Up System (Promega). Some DNA preparations had to be passed twice through the Clean-Up System to remove the brown color. The quality and concentration of the DNA were confirmed via electrophoresis on a 1% agarose gel.

PCR amplification of 16S rRNA genes. 16S rRNA genes were amplified from sediment- and water-extracted DNA for construction of bacterial and archaeal clone libraries. Each PCR reaction consisted of 1 Ready-to-Go PCR bead (Amersham Pharmacia), 0.8 μM of forward primer (either 27F for bacterial libraries [5'- AGA GTT TGA TCM TGG CTC AG -3'] or 21F for archaeal libraries [5' – TTC CGG TTG ATC CYG CCG GA – 3']), 0.8 μM of reverse primer (1492R [5' – GGT TAC CTT GTT ACG ACT T – 3']), 5-20 ng of DNA and enough water to bring the final volume up to 25 μL. Primers were synthesized at Integrated DNA Technologies (Coralville, IA). PCR was performed using the following conditions: initial denaturation at 95^{o}C for 5 minutes, 15 cycles of denaturation (1 minute at 94 ^{o}C), annealing (1 minute at 58 ^{o}C) and extension (2 minutes at 72 ^{o}C); and a final extension at 72 ^{o}C for 7.5 minutes. Twenty cycles were necessary to obtain PCR product of sufficient quantity when using the archaeal primer. Reactions were run in duplicate, combined and visualized on a 1% agarose gel. PCR products

were gel purified using the QIAquick Gel Extraction Kit (Qiagen). Resulting PCR product was quantified via gel electrophoresis prior to cloning.

Construction of clone libraries. Clone libraries of bacterial and archaeal PCR products were constructed using the TOPO TA cloning kit (Invitrogen) with plasmid pCR2.1. Successful transformants were inoculated into Luria-Bertani broth containing 100 μg/mL ampicilin, and allowed to grow overnight. Two duplicate libraries were prepared from each sediment and water sample. Clones containing putative 16S rRNA genes were screened by PCR amplification. Each PCR reaction consisted of 3 μL of Luria-Bertani broth containing overnight grown transformants, 0.6 μM each of M13 Forward and M13 Reverse primers, 2.5 μL of 10X reaction buffer, 1.5U of *Taq* polymerase (Promega) and water to a final volume of 25μL. PCR products were visualized on a 1% agarose gel to ensure the presence of inserts of the expected size. Clones that contained no insert or inserts of the incorrect size were excluded from sequencing.

Sequencing. Partial sequencing of 16S rRNA genes was accomplished using an ABI PRISM 3700/3730xl sequencer operated at the Agencourt Bioscience Corporation (Beverly, MA). A total of 1106 bacterial clones (704 from sediment samples and 402 from water samples) were sequenced using primer 27F. Six hundred and twenty four archaeal clones (380 from sediment samples and 244 from water samples) were sequenced with primer 21F. Selected clones were also sequenced with plasmid primers M13 Forward and M13 Reverse to obtain the nearly complete sequence of the 16S rRNA gene. Sequences were manually examined for quality, and those with multiple ambiguous bases or were too short (< 500 bp) were excluded from analysis. The remaining sequences were screened for chimeras by FASTA searches of the whole sequence and the 5' and 3' fragments of the sequences. Sequences whose 5' and 3' ends had close relatedness to different phylogenetic groups were considered chimeric and excluded.

Analysis of 16S rRNA data. Sequences were compared to a current database of rRNA gene sequences from GenBank (May 2006) and reference sequences were chosen based on BLAST similarities (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were also compared to the current database at the Ribosomal Database project using the RDPquery program (http://www.simo.marsci.uga.edu/public_db/rdp_query.htm). The program DOTUR (Schloss & Handelsman, 2005) with the average neighbor algorithm was used to group sequences in operational taxonomic units (OTUs). Sequences with identity ≥ 99% were grouped into OTUs.

All clone sequences were plotted on phylogenetic trees with reference sequences from known phyla. Alignments of clone and reference sequences were created with ClustalX (http://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Trees were constructed using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html). Distances were calculated using the Jukes-Cantor algorithm of DNADIST, and branching order was determined via the neighbor-joining algorithm of NEIGHBOR. Each tree was consensus of 100 replicate trees. Sequences which did not associate to any formally described lineages were classified to undescribed groups.

Statistical analysis and sequence population diversity. Coverage was calculated using the formula $C = 1 - (n1/N)$, where $n1$ is the number of OTUs with one representative and N is the total number of clones analyzed (Good, 1953). Rarefaction was used to evaluate OTU richness of the clone libraries. Shannon diversity was used to evaluate OTU evenness. The Chao1 richness estimator was used to estimate the total number of species in our samples (Schloss & Handelsman, 2005). The statistical significance in the compositions of pairs of libraries was calculated using the LIBSHUFF program (Singleton *et al.*, 2001).

Nucleotide sequence accession numbers. The sequences of cloned inserts were deposited in GenBank under accession numbers DQ432068-DQ432625. Only one representative for clones that were ≥ 99% identical was deposited.

Results

Physiochemical properties of the Wadi An Natrun lakes. Whereas the waters of lakes Fazda and UmRisha are similar, they greatly differ from that of Lake Hamra (Table 2.1). No vertical stratification was observed during the times of sampling. Measured sulfide, ammonium and phosphate concentrations were higher in lakes Fazda and UmRisha. Water in these 2 lakes also appeared to be anoxic as measured by the Hach kits. Exact concentrations of trace elements by ICP-MS of ICP-OES could not be determined due to the high NaCl content of the samples and the concentrations of trace elements were too low to dilute beyond 100X, but semi-quantitative results showed Na^+ present at concentrations 300-350 ppt (parts per thousand), K^+ at 10-50 ppt, and Ca^{2+} and Rb^{2+} at 1-5 ppm. Other elements such as Li^+, Sr^{2+}, Zn^{2+} and Cd^{2+} were present in the low ppb range.

Autosimilarity and sample representativeness. Prokaryotic communities are highly complex and all unique sequences are not sampled. To assess whether the clone libraries were representative samples of rRNA genes amplified from community DNA, autosimilarity (similarity among replicate samples of duplicate clone libraries) from each water and sediment sample was determined with the LIBSHUFF program (Singleton *et al.*, 2001). The results showed high autosimilarity with no significant difference in phylogenetic representation between duplicate libraries from the same sediment or water sample (P>0.191). This indicates the clone

libraries were representative samples of at least the most abundant phylogenetic diversity of sequences cloned from each sediment or water sample. Based on these results, we combined duplicate libraries from each community and performed all subsequent analyses on combined sequence sets.

Estimated richness and coverage. Good's Coverage and rarefaction were used for coverage, the non-parametric estimator Chao1 for richness, and the computed Shannon-Weaver to estimate evenness of community composition (Table 2.2, see Schloss & Handelsman, 2005). The total bacterial 16S rRNA gene sequences ($N = 769$) consisted of 428 unique sequences and 345 OTUs defined by a minimum threshold of 99% sequence identity. During definition of OTUs, a sharp increase in number of OTUs was noted at the 99% threshold in each clone library. This shows that a large fraction of the sequences (40-50%) have relatives from which they differ by < 1% but are not identical. This phenomenon, "microdiversity", has been observed previously with other environmental samples (Acinas *et al.*, 2004a).

Based upon the clone libraries, the diversity of bacterial communities in the sediment and water samples was high. The Shannon indices for 5 of the 6 clone libraries were close to the maximum values (Table 2.2). Even for the pooled library of 769 clones, the Shannon index remained at 0.82 of the maximum value, and the Chao1 estimator was 374. When OTUs were defined at 97% sequence identity, the Shannon index remained at 0.67 of its maximum value, and the Chao1 estimator was 272.

Diversity appeared higher in the water libraries of lakes Fazda and Hamra than the sediment libraries. The water libraries of these two lakes had higher H/Hmax values and Chao1

estimators than the sediment samples. However, there was overlap in the 95% confidence intervals of these clone libraries.

Rarefaction curves for all clone libraries did not plateau when OTUs were defined at 99% sequence identity (Figure 2.1). When all clones were pooled, the rarefaction curve still did not plateau. However, when OTUs were defined at 80% sequence identity, the curve did plateau over a range of 141 clones. This indicates that a large proportion of deep phylogenetic groups are represented in the clone libraries.

Comparison to known bacterial diversity. The relatedness of the Wadi An Natrun bacterial rRNA sequences to known rRNA gene sequences was determined by comparison to the 226,159 rRNA gene sequences in the Ribosomal Database Project (release 9, update 39) using the RDPquery program. Clones possessed a slightly higher similarity to other environmental clones than to cultured microorganisms in the RDP. Fifty seven percent of the Wadi An Natrun bacterial clones had less than 90% sequence identity to cultured microorganisms, and 55% of the clones had less than 90% sequence identity to environmental clones in the RDP. Nearly 42% of the Wadi An Natrun clones had less than 90% sequence identity to previously described sequences in the RDP, including both cultured and uncultured sequences.

Phylogenetic analysis. A total of 1106 clones were sequenced, resulting in 769 high quality sequences, of which 262, 258, and 250 were from lakes Fazda, UmRisha and Hamra respectively (Table 2.2). Sequences that were ≥ 99% identical were considered to be in the same OTU. All 6 bacterial clone libraries were diverse and contained sequences affiliated with phyla previously detected in hypersaline environments (Table 2.3 and Figures 2.2A-E). In terms of absolute

numbers, the *Firmicutes, α-proteobacteria* and the *Bacteroidetes* were the most prominent groups among the sediment samples from all three lakes. These three groups accounted for more than 70% of all clones for each sediment library. No single group was predominant among the clone libraries constructed from the water samples, although the *Firmicutes* and *α-proteobacteria* were predominant in Lake Fazda, the *Bacteroidetes* in Lake UmRisha and the α-*proteobacteria* in Lake Hamra (25%, 37% and 30% of sequences respectively).

Partial sequences of 16S rRNA genes fell into the following groups:

i. *α-Proteobacteria**:*** α-proteobacterial related OTUs were present in all clone libraries, but were less prominent in the library constructed from the water of Lake UmRisha (Figure 2.2A). Seventy four percent of the α-proteobacterial OTUs clustered with the *Rhodobacterales*. Thirty six OTUs (cluster 1) were related (90-98% identity) to *Rhodobaca bogoriensis, Roseinatronobacter thiooxidans, 'Natronohydrobacter thiooxidans'* and an uncultured α-proteobacterium clone ML602M-13 recovered from Mono Lake, an alkaline, hypersaline lake in California (Figure 2.2A, Table 2.4). Ten OTUs (cluster 2), 4 of which were from water of Lake Hamra forming 4% of all clones from this library, formed a phylogenetically coherent cluster, branching from the root of the *Rhodobacterales*. Seven OTUs had 90-95% identity to *Salipiger mucescens* and *Paracoccus zeaxanthinifaciens* (Figure 2.2A, cluster 3, Table 2.4). Seventeen OTUs, 7 of which formed 13% of all clones retrieved from water of Lake Fazda, formed two phylogenetically distinct clusters (clusters 4 and 5), also branching from the root of the *Rhodobacterales*, and did not cluster with previously documented sequences. Two OTUs, from water and sediment of Lake Fazda, clustered with the *Sphingomonadales* (Table 2.4).

ii. γ-*Proteobacteria*: Forty two OTUs were affiliated with the γ-proteobacteria, 38 of them belonging to the *Chromatiales*. Thirty of these OTUs fell into the *Ectothiorhodospiraceae*

(Figure 2.2B, clusters 1-4). Six OTUs could be assigned to the *Ectothiorhodospira* and *Halorhodospira* genera (Figure 2.2B, cluster 1, Table 2.5). This is consistent with enrichment cultures performed by Imhoff *et al*. (1978), which resulted in isolation of six strains of phototrophic bacteria belonging to the genus *Ectothiorhodospira*. Thirteen OTUs, 4 from Lake Fazda, 4 from Lake UmRisha and 5 from Lake Hamra (comprising 11% of the clone library constructed from water of Lake Hamra), formed two distinct clusters within the γ-proteobacteria (clusters 2 and 3) and were distantly related (87-93% identity) to an uncultured gamma proteobacterium clone from Mono Lake, CA (Humayoun *et al.*, 2003). Seven OTUs (only one from Lake UmRisha, cluster 4) were members of the genus *Thialkalivibrio*, which consists of haloalkaliphilic chemolithoautotrophic sulfur-oxidizing bacteria (Sorokin *et al.*, 2003) (Table 2.5).

Eight OTUs (only two from lakes UmRisha and Hamra) fell into cluster 5, and were related to the *Chromatiaceae*. Seven of these OTUs were distantly related (90-93% sequence identity) to the genus *Thiorhodovibrio*, which consists of chemolithotrophs which oxidize sulfide or thiosulfate with molecular oxygen. They are alkaliphilic and halotolerant (Imhoff, 2001).

Two OTUs one from sediment of Lake Fazda and one from water of Lake Hamra clustered within the *Oceanospirillales* (Figure 2.2B and Table 2.5). Two OTUs from sediment of Lake Fazda were associated with the *Thiotrichales*, and were closely related to *Thiomicrospira thyasirae*, a halotolerant, facultatively chemolithoautotrophic, sulphur-oxidizing bacterium isolated from the gills of *Thyasira flexuosa*, a bivalve mollusc (Wood & Kelly, 1993).

iii. δ-*Proteobacteria*: OTUs of the δ-*proteobacteria* were members of the predominantly sulfate-reducing groups such as the *Desulfovibrionales* and the *Desulfobacterales,* and were absent from waters of Lakes UmRisha and Hamra (Figure 2.2B and Table 2.6).

iv. *Bacteroidetes*: A total of 62 OTUs clustered with the *Bacteroidetes* phylum (Figure 2.2C and Table 2.6). Thirty OTUs, 13 from Lake Fazda, 10 from Lake UmRisha and 7 from Lake Hamra formed a separate lineage (Figure 2.2C, cluster 1), branching off from the root of the *Sphingobacteriales*. These were distantly related to clone ML623J-20 retrieved from the chemocline of Mono Lake, CA (Humayoun *et al.*, 2003). Only three OTUs from Lake Fazda, one from the sediment and 2 from the water, were distantly related (93% identity) to '*Rhodinella ikkaensis*', an alkaliphilic bacterium isolated from a cold alkaline environment in Greenland (GenBank description). Four OTUs clustered with the *Bacteroidales*, which is mainly composed of anaerobic microorganisms and are usually members of the gut microflora. Five OTUs, three from Lake Hamra and one from Lakes Fazda and UmRisha each, formed a separate cluster, cluster 2, that branched deeply within the *Bacteroidetes*. These only had a distant relation (85-92% identity) to a Bacteroidetes bacterium strain MO54, a halotolerant chemoorganotroph isolated from the Great Salt Plains of Oklahoma (GenBank description). Nineteen OTUs, 2 from Lake Fazda, 11 from Lake UmRisha and 6 from Lake Hamra clustered with *Salinibacter ruber*, an extremely halophilic red-pigmented bacterium isolated from salterns in Alicante, Spain (Anton *et al.*, 2002). The OTU WN-UWB-22 was distantly related (91% identity over positions 195-763) to *S. ruber*, and it comprised 62% of all the clones retrieved from the water of Lake UmRisha.

v. *Firmicutes*: *Firmicutes* affiliated OTUs dominated libraries constructed from the sediment of all three lakes. Of the 87 OTUs related to the *Firmicutes*, 28 of them formed a distinct cluster (Figure 2.2D, cluster 1) branching deep within the *Firmicutes* and did not associate with any sequences in the GenBank database. One sequence, WN-USB-255 (cluster 2) had 93% identity to '*Natronanaerobium salstagnum*', an obligately anaerobic alkaliphile isolated from salterns of

alkaline, hypersaline Lake Magadi, Kenya (Jones *et al.*, 1998). Fourteen OTUs (12 of them from Lake Fazda), fell into the *Clostridiales* and were distantly related (87-92% identity) to members of the genus *Alkaliphilus* which are found in subsurface alkaline environments (Table 2.6)(Cao *et al.*, 2003, Takai *et al.*, 2001). Eight OTUs (7 from Lake Hamra), clustered with the *Bacillales*. These OTUs were only distantly related (85-90% identity) to *Alkalibacillus haloalkalphilus*, an obligately alkaliphilic and halotolerant microorganism previously isolated from the Wadi An Natrun (Fritze, 1996).

Twenty-seven OTUs (only 4 from Lake Hamra) clustered with the *Halanaerobiales*, a phylogenetically coherent group of fermentative obligatory anaerobic halophilic bacteria abundant at the bottoms of hypersaline lakes, lagoons and solar salterns (Figure 2.2D, Table 2.6) (Rainey *et al.*, 1995). Twenty OTUs formed a separate cluster (Figure 2.2D inset, cluster 3) and were not related to previously documented sequences.

vi. *Spirochaetes:* Twelve OTUs, 9 from Lake Hamra, were related to mesophilic haloalkaliphilic *Spirochaeta* spp. (Figure 2.2E, Table 2.6) isolated from different hypersaline environments (Fracek & Stolz, 1985, Hoover *et al.*, 2003).

vii. Others groups: Five OTUs were retrieved from Lake Hamra water that clustered with the *Verrucomicrobiales* (Figure 2.2E). Four OTUs, 3 from Lake Hamra water, clustered with the *Actinobacteria*. Five OTUs were associated with the *Chloroflexaceae*, and 4 fell into candidate divisions. Seven OTUs (4 from Lake UmRisha) were related (90-97% identity) to a halotolerant cyanobacterium, *Euhalothece* sp. MPI95AH10 (Garcia-Pichel *et al.*, 1998).

Library comparisons. LIBSHUFF statistics was applied to determine the significance of differences between the different clone libraries (Singleton *et al.*, 2001). Pair-wise comparisons

of water and sediment samples from the same lake and individual water and sediment samples from different lakes showed that, with the exception of the water and sediment samples from Lake UmRisha, the bacterial community composition differed significantly between the different libraries (Table 2.7). Comparison of the bacterial community between the water and sediment of Lake UmRisha showed that the bacterial community of the water is not different from the sediment, but the community of the sediment is significantly different from the water, indicating that the water bacterial community is a subset of that of the sediment. Closer examination of the distribution of $(C_X\text{-}C_{XY})^2$ as a function of evolutionary distance showed differences between the libraries at evolutionary distances of 0.02-0.08. This result suggests that the differences were between closely related sequences. This conclusion is supported by the phylogenetic trees in which the sequences from the different clone libraries often grouped near each other but were rarely identical (Figures 2.2A-2.2E).

Despite this apparent microdiversity, the abundance of specific groups differed significantly among the clone libraries. Among the sediment clone libraries, the γ-proteobacterial OTUs varied significantly (Chi-squared analysis, $p \leq 0.025$), and were more abundant in the sediment of Lake Fazda. The δ-proteobacterial OTUs also differed significantly (Chi-squared analysis, $p \leq 0.05$). The numbers of δ-proteobacterial OTUs were higher in Lake UmRisha sediment than in any other clone library. The number of γ-proteobacterial and *Bacteroidetes* related OTUs were significantly greater in the water of Lake UmRisha, where they formed 30 and 37% of all observed OTUs respectively (Chi-squared analysis, $p . \leq 0.01$). Finally, in water of Lake Hamra, the abundance of OTUs affiliated with the *Spirochaetes*, *Actinobacteria* and *Verrucomicrobi*a was larger (Chi-squared analysis, $p \leq 0.01$).

Cloning and sequencing of archaeal 16S rRNA genes. Archaeal genes for 16S rRNA were amplified from DNA extracted from the sediment of all three lakes and from the water of Lakes Fazda and UmRisha. Several attempts failed to obtain 16S rRNA amplicons from DNA extracted from water of Lake Hamra. A total of 960 archaeal clones were sequenced, resulting in 588 high quality reads, 229 from Lake Fazda, 252 from Lake UmRisha and 107 from sediment of Lake Hamra. Autosimilarity among replicate samples of duplicate clone libraries was high ($P > 0.21$ in all 5 samples).

In comparison with the bacterial clone libraries, diversity in the archaeal clone libraries was low. Coverage for all clone libraries was greater than 0.78, indicating that a large proportion of the archaeal community has been sampled. This is also evident in the calculated diversity indices and rarefaction curves (Table 2.8 and Figure 2.3) All three sediment samples show similar patterns of diversity; UmRisha sediment has the most diverse archaeal community among the sediment samples which is reflected in both higher species richness (higher number of observed OTUs) and the higher H/Hmax. Based upon coverage and the Chao1 estimator, the archaeal community of the water of UmRisha appeared to be less diverse than that of Lake Fazda.

All of the archaeal OTUs were affiliated with the *Euryarchaeota*. Of the 198 archaeal OTUs detected, only 20 (none from Lake Hamra) were related to the *Methanosarcinales* (Figure 2.4B). Only 2 OTUs, WN-FWA-130 and WN-FWA-144, were 97-98% identical to *Methanolobus oregonensis*, a halo- and alkalitolerant microorganism (Boone, 2001). Thirteen OTUs formed a separate lineage within the *Methanosarcinales* (Figure 2.4B, cluster 1). The majority of the archaeal OTUs clustered with the *Halobacteria* (Figure 2.4A). Nineteen OTUs formed two separate lineages branching deep within the *Halobacteria*, (Figure 2.4A, clusters 4

and 5) and did not associate with any previously documented sequences. Seventy eight OTUs formed separate lineages within the *Halobacteriaceae* and did not associate with previously documented sequences (Figure 2.4A, clusters 1,2 and 3). Only 7 OTUs were closely associated (96-99% sequence identity) with uncultured MSP clones retrieved from a salt crystallizing pond at Lake Magadi, Kenya (Grant *et al.*, 1999). These clones formed a coherent clone-only cluster that branched deep within the *Halobacteriales*. Only 12 OTUs had 92-96% identity to *Natronomonas pharonis*, an extremely halophilic, alkaliphilic bacterium isolated from the Wadi An Natrun (Soliman & Truper, 1982). No sequences affiliated with the *Crenarchaeota* or *Korarchaeota* were detected.

Pair-wise comparisons of the 5 archaeal clone libraries by the LIBSHUFF program showed that, similar to the bacterial clone libraries, the archaeal community of Lake UmRisha water was a subset of that of the sediment (Table 2.9). Archaeal community of Lake Fazda sediment did not differ from that of UmRisha. Archaeal community compositions of the remaining samples were significantly different ($P< 0.05$). Examination of the distribution of $(C_X - C_{XY})^2$ as a function of evolutionary distance showed differences between the libraries at evolutionary distances of 0.02-0.07. This is also supported by the phylogenetic trees, where clones from different libraries are closely related but not identical.

Discussion

Even though PCR based analyses of microbial diversity are acknowledged to be less than fully representative due to biases introduced by factors such as differential DNA extraction, primer selectivity and variable rRNA gene copies (Wintzingerode *et al.*, 1997), they still remain an effective method to identify the most prominent microorganisms in a natural environment.

Although it is not possible to rule out systematic biases in this study, the low number of PCR cycles we used and the high autosimilarity observed between replicate clone libraries from the same sample indicates there was minimal random bias in the methods. The presence of roughly equal amounts of Gram-type positive and Gram-type negative clones indicates that the freeze-thaw method of cell lysis was effective while avoiding extensive shearing of DNA from Gram-type negative microorganisms. Statistical analysis of our results suggests that the clone libraries were representative of at least the most dominant phylogenetic groups present in the original samples. We assessed sample representativeness with a group diversity indices including coverage, rarefaction and evenness. Coverage for all the clone libraries exceeded 50%, and at the 80% 16S rRNA sequence identity level, rarefaction curves were flat. This indicates that the libraries were representative of the major phylogenetic groups present. Rarefaction curves constructed at the species level (99% 16S rRNA identity) showed similar patterns of diversity among the different samples and, while not flat, were also not linear, further indicating that the predominant phylogenetic groups have been sampled.

Results from this study indicate a diverse range of prokaryotes are present in three of the largest lakes of the Wadi An Natrun. A high proportion of the sequences retrieved (42% of bacterial sequences, 53% archaeal sequences) had less than 90% 16S rRNA sequence identity to any sequences deposited in the RDP or GenBank, representing a microbial assemblage different from what has currently been described, even in other hypersaline environments. Our results also indicate the presence of divergent groups of Gram-type positive bacteria, (Figure 2.2D, cluster 1) and Archaea (Figures 2.4A and B, cluster 1) with no close relatives. These clusters represent potential novel phylogenetic groups. We acknowledge, however, that the results presented in this study are based on a single sample taken, thus represent only a snapshot of the microbial

diversity present in three separate Wadi An Natrun lakes. This study does not fully assess microbial diversity nor does it identify which members of the community are functionally important.

Community composition and structure. The abundance of γ-proteobacterial sequences related to the *Chromatiales* is consistent with previous culture dependent studies of the Wadi An Natrun (Imhoff *et al.*, 1978, Imhoff & Truper, 1977). However, many of the clones retrieved were only distantly related (90-96% 16S rRNA sequence identity) to *Ectothiorhodospira* and *Halorhodospira* spp. previously isolated from the Wadi An Natrun (Imhoff *et al.*, 1978). Four OTUs, 2 from Lake Hamra were closely related to the nonphototrophic genus *Thialkalivibrio*. Members of the genus *Thialkalivibrio* can oxidize reduced sulfur compounds with either oxygen or nitrate as electron acceptors. It seems likely that the microorganisms from Lake Hamra share this metabolism, as the water of this lake undergoes fluctuations in oxygen content due to seepage from the freshwater well at its edge. We expect that mass developments of these phototrophic and nonphototrophic bacteria (*Chromatiales* and *Thiotrichales*) in the water and upper sediment of the lakes are major components involved in the oxidation of the sulfide produced by sulfate-reducers in the sediment.

α-proteobacterial sequences related to the order *Rhodobacterales* dominated in all clone libraries (with the exception of the water from Lake UmRisha), as would be expected in a hypersaline environment. OTUs of the δ-proteobacteria were present in the sediment of all lakes and water of Lake Fazda only. They were mainly associated with the sulfate-reducing *Desulfovibrionales* and *Desulfobacterales,* members of which are observed in hypersaline environments. It is interesting that no clones closely related to the δ-proteobacteria or the Gram-

type positive sulfate-reducers (*Desulfotomaculum* clade) were retrieved from Lake UmRisha water even though this lake had the highest sulfide levels (Table 2.1). However, the distant relationship of many of the clones retrieved from this sample to cultured representatives makes it difficult to predict the roles they are playing in the environment. It is possible that they represent novel groups of sulfate-reducers.

Clones affiliated with the *Halanaerobiales, Clostridiales* and unidentified *Firmicutes* were abundant in the sediments of all the lakes sampled. Cultured members of the *Halanaerobiales* are obligately anaerobic, moderately halophilic microorganisms that gain energy by fermentation of various organic compounds. Their abundance in the sediments of all the lakes suggests an affinity for anoxic environments, which is consistent with a fermentative mode of life. While it is possible that this increased abundance could be an artifact of multiple *rrn* copy number which is common among the low G+C Gram type positive group (Acinas *et al.*, 2004b), no clones affiliated with either the *Halanaerobiales* or the *Clostridiales* were retrieved from Lake Hamra water. The water of Lake Hamra is more oxygenated than that of either Lakes Fazda or UmRisha, thus not providing sufficient anaerobic conditions necessary for growth of this group of microorganisms. This is also supported by that the libraries from the anoxic waters of Lakes Fazda and UmRisha contained few *Bacillales* sequences, whereas the frequency of *Bacillales* sequences was higher in water of Lake Hamra (Figure 2.2D). *Bacillus* spp. are among the most commonly found aerobic, haloalkaliphilic bacteria in soda lakes and other hypersaline environments. The higher abundance of *Bacillales* sequences in water of Lake Hamra could be due to its higher dissolved oxygen content.

Forty percent of all *Bacteroidetes* affiliated clones formed a cluster (Figure 2.2C, cluster 1) distantly related to known groups of the *Bacteroidetes* and may represent a new lineage of

haloalkaliphilic microorganisms. OTU WN-UWB-22 was abundant in the water of Lake UmRisha where it clustered with *Salinibacter ruber*. *Salinibacter* is extremely halophilic requiring 15-23% NaCl for growth. It is a motile rod pigmented by a red pigment, possibly a carotenoid (Anton *et al.*, 2002). It has been shown that the pigment produced by *Salinibacter* spp. can account for up to 5% of the total prokaryotic pigment absorbance of saltern crystallizer ponds (Oren & Rodriguez-Valera, 2001). That and the fact that this particular OTU made up almost two-thirds of the clones retrieved from UmRisha water, indicates that it could be contributing to the bright red coloration that was only observed in Lake UmRisha.

The archaeal community of the Wadi An Natrun is novel and distinct from what has been currently described; with the majority of the clones forming deeply branching groups (Figure 2.4) with little relationship to cultured or uncultured sequences in the databases. Only 45 of the 198 archaeal OTUs detected had 93-97% 16S rRNA sequence identity to cultured members of the *Halobacteriales* and *Methanosarcinales*. Isolated strains of the *Halobacteriales* are aerobic halophiles growing at salinities up to NaCl precipitation, and require at least 9% NaCl for growth (Grant, 2001). This could explain the absence of archaeal clones from the more dilute Lake Hamra water (10% NaCl wt/vol). However, Purdy *et al*. (2004) reported a diverse community of haloarchaea in coastal salt marsh sediments with NaCl concentrations as low as 2.5% (wt/vol). It is possible that DNA extraction and/or PCR amplification could have selected against this group during construction of clone libraries, particularly if the abundance of *Archaea* in this sample was small compared with other groups.

Lake Hamra is distinct from both Lakes Fazda and UmRisha in that it is less alkaline, saline and contains a larger proportion of dissolved oxygen (Table 2.1). This is reflected in its microbial community composition. As stated above, we did not retrieve clones related to the

Halanaerobiales or *Clostridiales* from the water. This is not due to differential DNA extraction, as representatives of the aerobic Gram-type positive *Bacillus* group were obtained primarily from this sample. Lake Hamra also contains representatives of more phyla, such as the *Verrucomicrobia, Actinobacteria* and *Spirochaetes*. Thus it is possible that the less extreme conditions in this lake allowed for growth of microbial groups not adapted to alkaline, hypersaline conditions. On the other hand, this elevated phylogenetic diversity could be due to input of less saline water from the well at the lakes edge.

Comparison to other hypersaline environments. Consistent with previous studies on saline and alkaline lakes, the α- and γ-proteobacteria and the *Bacteroidetes* groups are dominant in the lake waters (Humayoun *et al.*, 2003, Jiang *et al.*, 2006, Rees *et al.*, 2004). However, the Wadi An Natrun lake waters have a relatively high abundance of OTUs affiliated with the *Firmicutes*, where they formed 11-25% of all OTUs observed in these samples. This is in contrast to water of Lake Chaka, China (salinity 32.5% wt/vol, pH 7.4), where *Firmicutes* related phylotypes accounted for only 7% of clones retrieved from the water (Jiang *et al.*, 2006), and no *Firmicutes* affiliated clones were retrieved from any of the lakes sampled in the Atacama desert (Demergasso *et al.*, 2004). The abundance of *Firmicutes* in the Wadi An Natrun waters is similar to that of the anoxic chemocline of athalassohaline Mono Lake, CA (22% of total phylotypes observed), situated at a depth 23m.

The sediments of the Wadi An Natrun lakes are dominated by clones affiliated with the low G+C Gram-type positive group, where they accounted for 34-39% of all OTUs observed. Rees *et al*. (2004) and Jones *et al*. (1998) reported Gram-type positive clones and isolates from soil/sediments in soda lakes of the Kenyan-Tanzanian Rift Valley. A similar pattern was

observed in sediments of Lakes Chaka and Qinghai, China (Dong *et al.*, 2006, Jiang *et al.*, 2006) where *Bacillus/Clostridia* related phylotypes dominated (20-60% of all phylotypes observed) sediment cores 10-40cm below the surface. However, the Wadi An Natrun sediments harbor a large proportion of α-proteobacterial OTUs (22-27% of all OTUs observed). This is greater than reported for alkaline, saline lakes in China (6-17% of all phylotypes observed, (Dong *et al.*, 2006, Jiang *et al.*, 2006)), even in cores collected 20-30 cm below the surface. This is similar to that reported in the soil/sediment of soda lakes in the Kenyan-Tanzanian Rift Valley and the Inner Mongolian Baer Soda Lake (Ma *et al.*, 2004, Rees *et al.*, 2004). Unlike other alkaline saline sediments, we did not retrieve any clones related to the β-proteobacteria.

Based on coverage, rarefaction and diversity analyses that we performed, the diversity of the bacterial community in the Wadi An Natrun is similar to other athalassohaline lakes (Dong *et al.*, 2006, Humayoun *et al.*, 2003, Jiang *et al.*, 2006). However, within each clone library, the diversity was high, with deeply diverging taxa, particularly among the *Firmicutes, Bacteroidetes* and γ-proteobacteria.

Archaeal communities in the water and sediments of the Wadi An Natrun appeared less diverse than archaeal communities in previously described hypersaline systems (Dong *et al.*, 2006, Jiang *et al.*, 2006, Maturrano *et al.*, 2006, Sorensen *et al.*, 2005). All clones belonged to the *Euryarchaeota,* no sequences affiliated with the *Crenarchaeota* were obtained. However, the majority of the clones formed deep branches within the *Euryarchaeota* and did not cluster with any previously identified sequences. This indicates an archaeal community different from what is currently known.

Conclusions. This study is the first culture-independent report on the microbial diversity in the alkaline, hypersaline lakes of the Wadi An Natrun. Despite high salinity, alkaline pH, in addition to anoxic conditions and intense solar irradiation, a rich microbial population exists. The Wadi An Natrun lakes are similar to other athalassohaline lakes in several aspects, the microbial diversity is low compared to marine and aquatic environments; the lakes are dominated by three groups of *Bacteria* (*Firmicute*s, *Bacteroidetes*, α- and γ-proteobacteria) and two groups of *Archaea*, *Halobacteriales* and *Methanosarcinales*. However, within each phylogenetic group, particularly the *Archaea, Firmicutes* and *Bacteroidetes*, extensive diversity exists, with the presence of unique clusters without close cultured of uncultured relatives. The majority of the clones retrieved from the Wadi An Natrun represent novel genera and species, that, given the extreme natural environment they inhabit, must posses novel physiological, metabolic and adaptive mechanisms.

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Table 2.1. Physiochemical properties of the water of the Wadi An Natrun lakes under study

Physical Property	Fazda	UmRisha	Hamra
NaCl (%wt/vol)	30.0	29.1	10.0
$pH^{25°C}$	9.3	9.8	8.5
S^{2-} (μM)	46	50	1.37
Dissolved Oxygen (ppm)	0.12	0.20	4.00
NH_4^+ (μM)	46.1	30.1	9.7
$P0_4^{2-}$ (μM)	633	515	3

Table 2.2. Diversity and richness indices for bacterial clone libraries

Type of Data	Sediment			Water			All Clones
	Fazda	UmRisha	Hamra	Fazda	UmRisha	Hamra	
N[a]	177	165	146	85	93	104	769
S[b]	76	69	56	57	27	60	345
Shannon (H)	3.90	3.92	3.58	3.86	1.85	3.87	4.82
H/Hmax	0.90	0.93	0.89	0.96	0.57	0.94	0.82
Coverage	0.78	0.79	0.76	0.51	0.80	0.63	0.86
Chao1	111	111	104	143	56	110	374
95% COI[c]	92-154	87-169	80-160	94-256	36-115	82-175	321-462

[a]*N*: number of clones in library
[b]*S*: OTUs defined at ≥ 99% sequence identity
[c]95% confidence interval for the Chao1 estimator

Table 2.3. Distribution of bacterial OTUs into phylogenetic groups.

	% OTUs in libraries					
	Sediment			Water		
Phylogenetic Group	Fazda	UmRisha	Hamra	Fazda	UmRisha	Hamra
Firmicutes	0.39	0.35	0.34	0.25	0.11	0.15
α- Proteobacteria	0.25	0.22	0.27	0.25	0.11	0.3
γ- Proteobacteria	0.17	0.04	0.04	0.08	0.30	0.15
δ- Proteobacteria	0.03	0.10	0.05	0.08	0	0
Bacteroidetes	0.1	0.23	0.18	0.23	0.37	0.15
Spirochaetes	0.03	0	0.07	0.02	0	0.08
Cyanobacteria	0.01	0.01	0	0	0.11	0.04
Chloroflexi	0.01	0.01	0.04	0.02	0	0
Actinobacteria	0	0	0	0.02	0	0.05
Verrucomicrobia	0	0	0	0	0	0.08
Unidentified Groups	0.01	0.04	0.01	0.05	0	0

Table 2.4. Summary of OTUs affiliated with the α-proteobacteria

OTU	GenBank Accession	Closest relative of BLAST search	% Identity	Characteristics of nearest BLAST hit	Reference
Rhodobacterales					
WN-HSB-177	DQ432280	*Rhodobaca bogoriensis* AF248638	98	Alkaliphilic, anoxygenic purple nonsulfur, isolated from soda lake in Kenya	(Milford *et al.*, 2000)
WN-FWB-13	DQ432431	'Natronohydrobacter thiooxidans' AJ132383	98	Alkaliphilic, autotrophic, isolated from sediment of soda lake in Kenya	GenBank description
WN-FSB-129	DQ432072	*Roseinatronobacter thiooxidans*, AF249749	96	Aerobic, alkaliphilic, isolated from soda lakes in Russia	(Sorokin *et al.*, 2000)
WN-HSB-23	DQ432265	*Salipiger mucescens* AY527274	95	Halophilic, alkaliphilic, aerobic, isolated from hypersaline soil in Spain	(Martinez-Canovas *et al.*, 2004)
WN-HSB-5	DQ432283	*Paracoccus zeaxanthinifaciens* AF461159	97	Halophilic, alkaliphilic, isolated from seaweed collected along coast of African Red Sea	(Berry *et al.*, 2003)
Sphingomonadales					
WN-FWB-119	DQ432400	*Erythrobacter aquimaris* AY461441	99	Halophilic, neutrophilic isolated from sea water of a tidal flat of the Yellow Sea in Korea	(Yoon *et al.*, 2004a)
WN-FSB-117	DQ432070	*Porphyrobacter donghaensis* AY559428	99	Halophilic, chemoorganotrophic, isolated from the East Sea in Korea	(Yoon *et al.*, 2004b)

Table 2.5. Summary of OTUs affiliated with the γ- proteobacteria

OTU	GenBank Accession	Closest relative of BLAST search	% Identity	Characteristics of nearest BLAST hit	Reference
Chromatiales					
WN-UWB-35	DQ432239	*Halorhodospira neutrophila* AJ318526	94%	Halophilic, neutrophilic, photolithoheterotrophic, isolated from benthic microbial mat in saltern, France	(Hirschler-Rea *et al.*, 2003)
WN-UWB-51	DQ432255	*Ectothiorhodospira halochloris,* M59152	95%	Halophilic, alkaliphilic, photolithoautotrophic, isolated from water of Wadi An Natrun lakes	(Imhoff & Truper, 1977)
WN-HWB-190	DQ432358	*Alcalilimnicola halodurans* AJ404972	93%	Alkaliphilic, halotolerant, chemoorganotrophic, isolated from soda-depositing Lake Natron, Tanzania	(Yakimov *et al.*, 2001)
WN-FSB-205	DQ432133	*Thialkalivibrio nitratireducens* AY079010	97%	Alkaliphilic, halophilic, anaerobic, obligately chemolithoautotrophic, isolated from sediment of Lake Fazda	(Sorokin *et al.*, 2003)
WN-FSB-61	DQ432099	*Thiocapsa rosea*, AJ002798	96%	Phototrophic sulfur bacterium, isolated from meromictic hypersaline lake in Switzerland	(Tonolla *et al.*, 1999)
Oceanospirillales					
WN-FSB-32	DQ432107	*Halomonas elongata* X67023	94%	Moderately halophilic, neutrophilic, common in aquatic and marine habitats	(Arahal *et al.*, 2001)

Table 2.6. Summary of OTUs affiliated with the δ- proteobacteria, *Bacteroidete*, *Firmicutes*, and *Spirochaetes*

OTU	GenBank Accession	Closest relative of BLAST search	% Identity	Characteristics of nearest BLAST hit	Reference
Δ- proteobacteria					
WN-FWB-161	DQ432411	*Desulfonatronum lacustre* AF418171	96%	Extremely alkaliphilic, halotolerant, reduces sulfate with H_2, formate or ethanol as electron donors .	(Pikuta *et al.*, 1998)
WN-FSB-188	DQ432085	*Syntrophobacter fumaroxidans* X82874	87%	Non-halophilic, neutrophilic, syntrophic, propionate-oxidizing bacterium, isolated from anaerobic granular sludge	(Harmsen *et al.*, 1998)
WN-FSB-6	DQ432146	*Desulfococcus multivorans* AF418473	89%	Anaerobic, halotolerant	GenBank description
Bacteroidetes					
WN-FSB-222	DQ432136	*Alkaliflexus imshenetskii* AJ784993	90%	Alkaliphilic, halotolerant, carbohydrate-fermenting, isolated from soda lakes in central Asia .	(Zhilina *et al.*, 2004)
Firmicutes					
WN-FSB-20	DQ432147	*Alkaliphilus crotonoxidans* AF467248	92%	Alkaliphilic, anaerobic, isolated from alkaline subsurface environments	(Cao *et al.*, 2003)
WN-FSB-102	DQ432068	*Halanaerobium saccharolyticum* L37424	94%	Neutrophilic, isolated from sediment of Lake Sivash, Crimea .	(Rainey *et al.*, 1995)
Spirochaetes					
WN-HSB-64	DQ432271	*Spirachaeta bajacaliforniensis* M71239	95%	Halophilic, neutrophilic, anaerobic, isolated from anaerobic mud in an evaporite flat in Baja, California	(Fracek & Stolz, 1985)

Table 2.7. LIBSHUFF comparisons of bacterial clone libraries

Comparison No.	Homologous (X) coverage data		Heterologous (Y) coverage data	
	Library	***N***	**Library**	***P***
1	Lake Fazda water	85	Lake Fazda sediment	0.001
	Lake Fazda sediment	177	Lake Fazda water	0.001
2	Lake UmRisha water	93	Lake UmRisha sediment	0.126
	Lake UmRisha sediment	165	Lake UmRisha water	0.001
3	Lake Hamra water	104	Lake Hamra sediment	0.001
	Lake Hamra sediment	146	Lake Hamra water	0.001
4	Lake Fazda water	85	Lake UmRisha water	0.001
	Lake UmRisha water	93	Lake Fazda water	0.001
5	Lake Fazda water	85	Lake Hamra water	0.001
	Lake Hamra water	104	Lake Fazda water	0.001
6	Lake UmRisha water	93	Lake Hamra water	0.001
	Lake Hamra water	104	Lake UmRisha water	0.001
7	Lake Fazda sediment	177	Lake UmRisha sediment	0.001
	Lake UmRisha sediment	165	Lake Fazda sediment	0.001
8	Lake Fazda sediment	177	Lake Hamra sediment	0.001
	Lake Hamra sediment	146	Lake Hamra water	0.001
9	Lake UmRisha sediment	165	Lake Hamra sediment	0.001
	Lake Hamra sediment	146	Lake UmRisha sediment	0.001

Table 2.8. Diversity and richness indices for archaeal clone libraries**.**

Type of Data	Sediment			Water		All Clones
	Fazda	UmRisha	Hamra	Fazda	UmRisha	
N[a]	110	134	107	119	118	588
S[b]	38	51	25	47	37	198
Shannon (H)	3.13	3.52	2.41	3.40	3.40	3.96
H/Hmax	0.86	0.90	0.75	0.88	0.94	0.75
Coverage	0.81	0.81	0.84	0.78	0.92	0.90
Chao1	80	84	70	80	43	194
95% COI[c]	52-166	63-137	38-182	59-133	38-61	158-267

[a]*N*: number of clones in library
[b]*S*: OTUs defined at ≥ 99% sequence identity
[c]95% confidence interval for the Chao1 estimator

Table 2.9. LIBSHUFF comparisons of archaeal clone libraries

Comparison No.	Homologous (X) coverage data		Heterologous (Y) coverage data	
	Library	***N***	**Library**	***P***
1	Lake Fazda water	119	Lake Fazda sediment	0.001
	Lake Fazda sediment	110	Lake Fazda water	0.006
2	Lake UmRisha water	118	Lake UmRisha sediment	0.001
	Lake UmRisha sediment	134	Lake UmRisha water	0.059
3	Lake Fazda water	118	Lake UmRisha water	0.001
	Lake UmRisha water	118	Lake Fazda water	0.001
4	Lake Fazda sediment	110	Lake UmRisha sediment	0.06
	Lake UmRisha sediment	134	Lake Fazda sediment	0.001
5	Lake Fazda sediment	110	Lake Hamra sediment	0.001
	Lake Hamra sediment	107	Lake Hamra water	0.001
7	Lake UmRisha sediment	134	Lake Hamra sediment	0.001
	Lake Hamra sediment	107	Lake UmRisha sediment	0.05

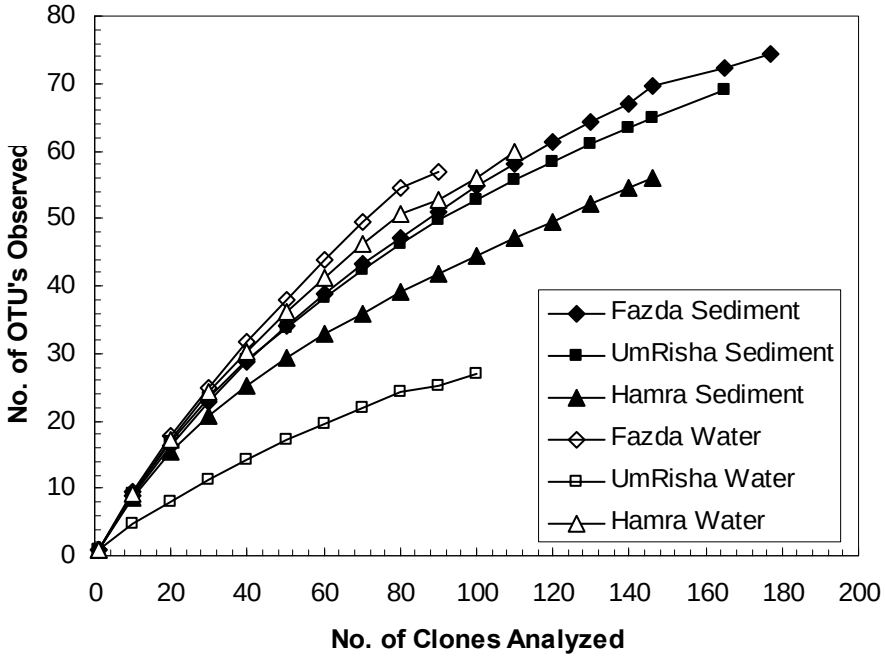
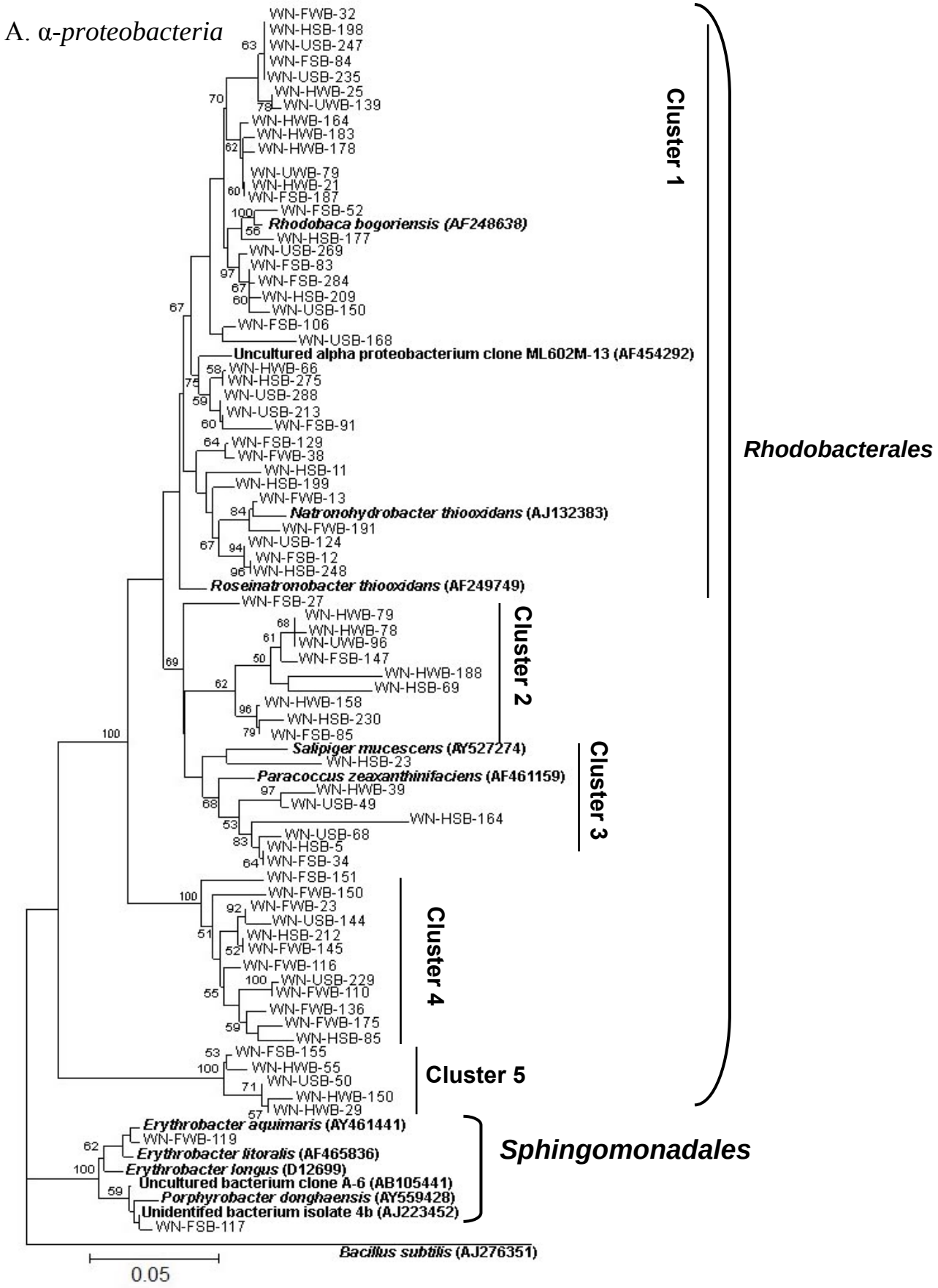


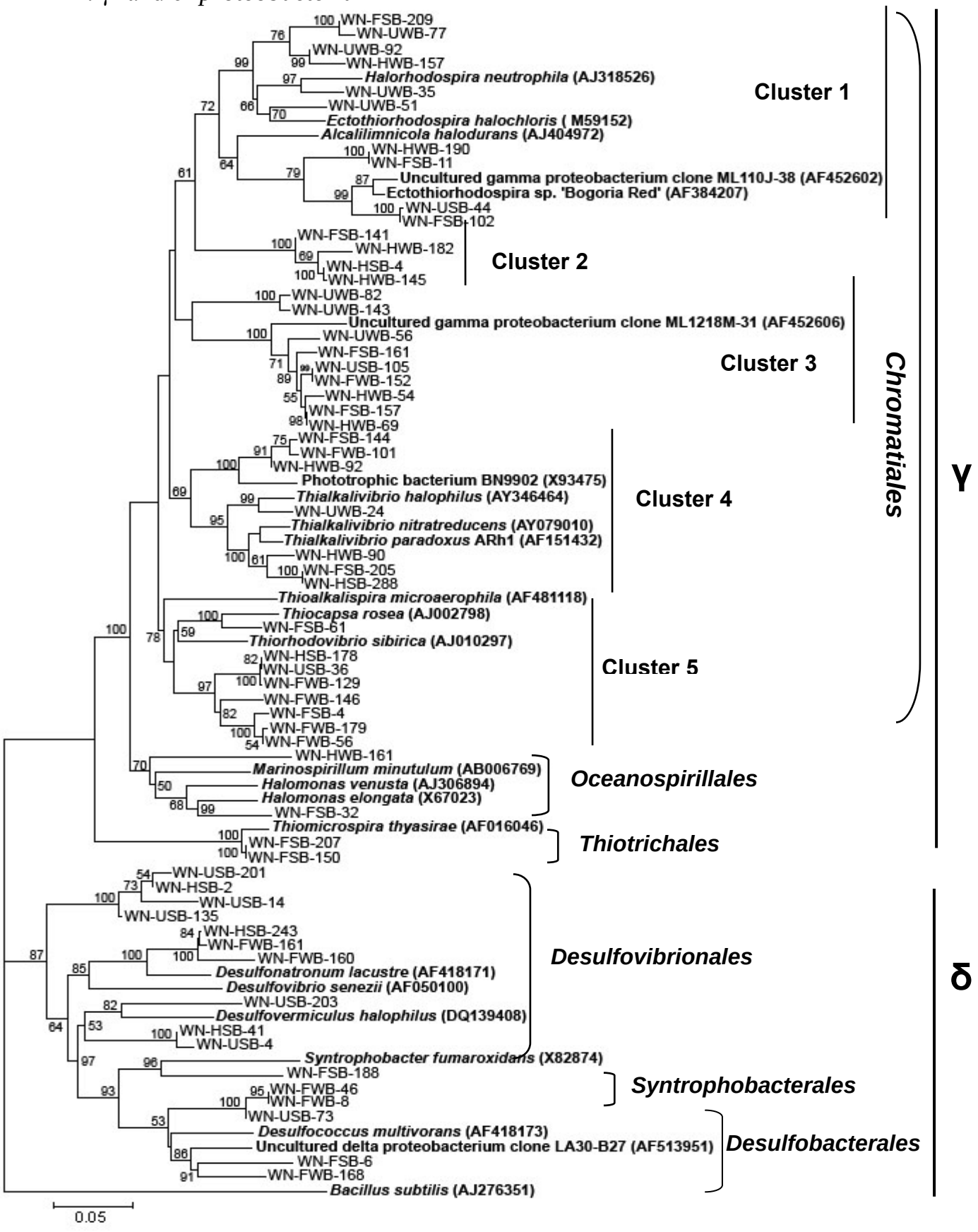
Figure 2.1. Rarefaction curves generated for bacterial 16S rRNA genes in clone libraries from sediment and water samples collected from Wadi An Natrun.

Figure 2.2. Phylogeny of bacterial OTUs. Clones from this study are coded as follows, with WN-FWB-32 as example: WN: Wadi An Natrun, FWB: Lake Fazda water bacterial 16S rRNA, 32: identifier assigned to that clone. Phylogenetic trees were constructed by the NEIGHBOR method in PHYLIP based upon 550 aligned positions. Bootstrap values are based upon 100 replicates, only values greater than 50% are shown. Trees are unrooted, with members of different phyla as outgroups. GenBank accession numbers for reference sequences are given in parentheses.

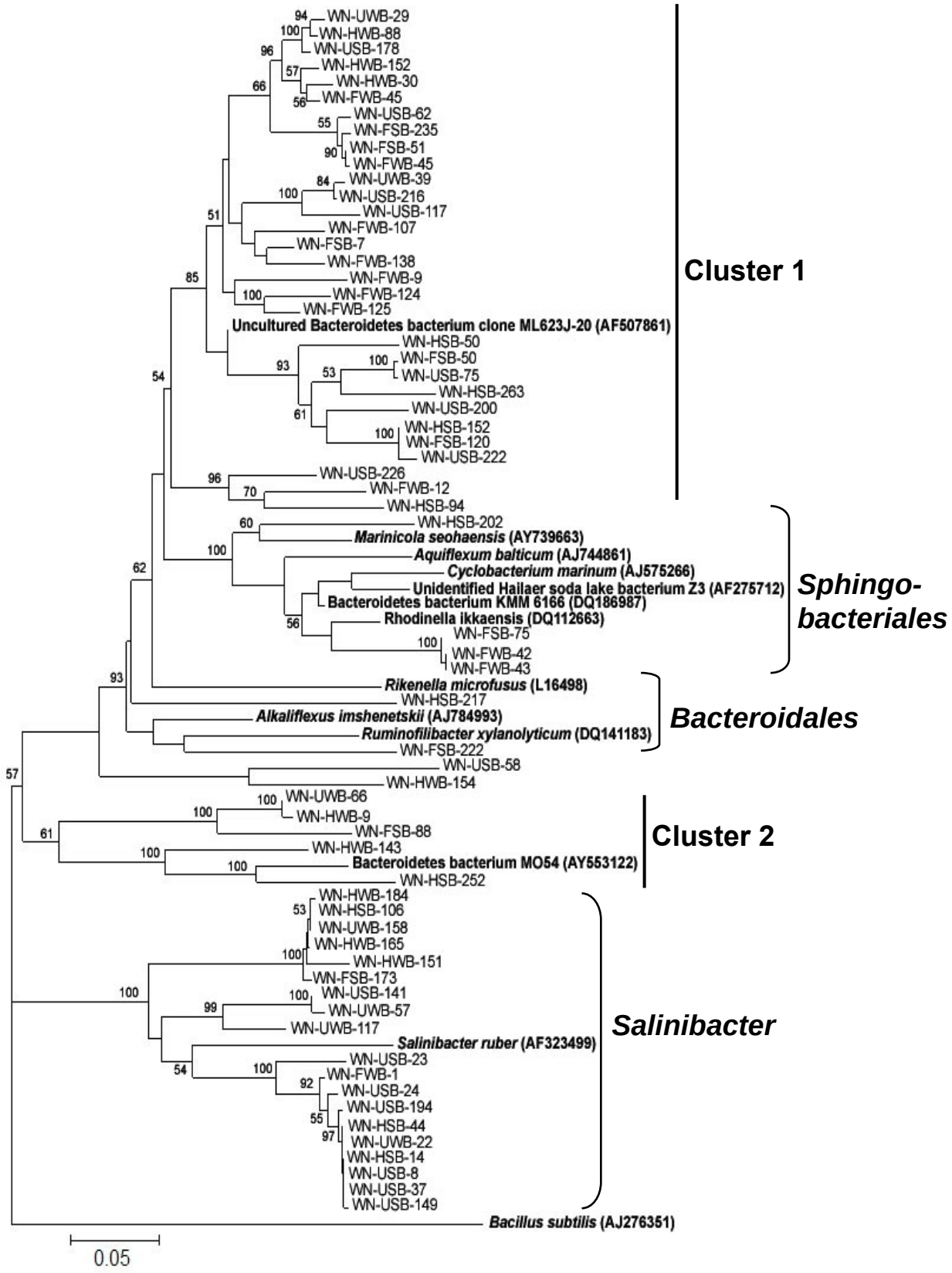
A. Phylogeny of α-proteobacterial clones. B. Phylogeny of γ- and δ-proteobacterial clones. C. Phylogeny of *Bacteroidetes* clones. D. Phylogeny of *Firmicutes* clones. Representative *Halanaerobiales* clones are shown in the inset. E. Phylogeny of clones affiliated with other taxonomic groups.


A. α-proteobacteria
WN-FWB-32
WN-HSB-198
WN-USB-247
WN-FSB-84
WN-USB-235
WN-HWB-25
WN-UWB-139
WN-HWB-164
WN-HWB-183
WN-HWB-178
WN-UWB-79
WN-HWB-21
WN-FSB-187
WN-FSB-52
Rhodobaca bogoriensis (AF248638)
WN-HSB-177
WN-USB-269
WN-FSB-83
WN-FSB-284
WN-HSB-209
WN-USB-150
WN-FSB-106
WN-USB-168
Uncultured alpha proteobacterium clone ML602M-13 (AF454292)
WN-HWB-66
WN-HSB-275
WN-USB-288
WN-USB-213
WN-FSB-91
WN-FSB-129
WN-FWB-38
WN-HSB-11
WN-HSB-199
WN-FWB-13
Natronohydrobacter thiooxidans (AJ132383)
WN-FWB-191
WN-USB-124
WN-FSB-12
WN-HSB-248
Roseinatronobacter thiooxidans (AF249749)
WN-FSB-27
WN-HWB-79
WN-HWB-78
WN-UWB-96
WN-FSB-147
WN-HWB-188
WN-HSB-69
WN-HWB-158
WN-HSB-230
WN-FSB-85
Salipiger mucescens (AY527274)
WN-HSB-23
Paracoccus zeaxanthinifaciens (AF461159)
WN-HWB-39
WN-USB-49
WN-HSB-164
WN-USB-68
WN-HSB-5
WN-FSB-34
WN-FSB-151
WN-FWB-150
WN-FWB-23
WN-USB-144
WN-HSB-212
WN-FWB-145
WN-FWB-116
WN-USB-229
WN-FWB-110
WN-FWB-136
WN-FWB-175
WN-HSB-85
WN-FSB-155
WN-HWB-55
WN-USB-50
WN-HWB-150
WN-HWB-29
Erythrobacter aquimaris (AY461441)
WN-FWB-119
Erythrobacter litoralis (AF465836)
Erythrobacter longus (D12699)
Uncultured bacterium clone A-6 (AB105441)
Porphyrobacter donghaensis (AY559428)
Unidentifed bacterium isolate 4b (AJ223452)
WN-FSB-117
Bacillus subtilis (AJ276351)
0.05
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Rhodobacterales
Sphingomonadales

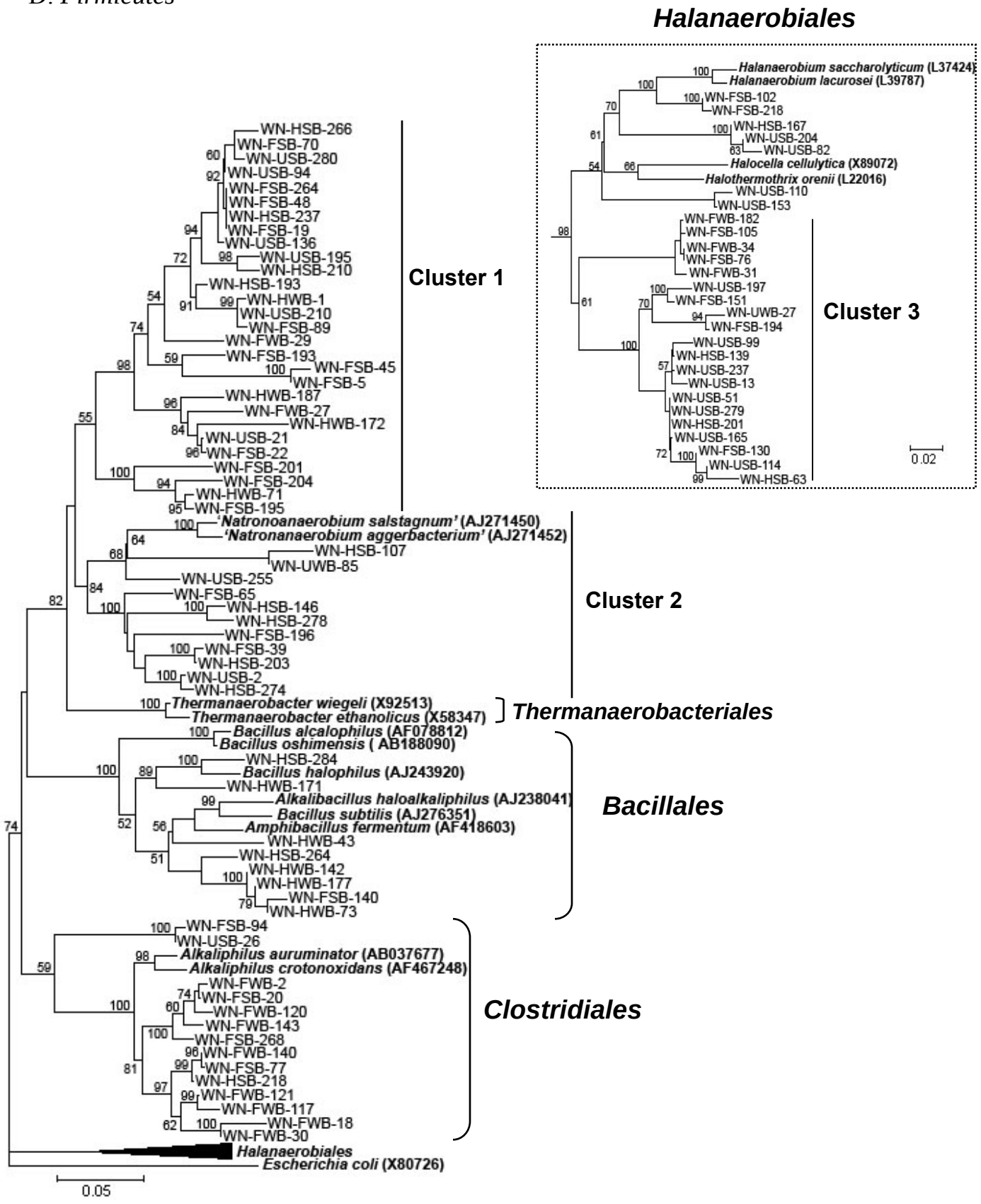
B. γ- and δ- *proteobacteria*



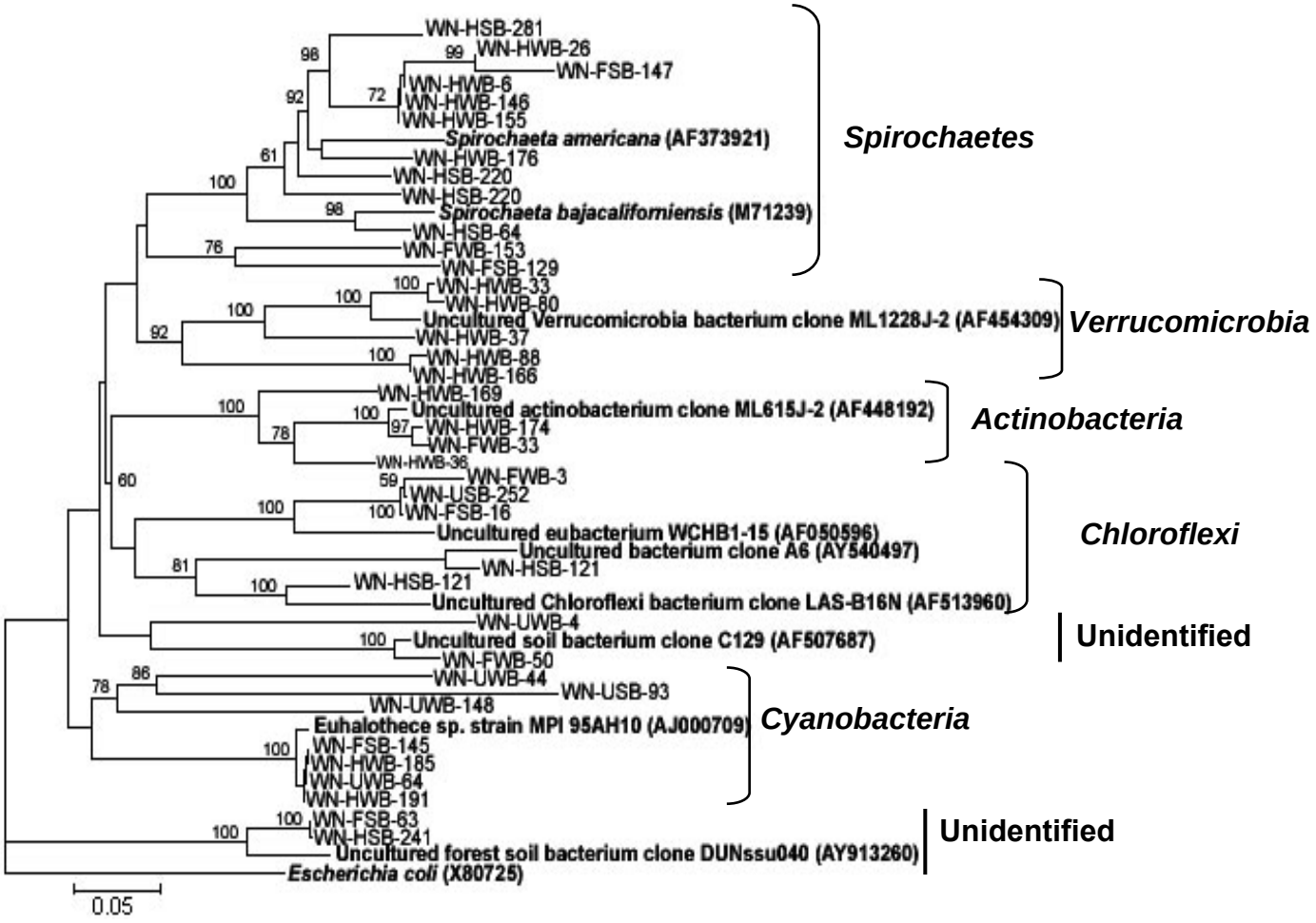
C. *Bacteroidetes*



D. *Firmicutes*



E. Other Groups



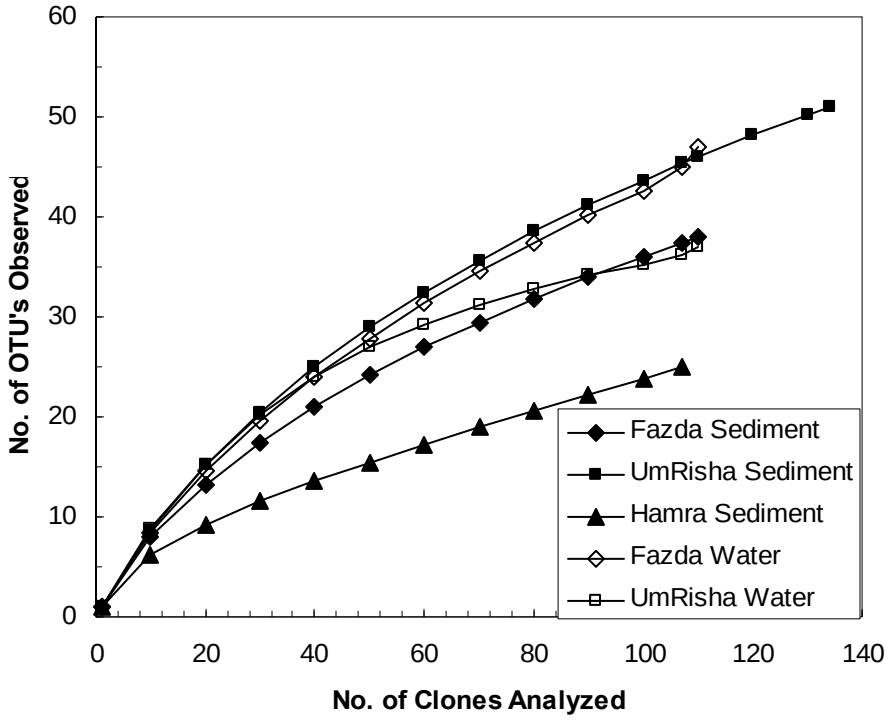
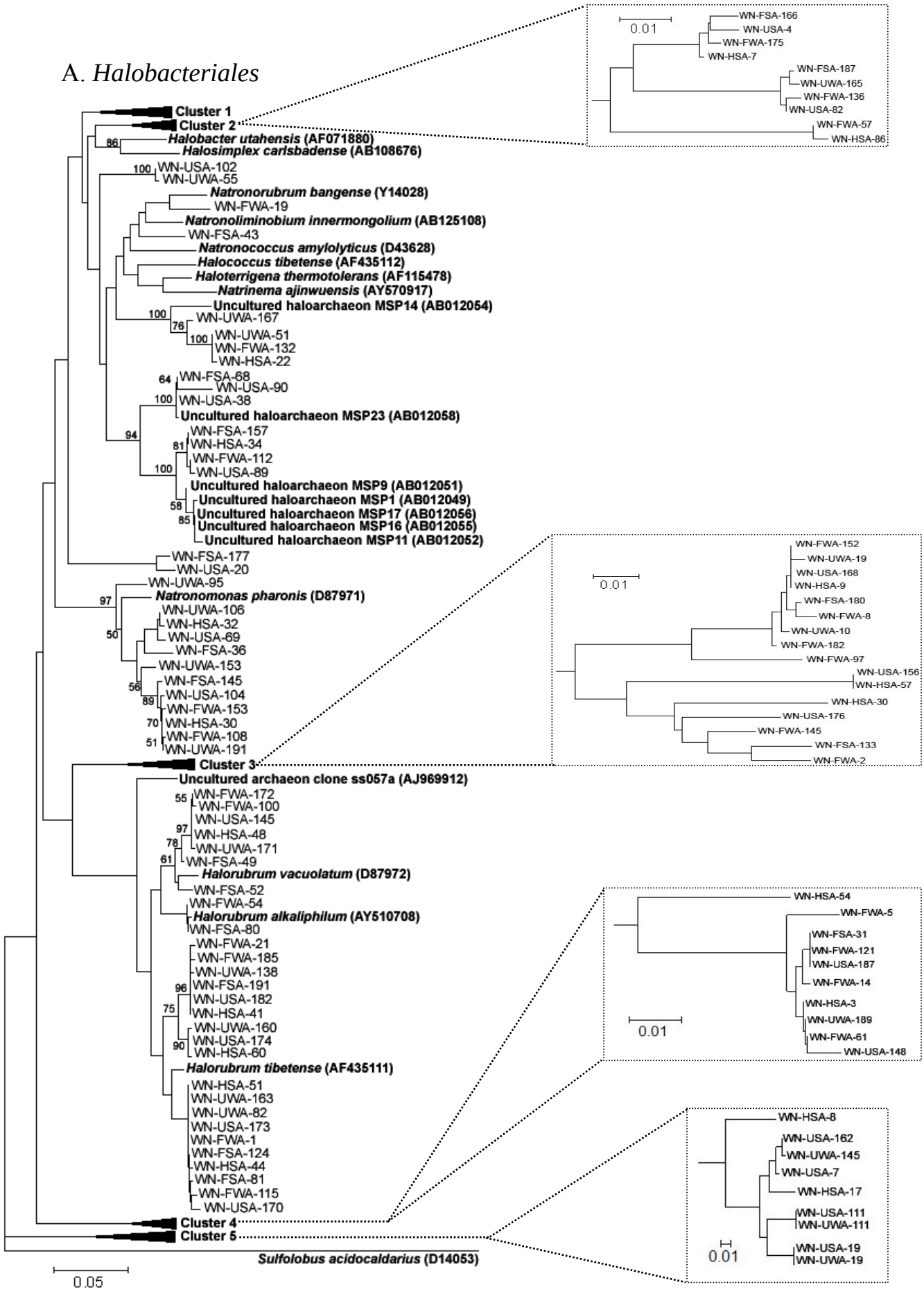
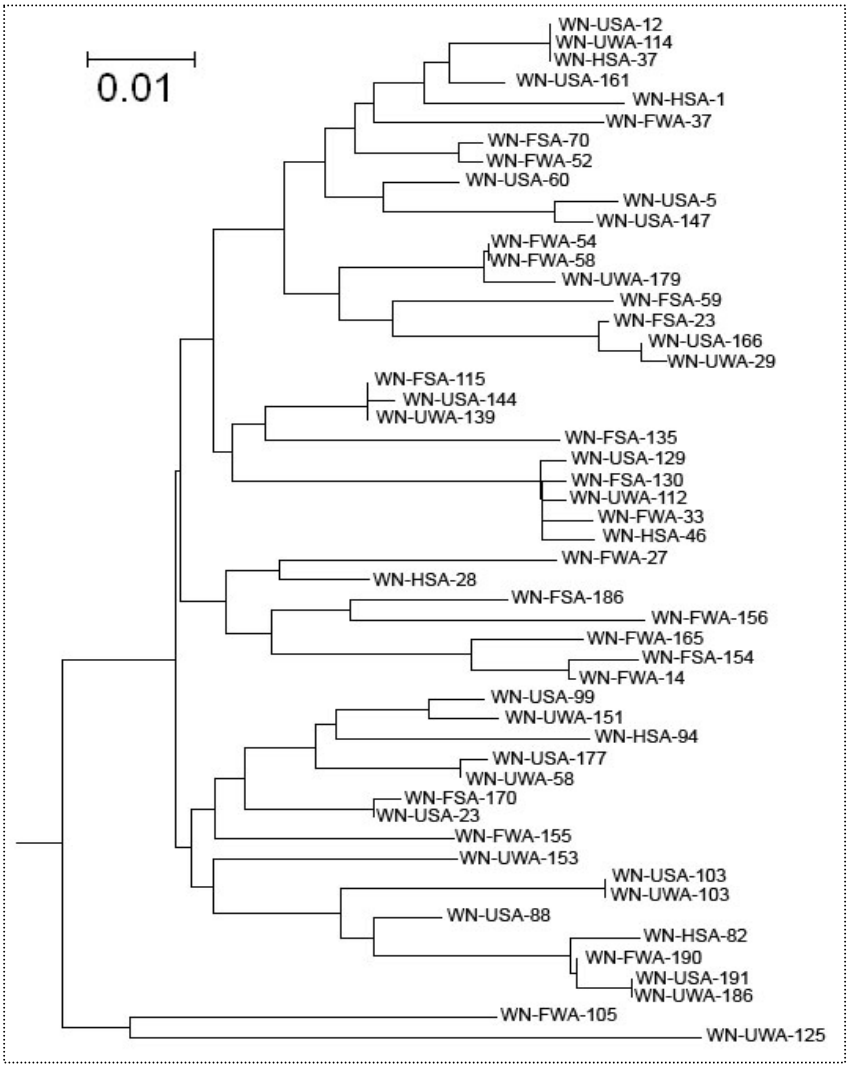


Figure 2.3. Rarefaction curves generated for archaeal 16S rRNA genes in clone libraries from sediment and water samples collected from Wadi An Natrun

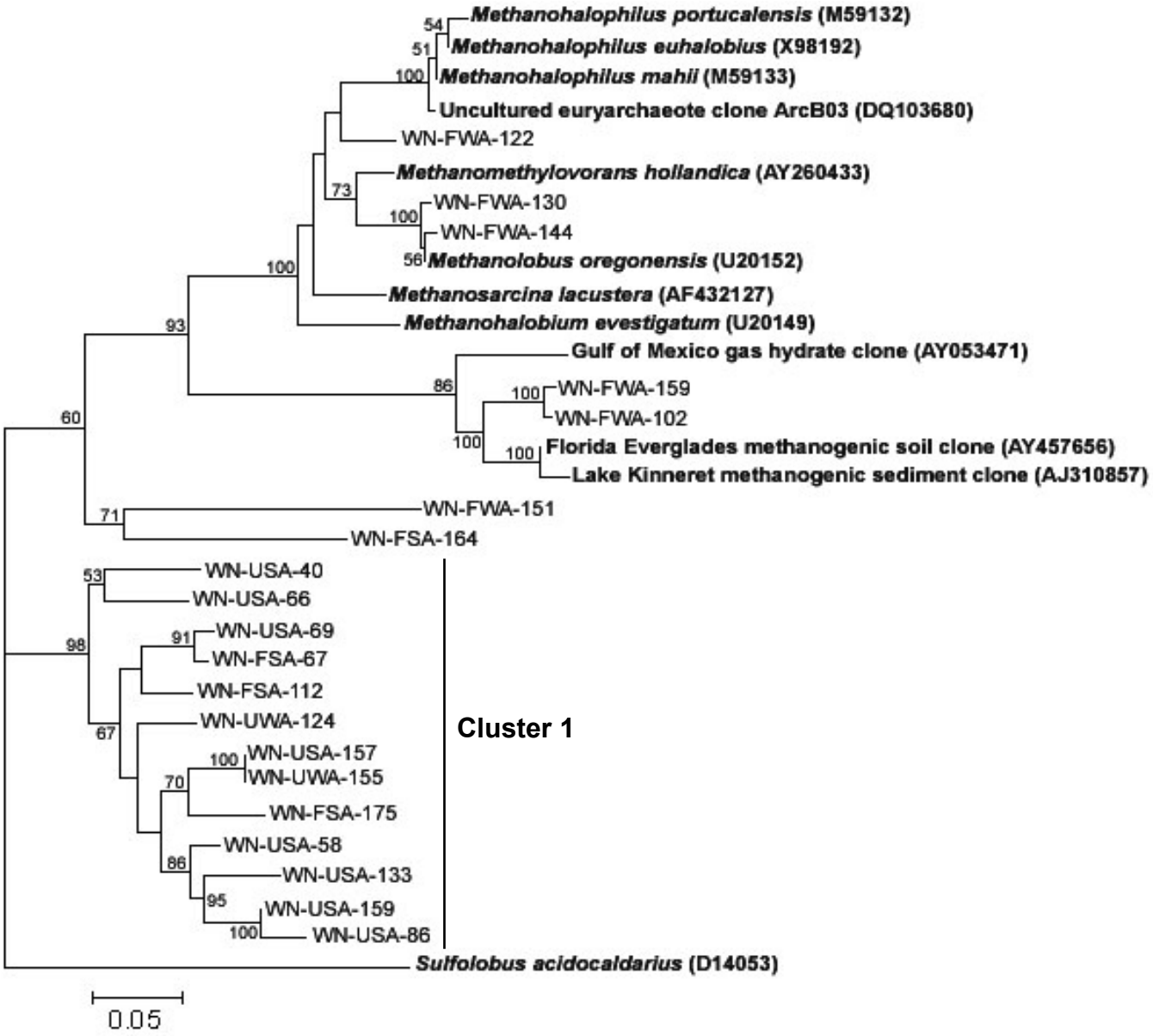
Figure 2.4. Phylogeny of archaeal OTUs. Phylogenetic trees were constructed by the NEIGHBOR method in PHYLIP based upon 600 aligned positions. Clones are coded as follows, with WN-FSA-43 as example, WN: Wadi An Natrun, FSA: Lake Fazda sediment archaeal 16S rRNA, 43: identifier assigned to that clone. Trees are otherwise as described for Figure 2. A. Phylogeny of *Halobacteriales* OTUs. Clones in Cluster 1 are shown as a separate inset. B. Phylogeny of *Methanosarcinales* clones.


A. Halobacteriales
Cluster 1
Cluster 2
Halobacter utahensis (AF071880)
Halosimplex carlsbadense (AB108676)
WN-USA-102
WN-UWA-55
Natronorubrum bangense (Y14028)
WN-FWA-19
Natronoliminobium innermongolium (AB125108)
WN-FSA-43
Natronococcus amylolyticus (D43628)
Halococcus tibetense (AF435112)
Haloterrigena thermotolerans (AF115478)
Natrinema ajinwuensis (AY570917)
Uncultured haloarchaeon MSP14 (AB012054)
WN-UWA-167
WN-UWA-51
WN-FWA-132
WN-HSA-22
WN-FSA-68
WN-USA-90
WN-USA-38
Uncultured haloarchaeon MSP23 (AB012058)
WN-FSA-157
WN-HSA-34
WN-FWA-112
WN-USA-89
Uncultured haloarchaeon MSP9 (AB012051)
Uncultured haloarchaeon MSP1 (AB012049)
Uncultured haloarchaeon MSP17 (AB012056)
Uncultured haloarchaeon MSP16 (AB012055)
Uncultured haloarchaeon MSP11 (AB012052)
WN-FSA-177
WN-USA-20
WN-UWA-95
Natronomonas pharonis (D87971)
WN-UWA-106
WN-HSA-32
WN-USA-69
WN-FSA-36
WN-UWA-153
WN-FSA-145
WN-USA-104
WN-FWA-153
WN-HSA-30
WN-FWA-108
WN-UWA-191
Cluster 3
Uncultured archaeon clone ss057a (AJ969912)
WN-FWA-172
WN-FWA-100
WN-USA-145
WN-HSA-48
WN-UWA-171
WN-FSA-49
Halorubrum vacuolatum (D87972)
WN-FSA-52
WN-FWA-54
Halorubrum alkaliphilum (AY510708)
WN-FSA-80
WN-FWA-21
WN-FWA-185
WN-UWA-138
WN-FSA-191
WN-USA-182
WN-HSA-41
WN-UWA-160
WN-USA-174
WN-HSA-60
Halorubrum tibetense (AF435111)
WN-HSA-51
WN-UWA-163
WN-UWA-82
WN-USA-173
WN-FWA-1
WN-FSA-124
WN-HSA-44
WN-FSA-81
WN-FWA-115
WN-USA-170
Cluster 4
Cluster 5
Sulfolobus acidocaldarius (D14053)
0.05
0.01
WN-FSA-166
WN-USA-4
WN-FWA-175
WN-HSA-7
WN-FSA-187
WN-UWA-165
WN-FWA-136
WN-USA-82
WN-FWA-57
WN-HSA-86
0.01
WN-FWA-152
WN-UWA-19
WN-USA-168
WN-HSA-9
WN-FSA-180
WN-FWA-8
WN-UWA-10
WN-FWA-182
WN-FWA-97
WN-USA-156
WN-HSA-57
WN-HSA-30
WN-USA-176
WN-FWA-145
WN-FSA-133
WN-FWA-2
0.01
WN-HSA-54
WN-FWA-5
WN-FSA-31
WN-FWA-121
WN-USA-187
WN-FWA-14
WN-HSA-3
WN-UWA-189
WN-FWA-61
WN-USA-148
0.01
WN-HSA-8
WN-USA-162
WN-UWA-145
WN-USA-7
WN-HSA-17
WN-USA-111
WN-UWA-111
WN-USA-19
WN-UWA-19
0.01

A. *Halobacteriales* Cluster 1



B. *Methanosarcinales*



CHAPTER 3

NATRANAEROBIUS THERMOPHILUS GEN. NOV. SP. NOV., A HALOPHILIC, ALKALITHERMOPHILIC BACTERIUM FROM SODA LAKES OF THE WADI AN NATRUN, EGYPT, AND PROPOSAL OF *NATRANAEROBIACEAE* FAM. NOV. AND *NATRANAEROBIALES ORD*. NOV.[*]

[*] Mesbah, N.M., D.B. Hedrick, A.D. Peacock, M. Rohde, and J. Wiegel. 2007. *International Journal of Systematic and Evolutionary Microbiology*. 57: 2507-2512. Reproduced with permission of publisher.

Abstract

Novel halophilic, alkalithermophilic Gram-type positive bacterial strains were isolated from sediment of alkaline, hypersaline lakes of the Wadi An Natrun. Cells of strain JW/NM-WN-LF^T were rod shaped, non-sporeforming, and non-motile. Strain JW/NM-WN-LF^T grew (at $pH^{55ºC}$ 9.5) between 35 and 56°C, with an optimum at 53°C. The $pH^{55°C}$ range for growth was 8.3-10.6, with an optimum at $pH^{55°C}$ 9.5 and no growth at $pH^{55ºC}$ 8.2 or below and at $pH^{55ºC}$ 10.8. or above. At the optimum pH and temperature, the strain grew in the Na^+ range of 3.1-4.9 M (1.5-3.3 M of added NaCl) and optimally between 3.3 and 3.9 M Na^+ (1.7-2.3 M added NaCl). Strain JW/NM-WN-LF^T utilizes fructose, cellobiose, ribose, trehalose, trimethylamine, pyruvate, casamino acids, acetate, xylose, and peptone as carbon and energy sources. Fumarate (20 mM), $S_2O_3^{2-}$ (20 mM), NO_3^- (20 mM), and Fe III citrate (20 mM) are utilized as electron acceptors. During growth on sucrose, the isolate produced acetate and formate as major fermentation products. Main cellular fatty acids are iso-branched 15:0, i17:0 dimethyl acetal and 16:0 dimethyl acetal. The G+C content of genomic DNA was 40.4 mol% (HPLC). On the basis of genotypic and phenotypic characteristics, it is proposed that strain JW/NM-WN-LF^T represents a novel genus and species, *Natranaerobius thermophilus* gen. nov. sp.nov. The type strain is JW/NM-WN-LF^T (= DSM 18059^T = ATCC BAA-1301^T). Based on 16S rRNA gene sequence analysis, the strain formed a novel lineage within the Class *Clostridia*, and clustered with uncultivated bacteria and unidentified strains retrieved from alkaline, hypersaline environments. The phylogenetic data suggest the lineage represents a novel family, *Natranaerobiaceae* fam. nov., and order, *Natranaerobiales* ord. nov.

Main Text

Extremophiles are microorganisms well adapted to one or two extreme environmental conditions. Halophilic alkalithermophiles are a novel physiological group that requires high salt concentrations, alkaline pH values, and elevated temperatures for growth. Halophilic alkalithermophiles must possess special adaptive mechanisms for survival under these three extreme conditions. As a result of their unique and extreme growth conditions, halophilic alkalithermophiles are of considerable commercial and biotechnological significance. The halophilic alkalithermophiles are also of evolutionary significance as they represent model organisms for evaluating theories on the origin of life. These include that life evolved in shallow heated saline and alkaline pools (Baross, 1998, Zavarzin, 1993). We describe in this report the characterization of, to our knowledge, the first true anaerobic halophilic alkalithermophile isolated from the sediments of the solar-heated alkaline, hypersaline soda lakes of the Wadi An Natrun, Egypt (criteria for defining halophiles, alkaliphiles and thermophiles are described by Oren (2000), and Wiegel (1998a)). On the basis of physiological and phylogenetic evidence presented, we propose a novel genus, *Natranaerobius*, to accommodate this microorganism. Further, the order *Natranaerobiales*, consisting of the family *Natranaerobiaceae*, is proposed to encompass *Natranaerobius* gen. nov.

Isolation and cultivation of strain JW/NM-WN-LF^T. Strain JW/NM-WN-LF^T was isolated from a mixed water-sediment sample collected from the sediment of Lake Fazda, Wadi An Natrun during May 2005. At the time of collection, the lake water had a salinity of 4.7 M and $pH^{25°C}$ 9.8. For initiating enrichment cultures, 5g of soil was inoculated into 80 mL of carbonate-buffered medium consisting of (g L^{-1}): KH_2PO_4, 0.2; $MgCl_2$, 0.1; KCl, 0.2; NH_4Cl, 0.5, NaCl,

100; Na_2CO_3, 68; $NaHCO_3$, 38; cysteine.HCl, 0.7; yeast extract; 5, tryptone, 5; sucrose, 5, trace element solution 1 mL (Kevbrin & Zavarzin, 1992), vitamin solution 10 mL (Wolin*, et al.*, 1963). The $pH^{55ºC}$ was adjusted to 9.5 with anaerobic 5 N HCl. The enrichment cultures became turbid after 48 hours of growth at 55ºC. Pure cultures were obtained in dilution rows in agar (1% w/v) shake-roll tubes (Ljungdahl & Wiegel, 1986). To ensure that colonies were derived from a single cell, the isolate was purified by four successive rounds of single colony isolation. The isolates were maintained in the carbonated buffered medium at pH 9.5 and 55ºC under anaerobic conditions (100 % N_2) using a modified Hungate technique (Ljungdahl & Wiegel, 1986).

Colony and cell morphology. Colonies of strain JW/NM-WN-LF^T appeared in agar-shake roll tubes after 3-4 days and were 1-2 mm in diameter, circular to irregular and opaque. Cell morphology was observed via light microscopy (Olympus VANOX phase-contrast microscope) and electron microscopy. Cells in liquid culture in exponential growth phase were straight to curved rods, 0.2-0.4 μm in diameter and 3-5 μm in length. Cells were either single or formed chains. No active motility was observed under phase-contrast microscopy; and accordingly flagella were absent in negatively stained samples (2 % uranyl-acetate). Cells exhibit a rod like appearance with variable length as shown by field emission scanning electron microscopy (Figure 3.1A; taken with a Zeiss DSM982 Gemini, Oerkochen, Germany). Ultrathin sections exhibit a Gram-positive like cell wall and no endospores were observed either with a Zeiss EM910 microscope (Oberkochen, Germany, Figure 3.1B) or by light microscopy after heat treatment (10 min at 80°C). (Figure 3.1). Cells stained Gram positive in both early exponential and stationery growth phases (Doetsch, 1981).

Cultural and physiological characteristics. The optimal conditions for growth of strain JW/NM-WN-LF^T were tested in carbonate buffered medium with 0.3 % yeast extract and tryptone, 640 mM Na_2CO_3 , and 320 mM $NaHCO_3$ (before pH adjustment, yielding a base Na^+ concentration of 1.6 M).Using a temperature-gradient incubator (Scientific Industries, Inc.), the temperature range for growth (at $pH^{55ºC}$ 9.5) was 35-56°C, with an optimum at 53°C; and no growth at 34°C or below and at 57°C or above.

The growth temperature profile revealed a broken Arrhenius plot, with two peaks and an intermediate plateau (Supplementary figure S3.1A). The initial peak in growth rate occurred at 37°C (doubling time 6h), the second, and larger peak in growth occurred at 53°C (doubling time 3.5 h). Such a pattern has been observed with other thermophiles (Wiegel, 1990, 1998b). Dilution to extinction, microscopy, and 16S rRNA sequence analysis all confirmed that the isolate was pure and was not contaminated with another mesophilic or thermotolerant microorganism that could have caused the initial growth peak at 37ºC.

The pH range for growth was determined at 55°C in the above mentioned carbonate buffered medium. All pH measurements were performed as described previously (Mesbah & Wiegel, 2006), with a microelectrode (Accumet® combination microelectrode with calomel reference, Cole-Palmer), calibrated at 55ºC with pH standards preheated to the same temperature. The pH of the medium was adjusted by addition of sterile anaerobic HCl or Na_2CO_3. The $pH^{55°C}$ range for growth was 8.3-10.6, with an optimum at $pH^{55\ °C}$ 9.5. There was no growth at $pH^{55ºC}$ 8.2 or below nor at or above $pH^{55ºC}$ 10.8. (Supplementary figure S3.1B)

The salinity range for growth was determined in carbonate buffered medium at $pH^{55ºC}$ 9.5. Strain JW/NM-WN-LF^T grew over total Na^+ concentrations (which includes 1.5-3.3 M of added NaCl) of 3.1-4.9 M (which would correspond to 18 - 28.5%(w/v) NaCl). No growth occurred

when the total Na^+ concentration was below 3.0 M. Optimal growth occurred at Na^+ concentrations between 3.3 and 3.9 M. Maximum Na^+ tolerance of strain JW/NM-WN-LF^T was not changed (either increased or decreased) by addition of 500 mM KCl to the growth medium. Strain JW/NM-WN-LF^T did not grow when equimolar amounts of K_2CO_3 and $KHCO_3$ were substituted for Na_2CO_3 and $NaHCO_3$, even in the presence of 1.7-3.1 M of NaCl. The doubling time at the optimal conditions, 3.5 M Na^+, $pH^{55°C}$ 9.5, 53°C, was 3.5 hours.

For substrate-utilization tests, cultures were incubated for up to 5 days and growth was judged positive if in the third successive transfer the optical density (at 600 nm) of the culture was twice that of a control culture incubated with only 0.2% yeast extract and tryptone. Utilization of possible substrates (0.5 % wt/vol) was tested in the presence of 0.2 % yeast extract and tryptone. Strain JW/NM-WN-LF^T used fructose, cellobiose, ribose, trehalose, trimethylamine, pyruvate, casamino acids, acetate, xylose, and peptone as carbon and energy sources. No growth was observed with glucose, mannose, formate, glycine betaine, ethanol, n-propanol or ribitol as carbon or energy sources. The use of electron acceptors was determined by measuring growth (increase in OD_{600}), sulfide, ammonium and nitrate production, and color change. In the presence of 0.2 % yeast extract, strain JW/NM-WN-LF^T utilized as electron acceptors fumarate (20 mM), $S_2O_3^{2-}$ (20 mM), NO_3^- (20 mM), and Fe III citrate (20 mM), determined by absorbance at 562 nm of Fe(II)-ferrozine complex (Stookey, 1970). None of the following electron acceptors were utilized: SO_4^{2-} (20 mM), SO_3^{2-} (20 mM), or MnO_2 (10 mM). The main organic fermentation products from 20 mM sucrose were acetate (17 mM) and formate (10 mM), and minor amounts of lactate (2.5 mM).

Strain JW/NM-WN-LF^T was negative for catalase and oxidase, gelatin liquefaction and casein degradation. Strain JW/NM-WN-LF^T was obligately anaerobic. Negative results were

obtained in an API ZYM enzyme assay (bioMérieux) for alkaline phosphatase, esterase C-4, esterase lipase, leucine-, valine- and cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-α,β-phoshpohydrolase, α- and β-galactosidase, α- and β-glucosidase, β-glucouronidase, α-mannosidase, α-fucosidase, and N-acetyl- β-glucosaminidase.

Chemotaxonomic characteristics. Attempts to purify peptidoglycan from cells of strain JW/NM-WN-LF^T failed, and no isomer of diaminopimelic acid was detected in the strain. It was concluded that the amount of peptidoglycan in the strain is below detectable amounts (Peter Schuman, person. comm.).

Phospholipid fatty acid (PLFA) analysis was performed on cells that had been grown at 53°C, pH$^{55°C}$ 9.5, 1.7 M NaCl, 640 mM Na_2CO_3 and 320 mM $NaHCO_3$. Lyophilized cell material was extracted using chloroform/methanol/water solvent system (Bligh & Dyer, 1959), with the modification of Peacock *et al*. (2000). The total lipid extract obtained was then fractionated into neutral lipid, glycolipid, and polar lipid fractions by silicic acid column chromatography (Guckert, *et al.*, 1985). The polar lipid fraction was prepared for gas chromatography/mass spectroscopy by transesterification to fatty acid methyl esters by mild alkaline hydrolysis (Guckert, *et al.*, 1985).The resulting mixed fatty acid methyl esters and dimethylacetals were separated and quantified using a Hewlett-Packard 5890 series 2 gas chromatograph interfaced with a Hewlett-Packard 5971 mass selective detector. The chromatographic column was a 50 m non-polar column (0.2 mm i.d., 0.11 mm film thickness). The amount of PLFA + dimethyl acetal (DMA) per g of cells was 13.6 nmole/g dry weight cell material. PLFA composition of strain JW/NM-WN-LF^T was dominated by branched-chain fatty acids (i15:0, i17:0), which formed 29 % of total PLFAs. PLFA analysis also showed a unique pattern of DMAs, which were

predominated by a branched chain DMA (i17:0DMA, 27.4 % of total PLFA) and an unbranched DMA (16:0DMA, 16.4 % of total PLFA). Small amounts of unsaturated PLFAs and unbranched DMAs were also present (Supplementary Table S3.1).

The DNA G+C content of the strain JW/NM-WN-LF^T was determined by HPLC according to Mesbah *et al*. (1989), with the modification of Lee *et al*. (2005), using S1 nuclease and 0.3 M sodium acetate (pH 5.0). The G+C content of genomic DNA was 40.4 mol% (mean of six replicate analyses).

Phylogenetic analysis. The nearly complete 16S rRNA gene sequence for strain JW/NM-WN-LF^T was determined by Macrogen Inc. (Seoul, Korea), and compared with all GenBank entries by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The partial 16S rRNA gene sequence of strain JW/NM-WN-LF^T formed a phylogenetic cluster consisting of uncultured bacterial clones from sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah*, et al.*, 2007). The pH in these sediments ranged from 9-11 and the NaCl concentration at the time of sampling was around 5 M. Strain JW/NM-WN-LF^T was also closely related (93-95% 16S rRNA similarity) to unpublished bacterial strains isolated from the alkaline soda lakes in the Kenyan-Tanzanian rift (Jones*, et al.*, 1998). No effective description of these isolates exists in the literature describing the temperature range. These isolates were retrieved from mixed water-sediment samples and grown at 37^{o}C (Owenson, 1997). The soda lakes of the Kenyan-Tanzanian rift are reported to have pH values ranging between 10-12 and salinity levels greater than 2.5 M. The lakes are solar heated; no source of geothermal heating has been reported. The three corresponding strains mentioned by Owenson (1997) were Gram-staining variable anaerobic heterotrophic rods of various sizes, able to use a variety of heterotrophic substrates including

glucose and forming acetate and isovalerate as major fermentation products. The NaCl range is given as 12 -26% (w/v) NaCl and the pH optima > 9.5. However the strains are presently not available for comparison.

Among validly published species, the highest 16S rRNA similarity levels were with members of the family *Peptococcaceae*, namely *Desulfotomaculum geothermicum* (~85% similarity) and the type species *Desulfotomaculum nigrificans* (84%) (Fig. 3.2). Strain JW/NM-WN-LF^T clearly belongs to the class *Clostridia*, but is not closely affiliated with any of the described lineages (Supplementary figure S3.2 shows the type genus *Natranaerobius* gen. nov., for the proposed novel family and order in a tree with the type species from the type genera for the Orders and Classes in the Phylum *Firmicutes*).

The 16S rRNA gene sequence of strain JW/NM-WN-LF^T was aligned with representatives within the class *Clostridia* (Figure 3.2). Multiple sequence alignments were created with the ClustalX program, (http://ftp-igbmc.u-strasbg.fr/pub/ClustalX). Trees were constructed using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html). Distances were calculated using the Jukes-Cantor algorithm of DNADIST, and branching order was determined via the neighbor-joining algorithm of NEIGHBOR. Each tree was consensus of 1000 replicate trees. Strain JW/NM-WN-LF^T, together with the African rift isolates and environmental clones, form a strongly supported cluster with gene sequences ranging from 92-96 % similarity. Interestingly, strain JW/NM-WN-LF^T was only 93 % similar to uncultured clone WN-FSB-108, which was retrieved from sediment of the same lake indicating that the genus *Natranaerobius* is represented by several species in the lakes and may be even closely related genera. The *Natranaerobius* cluster forms a separate lineage within the *Clostridia*. Additional phylogenetic analyses

performed with 16S rRNA sequences of the type genera of the *Firmicutes* and a different treeing method (Fitch-Margoliash), confirm the divergence of JW/NM-WN-LF^T and related sequences from representatives of described families and orders of the *Clostridia* (Supplementary figure S3.2).

Taxonomic conclusions. Phylogenetic analysis indicates that strain JW/NM-WN-LF^T belongs to the class *Clostridia* of the *Firmicutes*. Table 3.1 shows the phenotypic characteristics of strain JW/NM-WN-LF^T and its two, closest validly described species, *Desulfotomaculum geothermicum* and *D. nigrificans* belonging to the clostridial family *Peptococcaceae.* Similar to strain JW/NM-WN-LF^T, *D. geothermicum* has a growth temperature optimum of 53°C (temp. range 37-57°C), and also has i15:0 as a major cellular fatty acid. However, it clearly differs in its NaCl requirement for growth (0-0.7 M), and lack of DMAs in the PLFA profile. *D. geothermicum* is also neutrophilic; it does not tolerate pH values greater than 8.5. *D. nigrificans* is distinguished from strain JW/NM-WN-LF^T in that it is motile, has a different fatty acid content and different NaCl, pH and temperature ranges and optima (Table 3.1).

Altogether, phylogenetic and physiological data indicate that strain JW/NM-WN-LF^T is sufficiently divergent from all known bacterial species as to be described as a novel genus and species, *Natranaerobius thermophilus* gen. nov. sp. nov. (type strain JW/NM-WN-LF^T = DSM 18059^T = ATCC BAA 1301^T).

At present, the class *Clostridia* is represented by three orders, *Clostridiales, Halanaerobiales*, and '*Thermoanaerobacteriales*'. Based on the distinct phylogenetic position of *Natranaerobius thermophilus* gen. nov. sp. nov. within the class *Clostridia* and the differences

observed in physiological and cultural characteristics, a novel order, *Natranaerobiales* ord. nov., represented by the single family, *Natranaerobiaceae* fam. nov., is proposed.

Description of *Natranaerobius* gen. nov.

Natranaerobius (Natr.an. ae.ro.bi'.us. N. Gr. n. *natron*, derived from Arabic *natrun*, soda [sodium carbonate], Gr. pref. *an*, not, Gr. n. *aer*, air, Gr. masc. n. *bios* life, N. L. masc. n. *Natranaerobius*, being a soda requiring anaerobe.

Cells are Gram-type positive, endospores not observed. Obligately halophilic, growth requires at least 3 M Na^+, obligately alkaliphilic, no growth below pH 8.2. Thermophilic. The fatty acid profile is dominated by branched fatty acids with odd numbers of carbons; dimethyl acetals are also present. The DNA G+C content is around 40 mol%. Strictly anaerobic chemoorganotrophs. The type species is *Natranaerobius thermophilus* sp. nov.

Description of *Natranaerobius thermophilus* sp. nov.

Natranaerobius thermophilus (ther.mo'phil.us. Gr. n. *therme* heat, Gr. adj. *philos* friend, loving, N. L. masc. adj. *thermophilus* heat-loving, referring to its growth temperature).

Cells form irregular to circular, opaque colonies with a white color (when grown inside of 1 % agar). Cells are 3-5µm x 0.2-0.4µm in size, non-motile, catalase and oxidase negative. Cells are Gram-staining and Gram-type positive (Wiegel, 1981). Extremely halophilic, optimal growth occurs between 3.3 and 3.9 M Na^+ (1.7-2.3 M added NaCl), no growth occurs at Na^+ concentrations below 2.5 M or greater than 5 M. Obligately alkaliphilic, $pH^{55°C}$ range 8.3-10.6,

with an optimum at $pH^{55°C}$ 9.5. Thermophilic, temperature range for growth is 34-57°C (at $pH^{55°C}$ 9.5), with an optimum at 53°C. Obligately anaerobic. In the presence of 0.2% yeast extract and tryptone, fructose, cellobiose, ribose, trehalose, trimethylamine, pyruvate, casamino acids, acetate, xylose, and peptone are used as carbon and energy sources. The main organic fermentation products from 0.5 % sucrose are formate and acetate. Fumarate (20 mM), $S_2O_3^{2-}$ (20mM), NO_3^- (20 mM), and Fe III citrate (20 mM) are utilized as electron acceptors. Major cellular fatty acids include i15:0, i17:0DMA and 16:0DMA. The type strain lacks significant amounts of murein and m-diaminopimelic acid in the cell wall. The DNA G + C content of genomic DNA is 40.4 mol% (HPLC).

The type strain, JW/NM-WN-LF^T (= DSM 18059^T = ATCC BAA-1301^T), was isolated from sediment of Lake Fazda, Wadi An Natrun, Egypt.

Description of *Natranaerobiales* ord. nov.

Natranaerobiales (Natr.an.ae.ro.bi.a.les. N.L. masc. n. *Natranaerobius*, type genus of the order; suff. -ales, ending denoting an order; N.L. fem. pl. n. *Natranaerobiales*, the order of *Natranaerobius*)

Description is as for the family.

Description of *Natranaerobiaceae* fam. nov.

Natranaerobiaceae (Natr.an.ae.ro. bi. a. ce. ae. N.L. n. *Natranaerobius* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Natranaerobiaceae* the *Natranaerobius* family).

Cells are Gram-staining, Gram-type positive, endospores not observed. Straight or slightly curved, slender rods. Non-motile. Strictly anaerobic. Members are halophilic, alkaliphilic. Chemolitho- or organoheterotroph. Phylogenetically, the family belongs to the order *Natranaerobiales*. The type genus is *Natranaerobius*.

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Table 3.1. Differential characteristics of strain JW/NM-WN-LF^T and closely related species.

Strains: 1, JW/NM-WN-LF^T; 2, *Desulfotomaculum geothermicum* DSM 3669^T (data from Daumas *et al*. (1988)); 3, *Desulfotomaculum nigrificans* ATCC 19656^T (Nazina *et al*. (2005)); 4, African rift isolate G-M14CH-4 (Owenson, 1997). +, positive, -, negative.

Characteristic	1	2	3	4
Length of rods (µm)	3-5	2-3	3-6	0.6-10
Motility	-	+	+	-
pH range (optimum)	8.3-10.6 (9.5)	6.5-8.5 (7.3-7.5)	6.0-8.5 (7.0)	9.5-10.5 (>9.5)
NaCl range (optimum), % wt/vol	9-19 (10-14)	0-4 (2.5-3.5)	0-6 (1)	12-26 (20)
Temperature range (optimum), °C	35-56 (53)	37-57 (54)	45-75 (60-65)	Not determined
Major fatty acids (> 8%)	i15:0 (23%) i17:0DMA (27.4%) 16:0DMA (16.4%)	i15:0 (32.8%) 16:0 (10.1%)	i15:0 (29.6%) 16:0 (26.6%) i17:0 (14.2%) 18:0 (16.7%)	Not determined
DNA G + C content, mol%	40.4	50.4	50.8	Not determined

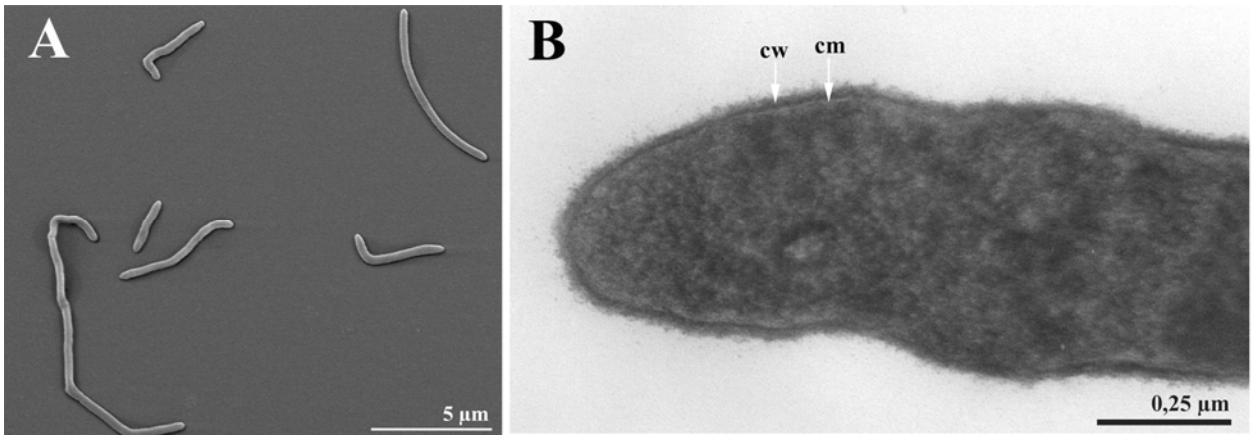


Figure 3.1. Electron microscopic images of strain JW/NM-WN-LF^T A) field emission scanning electron micrograph of aldehyde-fixed, acetone dehydrated and critical point-dried cells of strain JW/NM-WN-LF^T revealed a rod-like appearance with variable length of single cells, B) ultrathin section (embedded in LRWhite and counter-stained with uranyl acetate) exhibit the Gram-positive like cell structure (cm, cell membrane, cw, cell wall); furthermore, no endospores were detectable within the cytoplasm.

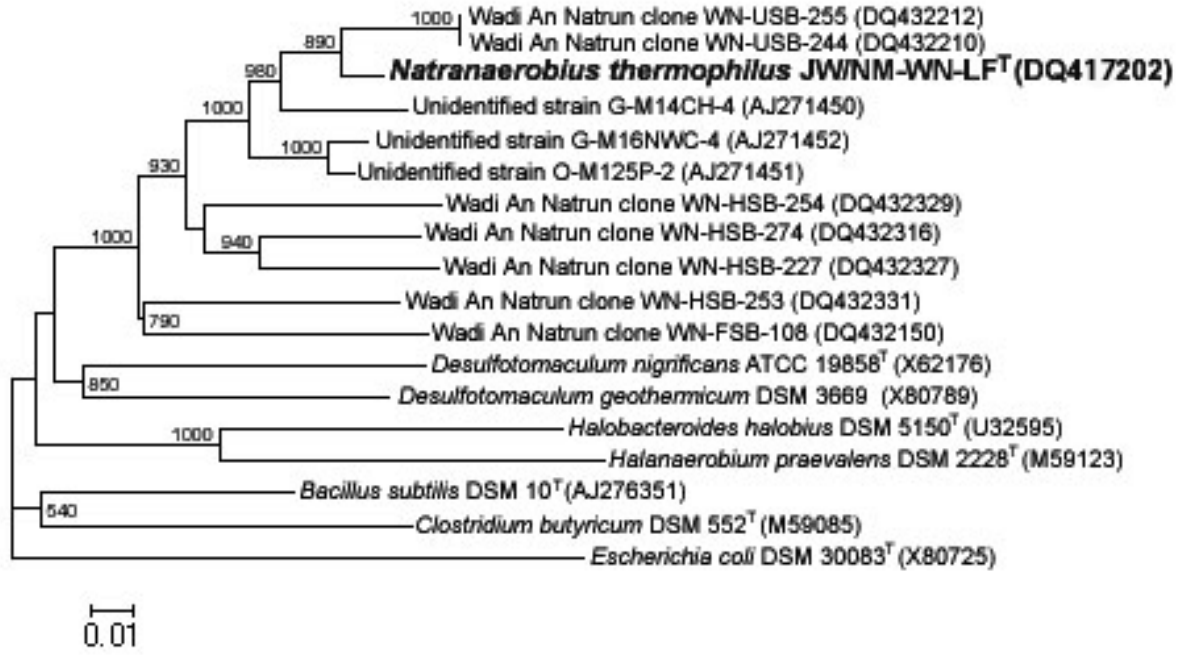


Figure 3.2. Neighbor-joining tree based on 16S rRNA gene showing the position of strain JW/NM-WN-LF^T in relation to its most closest relatives within the *Firmicutes*, unidentified strains from the Kenyan-Tanzanina Rift and uncultured clones from sediments of Wadi An Natrun lakes. GenBank accession numbers for the sequences are in the parentheses. The tree was rooted with the 16S rRNA gene of *Escherichia coli* DSM 30083^T as the outgroup. Numbers at nodes are bootstrap values based on 1000 replicates; only values greater than 500 are shown. Bar, 1 nucleotide substitutions per 100 nt.

Supplementary Data

Table S3.1. PLFA composition of strain JW/NM-WN-LF^{T}.

PLFA/DMA	Mole percent*
14:0	0.5
16:0	4.9
18:0	1.0
i15:0	23.0
a15:0	3.7
i17:0	6.1
a17:0	1.2
i19:0	0.8
16:1w7c	1.0
18:1w7c	0.6
i15:1	0.7
i17:1	1.2
i19:1	0.5
16:0DMA	16.4
18:0DMA	2.5
i15:0DMA	2.9
i16:0DMA	1.8
i17:0DMA	27.4
a17:0DMA	2.1
i19:0DMA	1.7

*****Values are percentages of total fatty acid

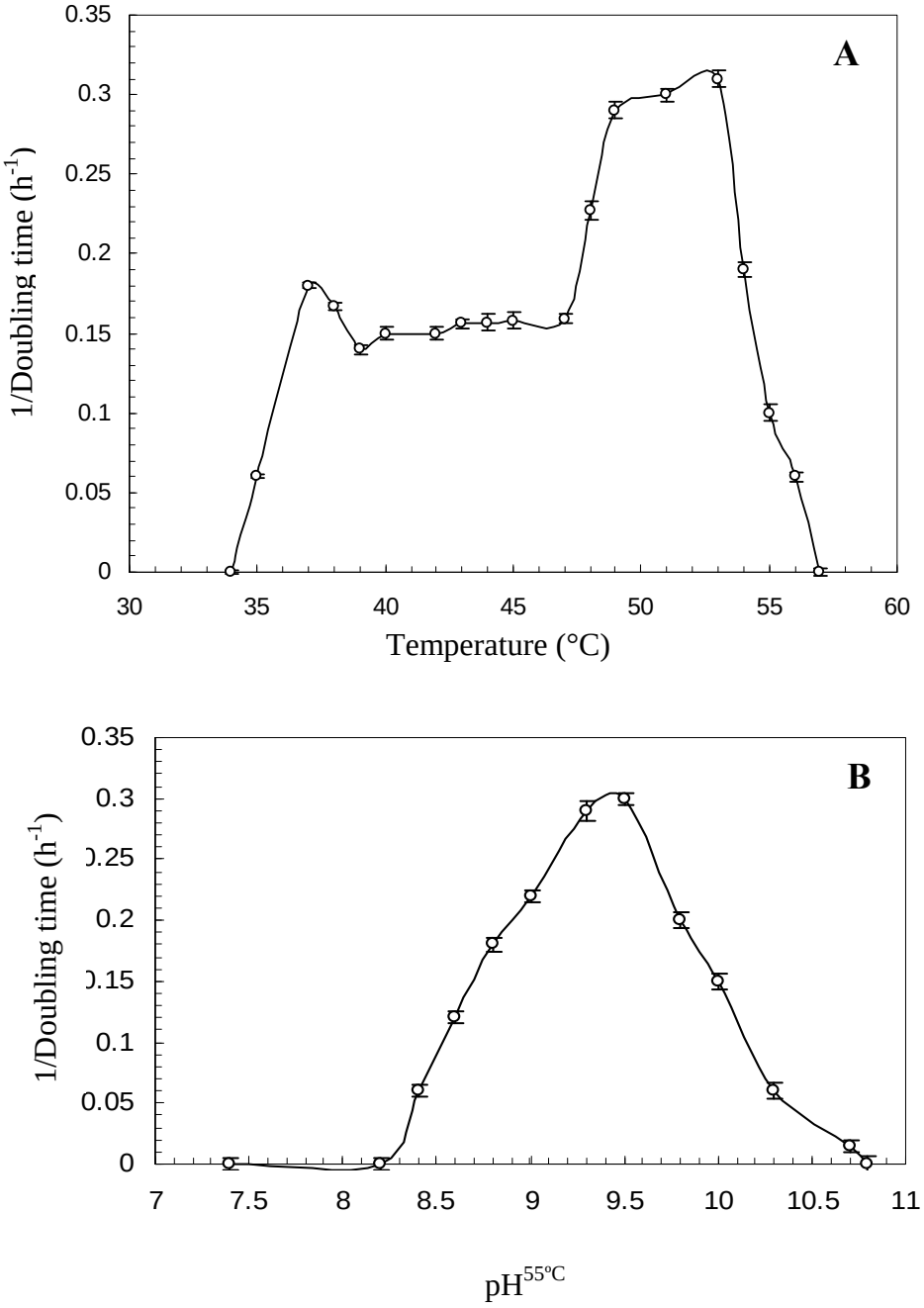
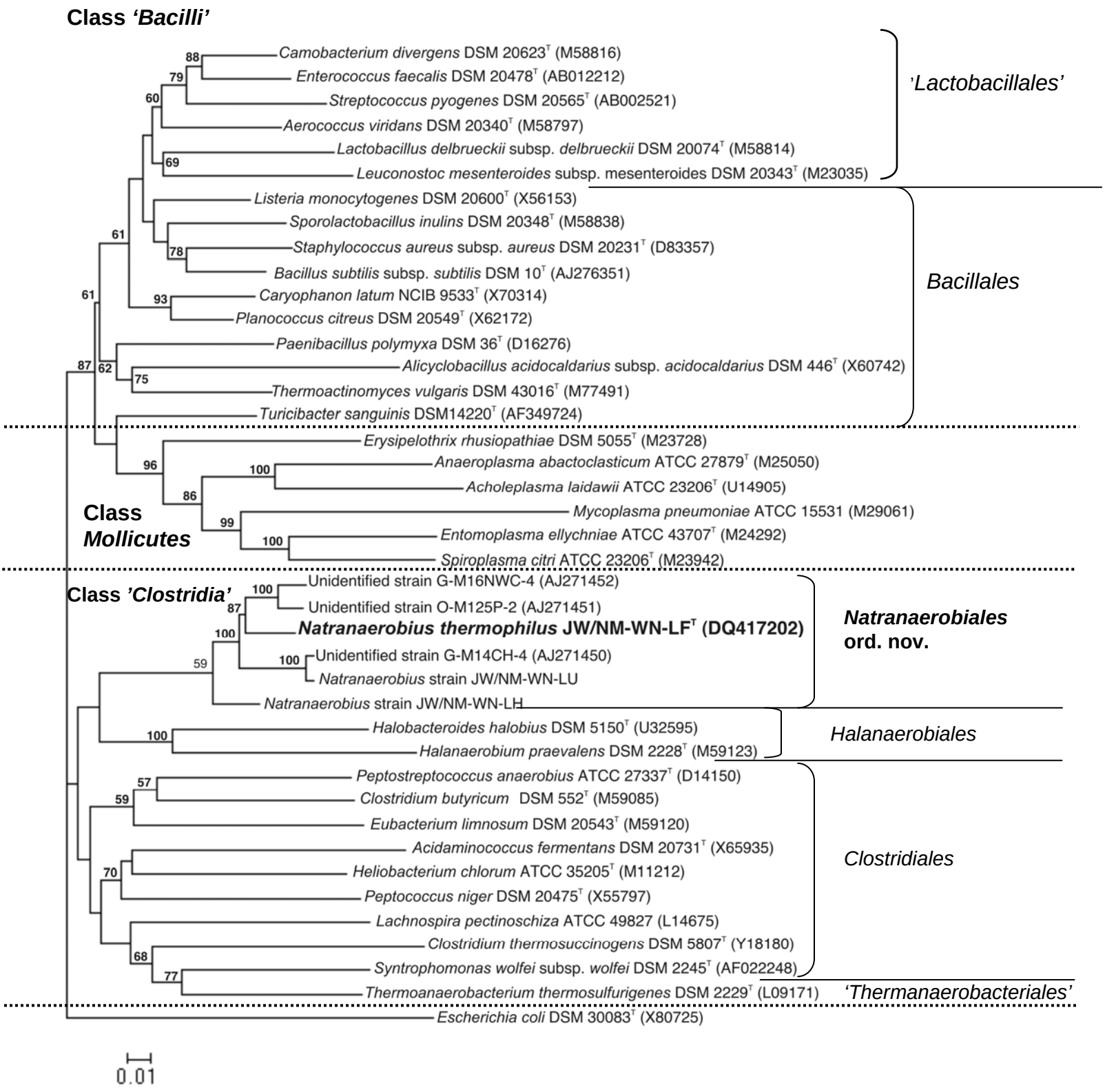


Figure S3.1. Dependence of growth rate of strain JW/NM-WN-LF^{T} on (A) temperature and (B) media pH^{55ºC}. Bars represent standard errors, n=3.

Figure S3.2. Fitch-Margoliash tree based on 16S rRNA sequences showing position of strain JW/NM-WN-LF^T in relation to additional *Natranaerobius* species isolated in our laboratory, unidentified Kenyan-Tanzanian Rift isolates, and type species of the type genera within the *Firmicutes*. GenBank accession numbers for the sequences are in parentheses. The tree was rooted with the 16S rRNA gene of *Escherichia coli* DSM 30083^T as the outgroup. Numbers at nodes are bootstrap values based on 100 replicates; only values greater than 50 are shown. Bar, 1 nucleotide substitutions per 100 nt.


Class 'Bacilli'
Camobacterium divergens DSM 20623T (M58816)
Enterococcus faecalis DSM 20478T (AB012212)
Streptococcus pyogenes DSM 20565T (AB002521)
Aerococcus viridans DSM 20340T (M58797)
Lactobacillus delbrueckii subsp. delbrueckii DSM 20074T (M58814)
Leuconostoc mesenteroides subsp. mesenteroides DSM 20343T (M23035)
'Lactobacillales'
Listeria monocytogenes DSM 20600T (X56153)
Sporolactobacillus inulins DSM 20348T (M58838)
Staphylococcus aureus subsp. aureus DSM 20231T (D83357)
Bacillus subtilis subsp. subtilis DSM 10T (AJ276351)
Caryophanon latum NCIB 9533T (X70314)
Planococcus citreus DSM 20549T (X62172)
Paenibacillus polymyxa DSM 36T (D16276)
Alicyclobacillus acidocaldarius subsp. acidocaldarius DSM 446T (X60742)
Thermoactinomyces vulgaris DSM 43016T (M77491)
Turicibacter sanguinis DSM14220T (AF349724)
Bacillales
Erysipelothrix rhusiopathiae DSM 5055T (M23728)
Anaeroplasma abactoclasticum ATCC 27879T (M25050)
Acholeplasma laidawii ATCC 23206T (U14905)
Mycoplasma pneumoniae ATCC 15531 (M29061)
Entomoplasma ellychniae ATCC 43707T (M24292)
Spiroplasma citri ATCC 23206T (M23942)
Class Mollicutes
Class 'Clostridia'
Unidentified strain G-M16NWC-4 (AJ271452)
Unidentified strain O-M125P-2 (AJ271451)
Natranaerobius thermophilus JW/NM-WN-LFT (DQ417202)
Unidentified strain G-M14CH-4 (AJ271450)
Natranaerobius strain JW/NM-WN-LU
Natranaerobius strain JW/NM-WN-LH
Natranaerobiales ord. nov.
Halobacteroides halobius DSM 5150T (U32595)
Halanaerobium praevalens DSM 2228T (M59123)
Halanaerobiales
Peptostreptococcus anaerobius ATCC 27337T (D14150)
Clostridium butyricum DSM 552T (M59085)
Eubacterium limnosum DSM 20543T (M59120)
Acidaminococcus fermentans DSM 20731T (X65935)
Heliobacterium chlorum ATCC 35205T (M11212)
Peptococcus niger DSM 20475T (X55797)
Lachnospira pectinoschiza ATCC 49827 (L14675)
Clostridium thermosuccinogens DSM 5807T (Y18180)
Syntrophomonas wolfei subsp. wolfei DSM 2245T (AF022248)
Clostridiales
Thermoanaerobacterium thermosulfurigenes DSM 2229T (L09171)
'Thermanaerobacteriales'
Escherichia coli DSM 30083T (X80725)
0.01

CHAPTER 4

NATRONOVIRGA WADINATRUNENSIS GEN. NOV. SP. NOV. AND *NATRANAEROBIUS TRUEPERI* SP. NOV., TWO HALOPHILIC, ALKALITHERMOPHILIC MICROORGANISMS FROM SODA LAKES OF THE WADI AN NATRUN, EGYPT[1]

[1] Mesbah, N.M. and J. Wiegel. Submitted to the *International Journal of Systematic and Evolutionary Microbiology*.

Summary

Novel strains of anaerobic, halophilic alkalithermophilic bacteria were isolated from sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. Strains JW/NM-WN-LU^T (and related strains) and JW/NM-WN-LH1^T were non-spore-forming, non-motile bacteria with a Gram-type positive cell wall. Optimal growth of strain JW/NM-WN-LU^T occurred at pH^{55ºC} 9.5, 52ºC and 3.7 M of Na^+ (2.2 M added NaCl). Optimal growth of strain JW/NM-WN-LH1^T occurred at pH^{55ºC} 9.9, 51ºC and 3.9 M of Na^+ (2.3 M added NaCl). Both strains were obligately anaerobic and chemoorganotrophic. The G+C contents of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T were 41.0 and 42.0 mol% respectively. Main cellular fatty acids in both strains were anteiso-branched 15:0 and iso-branched 15:0. Phylogenetic analysis showed that the isolates both belong to the family *Natranaerobiaceae* within the order *Natranaerobiales*. Based on genotypic and phenotypic data, strain JW/NM-WN-LU^T represents the type strain for the novel species, *Natranaerobius trueperi* sp. nov. (= DSM 18760^T = ATCC BAA-1443^T). Phylogenetic and phenotypic data indicate that strain JW/NM-WN-LH1^T represents a novel genus and species within the family *Natranaerobiaceae*, *Natronovirga wadinatrunensis* gen. nov. sp. nov. The type strain is JW/NM-WN-LH1^T (=DSM 18770^T = ATCC BAA-1444^T).

Main text

The Wadi An Natrun, Egypt, is a valley comprised of seven large, alkaline, hypersaline, athalassic lakes and a number of ephemeral pools. Salinities ranging between 1.5 and 5.0 M NaCl, pH values between 8.5-11 and temperatures up to 50ºC (Mesbah *et al.*, 2007a, Taher, 1999) make the Wadi An Natrun lakes excellent habitats for isolation of extremophiles. A rich microbial diversity exists in both the waters and sediments of the Wadi An Natrun lakes (Imhoff *et al.*, 1978, Imhoff *et al.*, 1979, Mesbah *et al.*, 2007a). These microbial communities are exposed to conditions of high salt and alkaline pH values, in addition to desiccation and intense solar irradiation. During a previous study, we described the halophilic, alkalithermophilic anaerobe, *Natranaerobius thermophilus*, that was isolated from the sediment of Lake Fazda in Wadi An Natrun (Mesbah *et al.*, 2007b). The order *Natranaerobiales*, consisting of the family *Natranaerobiaceae*, was proposed to harbor this novel species. In this article, taxonomic characterizations of two further novel anaerobic halophilic alkalithermophilic taxa from the Wadi An Natrun are presented. One of these isolates represents a novel species within the genus *Natranaerobius*; whereas the other isolate represents a novel genus and species within the family *Natranaerobiaceae.*

Isolation and cultivation of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T

Mixed water-sediment samples were collected from Lakes Hamra, UmRisha, Abu Dawood and Baida of the Wadi An Natrun during May of 2005. The pH values and salinity of the water at the time of collection are described in Mesbah *et al*. (2007a). The anaerobic, carbonate-buffered medium used for enrichment and initial cultivation contained (per liter): 0.2 g KH_2PO_4, 0.1g $MgCl_2$, 0.5 g NH_4Cl, 0.2 g KCl, 100 g NaCl, 68 g Na_2CO_3, 38 g $NaHCO_3$, 0.7 g

cysteine.HCl, 5 g yeast extract, 5 g tryptone, 5 g sucrose, 1 ml of trace element solution (Kevbrin & Zavarzin, 1992) and 10 mL of vitamin solution (Wolin *et al.*, 1963). The $pH^{55ºC}$ was adjusted to 9.5 with anaerobic 5N HCl. Incubation at 55ºC yielded turbid cultures within 48 hrs. Five pure cultures were isolated from repeated dilution rows of anaerobic agar (1% w/v) shake-roll tubes (Ljungdahl & Wiegel, 1986). On the basis of 16S rRNA gene sequence analysis and cell morphology, the 5 isolates formed two clusters (or groups) within the order *Natranaerobiales*. Four strains designated JW/NM-WN-LU, JW/NM-WN-LB2, JW/NM-WN-LA1 and JW/NM-WN-LA3, formed short thick rods (Figure 1A). They were isolated from sediments of Lakes UmRisha, Baida and Abu Dawood, respectively. Analysis of their complete 16S rRNA gene sequences indicated that their closest relative is *Natranaerobius thermophilus* (96.1-96.4 % sequence identity). Strain JW/NM-WN-LU^T was selected as a representative strain for detailed taxonomic characterization.

One strain designated JW/NM-WN-LH1, formed longer, thin rods and exhibited pleomorphic cell morphology (Figure 1B). It was isolated from sediment of Lake Hamra. Analysis of its complete 16S rRNA gene sequence showed *N. thermophilus* as its closest relative (93.8% sequence identity). This was designated strain JW/NM-WN-$LH1^T$, and was subject to further taxonomic characterization.

All strains were maintained in the carbonate-buffered medium described above, at $pH^{55ºC}$ 9.5 and 55ºC under anaerobic conditions (100% N_2) using a modified Hungate technique (Ljungdahl & Wiegel, 1986).

Colony and cell morphology

Colonies of strain JW/NM-WN-LU^T and related strains appeared in agar (1% wt/vol) shake roll tubes after 2-3 days and were 2-3 mm in diameter, circular to irregularly shaped and opaque. Colonies of strain JW/NM-WN-LH1^T appeared in agar shake roll tubes after 4-5 days and were 0.5-2 mm in diameter, ovoid and opaque. Cell morphology was observed via light microscopy (Olympus VANOX phase-contrast microscope) and electron microscopy. In exponential growth phase, cells of strain JW/NM-WN-LU^T and related strains were short rods, 0.6 μm wide and 2-3 μm long. Cells of strain JW/NM-WN-LH1^T were straight rods, 0.3-0.4 μm in diameter and 4-5 μm in length (Figure 1). Cells of both strains were single or formed chains upon entry to stationary phase. No active motility was observed for any of the strains under phase-contrast microscopy. Flagella were absent in negatively stained samples (2% uranyl acetate). Endospores were not observed either by field emission scanning electron microscopy or by light microscopy after heat treatment (5 min at 100ºC). Cells of both strains were not viable after heat treatment (5 min at 100ºC). Cells of both strains remained viable after 10 days of dessication (blue regenerated silica gel desiccant). Cells of all strains stained Gram-positive in both the early exponential- and stationary-growth phases (Doetsch, 1981).

Cultural and physiological characteristics

The optimal conditions for growth of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T were tested in carbonate buffered medium with 0.3% (wt/vol) yeast extract and tryptone, 640 mM Na_2CO_3 , and 320 mM $NaHCO_3$ (before pH adjustment, yielding a base Na^+ concentration of 1.6 M). The temperature ranges for growth were determined using a temperature gradient incubator (Scientific Industries, Inc.). Both species, and all their strains, were moderately

thermophilic, with growth temperature optima between 51 and 53ºC. There was no growth at 25ºC and below, or 57ºC and above.

The pH ranges and optima for growth of both strains were determined at 55ºC in carbonated buffered medium. All pH measurements were performed as described previously (Mesbah & Wiegel, 2006), with a microelectrode (Accumet ® combination microelectrode with calomel reference, Cole-Palmer), calibrated at 55ºC with pH standards preheated to the same temperature. The pH of the medium was adjusted by addition of sterile anaerobic HCl (5N) or Na_2CO_3 (3M). The $pH^{55ºC}$ for growth of strain JW/NM-WN-LU^T and its strains was 8.0 – 10.8, with no growth at $pH^{55ºC}$ 7.8 or below or $pH^{55ºC}$ 11.0 or above (Table 1). The optimal $pH^{55ºC}$ for growth was 9.5. Strain JW/NM-WN-$LH1^T$ was more alkaliphilic, growing optimally at $pH^{55ºC}$ 9.9, and within the range 8.5 – 11.5 (Table 1).

The salinity range for growth was determined in carbonate buffered medium at $pH^{55ºC}$ 9.5. Strain JW/NM-WN-LU^T grew over a NaCl range of 1.5 – 3.8 M, with an optimum at 2.2 M (Table 1). Taking into account the 1.6 M of Na_2CO_3/$NaHCO_3$ present in the medium, the total Na^+ ion concentration range for growth of strain JW/NM-WN-LU^T was 3.1 – 5.4 M; with an optimum at 3.8 M. Strain JW/NM-WN-$LH1^T$ grew over total Na^+ ion concentrations of 3.3 – 5.6 M (which includes 1.7 – 3.7 M of added NaCl). Optimal growth was at 3.9 M of Na^+ (2.3 M of added NaCl, Table 1). All strains did not grow when equimolar amounts of K_2CO_3 and $KHCO_3$ were substituted for Na_2CO_3 and $NaHCO_3$, even in the presence of 2.0 M of NaCl. The doubling times for strains JW/NM-WN-LU^T and JW/NM-WN-$LH1^T$ at optimal growth conditions were 3.0 and 2.0 h respectively.

For substrate utilization tests, cultures were incubated for up to 4 days and growth was judged positive if, in the third successive transfer, the optical density (at 600 nm) of the culture

was twice that of a control culture incubated with only 0.3% (wt/vol) yeast extract and tryptone. Utilization of possible substrates (0.5% wt/vol) was tested in the presence of 0.3% yeast extract and tryptone. Substrates utilized are presented in the species descriptions below. Strains JW/NM-WN-LU^T and related strains and JW/NM-WN-LH1^T were negative for catalase and oxidase, gelatin liquefaction and casein degradation. All strains were obligately anaerobic, no growth was observed in the presence of 0.02% (vol/vol) of oxygen.

Use of electron acceptors was determined by measuring growth (increase in OD_{600}, for all acceptors), by sulfide production (for SO_4^{2-},SO_3^{2-}, and $S_2O_3^{2}$,$CuSO_4$ spectrophotometric assay(Cord-Ruwisch, 1985)), and by measuring changes in visible color of the medium for MnO_2. Nitrate reduction was determined using the nitrate reductase test with sulfanilic acid and N,N-dimethyl-1-naphthylamine (Lennette *et al.*, 1980). The development of a red color was considered a positive reaction for nitrate reduction to nitrite. Negative results were confirmed with the addition of zinc dust. Failure of color development was considered as a positive test for nitrate reduction beyond nitrite, to ammonium.

In the presence of 0.3% yeast extract and tryptone, strain JW/NM-WN-LU^T reduced NO_3^- to ammonium. Sulfate, SO_3^{2-}, $S_2O_3^{2-}$ and MnO_2 were not utilized as electron acceptors, as evidenced by lack of sulfide production. Strain JW/NM-WN-LH1^T utilized MnO_2 as an electron acceptor as judged by color change and reduced NO_3^- to ammonium. Sulfate, SO_3^{2-}, and $S_2O_3^{2-}$ were not reduced.

In the presence of 0.3% yeast extract and tryptone, strain JW/NM-WN-LU^T produced from 10 mM pyruvate: 7.0 mM acetate, 1.5 mM lactate and 97 nM H_2 as fermentation products. Strain JW/NM-WN-LH1^T produced from 10 mM pyruvate 4.3 mM acetate, 5.0 mM lactate and

206 nM H_2. No fermentation products could be detected in the presence of 0.3% yeast extract and tryptone alone.

Biochemical characteristics were determined under anaerobic conditions using the API ZYM 20E system (bioMérieux). All strains tested positive for lysine decarboxylase, tryptophan deaminase and H_2S production, and tested negative for urease, indole and acetoin production. Biochemical characteristics that distinguish between strains JW/NM-WN-LU^T (and related strains) and JW/NM-WN-LH1^T are shown in Table 1.

For short-term preservation, cultures of both strains can be stored at room temperature and transferred every three weeks. For long-term preservation, cells in late exponential growth phase can be harvested, resuspended in fresh medium mixed with glycerol (50% vol/vol), and stored at -80ºC. Strict anaerobic techniques must be employed for both strains to avoid prolonged lag-phases and loss of viability.

Chemotaxonomic characteristics

Cell wall peptidoglycan of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T was isolated and the structure was analyzed according to the methods of Schleifer (1985), Rhuland *et al*.(1955) and Mackenzie (1987). Similar to *N. thermophilus*, strain JW/NM-WN-LU^T yielded on repeated attempts very low amounts of peptidoglycan. The total hydrolysate was free of isomers of diaminopimelic acid and contained the following proportions of amino acids: Orn, 1.1: Ala, 1.0: Gly, 0.8: Ser, 0.4: Asp, 0.9: Glu, 1.0. The structure of peptidoglycan could not be concluded (P. Schumann, personal communication).

Peptidoglycan hydrolysate of strain JW/NM-WN-LH1^T contained the amino acids ornithine, alanine, glycine, aspartic acid and glutamic acid as shown by thin layer

chromatography (Rhuland *et al.*, 1955). Gas chromatographic analysis of these amino acid derivatives (MacKenzie, 1987) showed them to be present with the following ratios: Orn, 0.9: Ala, 1.4: Gly, 0.8: Asp, 0.8: Glu, 1.0. Small amounts of serine were also detected. The identity of these amino acids was confirmed by GC-MS (320-MS, Quadropole GC/MS, Varian). These data suggest a peptidoglycan of the type α-4-β Orn-Gly-Asp. The peptidoglycan preparation also did not contain isomers of diaminopimelic acid.

Phospholipid fatty acid (PLFA) analyses were performed on cells that had been grown at optimal conditions: 52ºC, $pH^{55ºC}$ 9.5 and 1.7 M NaCl, 640 mM Na_2CO_3 and 320 mM $NaHCO_3$. Lyophilized cell material was extracted as described previously (Mesbah *et al.*, 2007b). Detailed fatty acid profiles are shown in Table 2. Polar and neutral fatty acid compositions for both strains were similar, with a predominance of iso- and anteiso-branched 15:0 fatty acids. Strain JW/NM-WN-LH1^T had roughly three times 16:0 and i17:1ω7c and twice as much i17:0 fatty acids among the neutral fraction (Table 2).

The DNA G+C content of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T was determined by HPLC according to (Mesbah *et al.*, 1989) with the modification of (Lee *et al.*, 2005), using S1 nuclease and 0.3 M sodium acetate (pH 5.0). The G+C contents of genomic DNA were 41.0 and 42.0 mol% for strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T respectively (Table 1). The results are the mean of six replicate analyses.

Phylogenetic analyses

The nearly complete 16S rRNA gene sequences from all five isolated strains were aligned with species of the orders *Halanaerobiales, Natranaerobiales, 'Thermanaerobacteriales'* and *'Clostridiales'* to verify their taxonomic positions (Figure 2). Multiple sequence alignments

were created with the Clustal X program (http://bips.u-strasbg.fr/fr.Documentation/ClustalX/). Trees were constructed by using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html). Distances were calculated by using the Jukes-Cantor algorithm of DNADIST, and branching order was determined via the neighbor-joining algorithm of NEIGHBOR. Each tree was a consensus of 1000 replicate trees. The neighbor-joining tree shows that strain JW/NM-WN-LU^T clusters with *Natranaerobius thermophilus*, and exhibits a 96.4% sequence identity to it (Figure 2). Strain JW/NM-WN-LH1^T formed a separate lineage within the order *Natranaerobiales*. Additional phylogenetic analyses performed with 16S rRNA gene sequences of type genera of the *Firmicutes* and different treeing methods (Fitch-Margoliash, Maximum Likelihood), confirmed the divergence of strain JW/NM-WN-LH1^T from other species within the *Natranaerobiales*. The 16S rRNA sequence identities of strain JW/NM-WN-LH1^T with the 16S rRNA sequences from JW/NM-WN-LU^T and *N. thermophilus* were 93 and 94% respectively.

Taxonomic conclusions

Phylogenetically, both strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T belong to the order *Natranaerobiales* and are within the confines of the family *Natranaerobiaceae*. Table 1 shows phenotypic characteristics differentiating strains JW/NM-WN-LU^T (and related strains) and JW/NM-WN-LH1^T from *Natranaerobius thermophilus*. Strain JW/NM-WN-LU^T differs from *N. thermophilus* in several traits. The main phenotypic differences are different patterns of amino acids in the cell wall, smaller cell size and narrow substrate utilization range. Strain JW/NM-WN-LU^T also has a different proportion of the fatty acid i15:0 in the cell wall, larger NaCl range and maximum and shorter doubling time. On the basis of phenotypic and

phylogenetic data, we propose that strain JW/NM-WN-LU^T represents the type strain of a novel species, *Natranaerobius trueperi* sp. nov.

Strain JW/NM-WN-LH1^T is distinguished from both *N. thermophilus* and JW/NM-WN-LU^T by having a more alkaline pH range and optimum, shorter doubling time and different cell morphology; the cells are longer in size. Strain JW/NM-WN-LH1^T has a cell wall of the α-4-β type, whereas no cell wall type could be deduced for the other two type strains of the genus *Natranaerobius*. Strain JW/NM-WN-LH1^T also has a larger proportion of i17:1ω7c, 16:0 and 18:0 fatty acids in the neutral fraction of its phospholipid fatty acid profile (Table 2). These physiological and chemotaxonomic differences in addition to distinctness of the 16S rRNA sequence and phylogenetic position differentiate strain JW/NM-WN-LH1^T from the other species of the genus *Natranaerobius*. Therefore we propose that strain JW/NM-WN-LH1^T be classified in a novel genus, *Natronovirga* gen. nov. The type species is *Natronovirga wadinatrunensis* sp. nov. Comparison of distances among genera of the order *Halanaerobiales* and *Natranaerobiales* confirm the placement of strain JW/NM-WN-LH1^T into a novel genus.

Description of *Natranaerobius trueperi* sp. nov.

Natranaerobius trueperi (true'pe.ri. N.L. gen. masc. n. trueperi, of Trüper, referring to Hans G.Trüper, in honor of his contributions to the field of physiology of halophiles and his earlier work on the microbial ecology of the Wadi An Natrun)

Cells form irregularly shaped to circular opaque colonies with a white color (when grown inside 1% agar). Cells are 2-3 X 0.6 μm in size, non-motile and catalase- and oxidase-negative. Cells are Gram-staining and Gram-type positive (Wiegel, 1981). Halophilic: optimal growth occurs at

3.7 M Na^+ (2.2 M added NaCl); no growth occurs at Na^+ concentrations below 3.1 M or above 5.4 M. Obligately alkaliphilic, $pH^{55ºC}$ range, 8.0-10.8, with an optimum at $pH^{55ºC}$ 9.5; no growth occurs at $pH^{55ºC}$ 7.8 or below and 11.0 or above. Thermophilic: temperature range for growth is 26-55ºC, with an optimum at 52ºC (at $pH^{55ºC}$ 9.5). No growth at 24ºC and below or 57ºC and above. Obligately anaerobic. Chermoorganotrophic. In the presence of 0.3% (w/v) yeast extract and tryptone, glucose, ribose, acetate, sucrose, Casamino acids, cellobiose, pyruvate and trimethylamine are used as carbon and energy sources (0.5% wt/vol, $pH^{55ºC}$ 9.5). Fructose, trehalose, betaine, formate, ribitol, ethanol, n-propanol and benzoate are not utilized. Organic fermentation products from pyruvate are acetate and lactate. Nitrate is utilized as an electron acceptor. Sulfate, MnO_2, SO_3^{2-} and $S_2O_3^{2-}$ are not reduced. Produces β-galactosidase, arginine dihydrolase, lysine decarboxylase, tryptophan deaminase and hydrogen sulfide in the presence of 0.3% yeast extract/ tryptone and 0.2% glucose. Does not utilize citrate or produce urease, indole or acetoin. Major cellular fatty acids include i15:0 and a15:0. The type strain of the species lacks detectable amounts of murein and meso-diaminopimelic acid in the cell wall. The DNA G+C content of genomic DNA is 41 mol% (HPLC).

The type strain, JW/NM-WN-LU^T = (DSM 18760^T = ATCC BAA-1443^T) was isolated from sediment of Lake UmRisha, Wadi An Natrun, Egypt. Strain JW/NM-WN-LB2 was isolated from sediment of Lake Baida and strains JW/NM-WN-LA1 and JW/NM-WN-LA3 were isolated from sediments of Lake Abu Dawood, Wadi An Natrun, Egypt.

Description of *Natronovirga* gen. nov.

Natronovirga [Na.tro.no.vir'ga. N.Gr. n. *natron* derived from Arabic *natrun* soda (sodium carbonate); L.fem. n. *virga* rod. N.L. fem. n. *Natronovirga* a soda requiring rod].

Cells are Gram-type positive (Wiegel, 1981); endospores are not observed. Extremely halophilic (growth requires at least 3.3 M Na^+); obligately alkaliphilic. Thermophilic. Fatty acid profile is dominated by branched fatty acids with 15 carbons. Cell wall peptidoglycan is of the α-4-β Orn-Gly-Asp type. The DNA G+C content is approximately 42 mol%. Obligately anaerobic chemo-organotrophs. The type species is *Natronovirga wadinatrunensis*.

Description of *Natronovirga wadinatrunensis* sp. nov.

Natronovirga wadinatrunensis (wa.di.na.trun.en'sis. N.L. fem. adj. *wadinatrunensis*, pertaining to the Wadi An Natrun, the source of isolation)

Cells form ovoid and opaque colonies with a yellow-white color (when grown inside 1% agar). Cells are 4-5 X 0.3-0.4 μm in size, non-motile and catalase and oxidase negative. Cells are Gram-staining and Gram-type positive (Wiegel, 1981). Extremely halophilic: optimal growth occurs at 3.9 M Na^+ (2.3 M added NaCl); no growth occurs at Na^+ concentrations below 3.1 M or greater than 5.3 M. Obligately alkaliphilic: $pH^{55ºC}$ range, 8.5-11.5, with an optimum at $pH^{55ºC}$ 9.9. No growth occurs at $pH^{55ºC}$ 8.3 or 11.7. Thermophilic: temperature range for growth is 26-56ºC (at $pH^{55ºC}$ 9.9), with an optimum at 51ºC, and no growth at 24ºC or 58 ºC. Obligately anaerobic. Chemoorganotrophic. When 0.2% (w/v) yeast extract and tryptone are present, glucose, fructose, trehalose, mannose, pyruvate, Casamino acids, acetate, galactose, sucrose and

lactose are used as carbon and energy sources. Cellobiose, ribose, formate, betaine, xylose, glucuronic acid, ethanol and n-propanol are not utilized (0.5% w/v final concentration at $pH^{55^{o}C}$ 9.9). The main organic fermentation products from pyruvate are lactate and acetate. MnO_2 and NO_3^- are utilized as electron acceptors. Sulfate, SO_3^{2-} and $S_2O_3^{2-}$ are not reduced. In the presence of 0.3% yeast extract/ tryptone and 0.2% glucose, produces ornithine decarboxylase, lysine decarboxylase, tryptophan deaminase, hydrogen sulfide and produces citrate. Does not produce β-galactosidase, arginine dihydrolase, urease, indole or acetoin. Major cellular fatty acids include i15:0 and a15:0. The type strain possesses peptidoglycan of type α-4-β Orn-Gly-Asp in the cell wall. The DNA G+C content of genomic DNA is 42 mol% (HPLC).

The type strain, JW/NM-WN-LH1^T (=DSM 18770^T = ATCC BAA-1444^T), was isolated from sediment of Lake Hamra, Wadi An Natrun, Egypt.

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Table 4.1. Selected characteristics that distinguish strains JW/NM-WN-LU^T (and related strains) and JW/NM-WN-LH1^T from *Natranaerobius thermophilus*

Strains: 1, JW/NM-WN-LU^T; 2, JW/NM-WN-LB2; 3, JW/NM-WN-LA1; 4, JW/NM-WN-LA3 5, JW/NM-WN-LH1^T; 6, *N. thermophilus*

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Length of rods (μm)	2-3	2-3	2-3	2-3	4-5	3-5
NaCl range (optimum) (M)	1.5-3.8 (2.2)	1.3-3.8 (2.2)	1.3-3.8 (2.2)	1.5-3.8 (2.3)	1.7-3.7 (2.3)	1.5-3.2 (1.6-2.3)
$pH^{55ºC}$ range (optimum)*	8.0-10.8 (9.5)	8.0-10.8 (9.5)	8.0-10.8 (9.5)	8.3-10.8 (9.6)	8.5-11.5 (9.9)	8.3-10.6 (9.5)
Temp. range (optimum) (ºC)	26-55 (52)	27-54 (52)	26-55 (52)	28-55 (53)	26-56 (51)	35-56 (53)
Doubling time (h)	3.0	3.0	3.0	3.0	2.0	3.5
Utilization of:						
Fructose	-	-	-	+	+	+
Ribose	+	+	+	+	-	+
Trehalose	-	-	-	-	+	+
Mannose	-				+	-
Cellobiose	+	+	+	+	-	+
Pyruvate	-	-	-	-	+	+
Trimethylamine	+	+	+	+	-	+
API ZYM tests:						
β-galactosidase	+	+	+	+	-	-

Arginine dihydrolase	+	+	+	+	-	-
Ornithine decarboxylase	-	-	-	-	+	-
Citrate utilization	-	-	-	-	+	-
DNA G+C content	41.0	41.3	40.8	40.9	41.7	40.4
Cell wall composition	could not be determined; amino acid ratios: Orn 1.1; Ala 1.0; Gly 0.8; Ser 0.4; Asp 0.9; Glu 1.0	n.d.	n.d.	n.d.	α-4-β Orn-Gly-Asp	could not be determined, amino acid ratios: Lys 1.0; Ala 1.5; Gly 0.8; Glu 1.0
Major fermentation product from 10 mM pyruvate	acetate	acetate	acetate	acetate	acetate, lactate	acetate

- pH was determined at 55ºC using a pH meter calibrated at 55ºC (Wiegel 1998)
- n.d.: not determined

Table 4.2. Polar and neutral fatty acid composition of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T.

1, strain JW/NM-WN-LU^T; 2, JW/NM-WN-LH1^T

Fatty acid	1	2
Polar Fatty Acids		
Terminally branched saturated fatty acids:		
i14:0	1.1	0.5
i15:0	81.1	80.4
a15:0	12.2	9.4
Branched monoenoic fatty acids:		
i17:1ω7c	0.8	2.2
Normal saturated fatty acids:		
14:0	2.5	2.6
16:0	0.6	1.4
Neutral Fatty Acids		
Terminally branched saturated fatty acids:		
i15:0	76.3	68.1
a15:0	10.0	7.5
i16:0	1.0	0.0
i17:0	2.3	5.3
Monoenoic fatty acids:		
16:1ω7c	1.1	0.0
Branched monoenoic fatty acids:		
i17:1ω7c	2.8	9.6
Normal saturated fatty acids:		
14:0	2.5	0.0
16:0	2.2	7.1
18:0	0.6	2.4

* results are in mol percent of total fatty acid

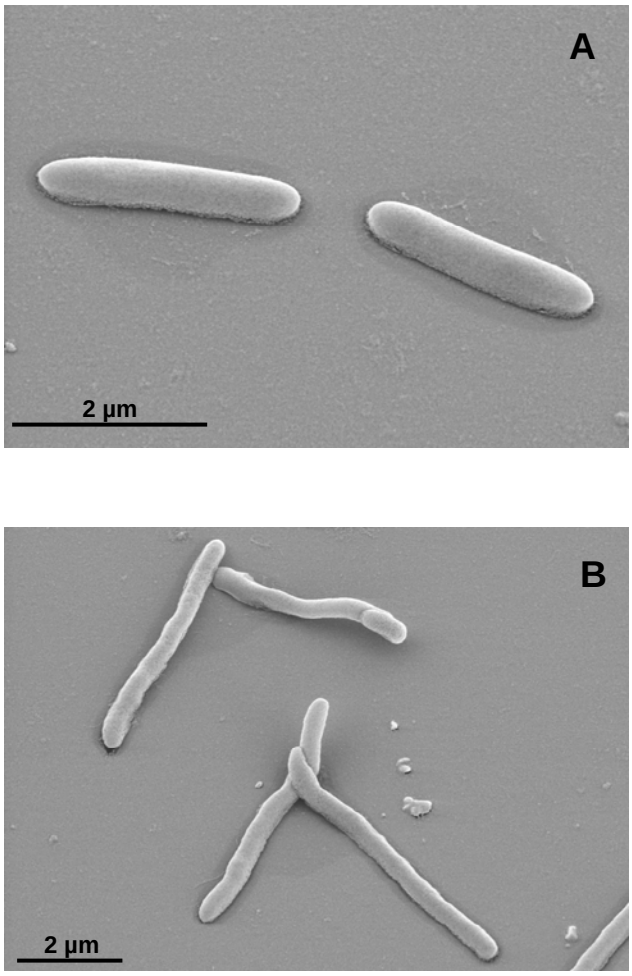

Figure 4.1. Field emission scanning electron micrographs of aldehyde-fixed, actetone-dehydrated and critical point-dried cells of (A) strain JW/NM-WN-LU^{T} and (B) strain JW/NM-WN-LH1$^{T.}$

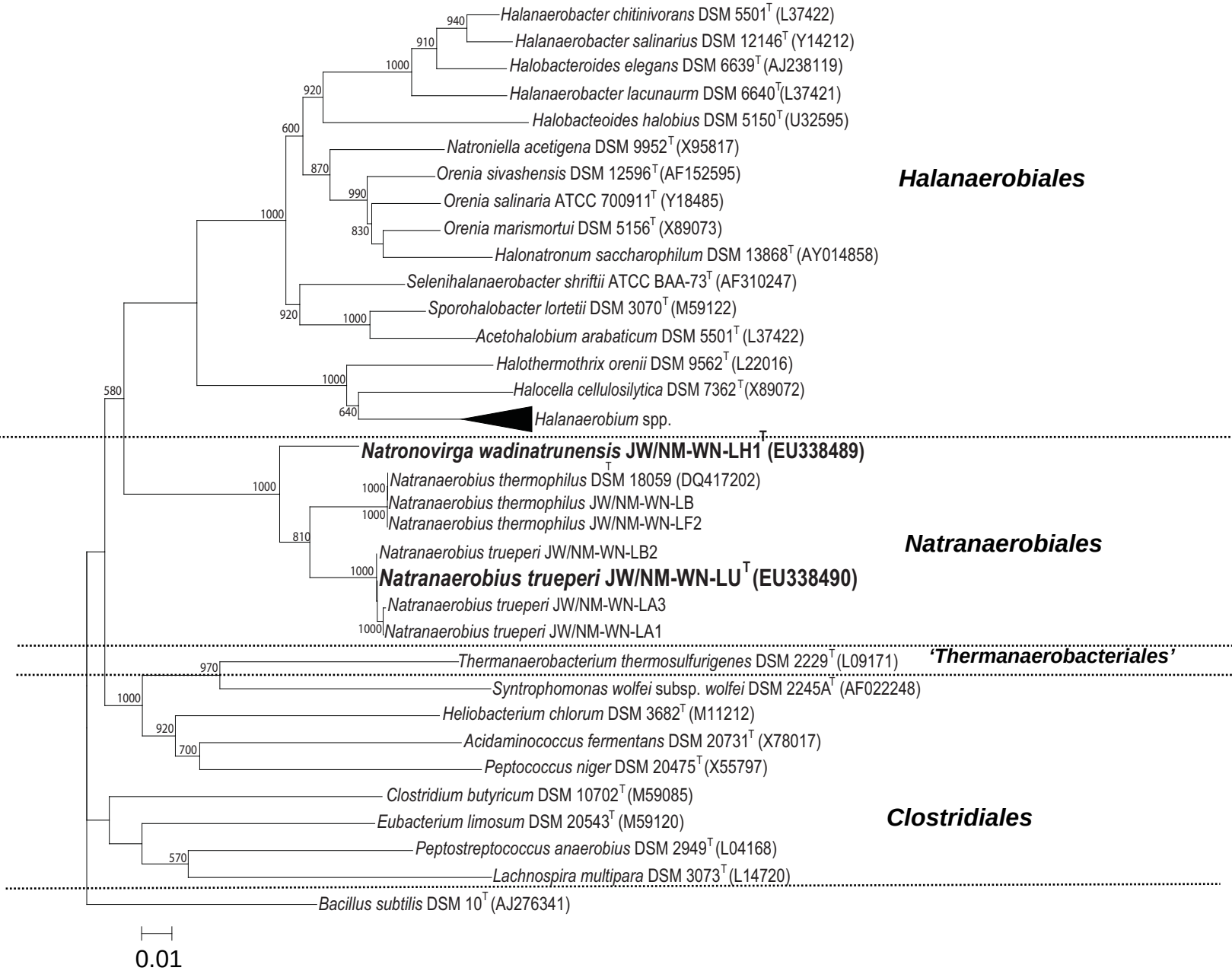

Figure 4.2. Neighbor-joining tree based on 16S rRNA gene sequences showing the position of strains JW/NM-WN-LU^T and JW/NM-WN-LH1^T in relation to type species of genera within the class *Clostridia*. GenBank accession numbers for the sequences are shown in parentheses. The tree was rooted with the 16S rRNA of *Bacillus subtilis* DSM 10^T as outgroup. Numbers at nodes denote bootstrap values based on 1000 replicates; only values greater than 500 are shown. Bar, one nucleotide substitution per 100 nt.

CHAPTER 5

BIOENERGETIC PROPERTIES AND INTRACELLULAR PH REGULATION IN *NATRANAEROBIUS THERMOPHILUS*, AN ANAEROBIC, HALOPHILIC, ALKALITHERMOPHILIC BACTERIUM[*]

[*] Mesbah, N.M., G.M. Cook and J. Wiegel. To be submitted to the *Journal of Bacteriology*.

Abstract

The extremophile *Natranaerobius thermophilus* grows in batch culture between 30 and 57°C, with an optimum at 53°C. *N. thermophilus* is an obligate alkaliphile; the $pH^{55°C}$ range for growth is 8.3-10.6, with an optimum at $pH^{55°C}$ 9.5. *N. thermophilus* is also an obligate halophile; it grows optimally between 3.3 and 3.9 M Na^+ (1.7-2.3 M added NaCl, remainder from Na_2CO_3/ $NaHCO_3$), the Na^+ range for growth being 3.1-4.9 M. *N. thermophilus* is obligately anaerobic and chemoorganotrophic. Growth of *N. thermophilus* at $pH^{55°C}$ 9.5 was inhibited by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone and the ionophores monensin, valinomycin and nigericin, suggesting that both proton and sodium motive forces exist across the membrane. When the extracellular $pH^{55°C}$ was raised from 8.0 to 10.2, *N. thermophilus* maintained a $pH^{55°C}$ gradient of 1 unit across the cell membrane (alkaline out). The membrane potential was maintained between -99 and -138 mV over the pH range 8.0-10.2. The proton motive force was -57 mV at $pH^{55°C}$ 9.5. However, a sodium motive force of –173 mV was maintained, and this compensated for the low proton motive force. The phosphorylation potential of *N. thermophilus* was maintained between -460 and -475 mV, and the calculated H^+/ATP stoichiometry of the ATP synthase was maintained between 8 and 10 over $pH^{55°C}$ values of 8-9.5, but then decreased to 5.5 as the extracellular $pH^{55°C}$ rose beyond 9.5.These data correlate dwell with growth kinetics of *N. thermophilus* over a range of increasing pH values.

Introduction

The recently isolated *Natranaerobius thermophilus* strain JW/NM-WN-LF^T is a Gram-staining and Gram-type positive (Wiegel, 1981), non-spore-forming, anaerobic halophilic alkalithermophile that grows optimally at 53ºC, an alkaline $pH^{55°C}$ of 9.5 and Na^+ ion

concentrations between 3.3 and 3.9 M (Mesbah *et al.*, 2007). The strain was isolated from sediments of the solar-heated, alkaline, hypersaline lakes of the Wadi An Natrun, Egypt, and represents the first isolated member of the novel order, *Natranaerobiales*, within the class *Clostridia* of the phylum *Firmicutes* (Mesbah *et al.*, 2007). Only one other halophilic alkalithermophilic bacterium has been validly published to date, the anaerobic *Halonatronum saccharophilum* (Zhilina *et al.*, 2004). A few other anaerobic non-halophilic alkalithermophiles are known, such as *Clostridium paradoxum* (Li *et al.*, 1993), *Clostridium thermoalcaliphilum* (Li *et al.*, 1994), *Anaerobranca horikoshii, A. gottschalkii* and *A. californiensis* (Engle *et al.*, 1995, Gorlenko *et al.*, 2004, Prowe & Antranikian, 2001), and the hyperthermohilic archaea *Thermococcus alcaliphilus* and *T. acidaminovorans* (Dirmeier *et al.*, 1998, Keller *et al.*, 1995). A number of moderately alkaliphilic, thermophilic methanogens are also known (Kevbrin *et al.*, 2004).

Since *Natranaerobius thermophilus* is an anaerobe, a thermophile, halophile and alkaliphile, its physiological and bioenergetic characteristics must fit these complex and extreme growth conditions. As an anaerobic, chemorganotrophic bacterium, it is faced with the problem that metabolic energy is mainly obtained by substrate level phosphorylation. The electrochemical ion gradient across the cytoplasmic membrane is usually generated by a H^+- or Na^+ coupled ATPase (Dimroth & Cook, 2004). The anaerobic alkalithermophiles studied to date have been shown to use Na^+ as a coupling ion for energy transducing processes (Ferguson *et al.*, 2006, Prowe *et al.*, 1996).

As a thermophilic and halophilic bacterium, *Natranaerobius thermophilus* is confronted with the problem of passive permeation of both H^+ and Na^+ through its cytoplasmic membrane. Membrane permeability to H^+ and Na^+ increases with temperature (Vossenberg *et al.*, 1995),

hence posing a problem for alkaliphiles which must acidify their cytoplasm (see below). The aerobic alkalithermophile *Bacillus* sp. TA2.A1, accounts for increased membrane proton permeability by increasing the H^+/ATP stoichiometry of its ATP synthase, maintaining a large inwardly directed sodium motive force (ΔpNa^+) of -100 mV and by coupling solute transport to Na^+ ions (Olsson *et al.*, 2003, Peddie *et al.*, 1999). Other anaerobic alkaliphiles use Na^+ as a coupling ion for energy transducing processes (Ferguson *et al.*, 2006, Prowe *et al.*, 1996, Speelmans *et al.*, 1993). This is advantageous since the permeability of membranes to Na^+ ions is less than permeability to H^+ (Konings *et al.*, 2002). However, it has been demonstrated that Na^+ permeability of liposomes increases as the NaCl concentration increases (Vossenberg *et al.*, 1999). This poses a large problem for halophiles surviving at NaCl concentrations greater than 1.5 M, as they will not be able to maintain cell shape and turgor pressure.

Given the considerations presented above, the bioenergetics of *Natranaerobius thermophilus* becomes an intriguing problem. Analysis of intracellular pH regulation of the anaerobic, non-halophilic alkalithermophile *Clostridium paradoxum* showed that it is capable of cytoplasm acidification; it increases its ΔpH across the cell membrane (pH_{out} — pH_{in}) by as much as 1.3 units, and the maximum ΔpH value observed corresponded to the optimal extracellular pH for growth. At pH values greater than 10.0, near its maximum pH range, the ΔpH and electrochemical membrane potential ($\Delta\psi$) gradually declined, and the intracellular pH significantly increased until it was almost equal that of the extracellular medium (Cook *et al.*, 1996). *C. paradoxum* has an absolute requirement for 50-200 mM of Na^+ for growth, and growth is inhibited by the sodium ionophore and an inhibitor of Na^+/H^+ antiporters, amiloride. These characteristics indicate the importance of a continuous Na^+ cycle in this alkalithermophile. For halophilic alkalithermophiles, the cell membrane would have to be highly impermeable to H^+

and Na^+ ions to maintain cytoplasmic pH and turgor pressure when growing at high salinity in addition to alkaline pH.

In this manuscript, we describe the ability of *Natranaerobius thermophilus* to grow and regulate its intracellular pH at external pH values ranging from 8.0 to 10.5. We also determine values for proton motive force (Δp), ΔpNa^+, and phosphorylation potential (ΔG_p) to investigate the roles of these parameters in energy production and solute transport under conditions of high temperature, high salt concentration and alkaline pH.

Materials and Methods

Abbreviations, chemicals and radiochemicals. Δp, proton motive force; ΔpNa^+, sodium motive force; Δψ, membrane potential, ΔpH, pH gradient, ZΔpH, transmembrane proton gradient; ΔGp, phosphorylation potential; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, 3,3',4',5-tetrachlorosalicylanilide. Valinomycin and nigericin were obtained from Sigma-Aldrich. The radiochemicals [^{3}H]methyltriphenylphosphonium (TPP^+), [^{14}C]methylamine hydrochloride, [^{3}H]water and [^{14}C]polyethylene glycol were obtained from Sigma-Aldrich. Luciferin, luciferase (E.C.1.13.12.7) and pyruvate kinase (E.C. 2.7.1.40) were also purchased from Sigma-Aldrich.

Media and culture conditions. *Natranaerobius thermophilus* strain JW/NM-WN-LF^T was routinely grown anaerobically under a nitrogen gas phase in carbonate buffered medium as previously described with 0.5% (wt/vol) sucrose as carbon source (Mesbah *et al.*, 2007). The pH of the medium was adjusted to 9.5 at 55ºC using sterile 5 N HCl. Unless otherwise stated, pH values of all media and buffers were measured at 55ºC. Cellular protein was determined by the

Biuret protein assay using bovine serum albumin as standard (Gornall *et al.*, 1949). The relationship between optical density and cellular protein was 320 mg of protein/ liter/ 1 OD unit.

Determination of bioenergetic parameters. For determination of ΔpH, Δψ and Δp, *Natranaerobius thermophilus* was grown in batch culture at $pH^{55°C}$ 9.5, 53ºC in the presence of 3.3 M Na^+. Cells were harvested during mid-exponential phase by centrifugation (6000 x *g*, 30 min, 24 ºC) and washed three times in sterile anaerobic carbonate-buffered medium, $pH^{55\ °C}$ 9.5. All washing steps were carried out anaerobically inside a Coy anaerobic chamber (Coy Laboratory Products Inc, Grass Lake, Michigan). Cells were resuspended to a final OD_{600} of 1.0 in anaerobic carbonate-buffered medium adjusted to the pH being studied. These cell suspensions (non-growing) were energized by the addition of sucrose (0.5% wt/vol) and incubation at 53ºC for 20 minutes. Energization caused an increase in gas pressure, but the pH value remained constant. Energized cell suspensions (2.0 mL) were then transferred by syringe to sealed serum vials (10 mL capacity, nitrogen in gas phase) containing one of the following isotopes: [^{14}C]methylamine (5.4 μM) or [^{3}H]TPP^+ (1 μM). After incubation for 5 min at 53ºC, 0.9 mL of the culture was rapidly centrifuged through 300 μL of silicone oil (mixture of Dow Corning 'DC 550', 36%, Dow Corning 'DC 200/200', 24%, and dioctyl phthalate, 40% vol/vol) in microcentrifuge tubes (13,000 x *g*, 3 mins, 24ºC). Twenty microliters of supernatant were removed. The tubes with remaining contents were then frozen at -80ºC for at least 2 hours. The bottoms of the tubes containing cell pellets were removed with dog-nail clippers; the supernatant and cell pellets were dissolved in scintillation fluid, and radioactivity (cpm) was determined with an LKB Wallac 1214 Rack-Beta scintillation counter.

Intracellular volume (5.6 ± 0.34 μL/ mg protein) was determined from the difference in partitioning of [^{3}H]water (1 mM) and [^{14}C]polyethylene glycol. Polyethylene glycol is not

metabolized by *N. thermophilus* (data not shown). The $\Delta\psi$ across the membrane was calculated from the uptake of [^{3}H]TPP^+ according to the Nernst relationship. Non-specific TPP^+ binding was estimated from valinomycin- and nigericin-treated cells (10 μM each). These inhibitors cause complete growth-arrest when added to cultures of *N. thermophilus* during the exponential growth phase, thus are membrane active with this bacterium (data not shown). The ΔpH was determined from the distribution of [^{14}C]methylamine with the Henderson-Hasselbach equation, and ZΔpH was calculated by 59 mV multiplied by the ΔpH. *N. thermophilus* does not metabolize or transport methylamine into its cells (data not shown).

Measurements of intracellular ATP, ADP and inorganic phosphate concentrations. For extraction of adenine nucleotides, 1 mL of exponentially growing culture was centrifuged quickly (13,000 x *g*, 30 sec, 24ºC), and the cell pellet was treated with ice-cold perchloric acid (14% vol/vol, 9 mM EDTA). The suspension was incubated on ice for 20 mins and then centrifuged for 5 min at 13,000 x *g*. The supernatant was neutralized with an equal volume of KOH/ $KHCO_3$ (1 M each), and frozen at -80ºC until analysis.

Prior to analysis, the samples were thawed on ice and potassium perchlorate was removed by centrifugation (13,000 x *g*, 3 min, 24ºC). The ATP and ADP concentrations were determined by the luciferin-luciferase method (Lundin & Thore, 1975). ATP was determined directly, ADP was determined after enzymatic conversion to ATP with pyruvate kinase. The enzymatic conversion of ADP to ATP was carried out by incubating the adenylate-containing extract in Tris-acetate buffer (pH 7.8), 1 mM $MgCl_2$, 0.1 mM phosphenolpyruvate, and 2 U of pyruvate kinase. After incubation at room temperature for 2 min, the samples were boiled for 30 sec, then stored on ice. This allowed rapid conversion of ADP to ATP. The samples were then diluted 50 to 100 fold in Milli-Q water.

The luciferase reaction was initiated by adding 25 µL of a purified luciferin-luciferase mix to 450 µL of the diluted extract. Light output was immediately measured with a luminometer (model LB953, EG & G Berthold) with ATP as standard. The concentration of inorganic phosphate was determined in neutralized cell extracts based on the formation of phosphomolybdate complex as described by Monk *et al*. (1991). All determinations were carried out in triplicate. The phosphorylation potential, ΔGp, was calculated with the equation: $\Delta G_p = \Delta G^o + 2.3RT \log[ATP]/ [ADP] [P_i]$. The value used for ΔG^o was 33.3 kJ/ mol, or the equivalent of -347 mV. The values for ΔGp were calculated with values of ΔG^o reported for the measured cytoplasmic pH and assuming an Mg^{2+} concentration of 1-25 mM (Rosing & Slater, 1972).

Measurement of intracellular sodium and potassium ion concentrations. Cells were harvested from $pH^{55°C}$ controlled batch culture, and resuspended in isotonic anaerobic medium adjusted to the pH value being studied to an OD_{600} of 1.0. This cell suspension was then centrifuged at 13,000 x *g* for 5 min at 24ºC. Five hundred microliters of supernatant was removed, and cell pellets were digested with 3N HNO_3 for 24 hours at room temperature. Sodium and potassium concentrations in cell digests and supernatants were analyzed by flame photometry. Corrections were made for extracellular contamination of the cell pellet by Na^+ and K^+. The sodium motive force, ΔpNa^+, was calculated from the equation 59 x log $([Na^+]_{in}/[Na_+]_{out})$, where in and out refer to the concentration of Na^+ inside and outside the cell, respectively.

Results and Discussion

Effect of external pH and metabolic inhibitors on the growth of *Natranaerobius thermophilus* in batch culture. *Natranaerobius thermophilus* is able to grow in the $pH^{55ºC}$ range of 8.3 – 10.6, with no growth at $pH^{55ºC}$ 8.2 or below nor at $pH^{55ºC}$ 10.8 or above (Figure 5.1A)(Mesbah *et al.*, 2007). The main organic fermentation products from 20 mM sucrose at $pH^{55ºC}$ 9.5, 53ºC and 3.5 M Na^+ were acetate (17 mM) and formate (10 mM), and minor amounts of lactate (2.5 mM). These products indicate mixed acid fermentation via the Embden-Meyerhof pathway. The addition of CCCP (50 μM), monensin (30 μM), valinomycin (10 μM) and nigericin (10 μM) to exponentially growing cells completely arrested growth, indicating that proton, sodium and potassium ion gradients exist and are important for growth under these conditions.

Intracellular pH in *Natranaerobius thermophilus*. To study intracellular pH homeostasis and Δp generation by *N. thermophilus*, the internal pH and membrane potential were determined in energized cell suspensions with the radioactive probes [^{14}C]methylamine (external pH 8.0 – 10.5) and [^{3}H]TPP^+, with corrections for non-specific binding. The energized cell suspensions were prepared from cultures in mid-exponential phase (grown at $pH^{55ºC}$ 9.5, 53ºC), and resuspended in carbonate-buffered medium adjusted to the experimental pH being tested. *N. thermophilus* showed the unusual feature of maintaining a ΔpH value of ~ 1 unit rather than cytoplasmic pH homeostasis. As the external pH was increased from $pH^{55ºC}$ 8.0 to 10.5, the intracellular pH increased from 7.2 to 9.9 (Figure 5.1B). The membrane potential (ΔΨ) increased from -99 mV at $pH^{55ºC}$ 8.0 to -138 mV at $pH^{55ºC}$ 10.0 (Figure 5.1C). The Δp increased from -50 mV at $pH^{55ºC}$ 8.0 to -89 mV at $pH^{55ºC}$ (Figure 5.1D). This occurred mainly due to an increase in

the membrane potential; the value for the ZΔpH only fluctuated by ~ 15 mV over the external pH range studied (Figure 5.1C).

The ΔpH values for *N. thermophilus* (~ 1 pH unit) were less than those reported for the anaerobic alkalithermophile *Clostridium paradoxum* (maximum ΔpH of 1.3 units)(Cook *et al.*, 1996). The ΔpH remained constant over the whole pH range tested and did not collapse even near the upper and lower pH boundaries for growth of *N. thermophilus*. In *C. paradoxum*, the ΔpH reached a maximal value at the optimal extracellular pH for growth and decreased at both higher and lower extracellular pH values until it collapsed at pH 7.0 and 10.8 (Cook *et al.*, 1996).

The increase in the Δp at super-optimal external pH values correlated with a decrease in growth rate and cell densites, thereby contradicting current hypotheses that optimal growth occurs at the largest value for the Δp. The Δp is a sum of the ΔpH across the membrane and the membrane potential. The ΔpH across the membrane remained nearly constant over the whole pH range tested, whereas the membrane potential increased (Figure 5.1 C). The increase in the value of the proton motive force mirrors that of the increase in ΔΨ.

It appears from the above results that growth inhibition of *N. thermophilus* as external $pH^{55°C}$ raises above 9.5 is not due to collapse of ΔpH, as has been reported for *Clostridium paradoxum*. It should be noted that the intracellular pH of *N. thermophilus* rises to > 8.8 as the external $pH^{55°C}$ value increases beyond the optimum of 9.5, and reaches a value of 9.9 at external $pH^{55°C}$ of 10.5. This is the highest value for intracellular pH reported thus far. Thus, it is likely that growth inhibition of *N. thermophilus* at alkaline pH is due the inability of the intracellular machinery to adapt to alkaline conditions.

Effect of metabolic inhibitors on intracellular pH of *Natranaerobius thermophilus*. To elucidate the mechanism of cytoplasm acidification in *N. thermophilus*, the effects of various protonophores and ionophores on intracellular pH were tested. Intracellular pH of *N. thermophilus* was increased almost to that of the external medium when energized cell suspensions were treated with the protonophore TCS, the sodium ionophore monensin and the potassium ion ionophore nigericin (Figure 5.2). This indicates that both Na^+ and K^+ ions are involved in intracellular pH acidification. Fluorescence-based antiport assays with inverted membrane vesicles prepared from cells of *N. thermophilus* did indeed show strong Na^+/H^+ and K^+/H^+ antiport activity. The centrality of monovalent cation/proton antiporters in cytoplasmic pH regulation and homeostasis has long been recognized (Padan *et al.*, 2005). However, as shown below, *N. thermophilus* accumulates K^+ inside the cytoplasm, where it functions as an osmoregulatory solute during growth in 3.2 M Na^+. Expulsion of K^+ ions from the cytoplasm by a K^+/H^+ antiporter seems to counteract this effect. The halophilic *Halomonas elongata* and halotolerant *Vibrio alginolyticus* have several active K^+ uptake systems (Kraegeloh *et al.*, 2005, Nakamura *et al.*, 1998). Thus, it follows that presence of similar K^+ uptake systems in *N. thermophilus* could function to replenish the cytoplasmic K^+ pool that is lost by K^+/H^+ antiport.

Phosphorylation potential and proton stoichiometry of ATP synthesis in *Natranaerobius thermophilus*. The ATP and ADP content, phosphorylation potential and H^+ stoichiometry of the ATP synthase (phosphorylation potential/proton motive force) were determined in energized cell suspensions at external $pH^{55°C}$ values 8.0-10.2. The intracellular ATP content of the energized cell suspensions increased from 7 mM at $pH^{55°C}$ 8.0 to 19 mM at $pH^{55°C}$ 9.5. The intracellular ADP concentration remained below 4.5 mM. The inorganic phosphate content was 15 mM at

$pH^{55°C}$ 8.0 and increased to 49 mM at $pH^{55°C}$ 9.5 (Figure 5.3). The phosphorylation potential of *N. thermophilus* was maintained between -477 and -491 mV over external $pH^{55°C}$ values 8.0-10.2.

To substantiate the above calculated values, the measurements were repeated in cultures of *N. thermophilus* growing at the optimum $pH^{55°C}$ of 9.5. The intracellular ATP content under these conditions was 4 mM, and the intracellular ADP concentration was 1 mM. However, the calculated phosphorylation potential under these conditions was -468 mV, and was not significantly different from that calculated in energized suspensions, indicating that the ratio of ATP:ADP is the same. The higher concentrations obtained for energized cell suspensions could be due to rapid turnover of ATP as has been observed for other anaerobes (Cook & Russell, 1994).

The proton stoichiometry of the ATP synthase decreased from 10.2 to 5.5 over the pH range tested. This decrease was primarily due to the increase in calculated proton motive force at higher pH values.

The calculated values of ATP, ADP and phosphorylation potential are similar to those reported for other alkalithermophiles and neutrophiles, indicating that ATP synthesis is not affected by the suboptimal proton motive force. *Natranaerobius thermophilus* is an obligate anaerobe and is not capable of growth even at 0.2 % vol/vol oxygen. Given the large value calculated for phosphorylation potential, it appears that most of the cellular ATP is synthesized at the level of the cytoplasm via substrate level phosphorylation.

Intracellular concentrations of sodium and potassium in *Natranaerobius thermophilus*. Cells from exponentially growing cultures had an intracellular K^+ concentration of 250 mM (extracellular K^+ concentration was 8.4 mM) and intracellular Na^+ of 8.0 mM (extracellular Na^+

was 3.2 M). The intracellular K^+ concentration increased from 208 mM at $pH^{55°C}$ 8.0 to 299 mM at $pH^{55°C}$ 10.2 in energized cell suspensions. The intracellular Na^+ concentration under these conditions remained below 9 mM. A ΔpNa^+ of -172 mV was calculated for actively growing cells at the optimum growth conditions. This large ΔpNa^+ is used to drive various cellular and metabolic processes. Initial data showed that uptake of the amino acid L-arginine is dependent on the presence of a sodium gradient, and was completely abolished in the presence of the Na^+ ionophore monensin (**Appendix B**).

Accumulation of K^+ in the cytoplasm is consistent with the halophilic nature of *N. thermophilus*. However, the highest intracellular K^+ concentration measured of 299 mM (at $pH^{55°C}$ 10.2) was not sufficient to osmotically balance the 3.2 M of Na^+ present outside the cell. Halophiles adapt to the high osmotic pressure exerted by their hypersaline environments in one of two ways: (1) the "salt-in" strategy, where cells accumulate K^+ and Cl^- ions to maintain osmotic balance, or the (2) "salt-out" strategy, where cells exclude salts from the cytoplasm as much as possible and accumulate low molecular weight organic compatible solutes to provide osmotic balance (Oren, 2008). Among the studied halophiles, both aerobic and anaerobic, only four aerobic members of the archaeal order *Halobacteriales* have been reported to use a combination of the "salt-in" and "salt-out" strategies. The closest phylogenetic relatives to *N. thermophilus*, members of the bacterial order *Halanaerobiales*, use the "salt-in" strategy for osmotic adaptation (Oren, 2008).

It should be noted that *N. thermophilus* compensates for its reversed ΔpH by maintaining a large membrane potential, positive out, relatively negative in, which increases as the extracellular pH increases (Figure 5.1C). Accumulation of a large concentration of K^+ inside the cytoplasm would decrease the membrane potential. Thus it follows that *N. thermophilus* must

use a combination of both "salt-in" and "salt-out" strategies, and does not accumulate iso-osmotic concentrations of K^+ to avoid a drastic decline in the overall value of the $\Delta\psi$.

Conclusions. This study has shown that the anaerobic halophilic alkalithermophile *Natranaerobius thermophilus*, like all other alkaliphiles, maintains an inverted pH gradient and has a suboptimal proton motive force. Despite these challenges, *N. thermophilus* maintains an optimal ATP/ADP ratio and ΔGp. *N. thermophilus* is capable of acidifying its cytoplasm, and showed the unusual feature of maintaining a ΔpH value of ~ 1 unit which does not vary over the whole extracellular pH range of 8.0-10.5. In contrast to the anaerobic alkalithermophile *Clostridium paradoxum*, the ΔpH does not collapse even at the upper and lower pH values for growth. These results indicate that failure of growth of *N. thermophilus* at pH values greater than 10.6 is not due to abolition of ΔpH, but rather due to over-alkalinization of the cytoplasm ($pH^{55ºC}$ > 9.9). *N. thermophilus* maintains a large inwardly directed ΔpNa^+, which is used for driving cellular processes such as solute transport. Despite the high salinity of its growth medium, *N. thermophilus* excludes Na^+ from its cytoplasm, and accumulates moderate amounts of K^+ which are used for both cytoplasmic acidification and to achieve a high osmotic pressure in the cytoplasm.

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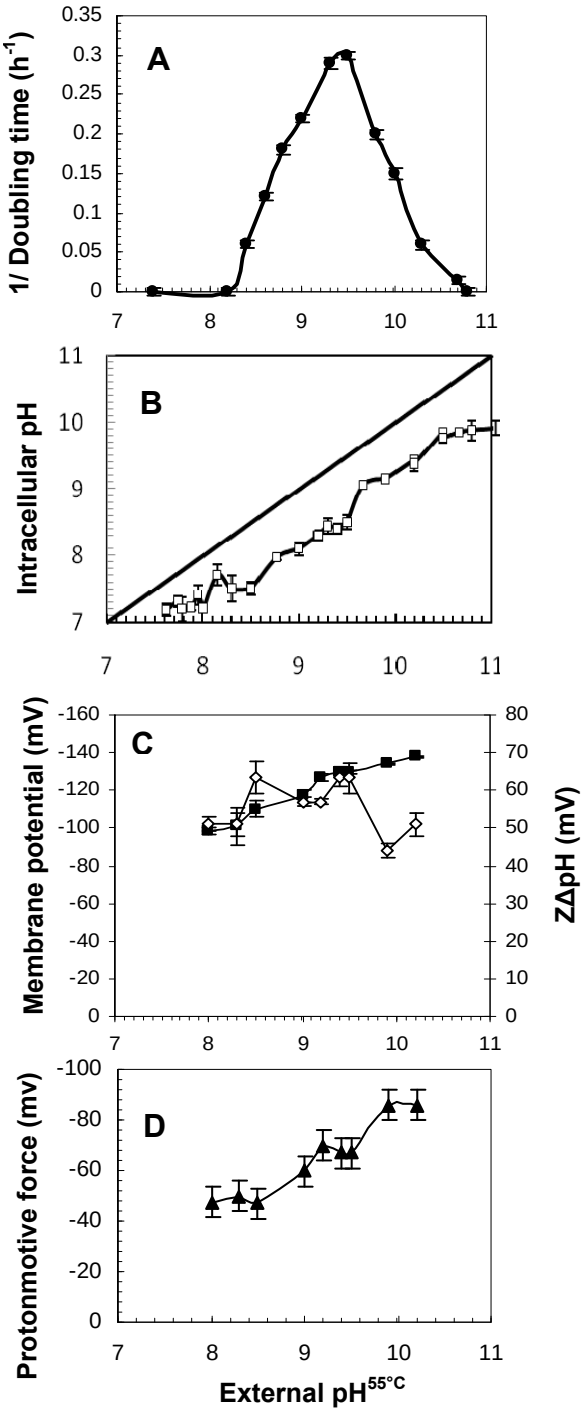

Figure 5.1. (A) Effect of external pH on growth of *N. thermophilus* in batch culture. The effect of external pH on (B) internal pH, (C) ΔΨ (■) and ZΔpH (○), and (D) proton motive force (▲). Values reported are the mean of three independent experiments; the standard error associated with the determinations is shown.

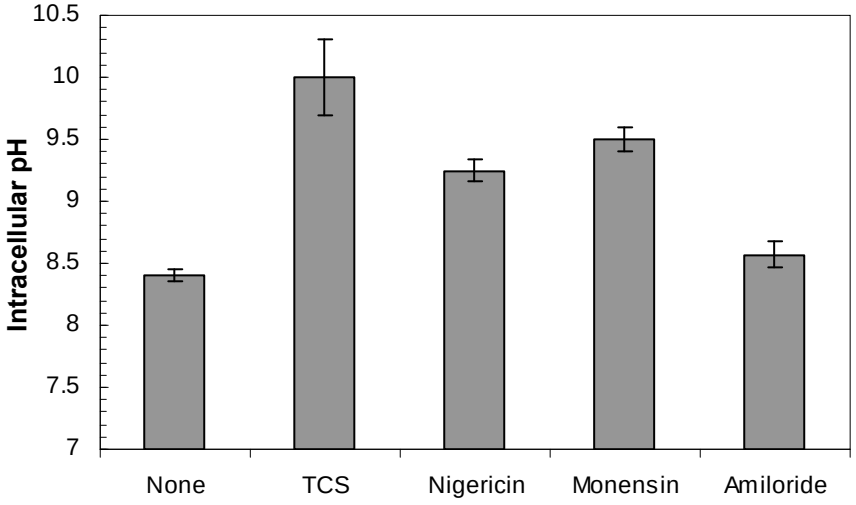

Figure 5.2. Effect on inhibitors on intracellular pH. Values reported are the mean of three independent experiments; the standard error associated with the determinations is shown. The values for intracellular pH were determined at an extracellular $pH^{55°C}$ of 9.5.

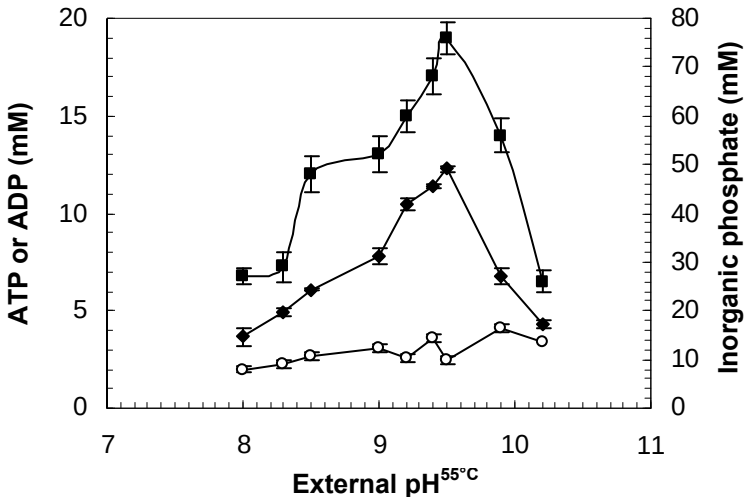

Figure 5.3. Effect of external pH on internal ATP (■), internal ADP (○), and inorganic phosphate (♦) in energized cell suspensions of *N. thermophilus*. Values reported are the mean of three independent experiments; the standard error associated with the determinations is shown.

CHAPTER 6

ACTIVITY PROFILES OF NA^+ (K^+)/H^+ ANTIPORTERS FROM THE HALOPHILIC, ALKALITHERMOPHILIC *NATRANAEROBIUS THERMOPHILUS* ARE ADAPTIVE TO THE EXTREME ENVIRONMENT[*]

[*] Mesbah, N.M. and J. Wiegel. To be submitted to *Proceedings of the National Academy of Sciences*.

Abstract

Natranaerobius thermophilus has twelve genes encoding for putative cation/ proton antiporters in its completed genome sequence. The deduced amino acid sequences of eight of these genes are homologous to antiporters of the NhaC family; the remaining four are homologous to the cation proton antiporter-1 and -2 families. We have successfully expressed eight of these twelve antiporter genes in *Escherichia coli* KNabc, a strain deficient in antiport activity. Inverted membrane vesicles prepared from *E. coli* KNabc harboring each individual antiporter protein exhibited Na^+ (Li^+) (K^+)/ H^+ antiport activity. One antiporter protein, Nt-NhaC3 showed exclusive K^+/H^+ antiport activity. Kinetic analysis of antiport activity revealed $K_{0.5}$ values for Na^+ that varied between 1.5 and 8.7 mM; indicating that active cytoplasm acidification is possible in *N. thermophilus* at both low and high concentrations of intracellular Na^+. All antiporters had alkaline pH optima between 8.5 and 8.8, which correlate with the intracellular pH of *Natranaerobius thermophilus* when grown at optimal conditions. The pH range over which antiporters were active is consistent with the intracellular pH profile of *Natranaerobius thermophilus*, indicating that active cytoplasmic acidification can occur over the whole pH range for growth. Using a fluorescent probe of the transmembrane electrical potential ($\Delta\psi$), cation/ proton antiport was shown to be $\Delta\psi$-consuming, from which it is inferred to be electrogenic. Collectively, the activity profiles of the antiporters correlate well with the unique intracellular conditions present in the halophilic, alkaliphilic, thermophilic *Natranaerobius thermophilus*.

Introduction

Alkaliphiles must maintain a cytoplasmic pH that is compatible with the functional and structural integrity of the cytoplasmic proteins that support growth. Alkaliphiles generally

maintain their intracellular pH between 7.8-9.8 at external pH values between 7.5 and 10.5 (Cook *et al.*, 1996, Ito *et al.*, 1997, Olsson *et al.*, 2003, Padan *et al.*, 2005). Thus, they are able to acidify the cytoplasm relative to the external milieu. Strategies involved in alkali-tolerance and alkaliphily include (i) increased metabolic acid production through sugar fermentation; (ii) changes in cell surface i.e. cell wall and cell membrane properties; (iii) increased ATP synthase activity that couples ATP synthesis to H^+ entry to the cell; and (iv) increased expression and activity of monovalent cation proton antiporters. Among these strategies, antiporters play a dominant role in cytoplasmic pH homeostasis in many bacteria (Padan *et al.*, 2005).

Microorganisms generally maintain an intracellular Na^+ concentration lower than the extracellular concentration. Na^+ stress is caused by an increase in the cytoplasmic concentration of Na^+, and this concentration varies over a wide range; from 20 mM in *Escherichia coli* to 3 M in the extremely halophilic archaea of the order *Halobacteriales* (Padan & Krulwich, 2000). Specific targets of Na^+-toxicity in bacteria have not been identified. Prokaryotic cells protect themselves from the adverse effects of Na^+ by primary and secondary Na^+ extrusion systems. Secondary Na^+/H^+ antiporters play a major role in Na^+-resistance as assessed by mutational disruption of antiporter genes (Padan & Krulwich, 2000, Padan *et al.*, 1989).

Monovalent cation/H^+ antiporters of bacteria extrude cytoplasmic monovalent cations in exchange for protons from the outside medium (Padan *et al.*, 2005). The exchange is driven by the transmembrane electric potential ($\Delta\psi$, negative inside relative to outside). Monovalent cation/H^+ antiporters that are energized in this way are found in several different families and superfamilies within the sequence-based transporter classification (TC) system (Busch & Saier, 2002), and they are assigned TC numbers (www.tcdb.org).

Most microorganisms have multiple monovalent cation/H^+ antiporters, often with several paralogues from the same antiporter family, in addition to antiporters from different families (Padan *et al.*, 2005). The precise role of each individual antiporter within any single microorganism has yet to be determined, but physiological roles for some cation/H^+ antiporters have been established. Antiporters play a major role in alkalitolerance, acidify the cytoplasm, protect bacterial cells from the cytotoxic effects of Na^+, and can also create an inwardly directed Na^+ gradient that can energize solute transport, motility and ATP synthesis via Na^+-coupled ATP synthase.

The role of cation/H^+ antiporters in cytoplasmic acidification under alkaline conditions was first demonstrated in extremely alkaliphilic bacteria, in which Na^+/H^+ antiporters are specifically required for maintenance of a cytoplasmic pH 1.0-2.5 units below that of the external medium (Kitada *et al.*, 2000, Krulwich *et al.*, 1996). In contrast, pH homeostasis in neutrophilic bacteria is achieved via both Na^+/H^+ and K^+/H^+ antiporters (Lewinson *et al.*, 2004, Radchenko *et al.*, 2006). Although the physiological role of Na^+/H^+ antiporters in aerobic alkaliphiles is well recognized (Padan *et al.*, 2005), identification and characterization of specific alkaliphile antiporter proteins has been focused on aerobic, mesophilic alkaliphilic *Bacillus* spp. Recently, putative cation/H^+ antiporters from the aerobic, mesophilic, haloalkaliphilic *Alkalimonas amylolytica* have been characterized (Liu *et al.*, 2005, Wei *et al.*, 2007). *A. amylolytica* possesses NhaD, CaCA, CPA2 and CPA1-type antiporters that confer modest Na^+ and alkali resistance onto the triple antiporter mutant strain of *E. coli*, KNabc. These studies also suggested the role of a K^+ (NH_4^+)/ H^+ antiporter that functions to extrude excess NH_4^+ from the cytoplasm during growth on amine-rich media.

Aerobic alkaliphilic bacilli studied have been shown to generate large ΔpH values across the cytoplasmic membrane (1.9-2.5 $pH^{25ºC}$ units, acidic inside) and are capable of maintaining this large ΔpH over broad external $pH^{25ºC}$ ranges (7.5-10.8 in *Bacillus pseudofirmus* OF4, (Sturr *et al.*, 1994). On the contrary, the anaerobic, alkalithermophilic *Clostridium paradoxum* generates a much smaller ΔpH (1.3 pH units) and can only maintain it over the much narrower $pH^{55ºC}$ range of 9.0-10.0 (Cook *et al.*, 1996). *Clostridium paradoxum*, as well as the anaerobic *Caloramator fervidus* and *Anaerobranca gottschalkii*, have all been shown to use Na^+ translocating ATPases for generation of sodium gradients across the cell membrane (Ferguson *et al.*, 2006, Prowe *et al.*, 1996, Speelmans *et al.*, 1993). If anaerobic alkaliphiles do use Na^+/H^+ antiporters for cytoplasmic acidification, the operation of these antiporters must be congruent with the Na^+-pumping activity of the ATPase as they both will be competing for the same substrate, which is intracellular Na^+. The possibility exists that intracellular pH in anaerobic alkaliphiles could be regulated by K^+/H^+ antiporters; however this will result in depletion of intracellular K^+, which is necessary for osmotic balance.

In this study, we present characterization of cation/H^+ antiporters from an anaerobic halophilic, alkalithermophilic bacterium. *Natranaerobius thermophilus* is a Gram-type positive bacterium of the *Firmicutes* that was recently isolated from sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah *et al.*, 2007). It is obligately anaerobic with a fermentative metabolism and grows at Na^+ ion concentrations between 3.1 and 4.9 M (optimal growth at 3.3-3.9 M Na^+) and at $pH^{55ºC}$ values between 8.3 and 10.6 (optimal growth at $pH^{55ºC}$ 9.5). Of the twelve putative cation/H^+ antiporters identified in the genome sequence of *Natranaerobius thermophilus*, eight have been functionally expressed in the triple antiporter deficient *E. coli* KNabc. Various aspects of the biological activity of these antiporters were

characterized, and their roles in physiology of growth at high Na^+ concentration and pH are discussed.

Materials and Methods

Bacterial strains and culture conditions. Strains used in this study are shown in Table 6.1. *Natranaerobius thermophilus* strain JW/NM-WN-LF^T was grown anaerobically at 52ºC in carbonate-buffered medium as described previously (Mesbah *et al.*, 2007). *E. coli* KNabc was grown routinely in LBK medium, pH 7.5 (Goldberg *et al.*, 1987) at 37ºC. *E. coli* JM109 was used for routine cloning procedures and was grown in LB medium. When antibiotics were added to the medium for selection or plasmid maintenance, concentrations used were 100 µg ampicillin mL^{-1}, 50 µg kanamycin mL^{-1} and 50 µg erythromycin mL^{-1}.

Genome sequencing and identification of transporter protein genes. The genome of *Natranaerobius thermophilus* strain JW/NM-WN-LF^T was sequenced by the random shot-gun method (see www.jgi.doe.gov for details of library construction and sequencing). The genome sequence of *Natranaerobius thermophilus* strain JW/NM-WN-LF^T is available at GenBank with accession number NC_010718. Complete predicted protein sequences were searched against a curated set of proteins with family assignment for similarity to known or putative transporter proteins and against a nonredundant general protein database using a semiautomated pipeline (Ren *et al.*, 2007). Manual annotation for final assignments was based on the number of hits to the transporter database, maximum, minimum, and average BLAST E values and cluster of orthologous group (COG) assignments. The annotated antiporter genes used in this study are shown in Table 6.2.

DNA extraction, cloning and plasmids. High molecular weight genomic DNA was isolated from *N. thermophilus* cells by the SDS/phenol/chloroform extraction method as previously described (Wilson, 1997). The twelve predicted antiporter genes and their native Shine-Dalgarno sequences were cloned behind the T7 promoter of pGEM-3Zf(+). The PCR primers listed in Table 6.1 were used to amplify the genes from *N. thermophilus* genomic DNA. The PCR reactions used 1 unit of Phusion^{TM} High-Fidelity DNA polymerase (New England Biolabs), 0.5 μM each of forward and reverse primer and ~ 50 of ng genomic DNA. The PCR cycling conditions consisted of an initial denaturation at 98ºC for 30 secs, followed by 25 cycles of: denaturation (98ºC for 10 secs), annealing (66ºC for 30 secs for *nt-NhaC1, nt-NhaC7, nt-NhaC8* and *nt-NhaC4*, 62ºC for 30 secs for remaining 8 genes), extension (72ºC for 45 secs), then a final extension at 72ºC for 5 minutes. The PCR products were purified, digested with *Bam*HI and *Xba*I *(nt-NhaC8* was digested with *Bam*HI and *Sal*I), and ligated into *Bam*HI- and *Xba*I (*Sal*1)-digested pGEM-3Zf(+) using T4 DNA Ligase (New England Biolabs). For all plasmid selections, blue-white screenings in *E.coli* JM109 were performed and complete DNA sequencing was used to ensure that the plasmids ultimately used were free of errors. The plasmids were transformed into *E. coli* KNabc by electroporation.

Complementation assays in *E. coli* KNabc. All complementation assays were done under anaerobic conditions. Recombinant plasmids were transformed into $Na^+(K^+)$ $(Ca^{2+})/ H^+$ antiporter-deficient *E.coli* KNabc. The plasmid pGEM-3Zf(+) was used as a negative control. For studies of complementation of the Na^+- and alkali-sensitive phenotypes of *E.coli* KNabc, the test and control transformants were cultured overnight in LBK medium, pH 7.5. Two hundred microliters of the overnight grown cultures were transferred into 5 mL of LBK adjusted to

different pH values with different NaCl concentrations. Cell growth was monitored by measuring optical density at 600 nm.

Preparation of inverted membrane vesicles. Inverted membrane vesicles were prepared from *E. coli* KNabc transformants in late-exponential growth phase. For cation/proton antiport assays, cell pellets from 1.5 L of culture were washed 3 times with 30 mL of buffer containing 10 mM Tris-chloride (pH 8.0), 140 mM choline chloride, 0.5 mM dithiothreitol and 10% (vol/vol) glycerol. Cells were then resuspended in 20 mL of membrane buffer (100 mM Tris-chloride [pH8.0], 140 mM choline chloride, 15 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10% glycerol [vol/vol]). DNase I (2 mg) was added and the cell suspension was shaken briefly. The following steps were performed at 4ºC: cell suspensions were disrupted by passage three times through a pre-cooled French pressure cell at 10,000 psi. Unbroken cell material was removed by two low speed centrifugations (8,000 x *g* for 10 mins), and the membranes were collected by ultracentrifugation at 250,000 x *g* for 1 hour. Membranes were washed in membrane buffer and centrifuged as described above. Washed membranes were resuspended in 1.5 mL of membrane buffer, and then were shock frozen in a slurry of dry ice and 100% ethanol and stored at -80ºC. Vesicles used in assays of membrane potential ($\Delta\psi$) generation were prepared in a similar way, but were washed and resuspended in membrane buffer composed of 10 mM bis-[tris(hydroxymethyl)-methylamino]-propane (BTP), pH 8.0, 10% glycerol and 1 mM PMSF. The final concentration was 30-50 mg protein/mL. Protein concentration was determined by the Bicinchoninic acid (BCA) assay using bovine serum albumin as standard.

Assays of ΔpH-dependent antiport activity in inverted membrane vesicles. Antiport assays were conducted in 10 mM Tris-Cl, 140 mM choline chloride, 15 mM $MgCl_2$, 0.75 μM acridine

orange and 500 µg protein/mL. The pH was adjusted to values between 7.5 and 10.0. Measurements were conducted using a Turner Designs TD-700 laboratory fluorometer with peak excitation 486 nm and peak emission 510 nm. Respiration was initiated by addition of Trizma-succinate (Sigma-Aldrich) to a final concentration of 2.5 mM. The high chloride content of the buffer ensured that the Δp established upon addition of an electron donor is entirely due to a ΔpH, acidic inside the inverted membrane vesicles (Rosen & Futai, 1980). Establishment of the ΔpH was monitored by quenching of the fluorescence of acridine orange. The dequenching of fluorescence that results from cation addition reflects cation-dependent proton movement out of the inverted membrane vesicles (Goldberg *et al.*, 1987). At the end of the assay, 12 mM of ammonium chloride was added to dissipate the remaining Δp and bring fluorescence back to baseline. All assays were performed under anaerobic conditions at room temperature.

Fluorescence-based assays of Δψ generation and antiporter dependent consumption. Δψ-dependent fluorescence of oxonol VI was used to measure the generation of a membrane potential, positive inside, with the addition of 0.6 mM of tetra(cyclohexylammonium)-NADPH. Electrogenicity of antiporter genes was evaluated by adding 4 mM NaCl to energized membranes and observing a reversal of the quench. Reversal of quenching represents antiporter-dependent consumption of Δψ, which reflects activity of an electrogenic antiporter (Padan *et al.*, 2005). Fluorescence was brought back to baseline by adding of 10 µM of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore that abolishes the Δψ. The assay mixture contained 10 mM BTP, 5 mM $MgSO_4$, 200 mM K_2SO_4, 1 µM nigericin, and 1 µM oxonol VI (pH 8.0-9.5). Measurements were made on a Turner Designs TD-700 laboratory fluorometer. The peak excitation and emission wavelengths were 523 nM and 630 nm, respectively. Final concentration of vesicle protein was 500 µg/mL.

Results

Identification of antiporter proteins in genome of *Natranaerobius thermophilus* JW/NM-WN-LF^T. The genome sequence of *N. thermophilus* was analyzed for transport systems using a semi-automated pipeline (Ren *et al.*, 2007). Twelve genes with significant homology to Na^+/H^+ exchangers were identified by these analyses (Table 6.2). Eight genes had closest homology to members of the NhaC Na^+/H^+ antiporter family (Saier *et al.*, 2006). Three genes were 38-48% identical to Na^+/H^+ exchanger proteins from '*Alkaliphilus metalliredigens*' and '*A. oremlandii'*, two obligately anaerobic, non-halophilic and moderately alkaliphilic bacteria (Fisher *et al.*, 2008, Ye *et al.*, 2004). Gene *nt-Nha* had 35% identity to ShaA (MrPA) gene of *Clostridium tetani*. The Mrp proteins belong to the monovalent cation:proton antiporter-3 protein family. This family is composed of multicomponent Na^+/H^+ and K^+/H^+ antiporters. Mrp antiporter systems are encoded by operons of six or seven genes, and all genes are required for full function in Na^+ and alkali resistance (Ito *et al.*, 2000). Sequence analysis of the regions surrounding gene *nt-Nha*, however, did not show that it was part of an operon.

***N. thermophilus* antiporter genes complement the Na^+ and alkali sensitivities of *E. coli* KNabc**. *E.coli* strain KNabc, is very sensitive to NaCl concentrations of 200 mM and above. Of the twelve cloned antiporter genes, eight were able to complement the Na^+-sensitive phenotype (Figure 6.1A). The KNabc-Nt-NhaC1, Nt-NhaC3 and -Nt-NhaC4 transformants exhibited Na^+ resistance up to 700 mM, while the remaining transformants supported resistance at up to 600 mM NaCl (Figure 6.1B). None of the transformants supported growth at NaCl concentrations of 750 mM or above. The same eight *N. thermophilus* antiporter genes supported modest alkali-resistance allowing growth at pH values up to 8.5 in the absence of added NaCl (Figure 6.1C).

***N. thermophilus* antiporters catalyze Na^+ (Li^+)/H^+ and K^+/H^+ antiport with high apparent $K_{0.5}$ for cations and alkaline pH optima.** Assays were performed to characterize the antiport properties of chromosomally encoded *N. thermophilus* antiporter proteins. Assays were first performed to characterize the cation specificity of antiport. Initial experiments were conducted at pH 8.5 in order to simulate the intracellular pH measured in energized cell suspensions of *N. thermophilus* (**Chapter 5**). With the exception of Nt-NhaC3, all *N. thermophilus* antiporter proteins exhibited significant Na^+-dependent antiport, with a dequenching percentage as high as 77.3% for NT-CPA1a (Table 6.3). Strong Li^+-dependent antiport was also observed, albeit less than that observed for Na^+. A K^+/H^+ antiport activity was observed for all 8 of the antiporter genes in concentration ranges between 3 and 31 mM K^+. Nt-NhaC3 was the sole protein that exhibited only K^+/H^+ antiport activity, and did not show any Na^+- or Li^+- dependent activities using a range of Na^+ or Li^+ concentrations from 2 to 100 mM (Table 6.3).

The antiport activity was examined over a range of NaCl concentrations. In contrast to the Michealis-Menten kinetics that have been reported for $Na^+(Li^+)/ H^+$ antiporters from Gram-type positive alkaliphiles, non-linear kinetics were observed for all the antiporter proteins studied. Antiport activity was inhibited for all proteins at high concentrations of the substrate. The NaCl concentration optima varied widely between the 7 Na^+-pumping antiporter proteins, and ranged between 1.5 - 8.7 mM. The K^+ optimum for the K^+/H^+ specific Nt-NhaC3, was larger, at 31 mM. Apparent $K_{0.5}$ values for the antiporter proteins were estimated by linear regression from Hill plots (Table 6.4). These calculated $K_{0.5}$ values are among the highest $K_{0.5}$ values to be reported thus far. The intracellular Na^+ concentration of *N. thermophilus* was measured to be between 7-9 mM. Thus the large estimated $K_{0.5}$ values for the antiporters allow them to function in cytoplasmic acidification at relatively high intracellular Na^+ concentrations.

The activity profiles of *N. thermophilus* antiporters as a function of pH indicated alkaline pH optima between 8.5 and 8.8 (Table 6.5). Activity was greatly reduced at pH 7.8 and no activity was detected at pH 7.6 or below. This is coherent with the intracellular pH values of *N. thermophilus*, which were measured to be between 8.3-8.8 at the optimal external $pH^{55^{o}C}$ for growth (pH 9.3-9.7). The pH profile for NT-NhaC2 is distinguished from the remaining antiporters in that it has an optimum at pH 9.5 and retained significant activity at pH 10.0. We were unable to test higher pH values due to the limitations of the heterologous *E. coli* system, and we do not know of any comparable multiple antiporter systems in alkaliphiles which could be used for the assays at higher pH values. Analysis of the intracellular pH profile for *N. thermophilus* showed that the intracellular pH increased from 7.2 to 9.9 over the external pH range 8-10.5 (**Chapter 5**). Thus the presence of an antiporter with an alkaline pH optimum of 9.5 could account for the ability of *N. thermophilus* to acidify its cytoplasm even at the upper end of its pH growth range.

Cation/proton antiport by *N. thermophilus* genes is electrogenic. When cation/proton antiporters are used for pH homeostasis, the antiport needs to be electrogenic rather than electroneutral (Padan *et al.*, 2005). During electrogenic antiport, the inward H^+ flux is larger than outward Na^+ flux during a single turnover, such that net positive charge is translocated into the cell. Thus, the antiport can be energized by the transmembrane electrical potential ($\Delta\psi$, positive outside, relatively negative inside in whole cells). As a result, antiport dissipates the $\Delta\psi$. Upon addition of NADPH, the anionic fluorescent probe Oxonol VI accumulates inside the inverted membrane vesicle upon development of a $\Delta\psi$, positive inside the vesicle system. The accumulation results in quenching of the probe fluorescence. Addition of nigericin to the assay

buffer prevents development of a ΔpH across the membrane. After the Δψ reaches steady state, NaCl or KCl are added, reversal of the quench indicates consumption of the Δψ, i.e. electrogenic antiport. Electrogenic antiport was observed in membrane vesicles containing the *N. thermophilus* antiporter genes (Figure 6.2). Interestingly, the membrane vesicle containing Nt-NhaC1 did not show a complete reversal of the quench on addition of NaCl. This could be due to a different stoichiometry of Na^+/H^+ exchange.

Discussion

Homologs of prokaryotic Na^+/H^+ antiporters in *Natranaerobius thermophilus*. Of the twelve genes that have been identified as homologs to antiporters in the genome sequence of *N. thermophilus*, eight of them have strong sequence identity to the Na^+/H^+ antiporter NhaC. NhaC was first described in *Bacillus pseudofirmus* OF4 (Ito *et al.*, 1997). In this bacterium, NhaC is one of the relatively high affinity Na^+/H^+ antiporters; its primary role is to extrude Na^+ from the cytoplasm at pH 7.5-9.0. It also plays a role in cytoplasmic pH homeostasis at pH 10.5. It has no required role in alkaliphily; a mutant strain of *B. psuedofirmus* in which *nhaC* is deleted shows only a modest growth defect at pH 10.5 and a slightly reduced capacity to acidify the cytoplasm at Na^+ concentrations less than 10 mM. It is hypothesized that the major role of NhaC in this bacterium is to extrude Na^+ and impart initial protection in the face of a sudden increase in external pH prior to full induction of additional antiporters (Ito *et al.*, 1997). It also has been reported that in the presence of a complement of antiporters, NhaC plays a role over a wide range of pH values (Ito *et al.*, 1997). This seems to be the case in *N. thermophilus*, the four NhaC antiporter genes that were expressed in inverted membrane vesicles all showed activity between pH 8.3 and 10.0. It is unclear however, whether they play a major role in pH resistance, salt

resistance, or both. Solute transport in *N. thermophilus* is Na^+ coupled (as shown for the amino acid L-Arginine, see **Appendix B**). In addition, the high extracellular Na^+ concentration (3.3-3.9 M) increases membrane permeability to Na^+ and some Na^+ will inevitably diffuse into the cell. Thus, *N. thermophilus* must have efficient means of extruding intracellular Na^+.

The four remaining genes had homology to members of the monovalent CPA1 and CPA2 families. Both these families contain transporters for which antiporter activity has been experimentally demonstated; e.g. Aa-NhaP, the K^+ (NH_4^+)/ H^+ antiporter of *Alkalimonas amylolytica* (CPA1), Vp-NhaP2, an exclusive K^+/H^+ antiporter from *Vibrio parahaemolyticus* (CPA1), the Na^+/H^+ antiporter NapA from *Enterococcus hirae* (CPA2), and the Na^+ (K^+)/ H^+ antiporter GerN from *Bacillus cereus*. All these antiporter play roles in cytoplasmic pH regulation, extrusion of intracellular Na^+ and cell volume regulation (Radchenko *et al.*, 2006, Southworth *et al.*, 2001, Waser *et al.*, 1992, Wei *et al.*, 2007).

Functional expression of *N. thermophilus* antiporter genes in *E. coli* under control of the T7 promoter. Over-expression of proteins with multiple transmembrane segments is expected to have a detrimental effect on the host cells. For example, the *E. coli* NhaA antiporter is normally expressed at low levels within the cell (0.2% of total cell proteins). When this protein is overexpressed under control of the inducible *tac* promoter, cell growth ceases (Taglicht *et al.*, 1991). We chose the T7 promoter of the pGEM-3Zf(+) vector as the promoter for expression of the various *N. thermophilus* antiporter genes in *E. coli*. *E. coli* strain KNabc does not produce a T7 RNA polymerase. As a result, this system produces very low levels of expression of membrane transport proteins that are toxic when expressed at higher levels (Southworth *et al.*, 2001).

Expression in *E. coli* KNabc of the *N. thermophilus* antiporter genes resulted in production of functional Na^+ (K^+) (Li^+)/ H^+ antiporters. In contrast, expression of the genes *nt-NhaC8, nt-NhaC7, nt-NhaC6*, and *nt-NhaC5* did not complement either the Na^+ or pH sensitive phenotype of *E. coli* KNabc. This may have been due to insufficient level of expressed protein, or the products of these genes may not be functional in this heterologous system. It is also possible that these proteins do not function as Na^+ extrusion systems, or that expression of these genes, even at low levels, has a detrimental effect on the host cells.

***N. thermophilus* antiporters are well adapted to the intracellular environment**. Nt-NhaC3 showed exclusive K^+/H^+ antiport activity. The only other exclusive K^+/H^+ antiporter found in bacteria is Vp-NhaP2 of *Vibrio parahaemolyticus*, where it is thought to provide protection against adversely high concentrations of K^+ at alkaline pH (Radchenko *et al.*, 2006). *N. thermophilus* has been shown to accumulate 250 mM of K^+ inside the cell (**Chapter 5**). It is hypothesized that accumulation of intracellular K^+ acts in part to osmotically balance the 3.3 M of Na^+ in the growth medium. The presence of a K^+/H^+ seems to counteract this function as it will cause depletion in intracellular K^+. Four homologs of K^+ uptake systems have been identified in the genome sequence of *N. thermophilus*. The products of these genes were able to complement the K^+ sensitive phenotype of the K^+ uptake deficient mutant *E. coli* TK2420 (data not shown). Thus, it follows that *N. thermophilus* possesses K^+ uptake systems that can replenish K^+ lost from the cell.

The remaining seven antiporter proteins showed Na^+ (K^+) (Li^+)/ H^+ antiport activity and functioned over a wide range of NaCl concentrations. The $K_{0.5}$ for Na^+ varied widely; from 1.5 to 8.7 mM (Table 6.4). The presence of both low and high affinity antiporters in *N. thermophilus* is

consistent with the ability of this microorganism to grow over a wide range of extracellular Na^+ concentrations (3.1-4.9 M). The lower affinity antiporters ($K_{0.5} > 3.0$) are able to function at high concentrations of Na^+ ions, similar to those measured in cells of energized suspensions of *N. thermophilus* (8-9 mM when grown in medium with 3.3 M Na^+). The presence of antiporters able to function at large concentrations of Na^+ is consistent with a role under extremely saline conditions where *N. thermophilus* is subject to periodic challenge by cytoplasmic Na^+, and needs antiporters that can function under these circumstances. The high affinity antiporters ($K_{0.5} < 3.0$) are able to function in cytoplasmic acidification even at low Na^+ concentrations. Thus the presence of a complement of antiporter proteins with various $K_{0.5}$ for Na^+ will enable *N. thermophilus* to maintain active cytoplasm acidification even if the intracellular Na^+ concentration changes.

The pH responses of the different antiporters reflect the intracellular pH profile of *N. thermophilus*. *N. thermophilus* shows the unique property of maintaining a ΔpH of 1 unit across the membrane throughout the whole pH range for growth. Over an external $pH^{55ºC}$ range of 8.0 to 10.5, the intracellular pH increased from 7.2 to 9.9. The intracellular pH at the optimal pH for growth ($pH^{55ºC}$ 9.5) was 8.3-8.8. This is consistent with the pH responses of antiporter proteins in inverted membrane vesicles (Table 6.5). With the exception of Nt-NhaC2, all antiporters function optimally at pH values between 8.5 and 8.8, similar to the intracellular pH of *N. thermophilus* when grown at optimal conditions. Antiport activity was detected at pH values as high as 10.0 for Nt-NhaC2, and as low as 7.8 for NT-CPA1b. Thus, in the natural setting of *N. thermophilus*, these antiporters can play a role in cytoplasmic acidification at, above and below the optimal pH for growth.

Dependence of antiporter activity on pH has been reported for the major Na^+/H^+ antiporter of *E. coli* NhaA. NhaA exhibits major pH-dependent changes in antiport activity; activation by alkaline conditions that depends on cytoplasmic pH involves a cytoplasmic loop between transmembrane segments VIII and IX (Padan *et al.*, 2004). A mechanism for participation of this cytoplasmic loop in activation of NhaA by alkaline pH has been suggested by the high-resolution structure of NhaA (Hunte *et al.*, 2005).

Electrogenicity is an important property for Na^+/H^+ antiporters that support alkali resistance by generating a pH gradient in which the cytoplasm is more acidic than the external medium (Padan *et al.*, 2005). All antiporters tested were shown to be electrogenic (Figure 6.2). Nt-NhaC1 was different in that it did not catalyze a complete reversal of the quenching of Oxonol VI. This is most likely due to a different stoichiometry of Na^+/ H^+ exchange, thus not completely consuming the $\Delta\Psi$. Alternatively, this particular protein may not be able to function optimally in the heterologous system used. It is not possible to accurately measure coupling stoichiometries for the antiporter proteins when expressed in inverted membrane vesicles as the phospholipid and heterologous protein complement of the membranes can change the antiporter stoichiometry (Padan *et al.*, 2005).

The aerobic halophilic alkaliphilic *Alkalimonas amylolytica* possesses a $Na^+(Li^+)/ H^+$ antiporter, NhaD, which can support low Na^+/H^+ antiport at pH 9.5 and 600 mM Na^+ (Liu *et al.*, 2005). *A. amylolytica* also possesses CaCA, CPA2 and CPA1-type antiporters that catalyze Na^+/H^+ antiport and possibly K^+ $(NH_4^+)/ H^+$ antiport (Wei *et al.*, 2007). No studies have been published on the ATPase of *A. amylolytica*, however aerobic mesophiles typically use H^+-coupled bioenergetics. Thus, there is no competition for the intracellular Na^+ substrate. On the other hand, the anaerobic alkalithermophiles studied thus far have Na^+-coupled bioenergetics

(Ferguson *et al.*, 2006, Prowe *et al.*, 1996, Speelmans *et al.*, 1993). As a result, Na^+-coupled ATPase will compete with the Na^+/H^+ antiporters for intracellular Na^+. In this case, it is beneficial to have Na^+ (K^+)/ H^+ and K^+/H^+ antiporters. For example, when Na^+/H^+ antiport activity sufficiently reduces the cytoplasmic Na^+ content, K^+/H^+ antiport activity can reduce the cytoplasmic pH further using the outwardly directed K^+ gradient to drive H^+ uptake. As stated above, homologs for K^+ uptake systems are present in the genome of *N. thermophilus*; these supposedly can act to replenish K^+ that is lost from the cytoplasm. These systems are yet to be completely characterized.

A model summarizing the bioenergetic processes presented and discussed in *N. thermophilus* is shown in Figure 6.3. *N. thermophilus* maintains a ΔpH of 1 unit across the cell membrane. The electrogenic cation/proton antiporters provide active cytoplasmic acidification over the whole pH range for growth. Electrogenic Na^+/H^+ antiporters also play a role in extruding cytotoxic Na^+ from the cytoplasm. Transport of solutes into the cell is coupled to Na^+ ions, this forms a route for Na^+ reentry into the cell hence providing the substrate for the action of Na^+/H^+ antiporters. Phylogenetic analysis of the *atp* operon in *N. thermophilus* showed high homology with other Na^+-coupled ATPases. Having Na^+ coupled bioenergetics is beneficial for a halophilic thermophile, as at high temperatures membrane permeability to Na^+ is much less than that of H^+ (Vossenberg *et al.*, 1995). At alkaline pH, the advantage of using Na^+ ions over H^+ is further magnified. The ATP synthase is fuelled by ATP produced in the cytoplasm via substrate level phosphorylation. Na^+-pumping outside the cell magnifies the $\Delta\psi$, which powers cation/ proton antiport, and develops a sodium motive force, which powers uptake of solutes inside the cell.

The presented data and model indicate that the activity profiles of *N. thermophilus* cation/ proton antiporters correlate well with its natural settings and can remain functional under suboptimal conditions for growth of this bacterium. Once genetic tools are available, disruption of individual genes in *N. thermophilus* and studies of mutant forms of the antiporters in their natural settings will be possible to further clarify the individual roles of these antiporters in alkali- and salt-resistance.

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Table 6.1. Bacterial strains, plasmids and oligonucleotides

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence[a]	Source or reference
Natranaerobius thermophilus JW/NM-WN-LF^T	Halophilic, alkalithermophilic, anaerobic, Gram-type positive	(Mesbah *et al.* 2007)
	E. coli strains	
KNabc	Δ*nhaA* Δ*nhaB* Δ*chaA*	(Nozaki *et al.* 1996)
JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15].	Promega
Plasmids		
pGEM-3Zf(+)	Cloning vector, Ap^r	Promega
pNMM1	*nt-CPA1a* (1.2 kb) PCR product (primers NT476/ BF1 and NT476/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM2	*nt-CPA1b* (1.2 kb) PCR product (primers NT1314/ BF1 and NT1314/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM3	*nt-CPA2* (1.2 kb) PCR product (primers NT1320/ BF1 and NT1320/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM4	*nt-Nha* (1.5 kb) PCR product (primers NT1499/ BF1 and NT1499/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM5	*nt-NhaC1* (1.3 kb) PCR product (primer NT1898/ BF1 and NT1898/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM6	*nt-NhaC2* (1.3 kb) PCR product (primer NT2084/ BF1 and NT2084/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM7	*nt-NhaC3* (1.4 kb) PCR product (primer NT2291/ BF1 and NT2291/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM8	*nt-NhaC4* (1.4 kb) PCR product (primer NT2693/ BF1 and NT2693/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM9	*nt-NhaC5* (1.4 kb) PCR product (primer NT855/ BF1 and NT855/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM10	*nt-NhaC6* (1.5 kb) PCR product (primer NT679/ BF1 and NT679/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
pNMM11	*nt-NhaC7* (1.4 kb) PCR product (primer NT1896/ BF1 and NT1896/ XR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study

pNMM12	*nt-NhaC8* (1.5 kb) PCR product (primer NT1910/ BF1 and NT1910/ SR1) in BamHI/ XbaI digested pGEM-3Zf(+)	This study
Oligonucleotides		
NT476/ BF1	CGGCGGATCCGGATTGAGGGGATTTAACTAATGTTGG	This study
NT476/ XR1	GGGGTCTAGACGTTACTCCTCTATGCAATCACAATT	This study
NT1314/ BF1	CCCCGGATCCCCTAGAGGTGACCTTAATTGACAATC	This study
NT1314/ XR1	GGGCTCTAGAGGTTACACCTCCTTAGTTGACG	This study
NT1320/ BF1	CCCCGGATCCGGTTTTTAAGAAAGGGATGATTTACAATG	This study
NT1320/ XR1	GGGCTCTAGAGGTTATTCTATTGAGGAAAGTTCAGCAGT	This study
NT1499/ BF1	GGCCGGATCCCCTAGGAGGTTTTAGTAGTGTCTTTT	This study
NT1499/ XR1	GGGCTCTAGAGGCTAGAAAGGATTACCATT	This study
NT1898/ BF1	CCCGGGATCCCCAGGAAGGAAAAGGAGGTGAAACTA	This study
NT1898/ XR1	CCCCTCTAGACCTTATAGCCGGGAGTAACCCC	This study
NT2084/ BF1	CCCCGGATCCTTATGGATCAAAAAGAAATTAAACCG	This study
NT2084/ XR1	CCCCTCTAGACCCTAAGCGCTATCTTCTGAAGGTTT	This study
NT2291/ BF1	CCCGGGATCCCCATGAAAAATGAAATTGACAAGGCT	This study
NT2291/ XR1	GGGCTCTAGAGGTTACCCTTCAATAGATTCTTCTTGTTC	This study
NT2693/ BF1	CCCCGGATCCCCATAGAAGGAGGTTTCAATATGAAT	This study
NT2693/ XR1	GGGCTCTAGAGGTTAATGTCTCTCCAGTTTCTTACC	This study
NT855/ BF1	CCGCGGATCCCCATGAACGAAAGTAATCAGAATTTT	This study
NT855/ XR1	CCCCTCTAGACCTTAAGCCTCTGTTTTCTCATAT	This study

NT679/ BF1	CCGCGGATCCCCAAGGAGGACATACACATGGAACAT	This study
NT679/ XR1	CCCCTCTAGACCTTAATAATTTAATTTACTGGATTCATT	This study
NT1896/ BF1	GGGCGGATCCGGAAAGGGGGGATTTTAATGGGTGAA	This study
NT1896/ XR1	CCCCTCTAGACCTTAATTGTCAGCTTCTTCAGTCTT	This study
NT1910/ BF1	CCCGGGATCCGGAGGAGGGATCTCTTATTGTCTGAC	This study
NT1910/ SR1	GGGGGTCGACGGTTAATCACCAGCTATTCCGGC	This study

[a] Ap^r, ampicillin resistance; oligonucleotide sequences are given 5' to 3'; restriction sites are underlined

Table 6.2. List of predicted antiporter proteins from *N. thermophilus*

Gene designation	GenBank gene designation	Gene size (kb)	Protein family	Closest homolog (GenBank accession no.)	Identity % (amino acids overlap)	Reported antiport activity
Nt-CPA1a	nther1135	1.2	CPA1	Na^+/H^+ exchanger *Alkaliphilus metalliredigens* QYMF (YP_001322502)	42	n.d.
Nt-CPA1b	nther0276	1.2	CPA1	Na^+/H^+ exchanger *Alkaliphilus oremlandii* OhILAs (YP_001513623)	38	n.d.
Nt-CPA2	nther0270	1.2	CPA2	Na^+/H^+ exchanger *Alkaliphilus metalliredigens* QYMF (YP_001322136)	48	n.d.
Nt-Nha	nther0107	1.5	CPA3	Na^+/H^+ antiporter ShaA *Clostridium tetani* E88 (NP_781965)	35	n.d.
Nt-NhaC1	nther2649	1.3	NhaC	Na^+/H^+ antiporter *Chromohalobacter salexigens* DSM 3043 (YP_575253)	56	n.d.
Nt-NhaC2	nther2454	1.3	NhaC	Na^+/H^+ antiporter *Fusobacterium nucleatum* subsp *nucleatum* ATCC 25586 (NP_604314)	51	n.d.
Nt-NhaC3	nther2242	1.4	NhaC	Na^+/H^+ antiporter *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953 (ZP_02240864)	55	n.d.
Nt-NhaC4	nther1834	1.4	NhaC	Na^+/H^+ antiporter *Bacillus pseudofirmus* OF4 (AAC45432)	36	Yes (Ito *et al*. 1997)

Nt-NhaC5	nther0736	1.4	NhaC	Na^+/H^+ antiporter *Alkaliphilus metalliredigens* QYMF (YP_001322347)	44	n.d.
Nt-NhaC6	nther0921	1.5	NhaC	Na^+/H^+ antiporter *Vibrio parahaemolyticus* (NP_796997)	40	No (Radchenko *et al*. 2006)
Nt-NhaC7	nther2636	1.4	NhaC	Na^+/H^+ antiporter *Alkaliphilus metalliredigens* QYMF (YP_001321248)	44	n.d.
Nt-NhaC8	nther2651	1.5	NhaC	Malate:$2H^+$/ Lactate:Na^+ antiporter YqkI *Bacillus subtilis* (ZP_00739085)	63	Yes (Wei *et al*. 2000)

n.d.; not determined

Table 6.3. Monovalent cation/proton antiport activity in inverted membrane vesicles of antiporter expressing *E.coli* KNabc transformants

Transformant	% Dequenching observed upon addition of[a]		
	Na^+	Li^+	K^+
Nt-CPA1a	77.3 ± 2.1	23.3 ± 1.48	20.2 ± 2.4
Nt-CPA1b	23.5 ± 1.7	19.5 ± 0.57	19.9 ± 0.3
Nt-CPA2	27.2 ± 0.14	27.2 ± 1.5	27.0 ± 2.1
Nt-Nha	28.5 ± 7.5	0	25.5 ± 6.5
Nt-NhaC1	20.9 ± 0.28	22.6 ± 0.85	28.5 ± 2.5
Nt-NhaC2	43.3 ± 0.02	24.7 ± 1.2	37.4 ± 0.3
Nt-NhaC3	0	0	25.7 ± 1.3
Nt-NhaC4	53.9 ± 4.3	43.9 ± 0.2	9.0 ± 1.7
Control	0	1.1 ± 0.1	2.4 ± 1.2

[a]Vesicles from transformants expressing vector [pGEM-3Zf(+)] and antiporter genes from *N. thermophilus* were assayed under anaerobic conditions in 4 mL containing 10 mM Tris-Cl, 140 mM choline chloride, 15 mM $MgCl_2$, 0.75 μM acridine orange and 500 μg protein/ mL. Anaerobic respiration was initiated by addition of Trizma-succinate to a final concentration of 2.5 mM. After steady-state fluorescence quenching was reached, NaCl, LiCl or KCl were added to final concentrations of 3 mM for NT-CPA1a, -CPA2, -NhaC1, -NhaC3, and NhaC4, 5.5 mM for NT-CPA1b and NT-Nha, and 8.5 mM for NT-NhaC2. KCl was added to a final concentration of 31 mM for NT-NhaC3. All assays were adjusted to pH 8.5, assays for NT-NhaC2 were done at pH 9.5.The values presented for the subsequent percent dequenching are from triplicate assays from two independent experiments. The percentages represent the average values of the calculated percent dequenching and are shown with the standard error of the values.

Table 6.4. Apparent $K_{0.5}$ values for *N.thermophilus* antiporter proteins

Transformant	$K_{0.5}$* (mM NaCl)
NT-CPA1a	2.1
NT-CPA1b	5.8
NT-CPA2	3.2
NT-Nha	4.4
NT-NhaC1	3.0
NT-NhaC2	8.7
NT-NhaC4	1.5
NT-NhaC3	30.20 mM KCl

*Apparent $K_{0.5}$ values were calculated by linear regression from Hill plots by the method described by Cook & Cleland (2007).

Table 6.5. Cation/proton antiport activity of *N. thermophilus* antiporters as a function of pH.

Transformant	% dequenching at pH*									
	7.8	8.0	8.3	8.5	8.8	9.0	9.3	9.5	9.8	10.0
NT-CPA1a	0	0	0	43.4 ± 2.1	**77.3 ± 2.1**	44.4 ± 1.2	23.8 ± 0.86	11.3 ± 2.7	0	0
NT-CPA1b	5.6 ± 0.25	11.6 ± 0.64	11.6 ± 1.4	**23.5 ± 1.7**	12.5 ± 1.2	0	0	0	0	0
NT-CPA2	0	0	23.0 ± 2.3	27.2 ± 0.14	17.3 ± 0.90	9.2 ± 0.61	0	0	0	0
NT-Nha	0	0	6.6 ± 0.9	**28.5 ± 7.5**	16.6 ± 0.57	11.7 ± 1.2	10.5 ±0 .36	9.0 ± 0.71	0	0
NT-NhaC1	0	0	9.05 ± 1.2	15.6 ± 1.3	**20.9 ± 0.28**	0	0	0	0	0
NT-NhaC2	0	0	0	18.0 ± 1.4	18.9 ± 1.3	27.25 ± .17	40.6 ± 1.9	**43.3 ± 0.02**	14.8 ± 2.2	6.8 ± 0.23
NT-NhaC3	0	0	12.4 ± 0.43	**25.7 ± 1.3**	17.6 ± 0.9	0	0	0	0	0
NT-NhaC4	0	0	7.0 ± 0.05	**53.9 ± 4.3**	11.1 ± 0.75	14.4 ± 0.64	8.5 ± 1.0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0

*The assay protocol was as described in the legend for Table 6.3, with the pH of the buffers adjusted to the values indicated. Values for standard error are from triplicate assays from two independent experiments. Optimal pH value for antiport activity for each protein is boxed.

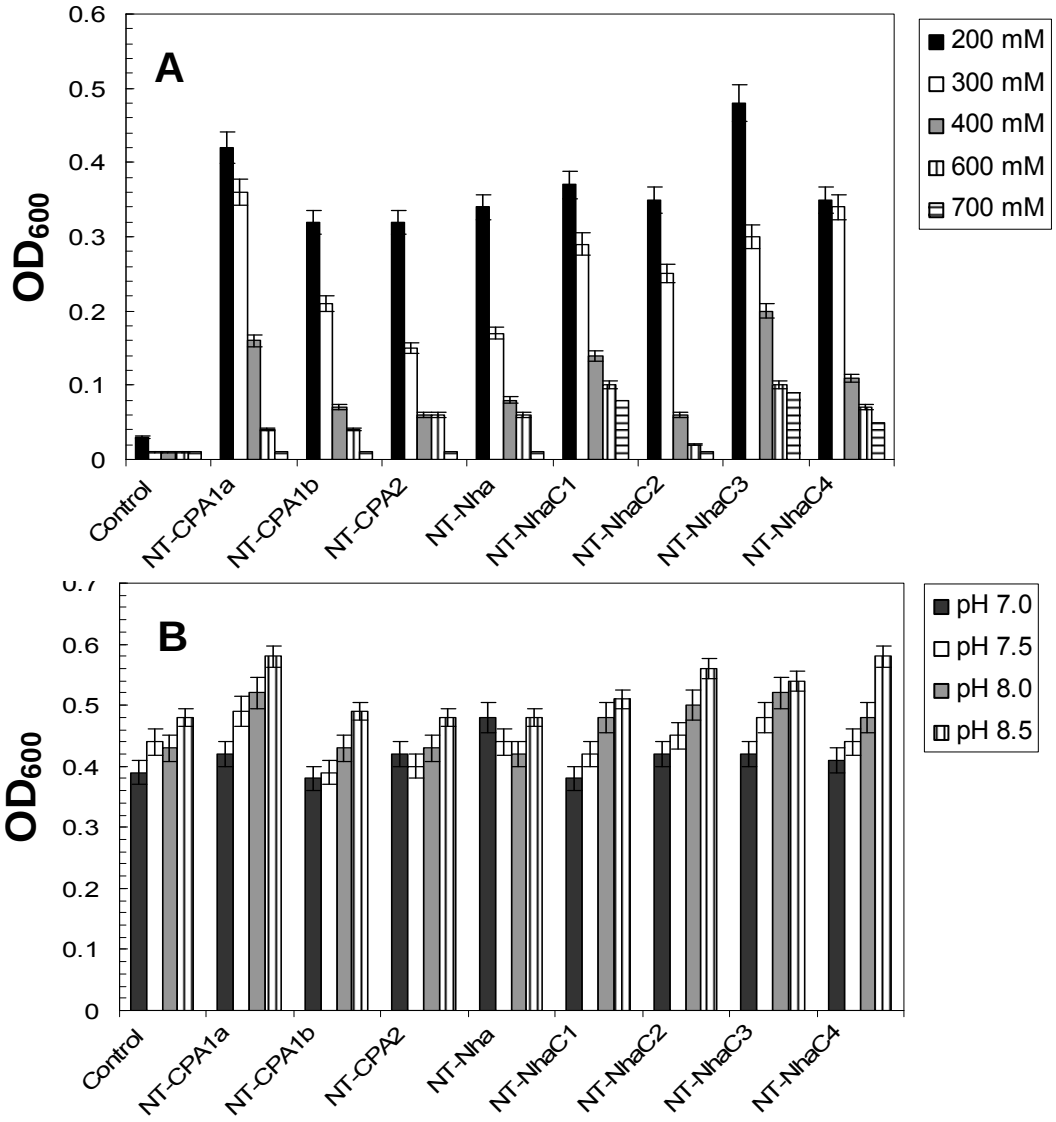

Figure 6.1. Effect of NaCl concentration and pH on the growth of *E. coli* KNabc transformants of *N. thermophilus* antiporters. (A) Transformants with vector control pGEM-3Zf(+), or expressing *N. thermophilus* antiporter genes, were grown on LBK medium, pH 7.5, containing indicated concentrations of NaCl. (B) LBK with no added NaCl was adjusted to the pH values indicated. Cells were grown anaerobically for 24 hrs at 37ºC with shaking after which the OD_{600} was measured. The error bars indicated standard errors from three independent experiments.

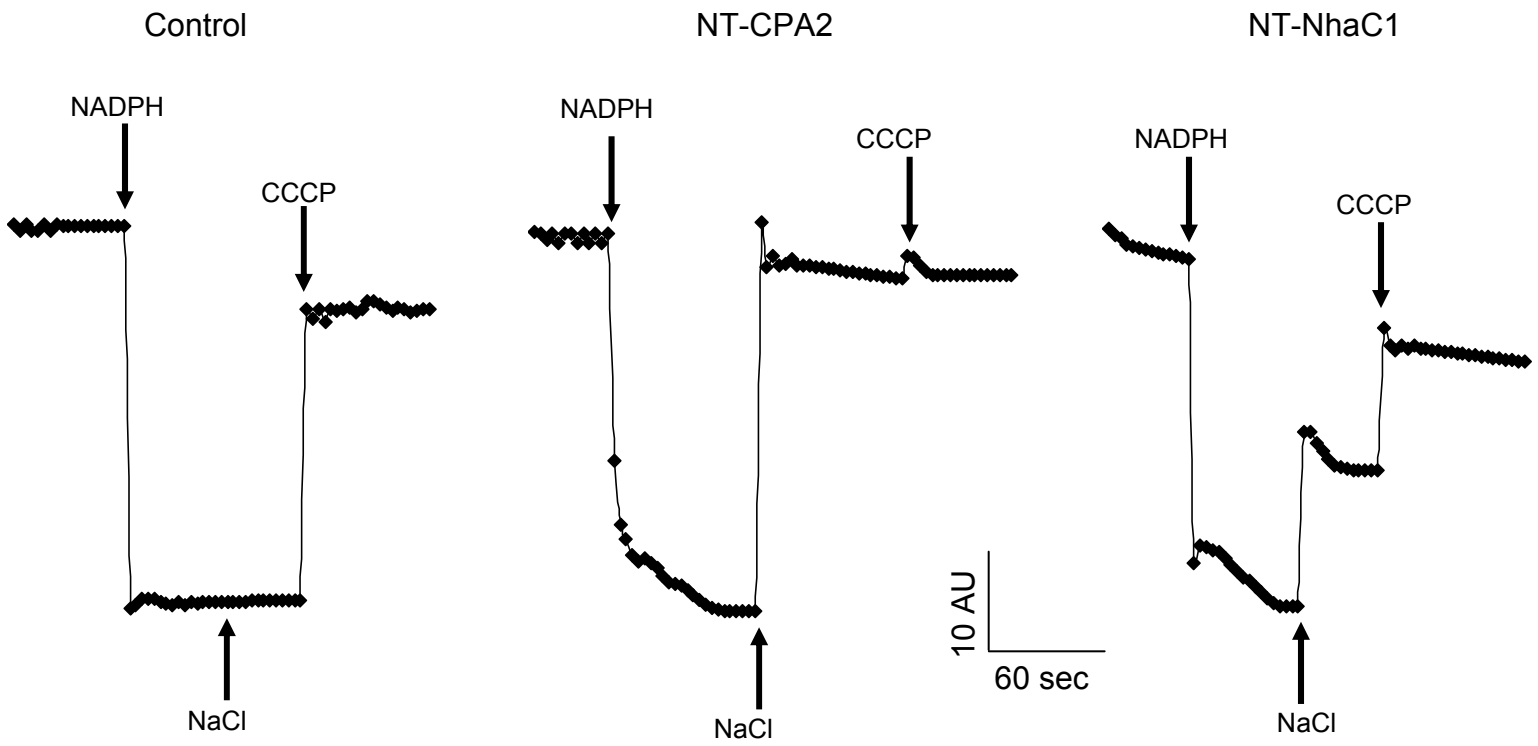

Figure 6.2. Electrogenicity of Na^+/H^+ antiport. Fluorescence-based assays of inverted membrane vesicles prepared from cells expressing the control plasmid [pGEM-3Zf(+)] and *N. thermophilus* antiporter genes. Assays were performed under anaerobic conditions in reaction mixtures containing 10 mM BTP-sulfate, 5 mM $MgSO_4$, 200 mM K_2SO_4, 1 µM nigericin, and 1 µM oxonol VI (pH 8.0-9.5). To initiate respiration, 0.6 mM of NADPH was added at the first arrow. Once the quenching reached steady state, 4 mM of NaCl (25 mM KCl for NT-NhaC3) was added at the second arrow. The final arrow indicates addition of 10 µM CCCP. The traces shown are representative of three independent experiments. The traces for the remaining six antiporter proteins were essentially identical to that of NT-CPA2, thus they are not shown.

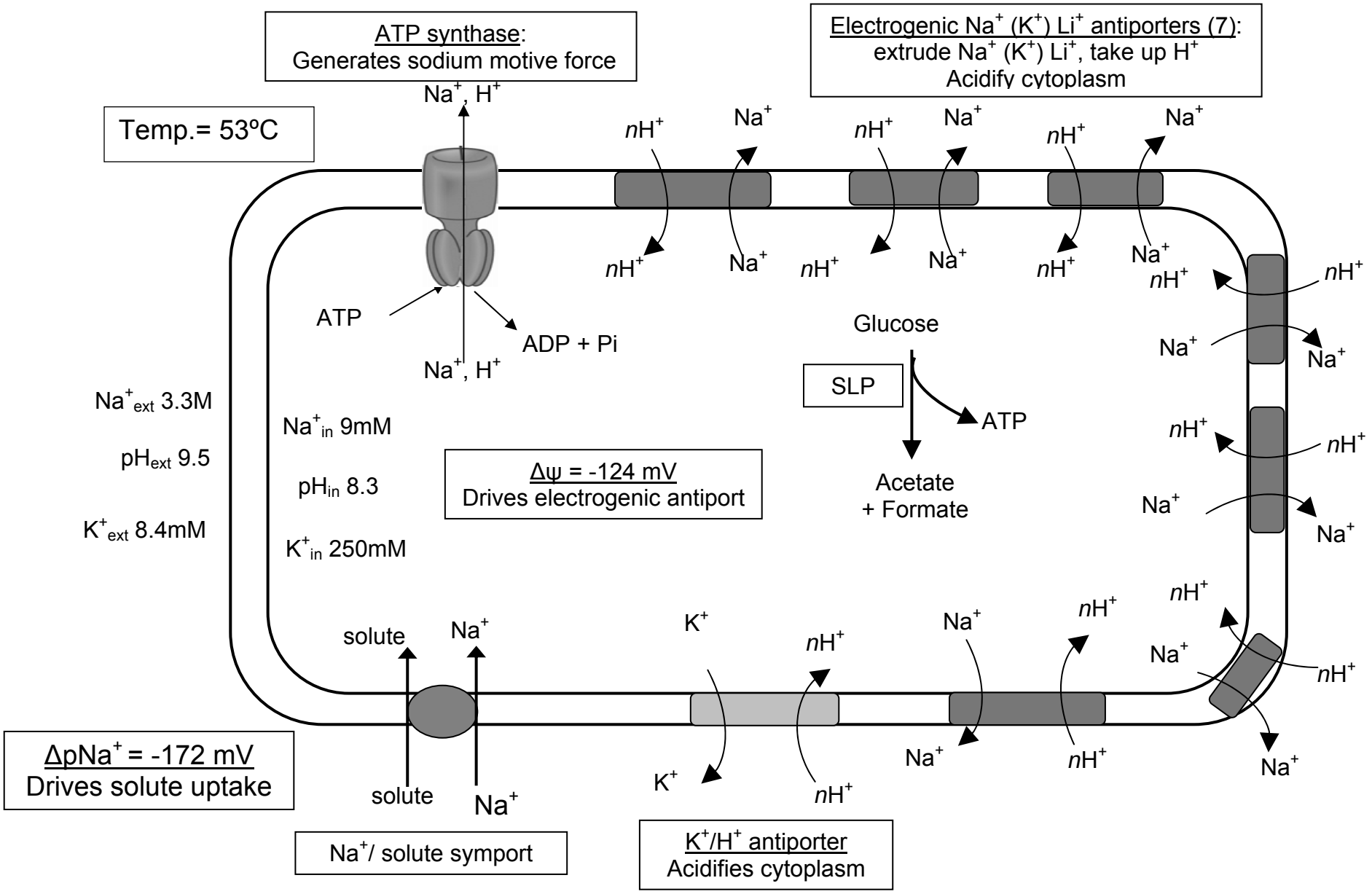

Figure 6.3. Schematic diagram of bioenergetic processes of *N. thermophilus*. SLP = substrate level phosphorylation.

CHAPTER 7

CONCLUSIONS

This dissertation has 1) provided the first detailed culture-independent study on the prokaryotic diversity of the alkaline, hypersaline lakes of the Wadi An Natrun in Egypt, 2) resulted in isolation and characterization of members of a unique and novel group of poly-extremophiles; the anaerobic halophilic alkalithermophiles, and 3) described bioenergetic aspects of these novel extremophiles with a focused exploration of the mechanism of cytoplasmic pH regulation, which is possibly applicable throughout the anaerobic halophilic alkaliphiles.

Reports on the isolation and characterization of poly-extremophiles are lacking when compared to those of extremophiles that are adapted to just one extreme. This could be due to lack of exploration of this area of prokaryotic diversity, or due to difficulty in designing suitable culture media and isolation schemes that yield poly-extremophiles. The group of poly-extremophiles studied during the course of this thesis is the halophilic alkalithermophiles; microorganisms that are adapted to survive and grow optimally under conditions of high salt concentration (≥ 1.5 M), alkaline pH values (≥ 8.5) and high temperature (≥ 50ºC). The alkaline, hypersaline lakes of the Wadi An Natrun, Egypt, are characterized by NaCl concentrations approaching saturation (5.6 M), alkaline pH as high as 11.0, and intense solar irradiation with temperatures as high as 60ºC below salt crusts. A culture-independent analysis showed that 3 of the largest lakes of the Wadi An Natrun harbor a rich and novel microbial community that is

dominated by three groups of *Bacteria; Firmicutes, Bacteroidetes*, α- and γ-proteobacteria, and two groups of *Archaea, Halobacteriales* and *Methanosarcinales* (**Chapter 2**). The extreme environmental conditions under which this microbial community survives make these lakes promising sources for isolation and characterization of novel halophilic alkalithermophiles.

Three novel anaerobic halophilic alkalithermophiles were isolated and characterized during the progress of this thesis (**Chapters 3 and 4**). These isolates were classified within a novel order, *Natranaerobiales*, which thus far is comprised exclusively of anaerobic halophilic alkalithermophiles. An investigation on the bioenergetics characteristics of one of these isolates, *Natranaerobius thermophilus*, revealed the unusual feature of the absence of cytoplasmic pH homeostasis. Rather, the intracellular pH rose continuously as the extracellular pH did and a ΔpH of 1 unit was maintained across the cell membrane throughout the whole pH range for growth (**Chapter 5**).

To investigate the mechanisms of cytoplasmic pH acidification in *Natranaerobius thermophilus*, homologs of cation/proton antiporter genes identified in the genome sequence of *N. thermophilus* were cloned and expressed in *Escherichia coli*. Cation/proton antiporters are known to be the primary defense mechanism employed by alkaliphiles to the threat of cytoplasmic alkalinization. Through these endeavors, 8 of the 12 identified homologs were functionally characterized. Their physiological characteristics are well suited to the intracellular conditions of *N. thermophilus*. Collectively, all 8 antiporter proteins are capable of ion transport within the pH range 7.8 to 10.0 and are electrogenic. Thus, all these proteins are capable of providing active cytoplasmic acidification throughout the whole pH range for growth of *N. thermophilus*. The specificity of the antiporter proteins for Na^+ varies, and they are capable of transporting both Na^+ and K^+, thus are capable of providing active cytoplasmic acidification at

various concentrations of Na^+ inside the cell, and will remain functional even if the intracellular Na^+ concentrations drop to very low levels (**Chapter 6**). Most of the antiporter proteins characterized belong to the NhaC family of antiporters, which are primarily employed in expulsion of Na^+ from the cell, and have not been shown to have an essential role in cytoplasmic acidification in the alkaliphilic bacilli. These data indicate that antiporters in *N. thermophilus* probably play a major role in adaptation to salt stress in addition to alkaline pH stress.

Work in this thesis is the first to directly address the subject of cytoplasmic pH acidification in anaerobic halophilic alkaliphiles and anaerobic alkaliphiles. The aerobic alkaliphilic bacilli maintain their cytoplasmic pH at constant 2.0-2.5 units below the extracellular pH, and they achieve this via the action of only 3-4 antiporters. On the other hand, the intracellular pH of *N. thermophilus* changes constantly with changes in the extracellular pH. *N. thermophilus* possesses at least 8 antiporter proteins capable of active cytoplasmic acidification, and these antiporters are able to function in the constantly changing intracellular environment of *N. thermophilus*.

The investigations reported here highlight future avenues of exploration in the area of physiological adaptations of anaerobes to multiple extreme growth conditions. Although the involvement of antiporter proteins in cytoplasmic pH acidification is not questioned, the extent of involvement of each individual protein, and transport efficiency remains to be determined in the natural host setting. In addition, the roles of antiporters in resistance to salt stress remains to be determined, as does the effect of high temperature on antiport activity. Involvement of other proteins in both cytoplasm acidification and resistance to salt stress cannot be ruled out. Changes in cellular structure, cell membrane composition and amino acid content must also play a role in adaptations to multiple extreme growth conditions. The availability of anaerobic halophilic

alkalithermophiles that can be cultured in the laboratory provides the platform for subsequent genomic, proteomic and biochemical studies that will provide a deeper understanding of the mechanisms of not only survival, but microbial growth and proliferation under multiple extreme conditions.

APPENDIX A

CALCULATION OF BIOENERGETIC PARAMETERS

Calculation of Intracellular pH

The intracellular pH of *Natranaerobius thermophilus* was calculated from the uptake of [^{14}C]methylamine into the cell. Methylamine permeates the cell membrane in unprotonated form, once in the cell, it becomes protonated and cannot diffuse out. The intracellular pH can be calculated from the equation:

$$pH_{in} = pH_{out} - \log_{10}\left(\frac{[B]_{in}}{[BH+]in}\right)$$

As described in **Chapter 5**, the intracellular pH of 0.9 mL of cells (OD_{600} 1.0) was calculated. The concentration of [^{14}C]methylamine in the cell pellet was determined by liquid scintillation counting. The total amount of base in the cell pellet = amount of base in intracellular aqueous volume + amount of base in the extracellular aqueous pellet volume

The steps involved in this calculation are outlined below:

(1) Determine concentration of undissociated methylamine in the extracellular space $[B]_{ex}$

(2) Determine intracellular volume (V_{in}) of the cell pellet: from the difference in partitioning of ^3H_2O and [^{14}C]polyethylene glycol

(3) Determine amount of methylamine in the intracellular space: $MET_{in} = MET_p - MET_{ex}$

(4) Determine intracellular pH:

$$pH_{in} = pH_{out} - \log_{10}\left(\frac{[B]_{in}}{[BH+]in}\right)$$

(1) Determination of the concentration of undissociated methylamine $[B]_{ex}$

$$pH_{out} = pKa\ methylamine + \log\left(\frac{[B]_{ex}}{[BH+]_{ex}}\right)$$

The pH_{out} is that at which the experiment is performed;

pKa of methylamine = 10.64

$[BH+]_{ex}$ = amount of methylamine in supernatant (METs), in counts per minute (cpm)

Rearranging for B_{ex}:

$$B_{ex} = \left(\frac{10^{pH_{ex} - pKa}}{1 + 10^{pH_{ex} - pKa}} \right) \text{ X METs}$$

(2) Determination of the intracellular volume

Intracellular volume = total pellet volume – extracellular volume

$$V_{in} = V_p - V_{ex}$$

a. **V_p**: measured from the amount of ^3H_2O in the cell pellet

$$V_p = \frac{H_2O_p}{H_2O_s} \text{ X volume of supernatant sample } (\mu L)$$

H_2O_p = amount of ^3H_2O in pellet (cpm)

H_2O_s = amount of ^3H_2O in supernatant (cpm)

b. **V_{ex}**: measured from accumulation of [^{14}C]polyethylene glycol in the cell pellet; polyethylene glycol is not taken into the cell, thus remains in the extracellular space

$$V_{ex} = \frac{PEG_p}{PEG_s} \text{ X volume of supernatant sample } (\mu L)$$

PEG_p = amount of [^{14}C]PEG in pellet (cpm)

PEG_s = amount of [^{14}C]PEG in supernatant (cpm)

(3) Determination of the amount of methylamine in intracellular space MET_{in}

$$MET_{in} = MET_p - MET_{ex}$$

a. **MET_p**= amount of methylamine in the pellet (cpm)

b. **MET_{ex}** = $\frac{METs}{\text{volume of supernatant}}$ X V_{ex}

MET_{ex} = amount of methylamine in extracellular space

MET_s = amount of methylamine in supernatant (cpm)

(4) **Determination of intracellular pH**

$$pH_{in} = pH_{out} - \log_{10}\left(\frac{[B]_{in}}{[BH+]in}\right)$$

The total amount of methylamine inside the cell includes both the protonated and unprotonated forms, thus:

$$MET_{in} = B_{in} + BH^{+}_{in}$$

At equilibrium, $B_{in} = B_{ex}$, thus

$$\mathbf{B_{in}} = \frac{B_{ex}}{\text{volume of supernatant sample}} \text{ X } V_{in}$$

$$\mathbf{BH^{+}_{in}} = (BH^{+}_{in} + B_{in}) - B_{in}$$

$$\text{Since } Met_{in} = BH^{+}_{in,} + B_{in}$$

$$\text{then: } \mathbf{BH^{+}_{in}} = Met_{in} - B_{in}$$

Met_{in} has been calculated in step 3

The intracellular pH is calculated from the equation:

$$pH_{in} = pH_{out} - \log_{10}\left(\frac{[B]_{in}}{[BH+]in}\right)$$

The ΔpH is calculated from:

$$\Delta pH = pH_{ex} - pH_{in}$$

Calculation of Electrochemical Membrane Potential (ΔΨ)

The Δψ across the membrane was calculated from the uptake of $[^3H]TPP^+$ according to the Nernst relationship. The Nernst equation relates the electrochemical equilibrium distribution of an ion to the membrane potential. The Δψ is positive outside the cell, negative inside. Diffusion of $[^3H]TPP^+$ into the cell proceeds till electrochemical equilibrium is attained.

To correct for non-specific binding of $[^3H]TPP^+$ to cells and the amount of $[^3H]TPP^+$ in the extracellular space, $[^3H]TPP^+$ "uptake" in cells that have been treated with 10 μM of both nigericin and valinomycin was measured. Valinomycin and nigericin both transport K^+ across the membrane till electrochemical equilibrium is reached, thus abolishing the Δψ. As a result, there is no driving force for TPP^+ uptake.

The Nernst equation:

$$\Delta\psi = \frac{2.3RT}{mF}\mathrm{Log}_{10}\frac{TPP+_{in}}{TPP+_{out}}$$

R: the gas constant 8.314 J mol^{-1} K^{-1}

T: temperature (Kelvin)

m: valency of ion

F: Faraday constant, 96.485 KC mol^{-1}

Total amount of TPP^+ accumulated in the pellet TPP^+pf:

$$TPP^+pf = TPP^+p - TPP^+p\ n/v$$

TPP^+p = amount of TPP^+ accumulated in cell pellet

$TPP^+p\ n/v$ = amount of TPP^+ accumulated in cell pellet treated with nigericin/ valinomycin

$$TPP^+_{in} = \frac{TPP + pf}{V_{in}}$$

$$TPP^+_{out} = \frac{TPP + s}{\text{volume of supernatant}}$$

TPP^+s = amount of TPP in the supernatant (cpm)

The value for $\frac{2.3RT}{mF}$ is 59.

Calculation of Proton Motive Force

$$pmf = Z\Delta pH + \Delta\psi$$

$$Z = \frac{2.3RT}{mF} = 59$$

The ΔpH is multiplied by the Z factor to convert it to units of charge: mV.

APPENDIX B

DEPENDENCE OF SOLUTE TRANSPORT ON NA^+

IN *NATRANAEROBIUS THERMOPHILUS*

Energized cell suspensions (3 mL) of *N. thermophilus* were incubated with 10 μM of [^{14}C]arginine, in the presence of either 100 mM NaCl or 100 mM NaCl + 25 μM monensin for 5 minutes. At 0, 0.5, 1, 3, and 5 minutes, 0.5 mL of cells were removed and rapidly filtered through a cellulose acetate filter. Transport was immediately terminated by addition of 100 mM LiCl. Filters were washed with 100 mM LiCl, immersed in liquid scintillation fluid and then counted.

Monensin is a Na^+ ionophore that abolishes the Na^+ gradient.

The cells were washed three times with sterile anaerobic Tris.EDTA buffer (pH 8.0) prior to energization to remove extracellular Na^+.

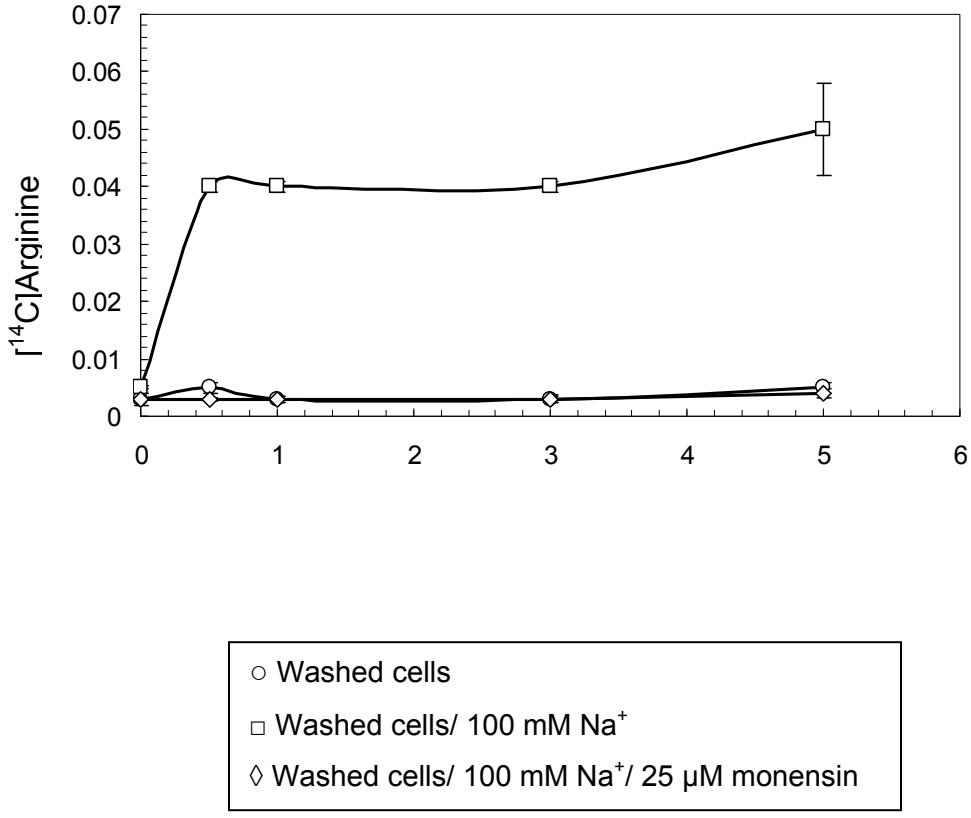

Figure B1. Effect of Na^+ on transport of [^{14}C]arginine into *Natranaerobius thermophilus*.

APPENDIX C

EFFECT OF PH ON PROTON PUMPING INTO INVERTED MEMBRANE VESICLES

The purpose of this experiment was to demonstrate that the activity of membrane proteins in inverted membrane vesicles does not change significantly at varying assay pH values. Assays in inverted membrane vesicles were performed as described in **Chapter 6**, in assay buffer adjusted to the indicated pH values.

Differences in the extent of quenching over the pH values used in experiments were not statistically significant. This implies that protein activity in inverted membrane vesicles is not affected by changes in assay pH. Thus, changes %dequenching observed at different pH values is due to changes in antiport activity, which is a physiological response, and is not an artifact of suboptimal protein function at any certain pH value.

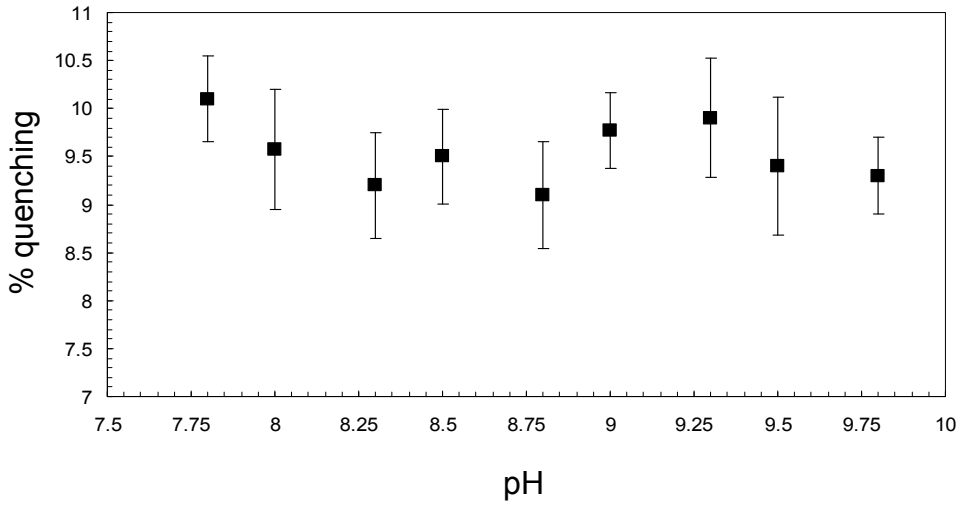

Figure C1. Effect of pH on proton pumping into inverted membrane vesicles prepared from *E.coli* KNabc transformants. These data represent averages from at least ten independent experiments

APPENDIX D

IN VIVO EXPRESSION OF *NATRANAEROBIUS THERMOPHILUS* ANTIPORTER GENES

RNA isolation

Natranaerobius thermophilus was grown at $pH^{55ºC}$ 9.5, 8.5, or 10.4 at 53ºC and 3.3 M Na^+. When the cultures had reached an OD_{600} of approximately 0.4, cells were harvested by centrifugation at 10,000 rpm for 20 min at 4ºC. Cells were lysed by resuspension in 1 mL of TRIZOL™ reagent (Invitrogen) and incubation at room temperature for 10 min. To 1 mL of TRIZOL lysate, 0.2 mL of chloroform was added and the lysate was incubated for a further 5 min. at room temperature. Aqueous and organic phases were separated by centrifugation at 10,000 rpm, 4 ºC for 15 min. Total RNA was precipitated from the aqueous phase by adding an equal volume of isopropanol and incubation at room temperature for 10 min followed by centrifugation at 10,000 rpm, 4 ºC. RNA pellets were washed three times with 75% ethanol and then dissolved in DEPC-treated water. Final RNA concentrations were 300-350 ng/ µL.

Quantitative Real-Time PCR

Total RNA was treated with RNase-free DNase I (amplification grade, Invitrogen). For each individual RNA sample (RNA extracted from cells grown at the different pH values), two reverse-transcriptase (RT) reactions were performed (SuperScript ™ III, Invitrogen). RT-reactions were primed with random hexamers.

Quantitative PCR was performed in 25 µL reaction mixtures containing 2 µL cDNA (synthesized from 200 ng total RNA), 200 – 700 nM of each primer (see table D1 for primer concentrations), and 12.5 µL of SYBR® GReenER™ qPCR supermix (Invitrogen). Primers for all 12 homologs of antiporter genes were designed using the OligoPerfect™ Designer program (http://www.tools.invitrogen.com). Samples were run in triplicate on an iCycler (Bio-Rad) with the following conditions: 10 min at 95ºC and 40 cycles of 95ºC for 15 s and 60ºC for 60 s,

followed by melt-curve analysis. A seven-point standard curve was run in parallel for each primer set to assess primer efficiency. A no-template-control (NTC) and – reverse transcriptase (-RT) control were run for each RNA sample to ensure absence of genomic DNA contamination. Primer amplification efficiencies were all within 5% of each other (Table D1).

Validation of Control Genes

Control genes were chosen based on the following criteria:

1. present in one copy in the genome
2. be expressed at the same level under the experimental conditions used

Following examination of the genome sequence of *N. thermophilus*, three genes were chosen for subsequent validation:

1. *recA*: DNA recombinase
2. *gyrB*: DNA gyrase subunit B
3. *rpoB*: RNA polymerase β-subunit

cDNA was synthesized from total RNA extracted from cells grown at $pH^{55^{\circ}C}$ of 8.5, 9.5 or 10.4 as described above, and was quantified by qRT-PCR. The values for the threshold cycle (Ct) were compared (Table D2). Based on these data, the *recA* gene was chosen as the control reference gene since it showed the least change in Ct value, i.e. is present in the same concentration in cells when grown under different growth conditions.

Normalization of transcript levels

For each experiment, the target mRNAs and control mRNA (*recA*) were reverse-transcribed together in one reaction primed with random hexamers. The resulting cDNAs were quantified by real-time PCR. To determine the relative amounts of target antiporter genes to one another, the target cDNA was normalized internally to the *recA* cDNA levels in the same sample. The results are expressed as 'normalized RNA level', where the 'normalized RNA level' is [Target mRNA] / [*recA* mRNA].

Results

Data from expression analyses are shown in Figure D1. The majority of antiporter gene transcripts are present with the greatest abundance at the optimal growth $pH^{55^{o}C}$ of 9.5, and appear to be under-expressed at the suboptimal pH values 8.5 and 10.4. This may be a part of a global repression of gene expression that occurs in *N. thermophilus* when grown at suboptimal conditions. It is also possible that changes in antiporter activity are not at the transcriptional level. However, since the cultures used for this experiment were grown in batch culture, it is possible that the cells grown at the suboptimal $pH^{55^{o}C}$ values of 8.5 and 10.4 were sampled during early exponential or stationary phases, and that this apparent decrease in transcript levels is due to unhealthy cells. Thus, antiporter transcripts will be quantified once again on cells that will be grown in continuous culture in a chemostat. This will ensure that cells used in the experiments will be harvested at the same growth stage and defined physiological conditions.

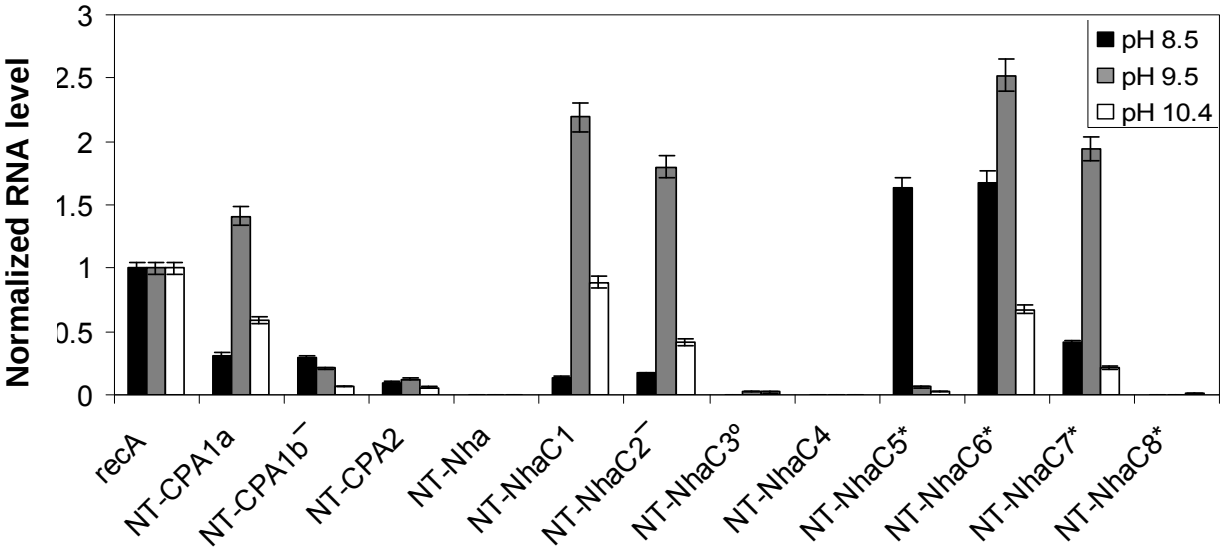

Figure D1. Expression of antiporter genes in *N. thermophilus* when grown at different pH values in batch culture.

* denotes the genes for putative antiporter proteins that did not complement the NaCl sensitivity phenotype of *E. coli* KNabc

º denotes the K^+ specific antiporter

¯ NT-CPA1b is the antiporter with the most acidic pH minimum, NT-NhaC2 is the antiporter with the most alkaline pH optimum and maximum

Table D1. Oligonucleotides used in qRT-PCR, product sizes, optimal primer concentrations used and PCR efficiency

Primer name	Sequence (5' → 3')	Product Size	Conc. used in qPCR (nM)	PCR Efficiency (%)
RecA-F	TTCATGCGGTAGCAGAAGCTCAGA	141	200	102.3
RecA-R	TGCTCGCCTGTATCTGGTTGTGAA		200	
NTCPA1a-F	ATATCCTAGCACGACTTGCGGGAA	141	200	104.5
NTCPA1a -R	AAATGGCTAGACCGATTGCTACGC		200	
NTCPA1b-F	GCCGGAGCTGAACTACACTT	119	600	102.9
NTCPA1b -R	TGTGACCATCTGGCACCTAA		600	
NTCPA2-F	TGAAATGGCCGACAGTGATA	92	200	105.3
NTCPA2-R	GTAGGCCGGCAACTTGTAAA		200	
NTNha-F	GTAACAGATGCCGGTGGACT	244	200	104.2
NTNha -R	GAGCCCCTTGCATTACACAT		200	
NTNhaC3-F	GTAACAGATGCCGGTGGACT	244	500	105.5
NTNhaC3-R	GAGCCCCTTGCATTACACAT		500	
NTNhaC2-F	GTTTGGTATTGGCTGGGCTA	168	200	106.7
NTNhaC2-R	AAAAGGCCAGTCCACCTTCT		200	
NTNhaC1-F	ACTTGCTATTGCATTGACCCTGGC	175	600	104.6
NTNhaC1-R	AGTCAGCACTGTAGCATTCCACCT		600	
NT-NhaC4-F	TTAGGAGTAGGTGGAGGACTTGGT	143	200	107.1
NTNhaC4-R	AGGAGCTGTCACCCACAACATTGA		200	

NTNhaC5-F	ATGAGAGTTCCCCCGATACC	86	200	103.7
NTNhaC5-R	GCGAATGAAGATCCCTGAAA		200	
NTNhaC6-F	TGGGCCTATTTTTGCAACTC	118	500	102.0
NTNhaC6-R	TCCCCAACCAACTATGGGTA		500	
NTNhaC7-F	GCTGGCTCTATCAGGAATCG	138	200	106.7
NTNhaC7-R	CCGGCTGTACTTGAGGCTAC		200	
NTNhaC8-F	ATGTAGGAACGGCACCAGAG	241	700	103.7
NTNhaC8-R	GCGAATCCATCATAGGCAGT		700	

Table D2. Validation of reference genes.

Gene	*recA*	*gyrB*	*rpoB*
Avg. Ct pH 8.5 (SE)	18.15 (0.17)	18.55 (0.31)	18.36 (0.19)
Avg. Ct pH 9.5 (SE)	18.7 (0.14)	14.92 (0.15)	14.50 (0.18)
Avg. Ct pH 10.4 (SE)	18.45 (0.17)	15.78 (0.10)	16.55 (0.19)

Avg. Ct is the average from three analyses. SE = standard error

APPENDIX E

EFFECT OF EXTRACELLULAR PH ON SOLUTE UPTAKE

IN *NATRANAEROBIUS THERMOPHILUS*

Natranaerobius thermophilus was grown in carbonate-buffered medium at optimal conditions, 52°C, 3.3 M Na^+ and pH55 ° C 9.5. Cells were harvested during mid-exponential phase by centrifugation (6000 x *g*, 16 min, 24 ºC) and washed three times in sterile anaerobic carbonate-buffered medium, $pH^{55\ ºC}$ 9.5. All washing steps were carried out anaerobically by a modified Hungate technique. Cells were resuspended to a final OD_{600} of 1.0 in anaerobic carbonate-buffered medium adjusted to the pH being studied. Cell suspensions were incubated at 52°C for 10 min before being used in the experiments.

Cell suspensions vials (nitrogen in gas phase) containing [^{14}C]sucrose. After incubation for 5 min at 53ºC, 0.9 mL of the culture was rapidly centrifuged through 300 µL of silicone oil. The tubes with remaining contents were then frozen at -80ºC for at least 2 hours. The bottoms of the tubes containing cell pellets were removed with dog-nail clippers; the supernatant and cell pellets were dissolved in scintillation fluid, and radioactivity (cpm) was determined with an LKB Wallac 1214 Rack-Beta scintillation counter

Results

Accumulation of [^{14}C]sucrose reached its highest level at low pH values, 7.4 – 7.9 (Figure E1). Accumulation decreased, and then gradually increased till reaching a maximum at pH values between 8.9 and 9.4. The ability to accumulate large concentrations of the substrate sucrose at lower pH values explains why the ΔpH across the membrane of *N. thermophilus* does not collapse towards the lower boundaries of the $pH^{55°C}$ range for growth. Accumulation of the substrate implies that the cells are energized; hence have energy to drive cytoplasm acidification processes.

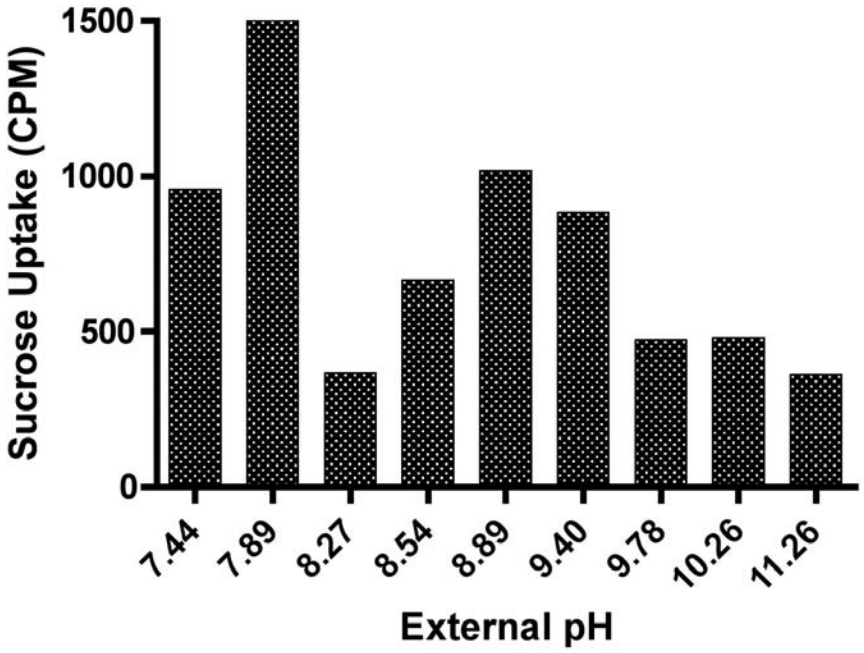

Figure E1. Effect of extracellular pH on transport of [^{14}C]sucrose into suspensions of *Natranaerobius thermophilus*. Uptake experiments were performed at 53°.

APPENDIX F

EFFECT OF EXTRACELLULAR PH ON INTRACELLULAR PH IN ENERGIZED AND NON-ENERGIZED CELLS OF *NATRANAEROBIUS THERMOPHILUS*

Natranaerobius thermophilus strain JW/NM-WN-LF^T was grown anaerobically under a nitrogen gas phase in carbonate buffered medium as described in **Chapter 3**. **.** For determination of ΔpH, *N.thermophilus* was grown in batch culture at $pH^{55°C}$ 9.5, 53ºC in the presence of 3.3 M Na^+. Cells were harvested during mid-exponential phase by centrifugation (6000 x *g*, 30 min, 24 ºC) and washed three times in sterile anaerobic carbonate-buffered medium, $pH^{55\ °C}$ 9.5. Cells were resuspended to a final OD_{600} of 1.0 in anaerobic carbonate-buffered medium adjusted to the pH being studied. A portion of the cell suspensions (non-growing) were energized by the addition of sucrose (0.5% wt/vol) and incubation at 53ºC for 20 minutes. The remaining cell suspensions were incubated at 53ºC in the absence of sucrose (non-energized). Cell suspensions (2.0 mL) were then transferred by syringe to sealed serum vials (10 mL capacity, nitrogen in gas phase) containing [^{14}C]methylamine (5.4 μM). After incubation for 5 min at 53ºC, 0.9 mL of the culture was rapidly centrifuged through 300 μL of silicone oil (mixture of Dow Corning 'DC 550', 36%, Dow Corning 'DC 200/200', 24%, and dioctyl phthalate, 40% vol/vol) in microcentrifuge tubes (13,000 x *g*, 3 mins, 24ºC). Twenty microliters of supernatant were removed. The tubes with remaining contents were then frozen at -80ºC for at least 2 hours. The bottoms of the tubes containing cell pellets were removed with dog-nail clippers; the supernatant and cell pellets were dissolved in scintillation fluid, and radioactivity (cpm) was determined with an LKB Wallac 1214 Rack-Beta scintillation counter. The ΔpH was determined from the distribution of [^{14}C]methylamine with the Henderson-Hasselbach equation.

Results

Energized cells of *N.thermophilus* maintained a constant ΔpH of approximately 1 unit throughout the entire extracellular $pH^{55°C}$ range for growth (Figure F1). Non-energized cells of *N.thermophilus* were not capable of cytoplasm acidification; intracellular pH was not significantly different from the extracellular $pH^{55°C}$ at extracellular $pH^{55°C}$ values of 9.5 and below. However, at extracellular pH values greater than 9.5, cytoplasm acidification was observed, and the intracellular pH of energized and non-energized cell suspensions of *N.thermophilus* was not different at extracellular $pH^{55°C}$ values of 10.5 – 11.5 (Figure F1). This indicates the presence of cytoplasm buffering in *N.thermophilus* during growth at alkaline extracellular $pH^{55°C}$ values (> 9.5). This phenomenon could account for the absence of ΔpH collapse towards the alkaline end of the extracellular $pH^{55°C}$ range for growth of *N.thermophilus*.

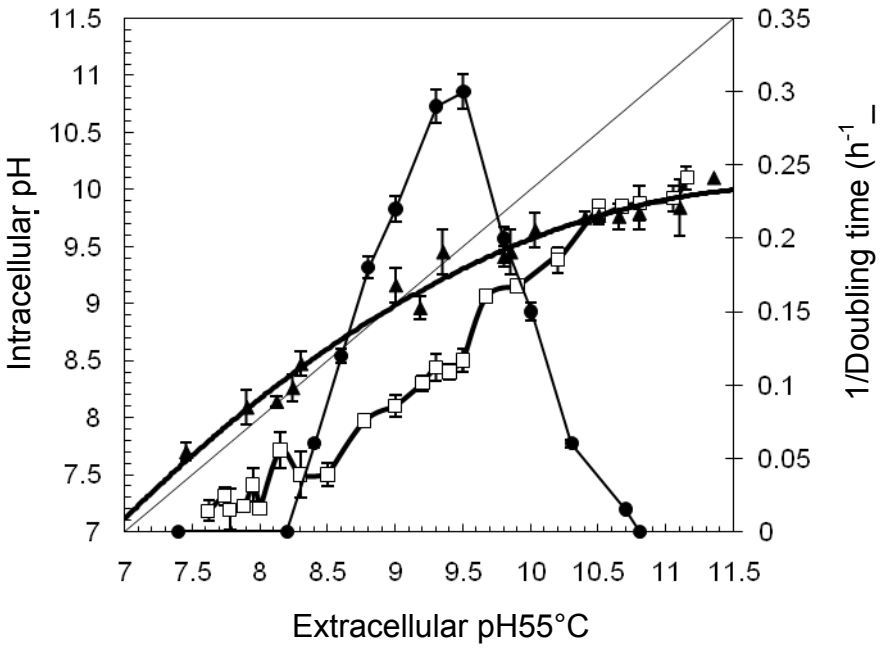

Figure F1. Effect of external pH on growth of *N.thermophilus* (●), intracellular pH in energized cell suspensions of *N.thermophilus* (□), and intracellular pH in non-energized cell suspensions of *N.thermophilus* (▲).