A NOVEL STRATEGY FOR HALOPHILICITY IN THE PHOTOAUTOTROPHIC PROTEOBACTERIUM *HALORHODOSPIRA HALOPHILA*.

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A NOVEL STRATEGY FOR HALOPHILICITY IN THE PHOTOAUTOTROPHIC PROTEOBACTERIUM *HALORHODOSPIRA HALOPHILA*.

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ABSTRACT

Halorhodospira halophila is an extremophilic photoautotrophic proteobacterium found in highly saline desert lakes. It is one of the most halophilic organisms known and provides a system to investigate adaptive mechanisms for survival of abiotic stress. This report describes genome-based experimental studies of halophilic adaptations in *H. halophila.* Two distinct strategies are known to be used by halophilic organisms to cope with high salt conditions, namely: 'High-salt-in-' where organisms accumulate KCl (up to 5 M) in their cytoplasm, which requires them to have an acidic proteome, and 'Low-salt, organic-solute-in': where compatible solutes are accumulated in the cytoplasm. The salt in cytoplasm strategy is mainly used by extreme halophiles, which gives them ability to grow in high salt environments (up to saturation levels) while the organic osmolyte strategy is often used by moderate halophiles, which gives them adaptability to grow over wide range of salt concentrations. In the work described here, it was found that *H. halophila* has an acidic proteome as examined by bioinformatics analysis and isoelectric focusing gel electrophoresis. In line with this, based on Inductively Coupled Plasma (ICP) and X-ray micro probe analysis revealed that *H. halophila* accumulates up to 3 M KCl in its cytoplasm. However it can grow over a broad range of NaCl concentrations (3.5-35% NaCl). When grown in 5% NaCl, it had KCl concentration similar to *E. coli* despite its acidic proteome. Determination of cellular glycine betaine content showed that *H. halophila* switches to accumulation of compatible solutes when grown in media containing high NaCl but a reduced KCl concentration. These data indicate that *H.*

halophila uses both halophilic strategies and can switch between them depending on the environmental conditions. This capability is likely to be important in enabling *H. halophila* to grow in high salt environments but also over wide range of salt concentrations. The potassium concentration at which *H. halphila* switches its halophilic strategy (1 mM KCl) is similar to that of its natural habitat (Wadi Natrun Lakes, Egypt), and therefore this osmoprotectant switch is likely to be ecologically relevant. Unexpectedly, the closely related organism *Halorhodospira halochloris* does not accumulate KCl but only glycine betaine. In line with this, isoelectric focusing gel electrophoresis revealed it does not have acidic proteome. This suggests recent rapid evolution in halophilic strategy in the genus *Halorhodospira*.

CHAPTER ONE

REVIEW OF LITERATURE

1.1 History of halophilic microorganism

One of the first accounts of halophilic microorganisms is found as far back as 2700 BC (8). It reports the red brines known to be caused by microbial communities adapted to hyper-saline environment. Such records have also been made during the ancient times in Egypt. Pierce in 1914 first isolated bacteria which could grow in saturated salt conditions (69). From the late 1920's to the early 1940 halophilic bacteria were isolated from a variety of materials such as fish, hides and anchovies (6). Amongst other researches, two research papers inspired future work in variety of salt-saturated environments. Both these PhD theses were carried out under the guidance of Albert Jan Kluyver. The first thesis was "*Over roode en andere bacterieen van gezouten visch*" (On red and other bacterial life in salted brines) by Helena Petter (68) and the second thesis was "Investigations concerning bacterial life in strong brines" by Trijntje Hof (30). Until the late 1930's the Dead Sea was considered to be a sterile environment. Inspired by the Petter and Hof theses, Benjamin Elazari's report of the isolation of microorganisms from this habitat ended the reputation of lifelessness of the Dead Sea (54). Benjamin Volcani isolated extreme halophiles like *Halobacterium marismortui* (now *Haloarcula marismortui*), *Halobacterium trapanicum and Micrococcus morrhhuae*, moderate halophiles like *Chromohalobacterium marismortui, Pseudomonas halestorgus* and *Flavobacterium halmephium* (82, 83). However this initial surge was not followed by other authors and Volcani himself changed his research efforts for almost 20 years. Since the 1980's

Aharon Oren and coworkers have been carrying out extensive research on the ecology, physiology and biochemistry of halophilic microorganisms and the microbiology of hypersaline environment (58, 61, 64). Antonio Ventosa and his group also have been carrying out extensive research on microbial life in the Dead Sea.

Recent advances in genome sequencing have been a big impetus for halophilic research. The first halophilic genome sequenced was of the archaeon *Halobacterium* NRC-1(78). Since then genome sequences of a number of halophilic archaea are available. However not many halophilic and halotolerant bacteria have been sequenced.

1.2 Ecology of halophilic organism:

Lourens Baas Becking, the Dutch botanist and microbiologist in his book *Geobiologie of inleiding tot de milieukunde* has famously hypothesized- "Everything is everywhere: but, the environment selects" (7). To explore this hypothesis in context of halophiles, a variety of different environments have been studied.

The oceans are the largest bodies of saline water with average salinities which are measured as Practical salinity scale (PSS) ranging from 32-35 (32,000-35,000 ppm) (45). Hypersaline environments, with salinities far above the normal seawater salinities, generally originate as a result of evaporation of seawater. Such environments are called thalassohaline environments and they have the ionic composition of seawater, and have nearly neutral or slightly alkaline pH. Solar salterns are examples of thalassohaline environment. Crystallization and precipitation of salts in the solar salterns occur in the following order: Calcite ($CaCO_3$), Gypsum ($CaSO_4.2H_2O$), Halite (NaCl), Sylvite (KCl) and Carnallite (KCl, $MaCl_2.6H_2O$) resulting in a brine more acidic than seawater. Deep

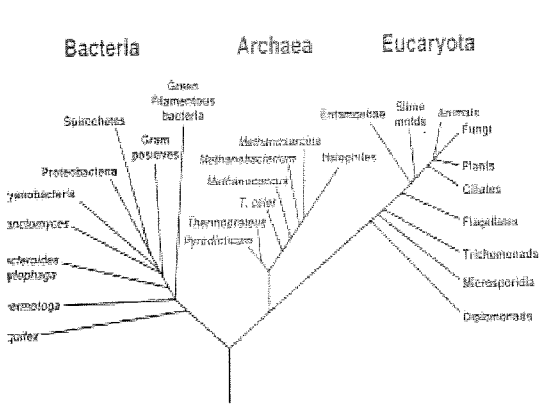
sea brines found on bottom of Red Sea, the Mediterranean Sea and Gulf of Mexico are examples of such brines. Athalassohaline environments are influenced by seawater but are reflection of the geology, geography and topography of the areas in which they develop. The brines are influenced by the solution of evaporate deposits from previous evaporative events (26). The Dead Sea in the Middle East and the Great Salt Lake in the Western United States, are the largest and most studied hypersaline lakes. Several evaporation ponds are found in the coastal areas where seawater enters through seepage or narrow inlets from the sea. Examples of such lakes or pools are found near the Red Sea coast, Baja California coast, Black Sea and Sharks Bay in Western Australia. Naturally occurring alkaline hypersaline soda brines are found in Egypt (Wadi Natrun Lakes), Kenya (Lake Magadi) and Western United States (Great Basin Lakes) (79).

Amongst terrestrial environment, soil is the most heterogeneous and exhibits wide range of salinities. Examples of hypersaline environments are salty soils in Death Valley (California, United States), the Great Salt Plains (Oklahoma, United States), Ribandar (Goa, India) and Kirkuk Plains (Northern Iraq). As freezing can also have same effect as evaporation, hypersaline ponds have been found in Antarctica (Deep Lake, Organic Lake and Lake Suribati).

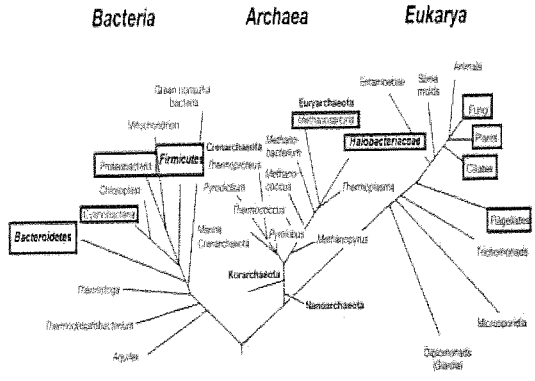
Some of the rarest places where halophiles have been found are domestic dishwashers, polar ice and spider webs in desert caves (29).

1.3 Physiology of Halophiles:

Halophilic microorganism can be defined as organism that can grow at salt concentrations equal to or higher than 100 g per liter (1.7 M NaCl). They are found in all the three domains of life: Archaea, Bacteria and Eukaryota [Figure 1.1 (a) and (b)]. They also vary considerably in their physiological properties. Halophiles include aerobic, anaerobic, chemoheterotrophs, photoheterotrophs, photoautotrophs and photoheterotrophic species. Thus different metabolic types exists which function in high salt conditions. Different classification schemes have been devised to describe microorganisms according to their behavior toward salt. The most widely used is that of Dr Donn Kushner, who defined moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt. Organisms able to grow in the absence of salt as well as in the presence of relatively high salt concentrations are classified as halotolerant (or extremely halotolerant if growth extends above 2.5 M). Microorganisms that can grow above 2.5M salt concentration but not in the absence of salt are classified as extreme halophiles.



(a) *Universal tree of life*



(b) *Distribution of halophiles in universal Tree of life*

Figure1.1: (a) Universal tree of life showing three domains and (b) Distribution of halophiles within three domains of life indicated in blue boxes
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1.3.1 Halophilic Archaea

The majority of prokaryotic organisms in hypersaline environments belong to members of the Haloarchaea, which form a distinct evolutionary branch of the domain Archaea. They belong to the family *Halobacteriaceae*. Two additional families in domain archaea contain halophilic organisms: *Methanospirillaceae* and *Methanosarcinaceae*. The family *Halobacteriaceae* includes 30 genera. Some of their physiological characteristics and evolutionary position has garnered a great deal of interest in sequencing genomes of these Haloarchaea. Most Haloarchaea grow best at salinities 3.4-5M NaCl and require 1-1.5M NaCl (26). They form varied shapes- rods, cocci, disc-shaped, triangular and even square-shaped (57). The intracellular K^+ concentration of these organisms has been found to be extremely high, up to 5 M (59, 62, 66). The gradient of K^+ is maintained by combination of an electrogenic Na+/H+ antiporter and two putative K^+ uniporters (40, 50, 51). Proteins of Haloarchaea require the presence of high salts for their activity (11, 18, 19,

21). One of the most well studied proteins of Haloarchaea is a retinal protein, bacteriorhodopsin, which is acts as a proton pump and captures light and uses it to pump protons across the membrane out of the cell. Other retinal proteins of Haloarchaea are halorhodopsin, which is an inward directed chloride pump driven by light and sensory rhodopsins which mediate phototaxis. Aerobic Haloarchaea living in environments of low molecular oxygen availability produce buoyant gas vesicles that are hollow proteinaceous structures surrounding a gas-filled space which help the cells to float to more oxygenated surface layers. They live in diverse environmental conditions such as fresh water sediments to brine ponds. Other unique features of Haloarchaea are their replication, transcription and translation machinery and their cell wall composition.

Due to their unique features and propensity to live in extreme environments, halophilic archaea have often been used to as test organisms for studies in astrobiology.

1.3.2 Halotolerant and Halophilic Bacteria

In the domain of Bacteria, halophiles are found in the following phyla: *Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes* and *Bacteroidetes*. Like the *Halobacteriaceae* family in Archaea, the anaerobic fermentative bacteria of orders *Halanaerobiales* (phyla- *Firmicutes*) consists of halophiles only. The microbial mats of hypersaline lakes are dominated by the planktonic mass of *Cyanobacteria*. Extensively studied halophilic *Cyanobacterial* genera are: *Aphanothece, Spirulina* and *Dactylococcopsis* (37, 85). Amongst the Proteobacteria, the sulfur reducers, the purple sulfur bacteria and *Halomonads* have been well studied (13, 32, 84). Halophilic purple sulfur bacteria that deposit sulfur granules inside the cells are mainly moderate halophiles

belonging to the family *Chromatiaceae*. The alkaline soda lakes of Egypt and Central Africa are dominated by halophilic anoxygenic photosynthetic sulfur bacteria of the *Halorhodospira-Ectothiorhodospira* group (33). Discovery of halophilic adaptation strategy of extreme halophile *Salinibacter ruber*, which belongs to phylum *Bacteriodetes* has garnered great deal of interest in halophilic adaptations in the bacterial domain bacteria who were thought to accumulate compatible solutes as their osmoprotectants (62).

Within the scope of this study, this report will be focusing on the extreme halophile *Halorhodospira halophila* of the family *Ectothiorhodospiraceae* and genus *Halorhodospira*.

1.4 Halophilic Adaptations

In order to survive hypersaline conditions, microorganisms show adaptations at structural, cellular and molecular level.

Structural adaptation: Archaeal cell membranes contain phosopholipids which are composed of branched isoprene units linked to glycerol by an ether group where as bacterial and eukaryal membranes have fatty acids linked to glycerol by an ester bond. These archaeal membranes are less permeable to ions and more resistant to high salts (73).

Cellular adaptation: In order to avoid excessive water loss, halophiles have developed two distinct strategies to increase the osmotic activity of their cytoplasm (60).

(i) The cytoplasmic accumulation of molar concentrations of KCl, which is called as 'high salt-in' strategy

(ii) The cytoplasmic accumulation of organic compounds, referred to as compatible solutes. These compatible solutes can either be taken up from the environment by specific transport systems, or can be biosynthesized by the halophilic organism. This strategy is called 'low-salt, organic-solute-in'.

Molecular adaptations: The presence of high intracellular salt requires adaptations of the whole enzymatic machinery of the cell. The Cytoplasmic accumulation of molar concentrations of KCl results in a sharp decrease in the distribution of protein isoelectric points (52).

1.4.1 'High- Salt-in' strategy:

'High-salt-in' strategy is adaptation in which halophiles accumulate inorganic ions intra-cellularly to balance the high salt concentration of the medium. Extreme halophiles (*Halobacteriaceae* family in archaea and *Halanaerbiales* in bacteria) use this strategy to maintain the osmotic balance (15),(22), (57), (60). A concerted action of the membrane bound proton-pump bacteriorhodopsin and proteins ATP synthase and Na^+/H^+ antiporter results in electrical potential ($\Delta\psi$) that drives the uptake of potassium ions. Potassium is taken up via a K+ uniport mechanism. To enable such a transport the electrical potential ($\Delta\psi$) has to be greater than the diffusion potential of K^+ ($\Delta\psi$ K). The counterion chloride is taken up either by primary or secondary transporters. In the dark, a light-independent Cl^-/Na^+ symporter is employed, but only little is known so far about this transport mechanism and the transporter is unknown.

Salinibacter ruber has also been reported to accumulate potassium ions to maintain the osmotic balance (62). No biochemical analyses for the protein responsible for the uptake

of K^+ and Cl^- are available for this organism. However genome analysis shows that potassium could be taken up via a TrkHA transport mechanism (52). The genome of *S. ruber* encodes two copies of a putative *trkH* gene that one thought to be result of lateral gene transfer events. TrkH is the membrane spanning translocating subunit and TrkA is the cytoplasmic membrane surface protein that binds $NADH/NAD^+$ and which is essential for the transport activity. Similar to the halobacteria, chloride can also be taken up by the chloride pump halorhodopsin. Four putative genes encoding a rhodopsin are identified in the genome of *S. ruber*. Two of these are sensory rhodopsins based on sequence similarity; one is a proton pump and one a chloride pump. Additionally, two copies of Na-K-Cl co-transporter – genes are identified that are common in eukaryotes but one rarely found in prokaryotes. Such a transporter can contribute to the accumulation of K^+ and Cl^- in *S. ruber*. It was confirmed through X-ray microanalysis using an electron microscope that the ratio of K^+/protein was as high as that found in the extreme halophilic *Halobacteriaceae* family in archae (5 mol/L). The chloride concentration was also found to be same as that of cations in the cell (20).

1.4.2 'Low-salt, organic solute-in' strategy:

The 'high-salt-in' strategy requires pronounced adaptation of the whole enzymatic machinery on high salt concentrations. Such a radical adaptation would be counterproductive for a moderate halophile thriving in habitats where salinities temporarily can reach molar concentrations but also can fall to freshwater concentrations after rainfalls. Hence a more widely used approach in maintaining the osmotic balance and establish proper turgor pressure in high salt conditions is accumulation of organic solutes. These are often called 'compatible solutes' or organic osmolytes, as they do not

have any inhibitory action on metabolic processes. They also protect the proteins from denaturation in low water activity. They have high adaptability as they can tolerate a sudden or dramatic increase or decrease of salinity depending on the environmental condition. This can be done either by taking up compatible solutes from the external milieu or can be synthesized (74). Many compatible solutes have been used by halophiles, they are generally polar, highly soluble and are mostly uncharged [compatible solutes of archaea can carry a negative charge at physiological pH (48)]. Compatible solutes used by halophiles include (72):

Sugars like sucrose and treshalose- often used to stabilize membranes

- Glycerol
- Glycosylglycerol
- Betaines
- Amino acids- proline, glutamate, glutamine(glutamine residues)
- Ectoines

Organic osmolytes can occur at high concentrations in the cell. They have been identified and quantitated using analytical techniques like column chromatography, high resolution NMR spectroscopy and HPLC (72).

Compatible solutes can be synthesized de novo. This is energetically more expensive than the accumulation of potassium or chloride ions (60). Presence of glycine betaine in

Halorhodospira halochloris is the first reported presence of compatible solutes in domain bacteria (23).

1.5 Genomes of Halophiles

Halobacterium NRC-1 was the first halophile genome to be sequenced (78). Since then a number a haloarchaeal genomes have been sequenced (16). The sizes of these genomes range between 2.6 and 5.4 Mb. Some of the common characteristics of halophilic archaeal genomes are- presence of large megaplasmids and minchromosomes. However the most dramatic feature is the extremely acidic nature of the encoded proteins.

Organism		Salt range for growth (gl^{-1})	Genome size	GC content (mol %)	Total proteins
Archaea	*Haloarcula marismortui*	10-30	4.3	62	4242
	Halobacterium sp NRC-1	15-35	2.59	68	2873
	Halobacterium sp R-1	15-35	2.7	63	2892
	Natronomonas pharanois	12-30	2.59	63.4	2661
	Halorubrum lacusprofundi	9-30	2.8	62.2	2993
	Haloquadratum walsbyi	15-30	3.2	47.9	2610
	Halorhabdus utahesis	9-30	3.1	64	3078
	Halomicrobium mukohataeti	15-26.5	3.3	63	3421
	Halogeometricum borinquense	8-30	3.9	59	3993
	Haloterrigena turkmenica	10-27.5	5.4	65.83	5351
	Haloferax volcanii	6-26	4	66.64	4209
Bacteria	*Chromohalobacter salexigens*	9-250	3.6	63.91	3319
	Salinibacter ruber M13	15-35	3.5	66.2	2833
	Halorhodospira halophila	5-35	2.7	68	2407
	Halothermothrix orenii	4-20	2.5	39.6	2451

Table 1.1: Features of genome sequences of halophilic archaea and bacteria

The acidic nature of the proteins is directly related to protein functioning in a hypersaline cytoplasm. The calculated isoelectric points (pIs) of the predicted proteins show an average pI of approximately 5 (52). In contrast, the average pI of non-halophile proteomes is close to neutral. The high G+C composition of the halophilic genomes is likely an adaptation to survive intense solar radiation, as it minimizes the thymine dimers.

Amongst the halophilic bacteria, the genomes of two extreme halophiles- (*Halorhodospira halophila* and *Salinibacter ruber* (52) and two moderate halophiles- (*Halothermothrix orenii* (49) and *Chromohalobacter salexigens* (63)) have been completely sequenced. *S. ruber* is know to have the 'high-salt-in' strategy for haloadaptation and thus possesses acidic proteome, with average pI of the proteome as

5.2 (62). *Halothermothrix orenii* is a thermophile which can grow in 20% NaCl [optimum growth at 68C and 10% NaCl] (14). It does not have acidic pI and has low G+C content. Even though the non-acidic pI and low G+C content is unexpected, but can be explained due to the fact that *H. orenii* requires protein that are active both high temperatures and high salinities unlike proteins of mesophilic halophiles, which require proteins stable only at high salinities (49). *Chromohalobacter salexigens* is a moderate halophile which employs the 'low-salt, organic solute-in' strategy and accumulates ectoine (77).

1.5.1 Genome of *Halorhodospira halophila*

Halorhodospira halophila is an anoxygenic photosynthetic halophile that was isolated from salt-encrusted mud along the shores of the Summer Lake in Oregon and later also identified in the hypersaline Wadi Natrun Lake in Egypt (31, 33). *Halorhodospira halophila* is only the second extremely halophilic bacteria whose genome has been sequenced. It is a phototrophic obligately anaerobic purple sulfur bacteria. The only other purple sulfur bacterium whose genome has been sequenced is *Allochromatium vinosium*.

The genome of *H. halophila* is 2.7 Mb long and consists of one circular chromosome with 67% G+C content. The main chromosome contains 2493 genes, 2407 of which are protein coding genes. Out of 2407 genes, 1905 have been assigned a putative function and others are annotated as hypothetical proteins.

1.6 Genus *Halorhodospira*

The genus *Halorhodospira* belongs to the gamma-subdivision of Proteobacteria (31). The genus *Halorhodospira* was formed by separating species *Halorhodospira halophila*, *Halorhodospira halochloris* and *Halorhodospira abdelmalekii* from genus *Ectothiorhodospira* based on their 16S rRNA sequences (36).

Species in the genus *Halorhodospira* are moderate and extreme halophiles. They are vibroid or spiral, motile by bipolar flagella and internal photosynthetic membranes as lamellar stacks. Growth is dependent on highly saline and slightly alkaline conditions. In agar media, red- or green-colored colonies are formed. Photosynthetic pigments of the red-colored species are bacteriochlorophyll-a and carotenoids of the normal spirilloxanthin series, with spirilloxanthin as the predominant component. The green-colored species, contain bacteriochlorophyll-b; the red species has bacteriochlorophyll-a (75). The carotenoid content of *H. halochloris* and *H. abdelmalekii* is low (80). The carotenoid composition of both of these species is quite similar. Mainly methoxyrhodopin glucoside (major), rhodopin glucoside, and rhodopin have been found in *H. halochloris* (31).

1.7 Wadi Natrun Lakes

Several alkaline soda water lakes are located in the interiors or rain-shadow zones of the tropical or subtropical locations. Alkaline soda water lakes are formed due to the following conditions:

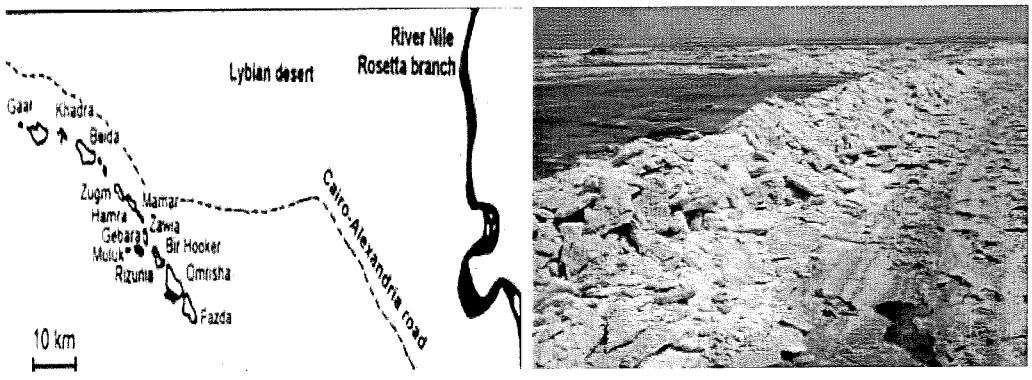
(i) Formation of alkaline drainage water

(ii) Outflow of surface water is restricted from a drainage basin

(iii) Evaporative concentration due climatic conditions

Examples of such alkaline soda water lakes are: Lake Magadi in Kenya, Wadi Natrun Lakes in Egypt, Soda Lakes in China, Mono and Big Soda Lakes in United States (27).

This report will be focusing on the Wadi Natrun lakes in Egypt.



(a) (b)

Figure 1.2: Wadi Natrun Lakes- (a) Location (1), (b) Red coloration due to photosynthetic bacteria producing bacteriochlorophyll-a.
Image obtained from: http://www.im.microbios.org/articles0203/2003/june/14%20BR%20Kunzel-Oren.jpg

The Wadi Natrun is situated about 100 km northeast of the capital city of Cairo, Egypt and extends in northeast by southeast direction. It forms a valley about 10-20 km wide in the Nile delta (Figure 1.2, panel a) (1). The valley contains a number of shallow lakes are fed by underground seepages from the Nile river and have become hypersaline by evaporative concentration. These lakes are eutrophic ecosystems. The geochemical composition of six lakes has been reported (29). The total salinity ranges from 91.9 to 393.9 g/L with average of 274.3 g/L. The organic and inorganic nutrients made up of

phosphates (116-6830 μM), nitrates (53-237 μM), ammonia (2-461 μM) and dissolved organic carbon (136-1552 mg/L). The microbial communities are made up by halophilic and alkaliphilic microorganisms (34). Due to the presence of photosynthetic puple bacteria, cyanobacteria and green algae, the water is often green, red or purple colored. The water column is generally dominated by extreme halophilic archaea of family *Halobacteriaceae*, like *Halobacterium salinarium*, *Halobacterium halobium*, *Halobacterium cutirubrum* and *Natronomonas pharonis*. The photosynthetic bacteria, *Halorhodospira halophila*, *Halorhodospira halochloris*, *Halorhodospira abdelmalekii* and *Ectothiorhodospira halalkaliphila* reside in the mats near the sediments along with cyanobacteria, *Spirulina plantesis* and *Oscillatoria limnetica*. Other microorganisms found in the Wadi Natrun lakes are- green alga *Dunaliella salina*, methanogenic archaea *Methanosalsus zhilinae* and aerobic heterotrophic bacteria *Bacillus haloalkaliphilus* (30).

1.8 Specific aims:

In this study the halophilic adaptation of extreme halophilic bacteria *Halorhodospira halophila* will be analysed. Extreme halophilicity in Bacteria is less common and less well studied, but has been described for the chemotroph *S. ruber,* but halophilic adaptations of photosynthetic extremely halophilic bacteria have not been examined. *H. halophila* exhibits unique extremophilic charactesistics as it grows in saturated salt solutions, can grow upto 47°C and is photoautotrophic. The genome sequence of *H. halophila* can provide clues about each of these characteristics. The approach to this study was to develope genome based hypotheses of the halophilic adaptations of *H. halophila* and experimentally verifiy these.

This study will address the following questions:

1) Does *H. halophila* use 'High-salt-in' strategy of halophilic adaptation?

2) Does *H. halophila* uses 'Low-salt, organic-solute-in' strategy of halophilic adaptation?

3) Is the osmoprotection strategy regulated by growth conditions?

CHAPTER TWO

BIOINFORMATIC ANALYSIS OF *HALORHODOSPIRA HALOPHILA* GENOME FOR HALOPHILIC ADAPTATIONS

2.0 Abstract

Extreme halophiles are able to thrive in media containing more than 15% NaCl and grow well at saturated NaCl concentrations. Identification and analysis of distinct genomic characteristics of halophiles can provide insight into the factors responsible for their adaptation to high-salt environments, and molecular signature indicating the type of halophilic adaptation used by the organisms. This chapter presents a comparative analysis of the genome and proteome composition of *H. halophila* with respect to other halophilic and non-halophilic microorganisms, with the aim of identifying such macromolecular signatures of haloadaptation in *H. halophila* that are experimentally testable.

2.1 Introduction

Proteins of extreme halophiles have been extensively studied as their peculiar environmental conditions require them to have distinguishing features from those of proteins from microorganisms living in 'normal' conditions. Proteins from extremely halophilic archaea are distinguished from their non-halophilic homologous proteins by their instability in low solvent salt concentration and by maintaining soluble and active conformations in high concentration of salt (11, 18, 19). Halophilic proteins bind significant amounts of salt and water, in solvent conditions similar to their physiological environment. This characteristic seems to be in contrast to that of non-halophilic proteins, which bind similar amounts of water but do not bind salt. Halophilic proteins maintain functionally active conformations in the presence of high concentrations of antichaotropic salts, whereas in the presence of chaotropic salts, their conformations become inactive (44). Statistical analysis of many halophilic proteins shows the acidic nature of the halophilic proteins (21, 86, 87). The relationship between acidic residues and salt binding has been suggested by a stabilization model proposed for the tetrameric malate dehydrogenase (MalDH) from *Haloarcula marismortui*. The amino acid residues located at the protein surface have been proposed to bind in a network of hydrated salt ions that cooperatively contribute to the stabilization (86, 87). The recent progress in genome sequencing has allowed comparison of primary structures of amino acids on a genome wide scale. A typical example of such a comparison would be the characterizing the proteins of thermophilic bacteria. Proteins from thermophiles are found to have a biased amino acid composition, with an abundance of charged residues and few polar residues

(21, 86, 87). The genome sequence allows a global analysis of the predicted isoelectric points (pI) of the proteome.

2.1.1 Acidic proteome

Organisms known to accumulate KCl have acidic proteins and the calculated Isoelectric point of the proteins is also in acidic range (15, 42, 52, 62). Organisms accumulating organic osmolytes have biosynthesis genes and transport systems for those osmolytes (5). It was checked if the *H. halophila* genome has acidic character.

2.1.2 Biosynthesis and Uptake of compatible solutes

Halophiles employ a variety of osmoprotectants (22). For three such osmoprotectants: Glycine betaine, Ectoine and Trehalose extensive information has been obtained, regarding the proteins involved in the biosynthesis and transport (4, 10, 12). This information allowed the bioinformatic analysis and identification of such systems in halophile genomes and is reported below. The remainder of this section provides summary of the current knowledge on the proteins involved on the biosynthesis and uptake of compatible solutes. In section 2.3 this published information is used to investigate the genome of *H. halophila* for the presence of similar proteins.

2.1.2a Glycine betaine

Biosynthesis:

Glycine betaine (*N,N,N*-trimethylglycine) is an osmoprotectant found in many bacteria. It is accumulated at high cytoplasmic concentrations in response to osmotic stress, to act as an osmoprotectant (35). In addition to its osmoprotectant activity, glycine betaine is also

a gives protection against mutagenic compounds and radiation-induced damage (38). Glycine betaine can either be taken up directly from the environment, or synthesized (54). Two pathways for the biosynthesis of glycine betaine are known:

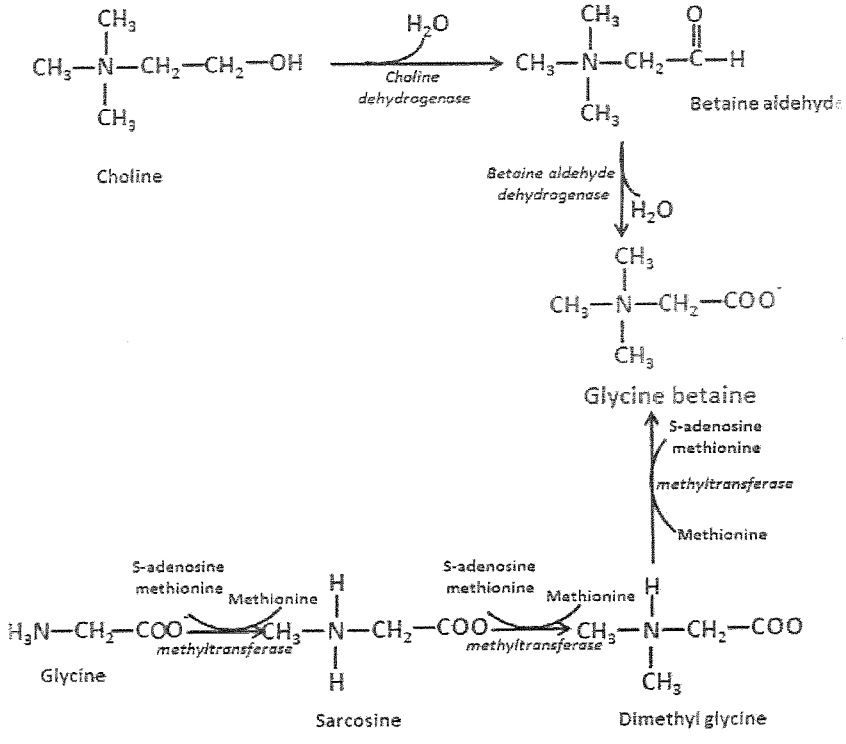


Figure 2.1: Pathways for biosynthesis of glycine betaine.

A common biosynthesis pathway for glycine betaine is from choline, (synthesized via a series of methylation steps from homocysteine) utilizing a two-step pathway with betaine aldehyde as intermediate. This pathway is conserved in bacteria and plants, but has enzymes involved. Gram-negative bacteria, Gram-positive bacteria and higher plants all

use a betaine aldehyde dehydrogenase to catalyze the conversion of betaine aldehyde to glycine betaine. Gram-negative bacteria such as *E. coli, Pseudomonas aeruginosa*, and *Synorhizobium meliloti* utilize a choline dehydrogenase, Gram-positive bacteria, such as *Bacillus subtilis*, use an alcohol dehydrogenase (10). There is also another pathway for the synthesis of glycine betaine, which is found in several halotolerant bacteria (see below). The pathway was found in *Halorhodospira halochloris* (previously known as *Ectothiorhodospira halochloris*) and several other organisms (54), which synthesize glycine betaine from glycine through a series of methylation reactions. However, since methylation reactions are among the most energy-consuming processes in nature (the regeneration of one active methyl group of S-adenosylmethionine costs the cell 12 ATP equivalents), this pathway is less common. This might be part of their adaptation to extreme conditions. Several organisms have been shown to synthesize glycine betaine from glycine through a series of methylation reactions. These include halophilic and halotolerant *Actinopolyspora halophila, Halorhodospira halochloris, Aphanothece halophytica*, and *Methanohalophilus portuclensis* (54, 55).The enzymes performing the methylation reactions have been characterized in *Actinopolyspora halophila* and *Halorhodospira halochloris*, and were found to be two methyltransferases, glycine-sarcosine methyltransferase (GSMT) and sarcosine-dimethylglycine methyltransferase (SDMT), with partially overlapping substrate specificity (Johnson, 1986 #103;Johnson, 1986 #141). The pathway is conserved with differences among the different organisms in the substrate specificity of the enzymes.

In *H. halochloris* and *A. halophila* both SDMT and GSMT have SDMT activity (accepting sarcosine as a substrate), so that they overlap in that respect. However, only

GSMT can accept glycine as a substrate, and only SDMT can accept dimethylglycine as a substrate. In *A. halophytica*, on the other hand, the first methyltransferase possesses both GSMT have SDMT activities, but the second one only catalyzes the methylation of dimethylglycine.

It has been shown that *Actinopolyspora halophila* (Actinobacteria) possesses both pathways for glycine betaine synthesis – the above *de novo* pathway and a pathway of choline oxidation (55).

Transport of glycine betaine:

In *Bacillus subtilis* the uptake of glycine betaine is mediated by three osmoregulated uptake systems belonging either to the ABC type (OpuA and OpuC) or to the class of secondary carriers (OpuD) (41). *C. glutamicum* is equipped with four secondary transporters for compatible solutes, namely BetP, PutP, ProP, and EctP. Two of these transport systems, BetP and ProP, are osmoregulated at the level of expression as well as on the level of activity, whereas EctP is constitutively expressed, but osmoregulated at the level of activity. PutP is an anabolic proline carrier and not involved in the process of salt adaptation. EctP with its broad substrate spectrum seems to be the emergency system for *C. glutamicum*. Its constitutive expression may protect the cells from unexpected changes of the external osmolarity (67).

2.1.2b Ectoine

Biosynthesis:

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is an osmoprotectant, and only a single pathway for its biosynthesis is known (12). The first two steps are shared with the biosynthesis of amino acids in the aspartate family. The ectoine synthesis genes of the moderately halophilic bacterium *Halomonas elongata* (*Chromohalobacter salexigens*) have been extensively studied (Figure 2.2). Aspartic-β-semialdehyde is converted to 2,4-diaminobutyrate by the enzyme, 2,4-diaminobutyrate aminotransferase. 2,4-diaminobutyrate is converted to γ-*N*-acetyl-α, γ -diaminobutyric acid by the enzyme diaminobutyrate acetyltransferase. The last step in ectoine biosynthesis is circularization of γ-*N*-acetyl-α, γ -diaminobutyric acid to ectoine by enzyme ectoine synthase.

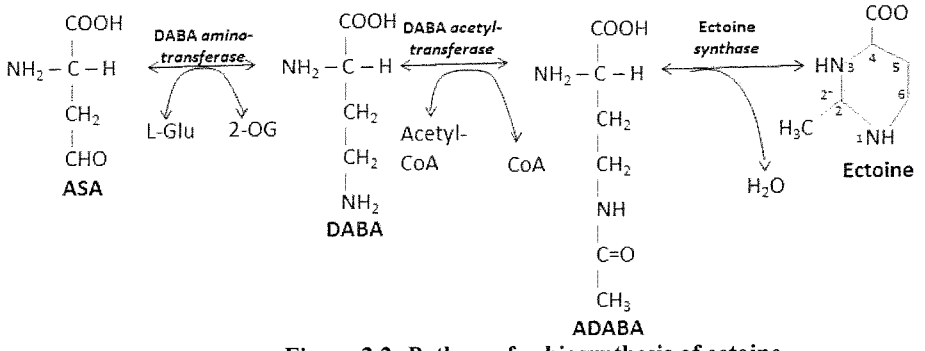


Figure 2.2: Pathway for biosynthesis of ectoine

Transport of Ectoine:

Two ectoine uptake systems found in extremophiles are TeaABC in *Halomonas elongata* (25) and Ota system in *Methanogenic archaea, Methanosarcina mazei* (76). TeaABC belongs to the family of TRAP transporters and as such it consists of two transmembrane proteins (TeaB, TeaC) and a periplasmic substrate-binding protein (TeaA). EctP with its broad substrate spectrum is the emergency system for *C. glutamicum* which is capable of ectoine uptake. Whereas the TeaABC is the only osmoregulated transporter for ectoines found in *H. elongata*, EctP's constitutive expression may protect the cells from unexpected changes of the external osmolarity.

2.1.2c Trehalose

Biosynthesis of Trehalose:

In prokaryotic organisms trehalose can be used as external carbon source, a compatible solute (in cyanobacteria and photosynthetic bacteria), a stress protector, and a structural component (part of the cord factor in mycobacteria) (3). In yeast and filamentous fungi, the main roles of trehalose are as a carbohydrate storage and as a stress protector. The enzymes for trehalose metabolism are present in higher animals, but their precise role is not well understood. Six different pathways for trehalose biosynthesis are known (4) (Figure 2.3). The enzymes for the biosynthesis of trehalose in *E. coli*, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), have been studied, and are encoded by the genes *otsA* and *otsB*. These genes are similar to the yeast genes TPS1 and TPS2 (39). In *S. cerevisiae* the two enzymes are combined into a single complex, called the trehalose synthase complex, which also includes the regulatory

subunits TSL1 and TPS3 (9). It should be noted that some authors use TPS3 as a redundant name for TSL1, while others use TPS3 for a different regulatory subunit.

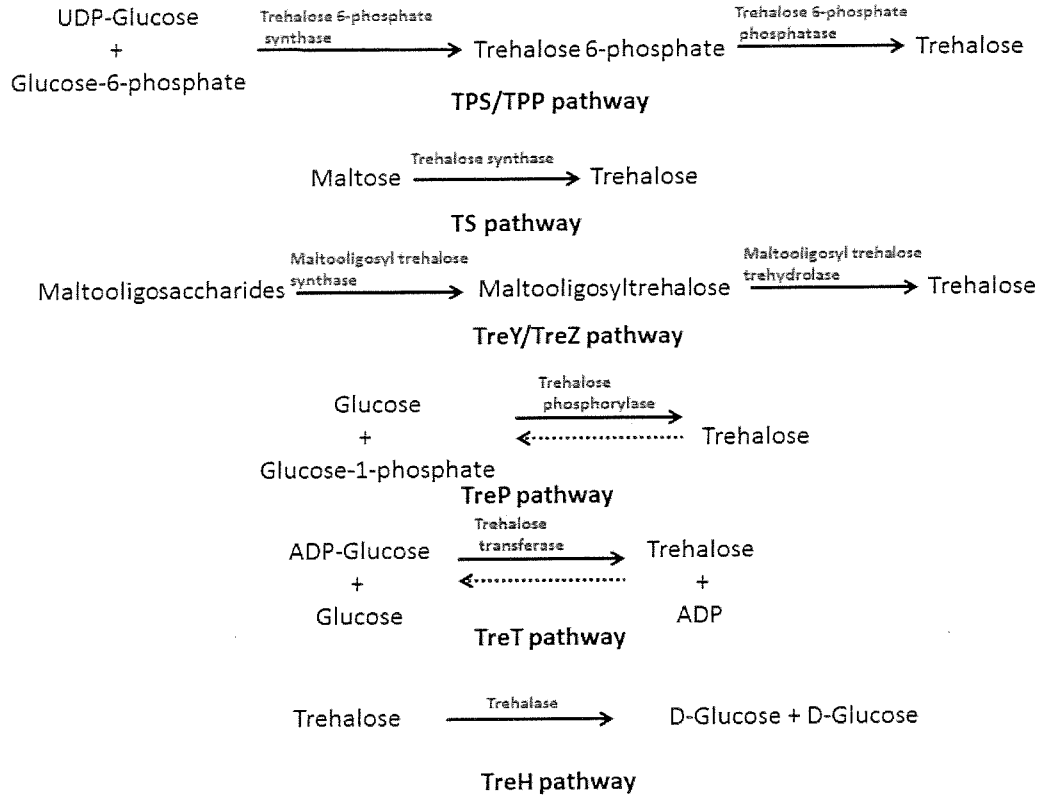


Figure 2.3: Pathways for biosynthesis of trehalose

Trehalose-6-phosphate synthases have been purified from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* that are capable of utilizing all five nucleoside diphosphate glucose derivatives (ADP-D-glucose, CDP-D-glucose,GDP-D-glucose, TDP-D-glucose and UDP-D-glucose) as glucosyl donors for generating trehalose-6-phosphate (17 and 65).

In Summary: The proteins for the biosynthesis of these compatible solutes (glycine betaine, ectoine and trehalose) and transport systems of glycine betaine and ectoine have

been reported. In addition, halophiles using KCl as osmoprotectant have been reported to exihibit an extremely acidic proteome. Below the presence of these properties in the *H. halophila* genome are investigated.

2.2 Methods

Genomes Compared: Proteins from the *H. halophila* genome were compared with those of the following organisms, which were selected as representative of the following lineages- From extreme halophilic archaea: *Haloarcula marismortui*, *Halobacterium* sp NRC-1, *Halobacterium* spR-1, *Natronomonas pharanois*, *Halorubrum lacusprofundi*, *Haloquadratum walsbyi*, *Halorhabdus utahesis*, *Halomicrobium mukohataeti*, *Halogeometricum borinquense*, *Haloterrigena turkmenica*, *Haloferax volcanii*. From extreme halophilic bacteria: *Salinibacter ruber* M13. From moderately halophilic bacteria: *Chromohalobacter salexigens* and *Halothermothrix orenii*. From non-halophilic archaea: *Archaeglobus fulgidus*. From non-halophilic bacteria: *Escherichia coli*, *Nitrosococcus oceanii* and *Alkalilimnicola ehrlichei* MLHE-1.

Protein dataset: FASTA sequences of proteins of selected organisms obtained through NCBI website.

Isoelectric points: Isoelectric points of the proteins were calculated by putting the FASTA sequences of the protein in the Expasy server: http://ca.expasy.org/tools/pi_tool.html. Percentage distribution of amino acid residues in the organisms were obtained by submitting FASTA sequences to- http://proteome.gs.washington.edu/cgi-bin/aa_calc.pl.

Transmembrane helices and Signal Peptide: The predictions of the trans-membrane regions and the signal peptides were performed by the programs TMHMM and SignalP, respectively.

2.3 Results:

2.3.1 pI distribution in *H. halophila*:

One of the most interesting results of sequencing of haloarchaea and *S. ruber* was identification of the extremely acidic nature of their encoded proteins, which was relates to the protein function in the hypersaline cytoplasm ('High-salt-in' strategy). Calculated isoelectric points (pIs) for the predicted proteins showed an average pI of approximately 5. In contrast, the average pIs of moderate halophiles like *C. salexigens* and non-halophiles like, *N. oceanii* that employ 'Low-salt, organic solute-in' strategy along are close to neutral (Table 2.1).

Organism	pI of the proteome
Haloarcula marismortui	4.6
Halobacterium sp NRC-1	4.6
Halobacterium sp R-1	4.7
Natronomonas pharanois	5.4
Halorubrum lacusprofundi	5.6
Haloquadratum walsbyi	5.1
Halorhabdus utahesis	5.8
Halomicrobium mukohataeti	5.7
Halogeometricum borinquense	5.7
Haloterrigena turkmenica	5.4
Haloferax volcanii	5.6
Salinibacter ruber M13	5.2
Halorhodospira halophila	5.5
Chromohalobacter salexigens	7.2
Halothermothrix orenii	7.8
Escherichia coli	7.4
Nitrosococcus oceanii	7.6
Alkalilimnicola ehrlichei MLHE-1	7.9

Table 2.1: pI's of halophilic archaea and bacteria

All the predicted proteins encoded *H. Halophila* were examined for their predicted pI using the Expasy server. An acidic proteome was predicted for the H. halophile genome with the average pI of the proteome as 5.5. This value is slightly less acidic as compared to haloarchaeal pIs like *Halobacterium* sp NRC-1 and *H. marismortui*. The predicted proteins of *H. halophila* were also compared with a non-halophilic, phylogenetic neighbor, *N. Oceanii* (Table 2.2)

pI	No. of proteins		
	Halorhodospira halophila	*Halbacterium sp NRC-1*	*Nitrosococcus oceanii*
2.01-3.0	1	2	0
3.01-4.0	93	439	33
4.01-5.0	821	1172	422
5.01-6.0	520	134	491
6.01-7.0	326	71	513
7.01-8.0	117	50	294
8.01-9.0	149	41	311
9.01-10.0	167	79	496
10.01-11.0	119	45	315
11.01-12.0	98	41	83
12.01-13	0	9	16
13.01-14	0	0	0
Total	**2407**	**2074**	**2974**

Table 2.2: Acidic and alkaline proteins in *H. halophila*, *Halobacterium* and *N. oceanii*

For the moderate halophile *C. salexigens* it has been reported that its secreted proteins are acidic, whereas its cytoplasmic and membrane proteins are nearly neutral (63). The pI value and amino acid composition of cytoplasmic, secreted and membrane proteins of extremely halophilic, moderate and non-halophilic archaea and bacteria, along with *H. halophila* were calculated (Table 2.3 and 2.4). Cytoplasmic and secreted proteins in *H. halophila* are more acidic then cytoplasmic and secreted proteins of its non-halophilic and phylogenetic neighbor *N. oceanii*. Predicted proteins in these fractions of *H. halophila* have more glutamic acid and aspartic acid residues as compared to proteins of *N. oceanii*.

Organisms	Proteins		
	Cytoplasmic	Secreted	Membrane
Haloarcula marismortui	4.1	3.92	6.89
Halobacterium sp NRC-1	4.29	3.98	7.22
Halorubrum locusprofundii	4.23	4.06	6.56
Natronomonas pharonis	4.38	4.27	6.45
Archaeglobus fulgidus	5.68	6.52	7.25
Salinibacter ruber	4.36	4.31	7.92
Halorhodospira halophila	5.12	4.57	8.56
Alkalimnicola ehrlichei MLHE-1	4.98	6.96	7.62
Nitrosococcus oceanii	5.78	7.22	8.21
Escherichia coli	5.66	6.96	8.83
Cytophaga hutchinsonii	5.45	7.24	7.92

Table 2.3: Average pI of different fractions of proteins in various halophilic and non-halophilic archaea and bacteria

Organisms	Cytoplasmic				Secreted				Membrane			
	Acidic	Basic	Ser+Thr	Hydrophobic	Acidic	Basic	Ser+Thr	Hydrophobic	Acidic	Basic	Ser+Thr	Hydrophobic
H. marismortui	15.2	11.5	9.2	30.5	17.2	8.7	11.2	37.1	12.3	10.5	11.6	35.8
H sp NRC-1	16.8	11.2	10.3	31.2	13.2	7.2	11.8	36.1	11.5	11.6	9.8	36.4
H. locusprofundii	14.9	12.3	10.5	32.3	14.1	7.6	10.5	35.8	10.8	12.5	9.2	36.2
N. pharonis	15.8	12.1	10.1	31.8	13.7	6.9	11.5	36.7	12.5	10.8	10.3	36.1
A. fulgidus	14.3	10.2	9.2	38.1	11.2	10.3	9.6	35.8	12.4	12.1	11.2	34.9
S. ruber	13.6	10.9	9.8	32.1	16.2	8.7	9.8	37.2	10.6	10.6	10.3	35.8
H. halophile	14.2	11.8	9.6	37.5	13.6	7.6	10.3	36.2	11.8	10.3	10.5	35.2
A. ehrlichei	13.2	15.2	9.4	37.2	13.4	17.2	10.2	34.9	10.3	9.9	9.8	35.1
N. oceanii	14.1	11.5	10.4	36.4	12.8	11.2	10.5	35.4	11.5	10.4	11.2	34.6
E. coli	13.2	12.7	10.6	38.1	15.2	13.2	11.3	34.9	13.5	10.2	10.5	35.5
C. hutchinsonii	12.9	10.3	9.8	37.6	14.3	11.8	10.8	35.2	12.8	12.3	10.7	38.3

Table 2.4: Amino acid composition of different fractions of the genomes of halophilic and non-halophilic archaea and bacteria

Extremely halophilic archaea of *Halobacteriaceae* family and *S. ruber* (bacteria) accumulate upto 5M KCl in their cytoplasm. As the genome sequences of these extremely halophilic archaea and *S. ruber* are available a bioinofrmatic analysis of the

predicted proteins can be carried out. The proteomic distribution of the predicted proteins in these organisms in strongly shifted to acidic values (Table 2.1). This is caused by a large excess of glutamic acid and aspartic acid residues, which allow the proteins to function in the hypersaline cytoplasm. The isoelectric point of the predicted proteins in *H. halophila* was calculated and compared with known extreme halophiles which accumulate KCl, and non-halophilic archaea & bacteria including *N. oceanii* a non-halophilic phylogenetic neighbor of *H. halophila*. The average pI value of *H. halophila* proteome is 5.5. *H. halophila* has an intermediate acidic character in comparison with the average pI value of 4.6 of the predicted proteins of *Halobacterium* spp NRC-1 and average pI value of 7.6 of the predicted proteins of *N. oceanii*. Out of 2407 predicted proteins of *H. halophila*, 1435 proteins are acidic in nature and 972 proteins are alkaline while in Halobacterium spp NRC-1 out of 2074 predicted proteins, 1747 are acidic in nature ans 327 are alkaline. This trend of higher acidic predicted proteins is not followed in N. oceanii in which has 946 acidic predicted proteins and 2094 alkaline proteins in the total of 2974 predicted proteins.

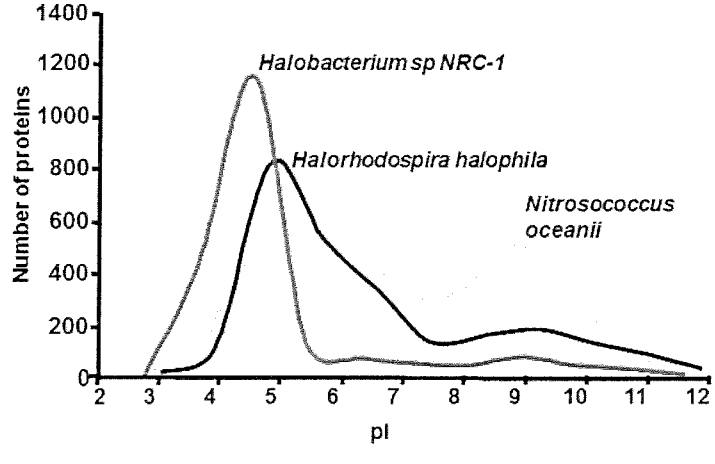


Figure 2.4: pI distribution of genome of *H. halophila, H. salinarium* and *N. oceanii*

H. halophila has a higher percentage of acidic residues compared to its non-halophilic neighbor, *N. oceanii* and its pI distribution is also similar to *Halobacterium* sp. NRC-1 (Figure 2.5). Comparison of pI and amino acid composition of the cytoplasmic, secreted & membrane proteins showed that *H. halophila* has a acidic proteome.

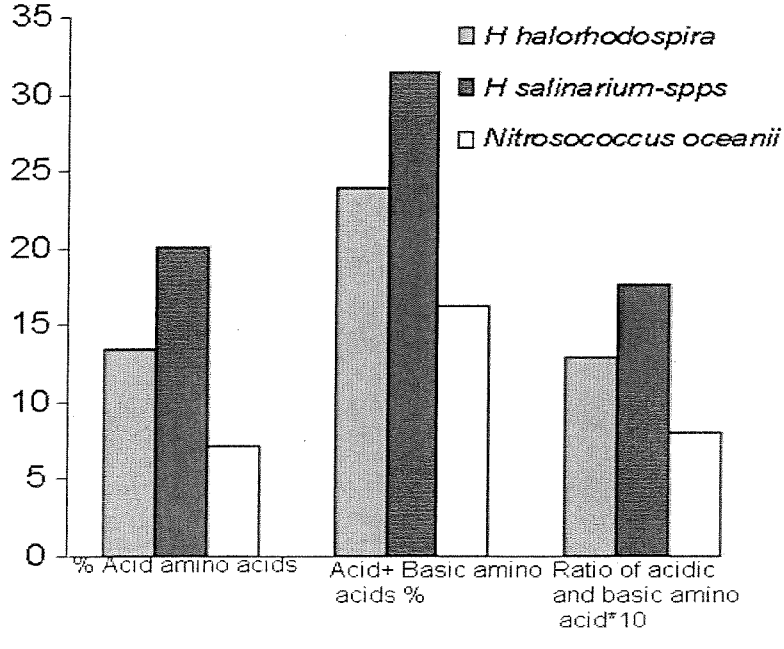


Figure 2.5: Acidic and Basic amino acids in *H. halophila*, *H. salinarium* and *N. oceanii*

2.3.2 Biosynthesis of compatible solutes:

The *H. halophila* genome was examined for presence of biosynthesis genes for glycine betaine, ectoine and trehalose using reference sequences from bacteria known to have uptake and biosynthesis genes for these compatible solutes. For glycine betaine, the best hits obtained were from GbsA and GbsB genes from *B. subtilis*, which uses pathway II and GSMT and SDMT genes from *H. halochloris* and *A. halophytica* (pathways II and III described in section 2.1.2a). BetA and BetB genes from *E. coli* did not yield any significant hits in the *H. halophila* genome. For ectoine the best hits obtained in *H. halophila* were that from EctA and EctB gene (described in section 2.1.2b) from *C. salexigens*, which is a moderate halophile. TPS1 and TPS2 from *E. coli* gave significant hits, but TreY/Z, TP, TreT and TreH genes for biosynthesis of trehalose in *S. cereviciae* and *M. tuberculosis* (described in section 2.1.2c) did not give a significant hit in *H. halophila* (Table 2.5).

Compatible solutes	Genes involved	Reference sequences used	Scores of hits obtained in *H. halophila* when compared to reference sequences		Presence in *H halophila*
			% Similarity	% Identity	
	Pathway I in gram negative bacteria :				-
	BetA		17	12	-
	BetB	*E. coli*	19	17	-
	Pathway II in gram positive bacteria				
	GbsA		48	30	+
	GbsB	*B. subtilis*	38	20	+
	Pathway III using glycine as a substrate				
	GSMT		94	90	+
	SDMT	*H. halochloris*	72	82	+
	GSMT		80	65	+
Glycine betaine	SDMT	*A. halophytica*	71	52	+
	EctA		48	36	+
	EctB		70	58	+
Ectoine	EctC	*C. salexigens*	67	43	+
	TPS1		64	45	+
	TPS2	*E. coli*	38	24	+
	TreY/Z		19	12	-
	TP		28	23	-
	TreT		30	27	-
Trehalose	TreH	*M. tuberculosis*	26	22	-

Table 2.5: Compatible solutes biosynthesis genes in *H. halophile.* Hits identified as significant are indicated with + sign

2.3.2a Glycine betaine biosynthesis:

Three genes for the biosynthesis of glycine betaine in *H. halophila* were identified. These were the hits obtained using the GbsAB genes from *B. subtilis* and GSMT and SDMT of *H. halochloris* and *A. halophytica.* The alignment of GbsAB from *B. subtilis* shows high percentage of identity and similarity (Figure 2.6 and Table 2.5) also alignment of the GSMT and SDMT from *H. halophila* with those from *H. halochloris* and *A. halophytica* (Fig. 2.6 and 2.5) revealed a high level of sequence similarity and the absence of gaps. Hence, these alignments were convincing. The *H. halophila* GSMT and SDMT have a high identity and similarity with *H. halochloris* (82% and 90% respectively)

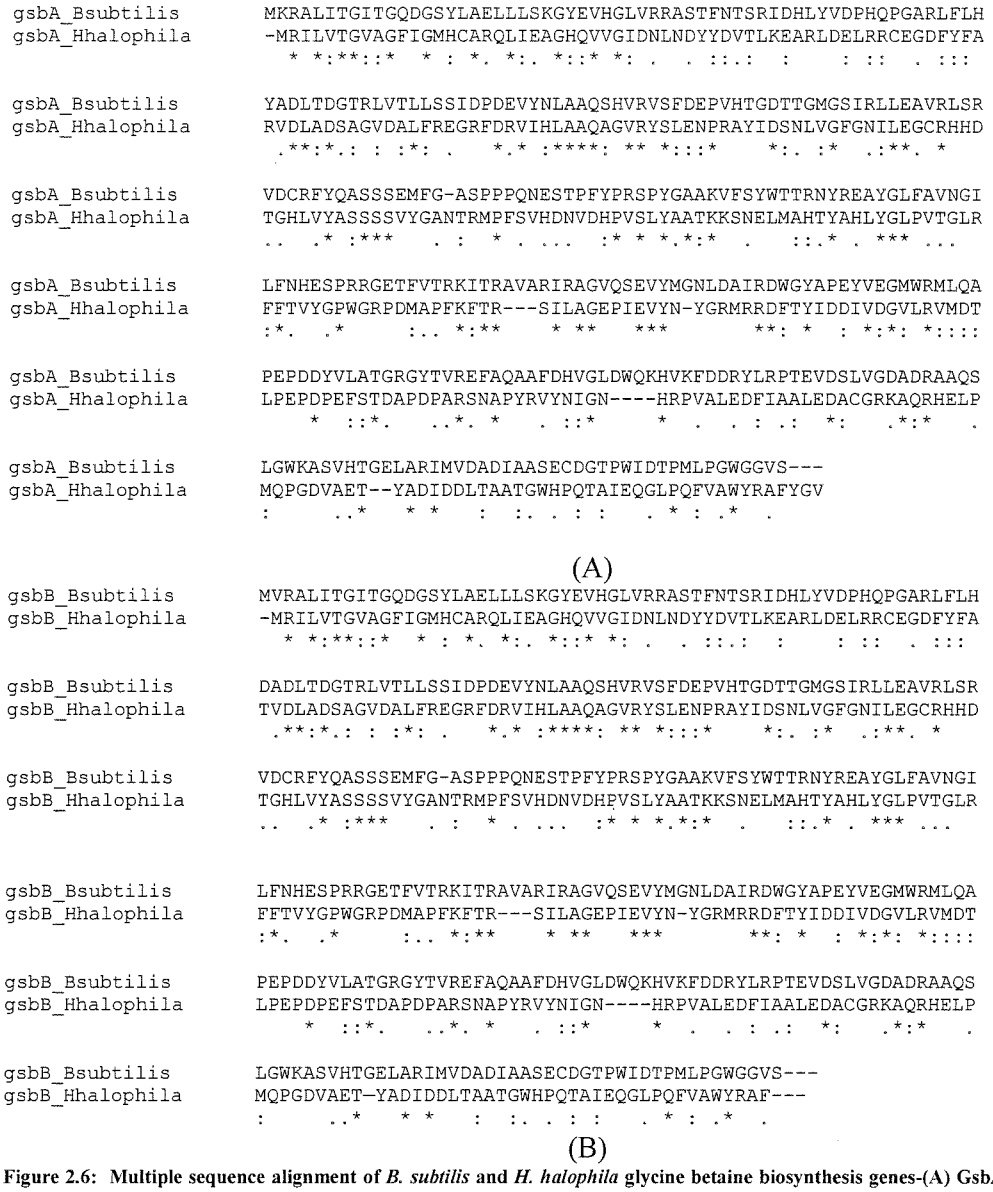


Figure 2.6: Multiple sequence alignment of *B. subtilis* and *H. halophila* glycine betaine biosynthesis genes-(A) GsbA and (B) GsbB

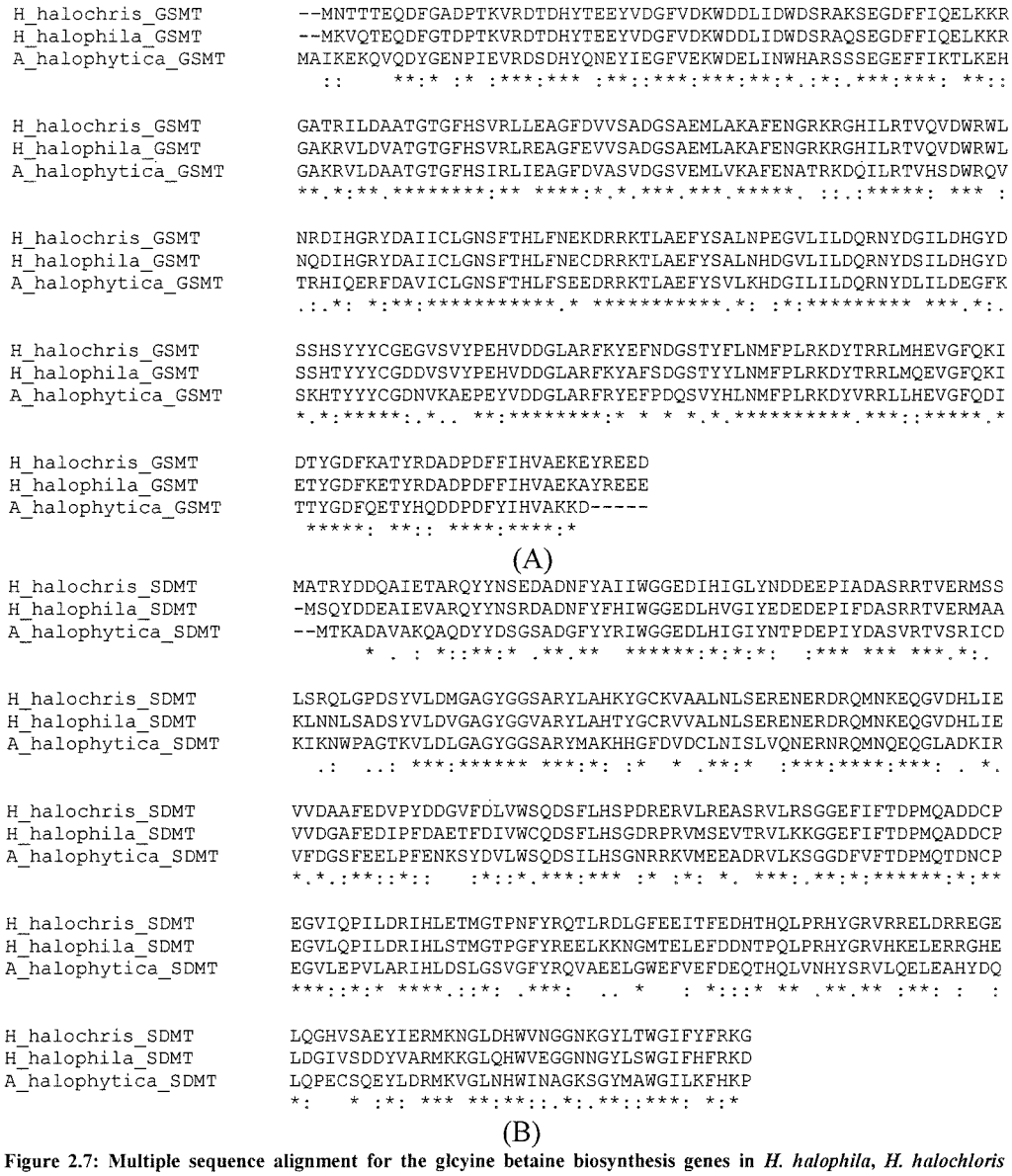


Figure 2.7: Multiple sequence alignment for the glcyine betaine biosynthesis genes in *H. halophila*, *H. halochloris* and *A. halophila*. (A) GSMT of *H. halophila*, *H. halochloris* and *A. halophila*. and (B) SDMT of *H halophila*, *H. halochloris* and *A. halophila*.

The genes encoding GSMT and SDMT from *H. halophila* are located immediately adjacent to each other in a likely operon (Fig 2.8). The two adenosyl methionine-related genes immediately upstream of the GSMT may be involved in biosynthesis of glycine betaine by providing two methyl groups required for methylation of sarcosine and dimethylglycine. The methyl group (CH_3) attached to the methionine sulfur atom in S-

adenosyl methionine (SAM) donated to Sarcosine and then dimethylglycine via transmethylation reactions. This multiple sequence alignment for the three methyltransferases from *H. halophila, A. halophytica* and *H. halochloris* are homologous. Hence we conclude that *H. halophila* synthesizes glycine betaine using same pathway as used by *A. halophytica* and *H. halochloris.*

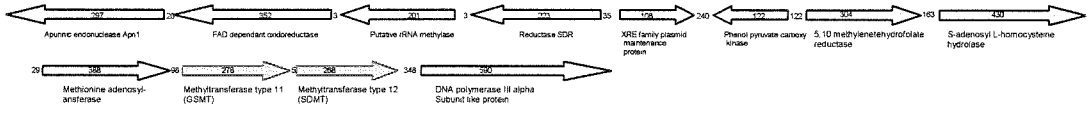


Figure 2.8: Probable glycine betaine synthesis operon in *H. halophila*. The proposed two-gene operon for glycine betaine synthesis is indicated in orange. The number of residues encoded by each gene is indicated, together with the number of intergenic nucleotides.

2.3.2b Ectoine:

Ectoine biosynthesis:

All the three genes used by *C. salexigens* for biosynthesis of ectoine were found in *H. halophila*. EctB (2,4-diaminobutyrate aminotransferase) of *H. halophila* has 58% identity and 70% similarity *C. salexigens*. EctA (diaminobutyrate acetyltransferase) of *H. halophila* has 48% identity and 36% similarity *C. salexigens*. EctC (ectoine synthase) of *H. halophila* has 67% identity and 43% similarity *C. salexigens*. The multiple sequence alignment of the three enzymes shows a high degree of sequence similarity and absence of sequence gap (Figure 2.10).

Figure 2.9: Sequence alignment of the final three ectoine biosynthesis genes of *H. elongata* and *H. halophila*.

Based on the multiple sequence alignment and high blast scores it can be concluded that *H. halophila* synthesizes ectoine using same biosynthetic pathway as *C. salexigens*. The following (Figure 2.11) is the proposed operon structure of ectoine synthesis in *H. halophila*.

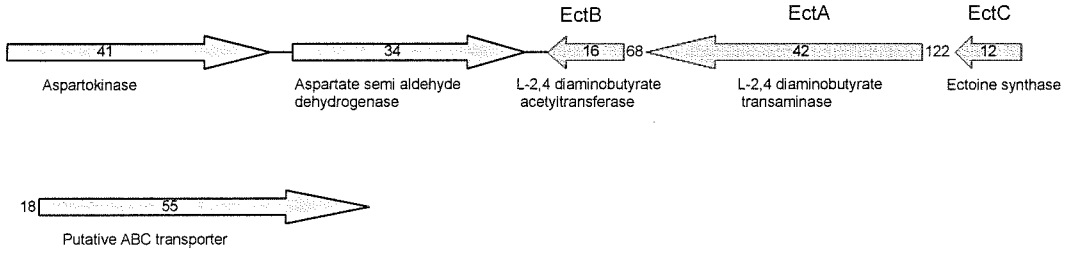


Figure 2.10: Proposed operon structure of ectoine synthesis in *H halophila*.

2.3.2c Trehalose:

Biosynthesis:

In *H. halophila* two genes similar to the otsAB genes responsible for trehalose biosynthesis in *E. coli* are present. The trehalose-6-phosphate synthase of *E. coli* and *H. halophila* have 64% similarity and 45% identity. The trehalose-6-phosphate phosphatases of *E. coli* and *H. halophila* have 38% similarity and 24% identity. The genes give a good sequence alignment (Figure 2.17)

```
TPS_Ecoli       MMTMSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNEDQPLK--K
TPS_Hhal        ---MSRLVTVSNRVALPSQLQAAQGGLAVGLRSALEESGGMWFGWDGGVDERIDGLRQPR
                   *****.****:* *.:  *: ******: .**: :**:****.* ..:. : *:  :

TPS_Ecoli       VKKGN-ITWASFNLSEQDLDEYYNKFSNAVLWPAFHYRLDLVQFQRPAWDGYLRVNALLA
TPS_Hhal        VQTANGVRYATLRLSRLEYDRYYLGYANQVLWPLFHYRMSFVHCRRERIEGYWEVNRLFA
                *:..* : :*::.**. : *.**  ::* **** ****:.:*: :*   :** .** *:*

TPS_Ecoli       DKLLPLLQDDDIIWIHDYHLLPFAHELRKRGVNNRIGFFLHIPFPTPEIFNALPTYDTLL
TPS_Hhal        EHLPPLLEGDEIIWVHDYHFIPLGQLLREQGVEAPIGFFLHTPFPPWDVFRALPGHEPLL
                ::* ***:.*:***:****::*:.: **::**:  ****** ***. ::*.*** ::.**

TPS_Ecoli       EQLCEYDLLGFQTENDRLAFLDCLSNLTRVTTRSAKSHTACGKAFRTEVYPIGIEPKEIA
```

```
TPS_Hhal        EALCRYDLVGFQTRIDRDNFLDCLTHYRPQLQRP----------RAEVFPISIDVDQVA
                * **.***:****. **  *****::      *.           *:**:**.*: .::*

TPS_Ecoli       KQAAGPLPPKLAQ-LKAELKNVQNIFSVERLDYSKGLPERFLAYEALLEKYPQHHGKIRY
TPS_Hhal        REAQRGYNSQQGRRLQQSLRDRRLMIGVDRLDYSKGLRNRFEAYEALLEQHSEHRGDVVF
                ::*     .: .: *: .*:: : ::.*:******** :** *******::.:*:*.: :

TPS_Ecoli       TQIAPTSRGDVQAYQDIRHQLENEAGRINGKYGQLGWTPLYYLNQHFDRKLLMKIFRYSD
TPS_Hhal        LQIAPVSRGDVPEYEEIRQYLEYLAGHINGRFAEYDWVPLRYLNRGFHRSNILGFLARSD
                 ****.*****  *::**: **  **:***::.: .*.** ***: *.*. :: ::  **

TPS_Ecoli       VGLVTPLRDGMNLVAKEYVAAQDPANPGVLVLSQFAGAANELTSALIVNPYDRDEVAAAL
TPS_Hhal        VGLITPMRDGMNLVAKEFVAAQDPGDPGALVLSRYAGAAEELDGAVLVNPYDVDQMVDAM
                ***:**:**********:******.:**.****::****:** .*::***** *::. *:

TPS_Ecoli       DRALTMSLAERISRHAEMLDVIVKNDINHWQECFISDLKQIVPRSAESQQRDKVATFPKL
TPS_Hhal        HQALTMPLGERRERWQQMMDALRRQDVHRWRKDFIQALHDAH-RARGSEAL---------
                .:****.*.** .*  :*:*.: ::*:::*:: **. *::   *:  *:

TPS_Ecoli       A
TPS_Hhal        -

TPP_ecoli       ----MIRPVTLTEPHFSQHTLNKYASLMAQGNGYLGLRASHEEDYTRQTR--GMYLAGLY
TPP_hhal        MTLPETEGWQLTYQGWLPEQQQHREAICVLGNGRFATRGAFEGAAPGDTHYPGTYMAGGY
                      .   **   :  .  ::  :: . *** :. *.:.*   . :*:  * *:** *

TPP_ecoli       HR-----AGKGEIN-ELVNLPDVVGMEIAING----EIFSLSREAWQRELNFASGELRRN
TPP_hhal        NRRTSTVSGRGVENEDLVNLPNWLCLNFRPAEGDWLDFDTVEWLDYRQTLDLRHGVLTWH
                :*     :*:*  * :*****: : :::        :: ::.   ::: *::  * *  :

TPP_ecoli       VVWRTSNGAGYTITSRRFVSADQLPLIALEITITPLDADALVLISTGIDATQTNHG----
TPP_hhal        LHFRDPAGRETRLTSRRLMHMGDSHLAAIHWELEPVNWTGALHIRSGIDGGVENLGVARY
                : :* . *    :****::  .:  * *:.  : *::  . : * :***.   * *

TPP_ecoli       ----RQHLDETQVRVFGQHLMQGIYTTQDGRSDVAISCCCKVSGDVQQCYTAKERRLLQH
TPP_hhal        RALETRHLDVLATEFFSDEAVLLRSMTNQSRIDLANAARTRAWQADGRGPEARER-----
                     :***   ...*.:. :     *::.* *:* :.  :.     :   *:**

TPP_ecoli       TCAQLHAGETLTLQKRVWIDWRDDRQAALDEWGSASLRQLEMCAQQS------YDQLLAA
TPP_hhal        VETDAYLGHDLTLQAEPGQPIRVEKVAALYSGRDRAICEPGIDATAAVERAPGFDELLRS
                . :: : *. **** .     * :: *** .  . :: :  : *  :      :*:** :

TPP_ecoli       STENWRQWWQKRRITVNG---GDAHDQQALDYALYHLRIMTPAHDE--RSSIAAKGLTGE
TPP_hhal        HAKAWERYWRGWDVTLHTDNNGDAEEQTVLRLHIFHLLQTTSLHTTDLDVGVPARGLHGE
                 :: *.::*:   :*::    ***.:* .*   ::**   *. *      .:.*:** **

TPP_ecoli       GYKGHVFWDTEVFLLPFHLFSDPTVARSLLRYRWHNLPGAQEKARRNGWQGALFPWESAR
TPP_hhal        AYRGHIFWD-ELFILPLLNLHSPEISRALLMYRYRRLPEARRAASAEGLRGAMYPWQSGS
                .*:**:*** *:*:**:  : .* ::*:** **::.** *:. *  :* :**::**:*.

TPP_ecoli       SGEEETPEFAAINIRTGLRQKVASAQAEHHLVADIAWAVIQYWRTTGDESFIAHEGMALL
TPP_hhal        SGREETQTLHLNPASG--RWLPDDTHRQRHVNAAIAHNIWRYYEVSGDTDFMAFAGAEMI
                **.***  :         *    .:: ::*: * **  : :*:..:** .*:*. *  ::

TPP_ecoli       LETAKFWISRAVRVNDR--LEIHDVIGPDEYTEHVN--------NNAFTSYMAYYNVQQA
TPP_hhal        LSIAQFWASLATYNPERQRYEIHGVVGPDEFHTRYPDSDTIGLSNNAYTNVMAAWCLHIA
                *. *:** * *.   :*   ***.*:****:  :          ***:*. ** : :: *

TPP_ecoli       LNIARQFG-CSDDAFIHRAEMYLKELL---------LPEIQPDGVLPQDDSFMAKPVINL
TPP_hhal        EQALEVIGPTPRNELLDRLEIDQAELARWQEIGQRMFIPFHGDGIISQFEGYEQLEELDW
                 :  . :*  . : ::.* *:   **          :  :: **::.* :.:     ::

TPP_ecoli       AKYKAAAG---KQTILLDYSRAEVNEMQILKQADVVMLNYMLPEQ------------FSA
TPP_hhal        AGYRERYGNIQRLDRILEAEGEDINRYQASKQADLLMLFYLFPRQQIESLLAEMGYELDA
                * *:   *   :   :*: .  ::*. *  ****::** *::*.*            :.*

TPP_ecoli       ASCLANLQFYEPRTIHDSSLSKAIHGIVAARCGLLTQSYQFWREGTEIDLG-ADPHSCDD
TPP_hhal        EAIPRNIAYYEARTSHGSTLSNIVHSWVLARS-DRQRSWDLFGNALISDLGDSQGGTTKE
                 :   *: :**.** *.*:**: :*. * **.    :*:::: :.   *** ::  : .:

TPP_ecoli       GIHAAATGAIWLGAIQGFAGVSVRDGELHLNPALPEQWQQLSFPLFWQGCELQVTLDAQR
```

```
TPP_hhal        GIHLGAMAGTVDLALRGYTGMEPQDGVLWLDPMLPDGLHEVEHKIHYRGHWIHLVINHTR
                *** .* ..    *::*::*:. :** * *:* **:  :::.. :.::*  :::.::  *

TPP_ecoli       IAIRTSAPVSLRLNGQLIYVAEESVFCLGDFILPFNGTATTHQEGE-
TPP_hhal        LTLRLEEGPWPPVR----------IGYRDRIHDLHQGETLELAYER
                :::* .      :.          :   * *  ::   * .   *
```

Figure 2.11: Multiple sequence alignment of trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP) of *E. coli* and *H. halophila*

The reported 3D structure of the *E. coli* TPS enzyme has allowed the identification of the amino acid residues involved in the binding of substrates and in catalysis (28). The residues involved in the binding of glucose 6-phosphate are Arg9, Trp40, Tyr76, Trp85 and Arg300, while Gly22, Asp130, His154, Arg262, Asp361 and Glu369 are involved in the binding of UDP-glucose (24). The presence of these active sites in *H halophila* TPS was detected.

Figure 2.12 Conserved active site residues in *H. halophila trehalose-6-phosphate synthase*

These two genes are located in a likely operon (Figure 2.18). The two enzymatic steps catalyzed by trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP).

TPS catalyzes the transfer of glucose from UDP-glucose to glucose 6-phosphate forming trehalose 6-phosphate and UDP, while TPP dephosphorylates to trehalose 6-phosphate trehalose and inorganic phosphate.

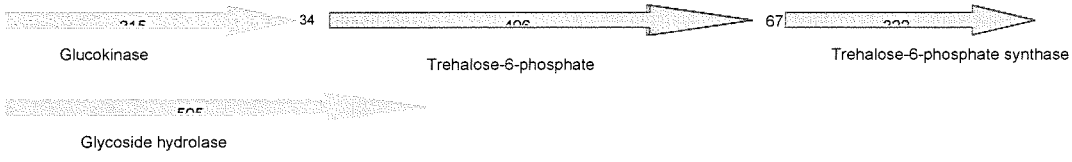


Figure 2.13: Probable operon structure for trehalose biosynthesis in *H. halophila*.

2.3.3 Transport of compatible solutes:

The *H. halophila* genome was examined for presence of biosynthesis genes for glycine betaine, ectoine and trehalose using reference sequences from bacteria knows to enable uptake of these compounds. For glycine betaine uptake systems BetP, OpuA and PutP (described in section 2.1.2a) yielded significant hits in *H. halophila* genome. For ectoine, uptake systems EctP and TeaABC (described in section 2.1.2b) gave significant hits in the *H. halophila* genome (Table 2.6).

Compatible solute	**Transporter used**	**Presence in *Halorhodospira halophila***	**Scores of hits obtained in *H. halophila* when compared to reference sequences**	
			% Similarity	**% Identity**
Glycine betaine	BetP	+	41	32
	OpuA	+	52	34
	PutP	+	62	41
Ectoine	EctP	+	65	39
	TeaABC	+	38	24
Amino acids	ProP	-	16	12
Trehalose	-	-	14	11

Table 2.6: Compatible solutes Uptake systems genes in *H. halophile*. Hits identified as significant are indicated with + sign

2.3.3a Transport of glycine betaine:

The genome of *H. halophila* was analyzed for presence of compatible solute transporters by using the amino sequences of known compatible solute transport system.

ref|YP_001002953.1| choline/carnitine/betaine transporter [Ha... 295
4e-81 G
ref|YP_001003417.1| choline/carnitine/betaine transporter [Ha... 290
9e-80 G
ref|YP_001001833.1| choline/carnitine/betaine transporter [Ha... 283
2e-77 G
ref|YP_001003929.1| choline/carnitine/betaine transporter [Ha... 275
5e-75 G

ref|YP_001001674.1|	choline/carnitine/betaine transporter [Ha...	274	9e-75 G
ref|YP_001001931.1|	BCCT transporter [Halorhodospira halophil...	169	2e-43 G
ref|YP_001002748.1|	membrane-flanked domain [Halorhodospira h...	27.3	1.8 G
ref|YP_001003903.1|	cytochrome c biogenesis protein, transmem...	26.9	2.4 G
ref|YP_001001945.1|	anion transporter [Halorhodospira halophi...	26.6	3.1 G
ref|YP_001003401.1|	lipoprotein signal peptidase [Halorhodosp...	25.4	7.0 G
ref|YP_001002181.1|	hypothetical protein Hhal_0593 [Halorhodo...	25.4	7.0 G
ref|YP_001002965.1|	cell divisionFtsK/SpoIIIE [Halorhodospira...	25.0	9.1 G
ref|YP_001002190.1|	ATP-dependent protease ATP-binding subuni...	25.0	9.1 G

(A)

```
H_hal4                  ---------------------------------------------MIDRKRAFRTTILA
H_hal5                  ---------------------------------------------MTGRRPHGGGVYA
H_hal1                  ---------------------------------------------MRAQKGPLKGLNI
H_hal2                  ---------------------------------------------MFNVATRGFFRGMSP
H_hal3                  ----------MTDPNNTDPKEVKKEIEELEQAYETDHEIGDQNISTEIKPIGLALDLHN
BetP_C_glutamicum       MTTSDPNPKPIVEDAQPEQITATEELAGLLENPTNLEGKLADAEEEIILEGEDTQASLNW
                                                                                :

H_hal4                  PVFFPAIAVALLLIIGAISSPDLAGAFFEDLLAFITETFGWFYMLAVAAFLVFLVAVAFT
H_hal5                  RVFLPAAALVVALVVSAAVWTEAVGDWIAELQTFIAVELGWVYTGVVAFLLGFVLVVLLR
H_hal1                  PLTGTATLIVLAFLIFGAWDPEYAETVFEGISGWVIETFKWYYIGVVAFFLLFALFLMFS
H_hal2                  RVTAISTFLVAAFALAGAIWPKHLEAVVTGWRESLTPFLQWYYVLVVAAFLLLVIWLGTG
H_hal3                  PVFIVSSALILVFLIGTLIFTAPAQEALEGVRGWATSSFDWFFLTAGNIFVLFCLLLIVL
BetP_C_glutamicum       SVIVPALVIVLATVVWGIGFKDSFTNFASSALSAVVDNLGWAFILFGTVFVFFIVVIAAS
                         :   :  :     :                          : * :      :: : : :

H_hal4                  -RWGHIKLGPEHGEPQYSFPAWFAMLFSAGYGIVLLFFGVAEPVLHY----ADPPRGEPE
H_hal5                  PDFRRLRLGPPDSYPEYSYLSWFAMLFSAGMGIGLLFYSVAEPLMHY----AEPPRAEPG
H_hal1                  -RFGDLKLGDDDRPPEFSYFAWFSMLFGAGMGIGLLFWSIAEPVWHFQ---GNPFIDEGE
H_hal2                  -RFKNVRLGQDHEVPEFRTFSWLTMLFAAGMGVGLIFWAVAEPISHFD---SNPFTVSGD
H_hal3                  -PLGSIRIGGQDAKPDFSRLSWFTMLFAAGMGIGLMFWAVAEPVGYYTEWFGSPFNIEGG
BetP_C_glutamicum       -KFGTIRLGRIDEAPEFRTVSWISMMFAAGMGIGLMFYGTTEPLTFYR------NGVPGH
                             :::*  .  *::   :*::*:*.** *: *:*:. :**: .:

H_hal4                  TIEAARQAMQIAFFHWGFHIWAIYGLVGLVLAYFSFRHGLPLSIRSALYPLIGDR-IYGP
H_hal5                  TPDAALEALQVTFFHWGLHPWAIYITVALSLAFFSYRHGLPLSLRSALYPLLGRR-IHGV
H_hal1                  TAAAADSAMRLTYFHWGMHPWAIYAIVALSLAFFCYRKKLPLAIRSALYPLIGNR-IYGP
H_hal2                  TTEAADTALRLAYFHWGLNGWAVFSLVALILAYFSFRRGLPLTMRSAFYPLIGKH-IHGP
H_hal3                  TDEAAKAAMGATMYHWGLHPWAIYGVMALALAFFTYNKGLPLTVRSVFYPLLGER-VWGP
BetP_C_glutamicum       DEHNVGVAMSTTMFHWTLHPWAIYAIVGLAIAYSTFRVGRKQLLSSAFVPLIGEKGAEGW
                            .  *:  : :** :: **::  :.* :*:  :.      : *.: **:* :   *

H_hal4                  IGHTVDVFAILGTLFGIATTLGLSVAQINAGLNYLWPSIPTSTTVQVIVIAVITALATIS
H_hal5                  IGDAVDTAAVVGTVLGVATSLGLGVMQVNSGLARVG-LLEESLTHQIGLIIAIMGAATLS
H_hal1                  IGHAADVLAVFGTIFGVATSLGFGAIQINTGLNELT-GLELSVTNQLLIVAVVTLIAVGS
H_hal2                  WGDAVDILAVLATVFGIATTLGLGIQQLNTGIGELT-GITAGTTGQIAIAITVMGIATIS
H_hal3                  LGHIIDTVAVLATIFGLATSLGFGAQQAASGLSYVFEAVPDTLGTQVAIIIGVTVAALVS
BetP_C_glutamicum       LGKLIDILAIIATVFGTACSLGLGALQIGAGLSAANIIEDPSDWTIVGIVSVLTLAFIFS
                         *.  *  *:..*::* * :**:.  *  :*:             : :   :      *

H_hal4                  VVAGLDKGIKRLSILNMILAAALMLFVFLVGPSILIVETFLQNTGSYVSGIVERTFNLEA
H_hal5                  VVSGLNNGIRLLSRANLFLGAALMLFVLIAGPTRLVLAGFFESVGHYVDGLVELTFRTDA
H_hal1                  VISGVGRGVKVLSQLNLILSAVILLFFLSFGPTLYLLSSFVQGIGDYLQNVVYLSFWTDA
```

(B)

Figure 2.14: Sequence similarity between glycine betaine uptake system in *C. glutamicum* and *H. Halophila* (A) Blast hits from BetP of *C. glutamicum* in *H. halophila* genome (B) Multiple sequence alignment of BetP of *C glutamicum* and highest hits in *H. halophila*

The top blast hits for sequence similarity with the known BetP system of *C. glutamicum* was analyzed through a multiple sequence alignment (Figure 2.9, panel B). The high bit

scores and the sequence alignment indicate the presence of BetP uptake system in *H. halophila.*

2.3.3b Transport of Ectoine:

The *H. halophila* genome was analyzed for the presence of both the EctP and TeaABC system for uptake of ectoine from *H. elongata.*

YP_001003929.1	Ectoine transporter [Halorhodospira halophila SL1]	331	331
YP_001002953.1	Ectoine transporter [Halorhodospira halophila SL1]	318	318
YP_001001674.1	Ectoine transporter [Halorhodospira halophila SL1]	301	301
YP_001003417.1	Ectoine transporter Halorhodospira halophila SL1]	265	265
YP_001001833.1	Ectoine transporter [Halorhodospira halophila SL1]	264	264
YP_001001931.1	BetP transporter [Halorhodospira halophila SL1]	187	187
YP_001003363.1	O-succinylhomoserine sulfhydrylase [Halorhodospira halophila SL1]	30.8	30.8
YP_001001730.1	phosphomethylpyrimidine kinase [Halorhodospira halophila SL1]	27.3	27.3
YP_001002831.1	peptidyl-arginine deiminase [Halorhodospira halophila SL1]	25.8	25.8
YP_001002268.1	valyl-tRNA synthetase [Halorhodospira halophila SL1]	25.4	25.4

Figure 2.15: Blast hits in *H. halophila* for EctP from *H. elongata*

The top blast hits from *H. halophila* (Figure 2.16) were used to make a multiple sequence alignment (Figure 2.13)

```
H_hal4                  ---------------------------------------------MIDRKRAFRTTILA
H_hal5                  ---------------------------------------------MTGRRPHGGGVYA
H_hal1                  ---------------------------------------------MRAQKGPLKGLNI
H_hal2                  ---------------------------------------------MFNVATRGFFRGMSP
H_hal3                  ----------MTDPNNTDPKEVKKEIEELEQAYETDHEIGDQNISTEIKPIGLALDLHN
EctP from H. elongata MTTSDPNPKPIVEDAQPEQITATEELAGLLENPTNLEGKLADAEEEIILEGEDTQASLNW
                                                                                :

H_hal4                  PVFFPAIAVALLLIIGAISSPDLAGAFFEDLLAFITETFGWFYMLAVAAFLVFLVAVAFT
H_hal5                  RVFLPAAALVVALVVSAAVWTEAVGDWIAELQTFIAVELGWVYTGVVAFLLGFVLVVLLR
H_hal1                  PLTGTATLIVLAFLIFGAWDPEYAETVFEGISGWVIETFKWYYIGVVAFFLLFALFLMFS
H_hal2                  RVTAISTFLVAAFALAGAIWPKHLEAVVTGWRESLTPFLQWYYVLVVAAFLLLVIWLGTG
H_hal3                  PVFIVSSALILVFLIGTLIFTAPAQEALEGVRGWATSSFDWFFLTAGNIFVLFCLLLIVL
EctP from H. elongata   SVIVPALVIVLATVVWGIGFKDSFTNFASSALSAVVDNLGWAFILFGTVFVFFIVVIAAS
                         :   :  :     :                          : * :      :: : : :

H_hal4                  -RWGHIKLGPEHGEPQYSFPAWFAMLFSAGYGIVLLFFGVAEPVLHY----ADPPRGEPE
H_hal5                  PDFRRLRLGPPDSYPEYSYLSWFAMLFSAGMGIGLLFYSVAEPLMHY----AEPPRAEPG
H_hal1                  -RFGDLKLGDDDRPPEFSYFAWFSMLFGAGMGIGLLFWSIAEPVWHFQ---GNPFIDEGE
H_hal2                  -RFKNVRLGQDHEVPEFRTFSWLTMLFAAGMGVGLIFWAVAEPISHFD---SNPFTVSGD
H_hal3                  -PLGSIRIGGQDAKPDFSRLSWFTMLFAAGMGIGLMFWAVAEPVGYYTEWFGSPFNIEGG
EctP from H. elongata   -KFGTIRLGRIDEAPEFRTVSWISMMFAAGMGIGLMFYGTTEPLTFYR------NGVPGH
                             :::*  .  *::   :*::*:*.** *: *:*:. :**: .:

H_hal4                  TIEAARQAMQIAFFHWGFHIWAIYGLVGLVLAYFSFRHGLPLSIRSALYPLIGDR-IYGP
H_hal5                  TPDAALEALQVTFFHWGLHPWAIYITVALSLAFFSYRHGLPLSLRSALYPLLGRR-IHGV
H_hal1                  TAAAADSAMRLTYFHWGMHPWAIYAIVALSLAFFCYRKKLPLAIRSALYPLIGNR-IYGP
H_hal2                  TTEAADTALRLAYFHWGLNGWAVFSLVALILAYFSFRRGLPLTMRSAFYPLIGKH-IHGP
H_hal3                  TDEAAKAAMGATMYHWGLHPWAIYGVMALALAFFTYNKGLPLTVRSVFYPLLGER-VWGP
EctP from H. elongata   DEHNVGVAMSTTMFHWTLHPWAIYAIVGLAIAYSTFRVGRKQLLSSAFVPLIGEKGAEGW
                             .  *:  : :** :: **::  :.* :*:  :.      : *.: **:* :   *

H_hal4                  IGHTVDVFAILGTLFGIATTLGLSVAQINAGLNYLWPSIPTSTTVQVIVIAVITALATIS
H_hal5                  IGDAVDTAAVVGTVLGVATSLGLGVMQVNSGLARVG-LLEESLTHQIGLIIAIMGAATLS
H_hal1                  IGHAADVLAVFGTIFGVATSLGFGAIQINTGLNELT-GLELSVTNQLLIVAVVTLIAVGS
H_hal2                  WGDAVDILAVLATVFGIATTLGLGIQQLNTGIGELT-GITAGTTGQIAIAITVMGIATIS
H_hal3                  LGHIIDTVAVLATIFGLATSLGFGAQQAASGLSYVFEAVPDTLGTQVAIIIGVTVAALVS
EctP from H. elongata   LGKLIDILAIIATVFGTACSLGLGALQIGAGLSAANIIEDPSDWTIVGIVSVLTLAFIFS
                         *.  *  *:..*::* * :**:.  *  :*:              : :   :      *

H_hal4                  VVAGLDKGIKRLSILNMILAAALMLFVFLVGPSILIVETFLQNTGSYVSGIVERTFNLEA
H_hal5                  VVSGLNNGIRLLSRANLFLGAALMLFVLIAGPTRLVLAGFFESVGHYVDGLVELTFRTDA
H_hal1                  VISGVGRGVKVLSQLNLILSAVILLFFLSFGPTLYLLSSFVQGIGDYLQNVVYLSFWTDA
H_hal2                  VLYGVQSGVRLISEANFWMSAAVLLFFLLWGPTQYLLALIVQSTGDYLQNLFTLSFHTHA
H_hal3                  VLRGIDGGIKLLSNLNISLAGLLMLFVIIAGGAIAFVTQLWHTTSAYAGDFFALSNPVGR
EctP from H. elongata   AISGVGKGIQYLSNANMVLAALLAIFVFVVGPTVSILNLLPGSIGNYLSNFFQMAGRTAM
                        .: *:  *:: :*  *: :.. : :*.:  * :  .:  :    . *  ...  :

H_hal4                  Y---ERREWIGNWTLFIFGWTIAWAPFVGMFIAKISRGRTIRQFVVGVMLVPTLFTFLWF
H_hal5                  F---RSPDWQADWTLFYWGWWISWCPFVGMFIARVSRGRTVGEFILGVLLVPTLFTFVWL
H_hal1                  SGAREAGDWQLSWTAFYWGWWIAWAPFVGMFIARISRGRTIREFLGGVLLVPTLLALGWL
H_hal2                  N---ALGDWQAEWTLFYWGWWLAWAPFVGIFIARISRGRKLREFVMGVLLVPTGITIVWI
H_hal3                  E----DETFLQGWTAFYWAWWISWSPFVGMFIARVSRGRTVREFMTAVLIVPTVVTIFWM
EctP from H. elongata   SADGTAGEWLGSWTIFYWAWWISWSPFVGMFLARISRGRSIREFILGVLLVPAGVSTVWF
                               :   ** * :.* ::*.****:*:*::****.: :*: .*::**: .:  *:

H_hal4                  SIFGGTGLNLIMNEGYEQLIGLVQEDEAVALFQLYDILP---WSALASFVTVILIMTFFV
H_hal5                  TAFGAG--ALHLEEAGAGISAVVQESVPQALYAMLEALP---LAAITVPLATAVVVGYFV
H_hal1                  TVFGGTGLYQELFGAGG-LVEAVSEDETIALYYTIEAVAPGVIATIFAAIATVLIATYFI
H_hal2                  GLFGGNAIHIELFGPGG-VVDATREEVSTAVFRTIELMDVGIWATAASILVTVLIATYLI
H_hal3                  SAFGGVGLQQAIEGIGA-LADGIGADESMALFHMLEQLP---WTLLTASVAVFLVLVFFV
EctP from H. elongata   SIFGGTAIVFEQNGESIWGDGAAEEQ----LFGLLHALP---GGQIMGIIAMILLGTFFI
                          **.                    .    ::   . :          :.  ::  :::
```

```
H_hal4                  TSSDSGSLVIDQLASGGASVTPVWQRVFWAVLEGAVAAVLLIAGG---LAALQTMAVTSA
H_hal5                  TSADSGALVMNVLASGGNPNPPLLQKIFWSSMTGAVAAVLLIAGG---LQALQTVTIAAA
H_hal1                  TSSDSATLVVTMLLSVGNTEPPTYQRAFWGVAEGCVAAVLLVAGG---LVALQAAAIVAA
H_hal2                  TSANAGILVTQTLLSNGSTEISRLHTVIWGTVITLVTIVLLTAGG---LTTLQGAVIAAA
H_hal3                  TSSDSGSLVIDSITAGGKTDAPDAQRVYWVVMEGLIAGVLLFIGGDAALSALQAGAVSAG
EctP from H. elongata   TSADSASTVMGTMSQHGQLEANKWVTAAWGVATAAIGLTLLLSGGDNALSNLQNVTIVAA
                        **:::.  *   :  *          *      :  .**  **   *  **  .: :.

H_hal4                  LPFAVIMLIAAGGLWRALIIESHHDTSLQNHVQRRQRYGTLLWKKRLYELFDFPTRDDVM
H_hal5                  LPLSLILVLMAWGLWTAFRADAQQ-SDLASPIPEPK-----------------------
H_hal1                  LPFSLLMLLMCYALIRGLQEE-------KR------------------------------
H_hal2                  VPFSFIIIGMVVGLLKALEQEAFAPRPGERSGAPME------------------------
H_hal3                  LPFTVVLLLVCLSLLIGLRHER--------------------------------------
EctP from H. elongata   TPFLFVVIGLMFALVKDLSNDVIYLEYREQQRFNAR-----------------------
                         *: .:::    .*   :  :

H_hal4                  AFIRGPVVQALEHVQKALDQRGWPAKVVLDEDHGRVYLAVHRDGLMDFLYDVRLTERPRP
H_hal5                  ----------LERLLRYLDER-----------------------------------RRRR
H_hal1                  ------------------------------------------------------------
H_hal2                  ------------------------------------------------------PWA
H_hal3                  ---------------------------------------------------------R
EctP from H. elongata   ----------------------------------------------------LARER

H_hal4                  AFAYPSIDPSGGPAEVYYRPEVYLRRGGQSYSVYEYNEQEIIDDVLDHFESYLQFLDSAP
H_hal5                  RGGAPQGDSNSGPGKRSKRL---------------------------------------
H_hal1                  RMQLSWQPGQGPPAAPHL-----------------------------------------
H_hal2                  QVESDWHTSETHTDTATDRTED-------------------------------------
H_hal3                  LIKLTQQA---------------------------------------------------
EctP from H. elongata   RVHNEHRKRELAAKRRRERKASGAGKRR-------------------------------

H_hal4                  ATLPWATEAHDEMIDAPVGGKGRGRG
H_hal5                  --------------------------
H_hal1                  --------------------------
H_hal2                  --------------------------
H_hal3                  --------------------------
EctP from H. elongata   --------------------------
```

Figure 2.16: Multiple sequence alignment of top blast hits of EctP from *H elongata* in *H. halophile genome*

When the three genes of TeaABC belonging to the TRAP transpoter system (section 2.1.2b) were analyzed in *H halophila* individually, the three genes did give any significant hits. However if the sequences are joined together high blast hits are obtained (Figure 2.14)

```
ref|YP_001002040.1|  TRAP dicarboxylate transporter, DctM subu...   435
5e-123 G
ref|YP_001002038.1|  TRAP dicarboxylate transporter- DctP subu...   195
9e-51  G
ref|YP_001002039.1|  Tripartite ATP-independent periplasmic tr...   125
8e-30  G
ref|YP_001002037.1|  TRAP dicarboxylate transporter- DctP subu...   109
5e-25  G
```

ref|YP_001001955.1|	TRAP transporter, 4TM/12TM fusion protein...	45.8	8e-06 G
ref|YP_001002051.1|	Na+/Pi-cotransporter [Halorhodospira halo...	29.6	0.62 G
ref|YP_001001696.1|	hypothetical protein Hhal_0100 [Halorhodo...	29.6	0.62 G
ref|YP_001002050.1|	ATPase, P-type (transporting), HAD superf...	29.3	0.81 G
ref|YP_001003840.1|	NAD(P) transhydrogenase, beta subunit [Ha...	28.9	1.1 G
ref|YP_001002712.1|	protein of unknown function UPF0118 [Halo...	27.3	3.1 G
ref|YP_001002272.1|	hypothetical protein Hhal_0688 [Halorhodo...	27.3	3.1 G
ref|YP_001003572.1|	hypothetical protein Hhal_2006 [Halorhodo...	26.6	5.3 G
ref|YP_001003269.1|	Redoxin domain protein [Halorhodospira ha...	26.6	5.3 G
ref|YP_001002053.1|	DNA polymerase III, alpha subunit [Halorh...	26.2	6.9 G
ref|YP_001003487.1|	ABC transporter related [Halorhodospira h...	26.2	6.9 G
ref|YP_001001847.1|	C4-dicarboxylate transporter/malic acid t...	25.8	9.0 G

Figure 2.17: Blast hits in the *H. halophila* genome for TeaABC from *H elongata*

Also a convincing multiple sequence alignment was not obtained if the top hits were analyzed individually. However if the first two top hits were joined and then checked the alignment, the alignment was much better (Figure 2.15). Other hits were also checked but had poor sequence alignments.

```
TeaABC_halomonas     MTDEEEAEKHYHSGLPGILGTIDTLISKLEAIILALGVLLMATNTVANVIGRFALGESLF
TRAP_hhal            ------------------------------------------------------------

TeaABC_halomonas     FTGEVNRILIIMITFAGIGYAARHGRHIRMSAIYDALPVGGRRALMIVISLFTSLVMFFL
TRAP_hhal            ------------------------------------------------------------

TeaABC_halomonas     MYYSVHYVLDLYDKGRILPALGFPIFIIYVWVPLGFLITGIQYLFTAIKNLTSRDVYLST
TRAP_hhal            ------------------------------------------------------------

TeaABC_halomonas     SVVDGYKDTETEVMTTIMVATMIGLLLLGFPMMIPLATASIIGFFMMFGGLGQMETLIQQ
TRAP_hhal            ------------MTTLVITVMLILLLLGFPLMVPLLAGALLILIVELPFIG-ADALVRQ
                                 ***:::::.*: *******:*:** :.::: ::: :  :*  ::*::*

TeaABC_halomonas     LMAGIRPASLIAVPMFILAADIMTRGQSANRLINMVMAFIGHIKGGLAVSTAASCTLFGA
```

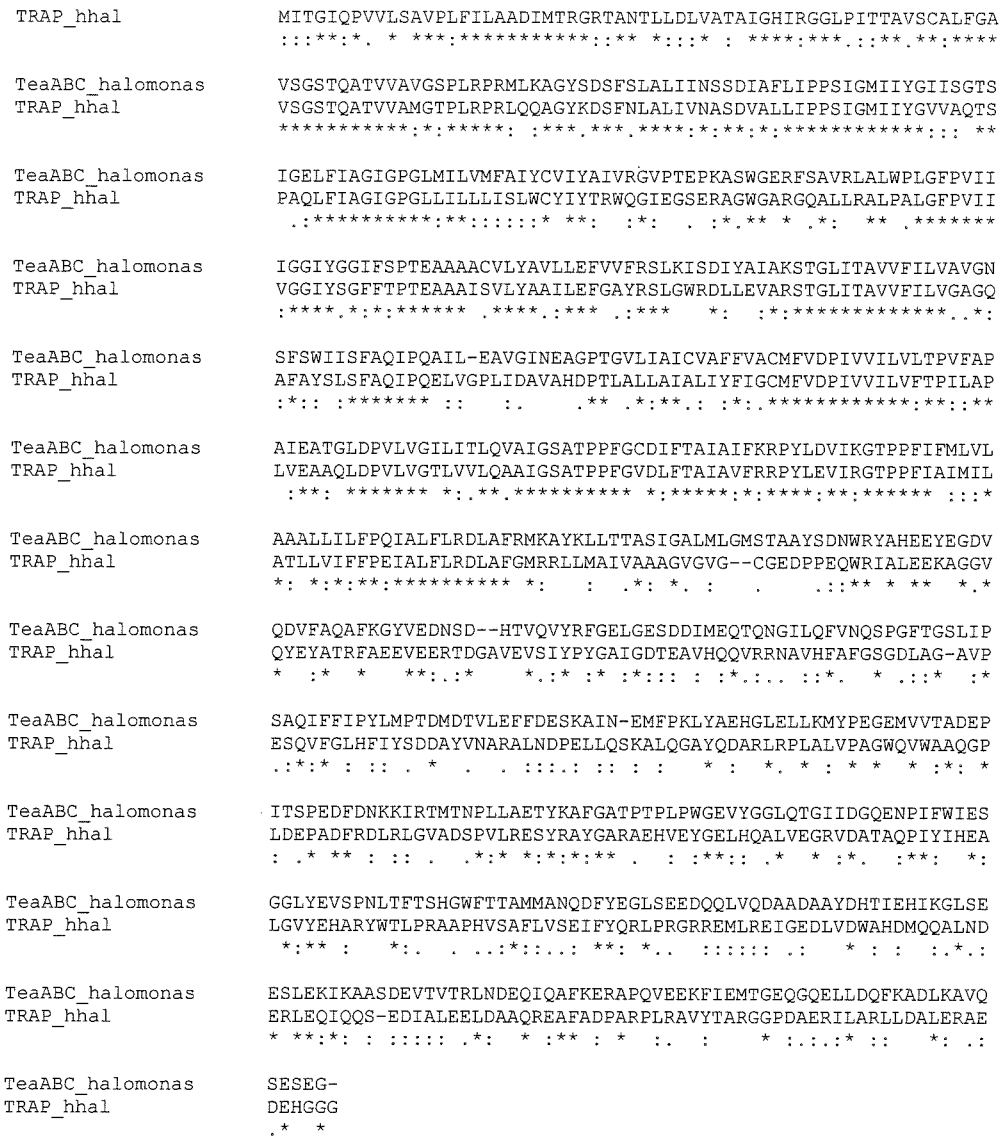


Figure 2.18: Multiple sequence alignment of first two top hit of *H. halophila* (joined together) and *H. elongata*

The joined top hits of *H. halophila* were checked for transmembrane helices individually and together. And only when they are together they have transmembrane helices which form substrate binding part of the protein (Figure 2.16).

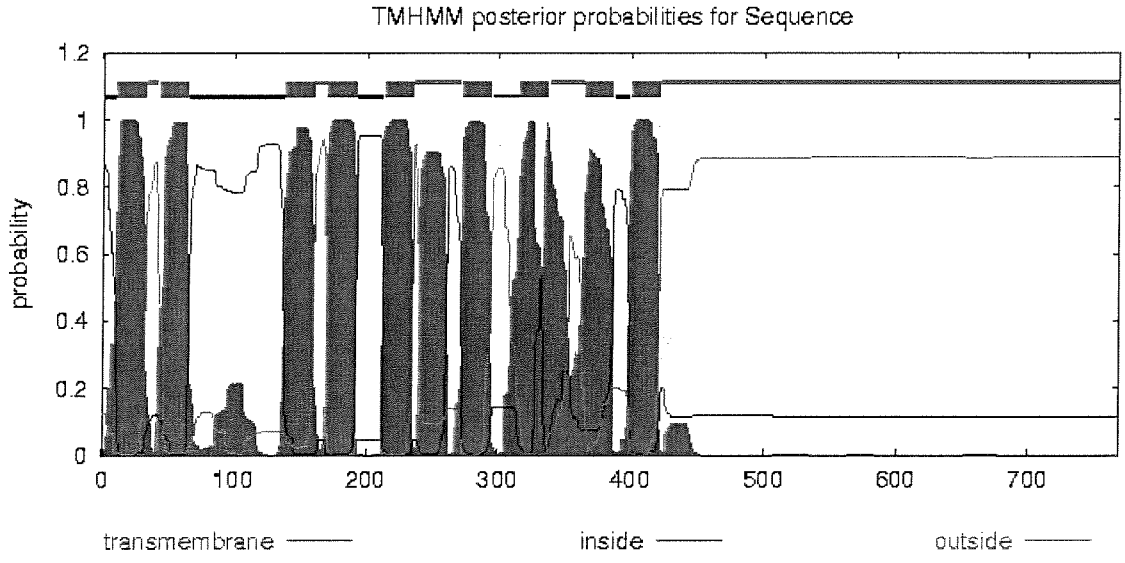


Figure 2.19: TMHMM of the two *H. halophila* hits joined together

2.4 Discussion

H. halophila is the first genome of an anoxygenic, photoautotrophic, extremely halophilic bacteria and its analysis can provide insights into the halophilic adaptations. *H. halophila* has been isolated from hypersaline lakes and therefore exposed to high salinity. Two halophilic adaptations strategies are observed in halophilic organisms. The 'high-salt-in' strategy (discussed in section 1.4.1) which involves a shift in amino acid composition, with an increased number of negatively charged residues coupled to the uptake of K^+ and Cl^-. The 'low-salt, organic solute-in' (discussed in section 1.4.2) strategy involves the production or uptake of large amounts of specific organic osmolytes (compatible solutes) which can be accumulated to high concentrations without disturbing cellular functions.

The amino acid composition of the predicted proteins in *H.halophila* resembles the profile of extremely halophilic archaea (and *S. ruber*) and is quite distinct from that of the

non-halophilic profiles (Figure 2.4) suggesting that its proteins have been adapted to high salt and to the 'high-salt-in' strategy. *H. halophila* also contains high percentage of acidic amino acids necessary for a 'high-salt-in' survival strategy. This finding was unexpected as immediate phylogenetic relative *H. halochloris* employs 'low-salt, organic solute-in' strategy of osmoprotection by accumulating glycine betaine. Hence *H. halophila* genome was examined for presence of biosynthesis and transport of compatible solutes like glycine betaine, ectoine and trehalose. GSMT and SDMT proteins from *H. halochloris* and *A. halophytica* which involved in biosynthesis of glycine betaine produced significant hits in *H. halophila* genome (H_hal1677 and H_hal1678) thus suggesting that *H. halophila* can biosynthesize glycine betaine. Gene encoding for glycine betaine transport across the membane were also found (H_hal 1384), suggesting that *H.halophila* is able to synthesize glycine betaine and can acquire it from the environment. Similarly EctA, EctB and EctC proteins from C. salexigens which are involved in biosynthesis of ectoine produced significant hits in *H. halophila* (H_hal1732, H_hal1733 and H_hal1734). Genes involved in ectoine transport were also identified (H_hal0449). Trehalose biosynthesis proteins TPS1 and TPS2 from *E. coli* also gave a significant hit in *H. halophila* (H_hal1120).

Presence of biosynthesis genes for glycine betaine, ectoine and trehalose and uptake genes for glycine betaine and ectoine indicates *H. halophila* is also capable of employing 'low salt, oraganic solute-in' strategy of osmoprotection.

2.5 Genome based prediction of the osmoprotectant strategy of *H. halophila*

The pI analysis of the proteome and the higher percentage of acidic residues in *H. halophila* resemble the same pattern as found in *Halobacterium* sp NRC-1 and *S. ruber*, which both accumulate molar concentrations of KCl ions in the cytoplasm as their osmoprotective strategy. This pI distribution appears to be unique to the halophiles that use KCl as their main osmoprotectant. This suggests that *H. halophila* also accumulates KCl.

The genome analysis indicates that *H. halophila* is capable of biosynthesis of glycine betaine, ectoine and trehalose and has transport systems for glycine betaine and ectoine. Hence it appears to be capable of accumulation of compatible solutes. Therefore *H. halophila* appears to be capable of using both KCl and compatible solutes as osmoprotectant.

In the subsequent chapters of the thesis, the predicted accumulation of KCl and glycine betaine is experimentally examined.

CHAPTER THREE

INTRACELLULAR SALT CONCENTRATIONS

3.0 Abstract

Extreme halophiles that employ 'High-salt-in' strategy of osmoprotection thrive in hypersaline environments by accumulating molar amounts of potassium chloride in their cytoplasm. They have evolved halophilic enzymes that function in the presence of high salt concentrations. They exhibit a proteome-wide adaptation in which all proteins have an acidic isoelectic point due to excess of Glu and Asp residues. In this chapter it is experimentally verified that (i) *H. halophila* has a acidic proteome by isoelectric gel focusing and (ii) it accumulates molar concentration of KCl when grown in high salt media. However, upon growth at 5% NaCl its cytoplasmic KCl content matches that of *E. coli*. These results demonstrate the use of KCl as an osmoprotectant in *H. halophila*, and reveal an acidic proteome that can function in the absence of high salt. In contrast the highly related organism *H. halochloris* does not exhibit an acidic proteome, matching its inability accumulate KCl. This indicates recent rapid evolutionary changes in halophilic strategy in these organisms

3.1 Introduction

Most extreme halophiles are members of the Haloarchaea, and particularly *Halobacterium* sp NRC-1 has been studied extensively (42, 46, 53). Extreme halophilicity in Bacteria is less common and less well studied, but has been described for the chemotroph *Salinibacter ruber* and the photosynthetic purple bacterium *H. halophila* (2, 31, 52, 62), A key factor in the halophilic adaptations of *H. salinarum* and *S. ruber* is that they accumulate up to 5 M KCl in their cytoplasm (15, 62). In both of these organisms the proteomic distribution of isoelectic points (pI) is strongly shifted to acidic values. This is caused by a large excess of acidic amino acid residues, which is thought to allow protein function in the saline cytoplasmic environment (44). Therefore, this halophilic strategy involves significant genome-wide modifications. The taxonomic distribution of the use of KCl as a major osmoprotectant is quite limited: it has only been reported in the Haloarchaea, in *S. ruber* and to a somewhat lesser extent in the *Haloanaerobiales* (42, 52, 57)

Another factor in the adaptations of extreme halophiles is that proteins from these organisms usually require the presence of at least 1 M salt to be stable and active (11, 44). The exact molecular origin of the halophilic character of the proteins from extreme halophiles is not fully understood. The negative charges on the surface of the proteins in these organisms interact favorably with the abundant cytoplasmic K^+ ions (21). Protein halophilicity makes it necessary for the organism to maintain a high cytoplasmic salt concentration, and is likely to be a factor in the minimum requirement of ~15% NaCl for growth of *H. salinarum* and *S. ruber*.

Here the possible occurrence of the 'high-salt-in' strategy of halophilicity in the two closely related organisms *H. halophila* and *H. halochloris* is examined. The growth of these obligately anaerobic and phototrophic organisms over wide range of medium NaCl concentration was determined. An analysis of the genome of *H. halophila*, revealed that it has a highly acidic proteome (Chapter Two- Bioinformatic analysis). These predictions are experimentally verified by isoelectric focusing gel electrophoresis of total protein extracts. The pI profile of H. halophile genome suggested it employed the 'high-salt-in' strategy for osmoprotection, hence the cytoplasmic KCl content of *H. halophila* was examined. Cells grown at 5% and 35% NaCl were used in electron microscopy microprobe analysis, together with *H salinarium*, *H. halochloris* and *E. coli* cells for comparison. To quantify the results; the cytoplasmic KCl concentrations were measured using plasma emission spectrometry. The dependence of the cellular KCl content of *H. halophila* on the NaCl concentration of the growth medium was also examined.

3.2 Methods

Cell growth: *H. halophila* and *H. halochloris* were grown in DSMZ 253 medium without yeast extract containing different salt concentrations (5%-35% NaCl). *E. coli* B culture was grown in 5 g/l nutrient broth. *Halobacterium* spp NRC-1 was grown in DSMZ 671 medium. The cultures were grown until late exponential phase and harvested at OD_{660} 1-1.2.

Determination of cellular potassium and chloride content: For plasma emission spectrometry 20 ml of cell culture was centrifuged (3,750 rpm, 25 minutes). Cell pellets were suspended in isotonic NaCl or Ammonium sulfate solutions, again pelleted, and

dried for 48 hours at 60°C. The dried pellets were divided in two halves. First half was used to measure potassium and sodium content using inductively coupled plasma emission spectrometry (Spectro Arcos). The second half was used for the colorimetric estimation of chloride using the Lachat 8000 Quick Chem flow injection analyzer. Cytoplasmic concentrations of the ions were calculated by using *E. coli* as a standard.

Electron microprobe analysis: *H. halophila*, *H. halochloris*, and *E. coli* cultures (20 ml) were pelleted (centrifugation at 3,750 rpm for 25 minutes), washed in isotonic NaCl or ammonium sulfate solutions, again pelleted, and spread on a carbon planchet. The planchet was immediately plunged into isopentane chilled in liquid nitrogen at -150°C. The preparation was then transferred to a vacuum pump and the sublimed water vapor was removed. The freeze-dried cells were then exposed to anhydrous paraformaldehyde vapor for 1 h to minimize the electron beam and carbon coating degradation.The samples were examined in a FEI Quanta 600^{TM} field emission gun environmental scanning electron microscope (SEM) using an Evex energy dispersive X-ray spectroscopy. The SEM was operated at an accelerating voltage of 20 kV and the gun current was 50-55 μA. The analysis was confined to a field of overlaying organisms at magnification of 5000X and covering an estimated area of 1-2 μm^2. Counts were accumulated over 180 s and 5 observations were made for each sample for 3 independent samples. This analysis was carried out OSU Microscopy laboratory facilty at Venture 1.

Isoelectric focusing gel electrophoresis: Cells were washed with lysis buffer, placed on ice, and sonicated (three times for 10 seconds). The cell extracts were then centrifuged (15 min at 15,000 rpm). The supernatant was dialysed for 24 hours against lysis buffer using Slide-a-Lyzers, with three changes of buffer. The volume of the sample was

adjusted with the lysis buffer to equal amounts of cell material based on their original OD_{660}. The sample is extracted in Urea buffer, which contains 9M Urea, 4% Triton, 2% Mercaptoethanol at pH 9 and centrifuged. The supernatant is used for both SDS PAGE, isoelectric focusing.

H. halophila and *H. halochloris* cells are grown to exponential phase. Cells of OD value 1 at 660nm are centrifuged for 15 min at 30,000 rpm. The pellets were re-suspended in 200 µl of 20mM Tris-buffer (pH-6.8). Cells were then micro-dialyzed. The samples were removed from the cassette and measured. 1 ml Sample buffer is added to 0.5 ml of sample. Electrophoresis was carried using MINI Protean apparatus at 500V with BIORAD precast IEF gels. Two IEF markers – BIORAD IEF standard mix (Broad range: pI 4.45-9.6) and Serva Liquid mix (pI 3-10) was used (Detail protocol in Chapter 6, Section 6.2.1).

3.3 Results

Growth characteristics of *H. halophila* and *H. halochloris* grown at different NaCl concentrations

H. halophila and *H. halochloris* exhibit a broad range of salt concentrations at which they can grow. For *H. halophila* the optimum salt range is between 15 (g/l) NaCl to 35 (g/l) medium NaCl. The doubling time is approximately 18 hours for medium NaCl concentrations of 15-35 (g/l). For *H. halochloris* the optimum medium NaCl concentration is 15-25 (g/l). Its doubling time is approximately 17 hours at the optimum medium NaCl concentration. However the doubling time increases below 15 (g/l) medium NaCl and above 25 (g/l) medium NaCl (Figure 3.1).

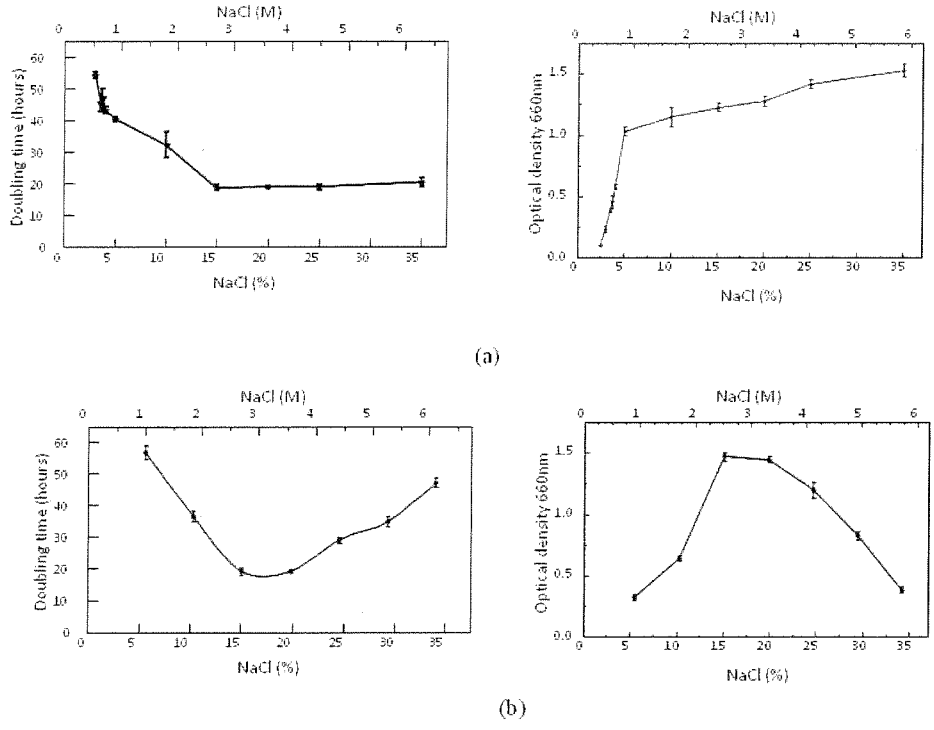


Figure 3.1: Final OD and doubling time of *H halophila* (panel a) and *H halochloris* (panel b). Effect of salt concentration on bacterial growth: The dependence of the doubling time (left) and final OD (right) of *H. halophila* (panel a) and *H. halochloris* (panel b) on the salinity of the growth medium are shown. **The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.**

Isoelectric gels of total protein extracts:

To verify the acidic nature of the *H. halophila* proteome, total protein extract from the cells grown in medium with high NaCl (35 g/l) concentration were prepared and isoelectric focusing gel electrophoresis was carried out. For comparison *H. halochloris*, which is a phylogenetically closely related organism which is known to accumulate glycine betaine as its osmoprotectant (23) and *Halobacterium* sp NRC-1 which accumulates KCl as its osmoprotectant and has a acidic proteome were used (15). The acidic proteins of Halobacterium sp NRC-1 culster between pI 4.5 and 5.1 (Figure 3.2, lane 3) *H. halophila* proteome does exhibit acidic character as majority of its proteins

have pI between 4.5 to 6.0 (Figure 3.2, lane 4) similar to that of *Halobacterium* sp NRC-1 whereas *H. halochloris* has a neutral preoteome (Figure 3.2, lane 2)

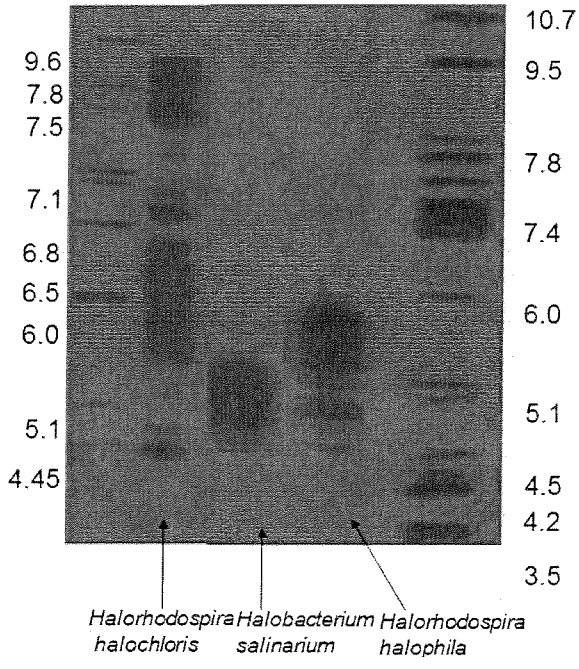


Figure 3.2: Isoelectric focusing gel electrophoresis of total cell extracts from *H. halophila* (lane 4), *H. salinarium* (lane 3) and *H. halochloris* (lane 2). Lane 1 and 5 are pI standards. pI of *Halorhodospira halophila* compared to extreme halophiles known to utilize K^+ and oraganic osmolyte as an osmoprotectant.

Comparison of pI of *H. halophila* at different NaCl concentrations:

The effect of medium NaCl concentration on the pI distribution in the *H. halophila* proteome was determined at 5%, 15% and 35% NaCl. *E.coli* was used for comparison. At all the three salt concentrations, low (5% NaCl, lane 3, figure 3.3), moderate (15% NaCl, lane 4, Figure 3.3) and high (35% NaCl, lane 5, Figure 3.3), *H. halophila* has an acidic proteome.

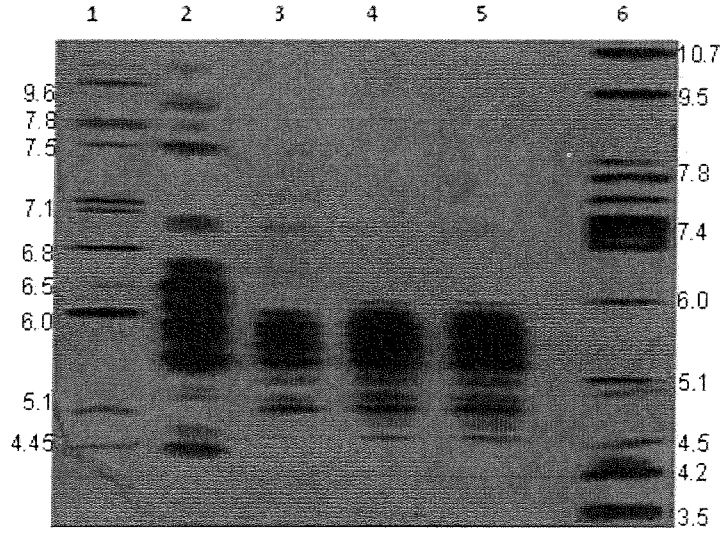


Figure 3.3: Effect of medium NaCl concentration on proteome acidity of *H. halophila* as detected by isoelectric focusing gel electrophoresis. pI distribution of *H. halophila* grown at different medium NaCl concentrations. 5% NaCl (lane 3), 15% NaCl (lane 4) and 35% NaCl (lane 5) NaCl. Lane 2 is *E.coli* total proteins Lane 1 and 6 are pI standards.

Cytoplasmic K^+ and Cl^- content of *H. halophila* using electron microprobe analysis:

As the pI distribution of the predicted proteins and the IEF gels confirmed the acidic nature of the *H. halophila* proteome, the intracellular concentration potassium and chloride concentration was measured using electron microprobe analysis. *Halobacterium* sp NRC-1, which is known to employ the 'High-salt-in' strategy of osmoprotection and accumulate upto 5 M KCl (15), *H. halochloris*, a closely related organisms known to employ 'Low-salt, osmolyte-in' strategy of osmoprotection (23) and *E. coli* cells were used for comparison. Prominent K^+ and Cl^- peaks were observed in the *H. halophila* samples (panel 3a, figure 3.4). The K^+ and Cl^- peaks were very small in the *E. coli* and *H. halochloris* cell material (panel 1a and 2a, Fig. 3.4), indicating KCl accumulation in *H. halophila.*

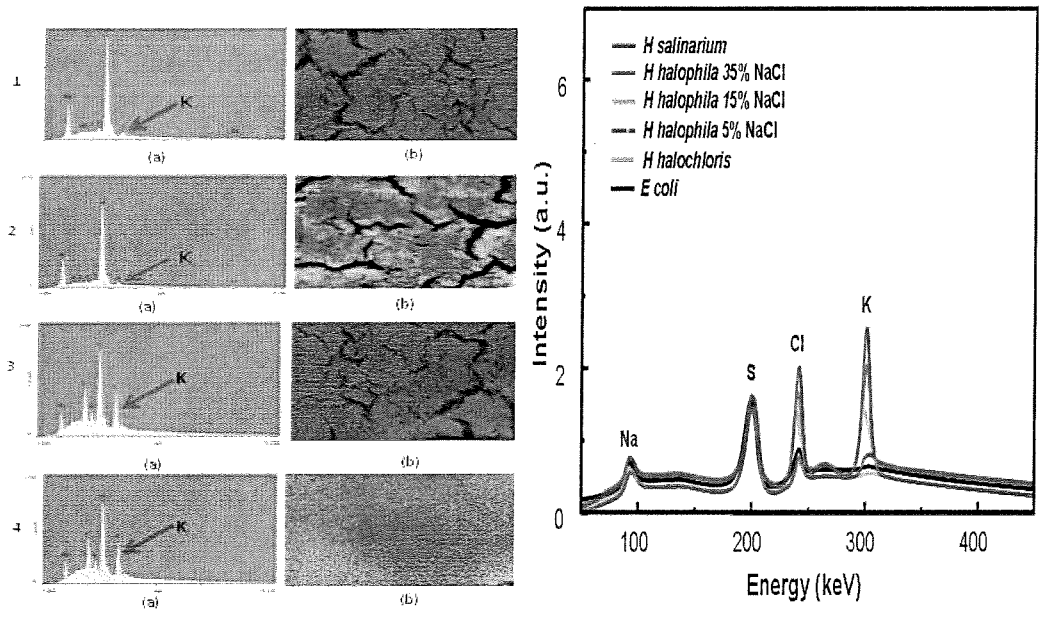
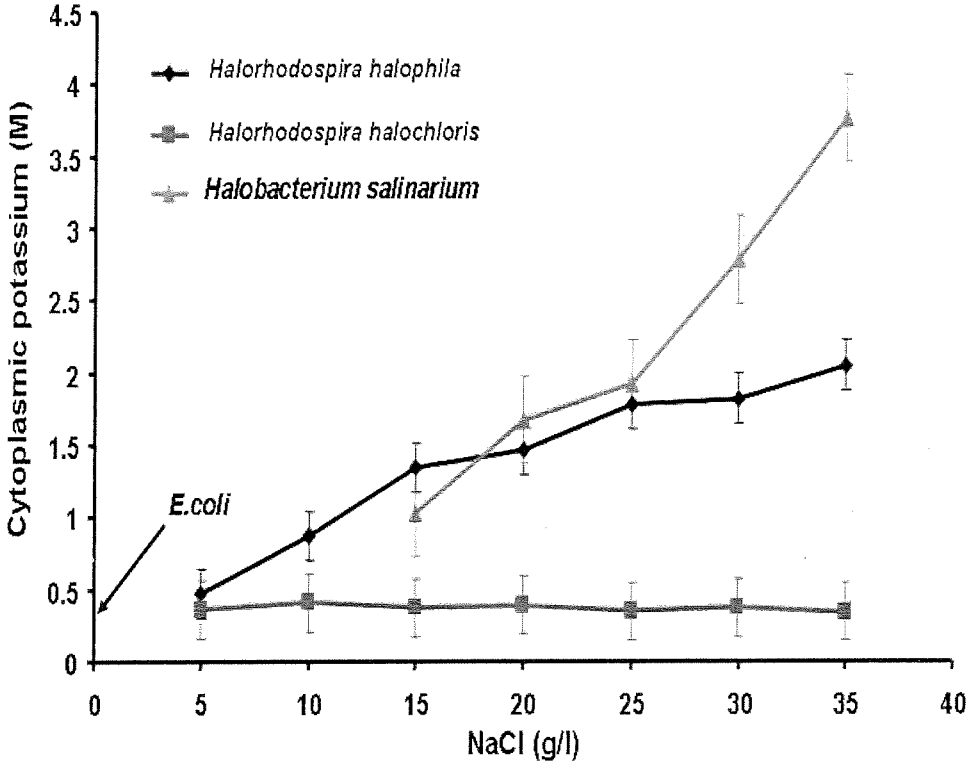


Figure 3.4: (A) Electron microprobe analysis of *H. halochloris* (panel 1), *E. coli* (panel 2), *H. halophila* (panel 3) and *Halobacterium* sp NRC-1 (panel 4). In each panel, (a) intensities of the intracellular ions and (b) is the cells. (B) Normalized intensities

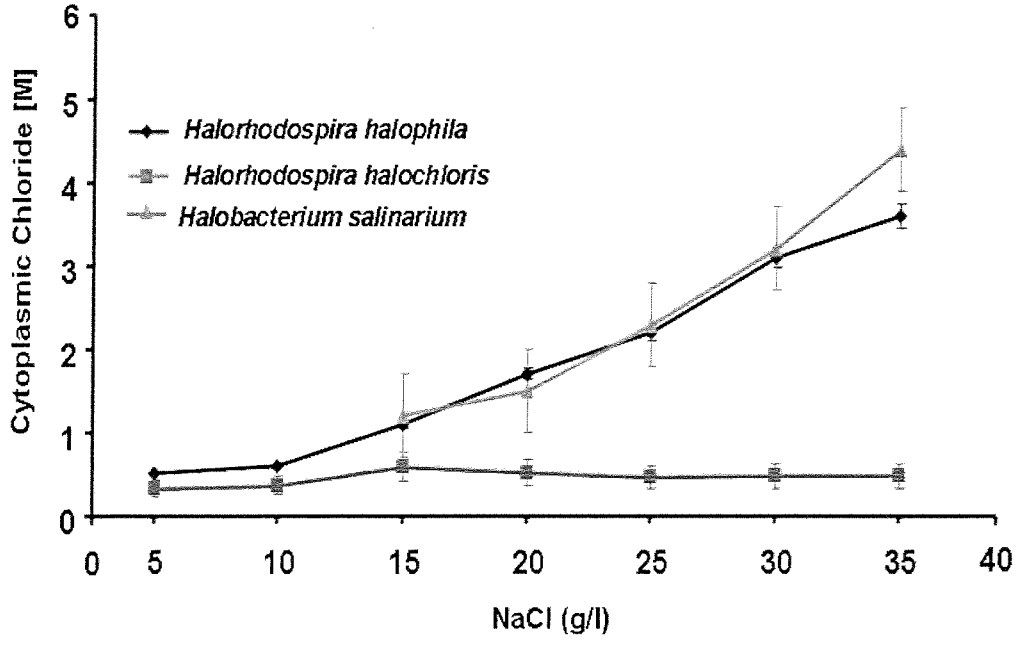
Plasma emission spectrometry measurements:

To quantify the intracellular potassium and chloride concentrations, MS-ICP (Inductively coupled plasma mass spectroscopy) measurements of *H. halophila*, *H. halochloris* and *Halobacterium* sp NCR-1 grown from low medium salt (5 g/l) to high salt (35 g/l) concentrations were carried. For comparison *E. coli* cells were used. The MS-ICP data indicated that the cytoplasmic K^+ content of *H. halophila* is 2.1 ± 0.6 M and *Halobacterium* sp NRC-1 is 4.3 ± 0.2M when grown at high salt concentration (Fig. 3.5). For comparison and as an internal standard, a typical cytoplasmic K^+ concentration in *E. coli* was taken as 211 mM (88). Similarly, the cytoplasmic Cl^- of *H. halophila* cells at

high medium NaCl was also found to be high. At 35% NaCl, the cytoplasmic Cl^- concentration was 3.3 ± 0.4 M (Figure 3.5). In contrast *H. halochloris* does exhibit high cytoplasmic K^+ and Cl^- concentration at any medium salt concentrations.



(a)



(b)

Figure 3.5: Cytoplasmic K^+ (panel a) and Cl^- (panel b) of *H. halophila*. *H. halochloris*, *H. salinarium* and *E. coli*. Dependence on medium salinity of cytoplasmic K^+ and Cl^- content of *H. halophila* (circles), *H. halochloris* (squares), and *Halobacterium salinarum* (triangles) as determined by plasma emission spectrometry and calorimetrically, respectively. **The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.**

Dependence of growth of *H. halophila* on the medium KCl concentration:

The mechanism of haloadaptation cannot be understood without taking into account the environment and its physical chemistry. The dependence of the growth of *H. halophila* on the KCl concentration of the growth medium was examined. High final OD readings indicating good growth were observed when the cells were supplied with higher KCl concentrations (0.1 g/l to 10 g/l KCl). However more than 10 g/l KCl concentration was

found to be toxic for *H. halophila* cells when grown in 5% NaCl (Fig 3.6).

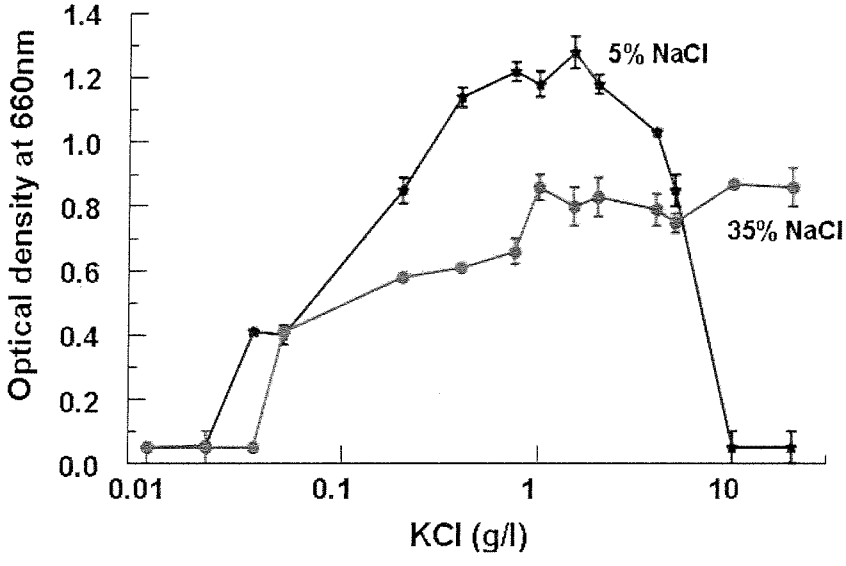


Figure 3.6: Potassium dependance of *H. halophila* at 5% and 35% NaCl. The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.

3.4 Discussion

Understanding of halophilic adaptations requires a strategy of complementary experiments combining bioinformatics analysis and physiological experiments. In an attempt to understand halophilic adaptations in *Halorhodospira*, a study to understand the response to different osmotic conditions in *Halorhodospira* was carried out. *H. halophila* and *H. halochloris* exhibit optimal growth over a wide range of medium NaCl concentration (Figure 3.7), and are capable of growth down to 3.5% NaCl (the salinity of sea water).

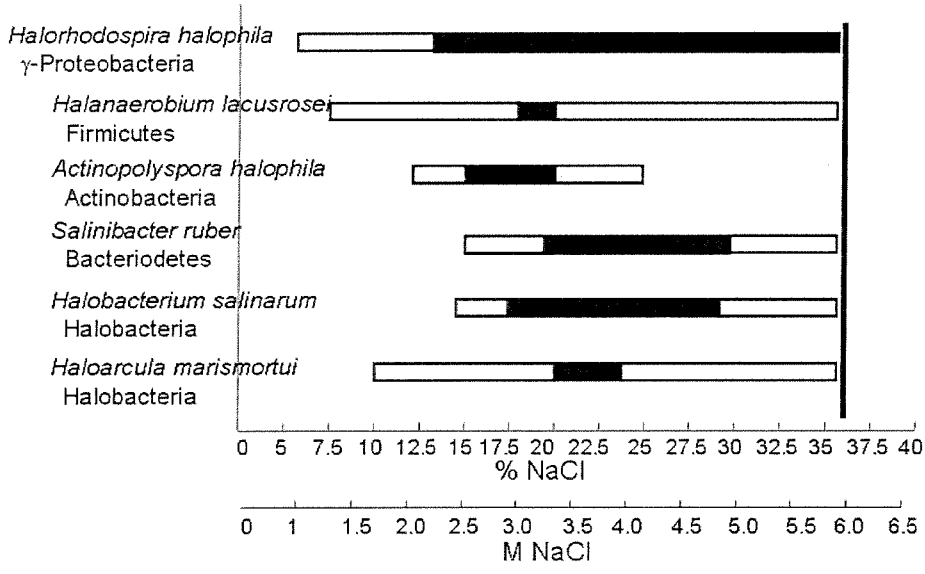


Figure 3.7: Range (white boxes) and optimum (black boxes) NaCl growth medium concentrations for growth of halophilic microorganisms belonging to different taxonomic groups: Archae: Halobacteria (*Haloarcula marismortui* and *Halobacterium salinarum)* and Bacteria: Bacteroidetes *(Salinibacter ruber),* Actinobacteria *(Actinopolyspora halophila),* Firmicutes *(Halanaerobium lacusrosei)* and Proteobacteria *(Halorhodospira halophila)*

Cytoplasmic KCl content of *H. halophila* and *H. halochloris*:

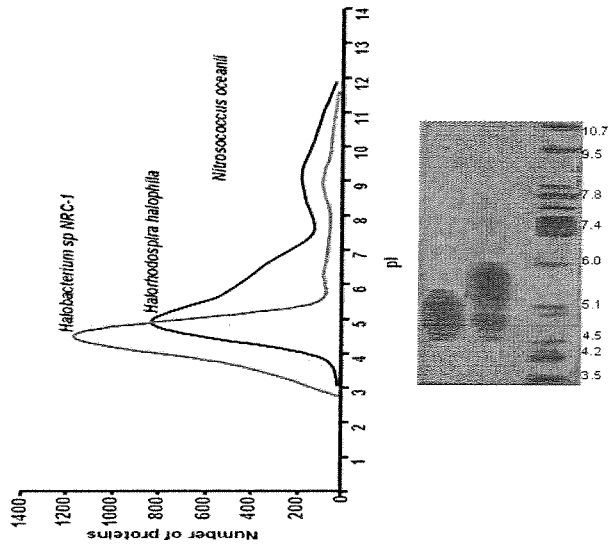


Figure 3.8: Comparison of predicted pI and IEF gel: Effect of medium NaCl concentration on proteome acidity of *H. halophila* as detected by isoelectric focusing gel electrophoresis.

The acidicity of the *H. halophila* proteome was examined by isoelectric gel focusing and was comparable to the average pI profile abtained through bioinformatics analysis (Figure 3.8).The cytoplasmic K^+ and Cl^- content of *H. halophila* were measured. Cells grown at 35% NaCl were investigated using electron microscopy microprobe analysis, together with *H. halochloris*, *H. salinarium* and *E. coli* cells for comparison. A prominent K^+ and Cl^- peaks were observed in the *H. halophila* and *H salinarium* samples, while these peaks were very small in the *H halochloris* and *E. coli* cell material, indicating a high degree of KCl accumulation in *H. halophila*. To quantify this observation, MS-ICP measurements were made. These experiments confirmed that the cytoplasmic KCl content of *H. halophila* is high. Comparison with *E. coli* cell material indicates a cytoplasmic K^+ concentration of 2.1 ± 0.2 M and Cl- concentration of 3.3 ± 0.4 M in *H.*

halophila under the growth conditions used. For comparison, a typical cytoplasmic K^+ concentration in *E. coli* was taken as 211 mM (20). These results show that KCl is a major osmoprotectant in *H. halophila*, and extends the use of KCl as a major osmoprotectant from the select group of the *Haloarchaea*, *Haloanaerobiales* (Firmicutes) (14) and *Bacteriodetes* (*S. ruber*) (62) to the γ-Proteobacteria. The highest reported K^+ concentration in these organisms is ~5.0 M for *H. salinarium* (14) and *S. ruber* (62). In contrast with the situation in *H. halophila*, *H. halochloris* was found to not accumulate KCl beyond levels present in *E. coli*. This is matched by the broad distribution of pI values of its proteome as revealed by isoelectric focusing gel electrophoresis (Figure 3.3). Interestingly, *H. halochloris* uses the organic osmolyte glycine betaine as a main osmoprotectant (21). The striking difference in the cytoplasmic KCl content of *H. halophila* and *H. halochloris* indicates a quite recent divergence is osmoprotectant strategy. A similar recent change in osmoprotectant strategy is suggested by the absence of an acidified proteome in *H. orenii*, while closely related members of the *Halanaerobiales* have an acidic proteome and utilize KCl as an osmoprotectant (49).

The dependence of the cellular KCl content of *H. halophila* on the NaCl concentration of the growth medium was examined. This revealed that the cytoplasmic KCl concentration of *H. halophila* is strongly regulated by the salinity of the growth medium: a ~10-fold reduction in cellular KCl content was observed upon a reduction in medium NaCl concentration from 35 to 5% (Figure 3.6). Thus, at 5% NaCl the cytoplasmic KCl concentration of *H. halophila* is very similar to that in *E. coli*. This observation was confirmed by electron microscope microprobe analysis (Figure 3.4). These results show

that while *H. halophila* possesses a strongly acidic proteome, it is capable of reducing its cellular KCl concentration to low values.

The process of adaptation to changing osmotic condition depends on the acquisition of osmotic balance, which is the main limiting factor for adaptation to hyper or hypo saline conditions. While haloarchaea, *Haloanaerobiales* and *S. ruber* withstand high salinities, low salt conditions are lethal for these organisms. Either in long term adaptation to low salinities or an immediate response to a sudden salt dilution on down shift which causes protein destabilization and cell lysis. This is not the case in *H. halophila* which can grow in significantly low salt concentrations as shown with its range and optimum (Figure 3.7).

The non-requirement for high NaCl concentrations for maintaining the optimally folded structure of *H. halophila* protiens suggests the possibility that these proteins behave differently than halophilic proteins which require high salt. However these proteins cannot be considered simply as a set of well folded polypeptide chains interacting with each other in high salt environment (47). The complexity of these proteins does not allow any single explanation for the salt discrepancy observed. Electrostatic shielding of charged groups takes place at salt concentrations below 0.5M ((44). This shielding decreases the stability of charge interactions. It also affects the hydrogen bonding. However in halophiles which accumulate potassium and have higher acidic amino acids in their proteins the effect of these salts is structural stabilization. At higher concentrations of salt, new hydrophobic interactions are formed and the proteins assume a tight folded conformation as compared to its stability in water. However the proteins of *H. halophila* which show acidic proteome even at low salt concentrations are capable of carrying out same function as that of halophilic proteins.

3.5 Conclusion

The current understanding of the 'high-salt-in' strategy halophilic adaptations is that the use of KCl as the main osmoprotectant is an energetically attractive strategy, since it does not involve the biosynthesis of molar amounts of organic osmoprotectants (56). This strategy requires the organism to modify all of its proteins to be compatible with the presence of high salt concentrations. It is believed that this results in the halophilic nature of the proteins from extreme halophiles, and therefore necessitates the permanent presence of high cytoplasmic salt concentrations.

The data reported here for *H. halophila* do not fit this paradigm, since its acidic proteome can apparently function in the absence of increased cytoplasmic KCl concentrations upon growth in 5% NaCl. *H. halophila* grows in a hypersaline lake, the water of which evaporates during summer thus having fluctuating salinity. Having a proteome adaptable to these fluctuating conditions can explain the unusually broad range of NaCl concentrations at which *H. halophila* is able to grow. In addition, it indicates that while proteome acidity is needed to allow protein function in the presence of high cytoplasmic salt concentrations, it does not necessitate enzyme halophilicity. Thus, it is not clear what causes the acidic proteins from *H. salinarum* to be halophilic, while the acidic proteins from *H. halophila* are not.

CHAPTER FOUR

INTRACELLULAR COMPATIBLE SOLUTE CONCENTRATION

4.0 Abstract

Extreme halophiles that employ the 'Low-salt, organic solute-in' strategy of osmoprotection thrive in saline environments by accumulating organic osmolytes in their cytoplasm. These organic osmolytes do not disturb vital cellular functions or correct folding of proteins. Hence they do not exhibit a proteome-wide adaptations in which all proteins have an acidic amino acid residue bias, as seen in 'High-salt-in' strategy of osmoprotection. In this chapter it is experimentally verified that *H. halophila* aacumulates glycine betaine when grown in high NaCl concentrations in absence of KCl. The glycine betaine accumulation pattern of *H. halophila* and *H. halochloris* were also compared. It is shown that *H. halophila* prefers KCl over glycine betaine, which is not the case for *H. halochloris* which prefers glycine betaine as its primary osmolyte. We also explain the possible ecological relevance of the observed osmoprotectant switch *in H. halophila*.

4.1 Introduction

In the 'Low-salt, organic solute-in' strategy of halophilic adaptation, microorganisms accumulate large quantities of a particular group of organic osmolytes in hyperosmotic conditions (Chapter 1, Section 1.4.2). This accumulation takes place either by de novo synthesis or direct uptake of the compatible solutes from the environment. In some cases it has been shown that halophiles expel these compounds when exposed to hypoosmotic circumstances (81). Organic osmolytes are also called compatible solutes as they do not disturb the vital cellular functions and correct folding of the proteins. As microorganisms lack the ability to actively transport water in and out of the cell, the osmotic conditions determine the water content of the cell. Compatible solutes help prevent cytoplasmic dehydration and maintain turgor pressure under conditions of low water activity by counteracting the efflux of water from the cell. They are also known to have a stabilizing influence on native structures of proteins and cell components under both in vivo and in vitro conditions.

Compounds that serve as compatible solutes are highly soluble and do not carry a net charge at physiological pH, except in case of some archaea (43). Examples of widely used compatible solutes are the amino acid proline, the quaternary ammonium compound glycine betaine, sugars like trehalose and the tetrahydropyrimidine ectoine (66). Accumulation of glycine betaine is widespread amongst these halotolerant organisms: *H. halochloris, A. halophytica* and *M. portuclensis* (35). The extreme halophile *H. halochloris* is also known to synthesize glycine betaine (22).

A typical response of halotolerant bacteria to a sudden increase in the external osmolality is the rapid uptake of mmol quantities of KCl to counteract the immediate outflow of water from the cell. However, this high intracellular concentration of high salt has toxic effects on physiological functions. Hence these ions are replaced by synthesizing large amounts of compatible solutes via the de novo synthesis pathways or uptake from the environment by the transport systems (70, 71).

The glycine betaine accumulation in *H. halochloris* under osmotic stress and identification of biosynthesis genes and uptake system in *H. halophila* (Chapter 2) prompted the question whether *H. halophila* accumulates glycine betaine.

A physiological analysis of glycine betaine accumulation in *H halophila* and *H. halochloris* under various NaCl and KCl concentrations was conducted. It was also examined if this accumulation of glycine betaine may have any ecological relevance for survival of *H. halophila*.

4.2 Methods

Glycine betaine uptake: One set of *H. halophila* cells were grown in DSMZ medium high salt concentration (35% NaCl) and 3 KCl concentrations- 1 g/l (normal) and 0.4 and 0.02 g/l (reduced), until late exponential phase. The OD_{660nm} was measured during growth using Shimazu spectrophotometer and a specially made round tube adapter. A second set of *H. halophila* cells were grown under same conditions but in the presence glycine betaine concentrations from 0 mM to 20 mM. The OD_{660nm} was measured during the growth of both set of cells.

Intracellular concentration of glycine betaine:

Cell growth: *H. halochloris* was grown in 0.035g, 0.2g ,1g, 2g, 4g and 10g KCl and at different NaCl concentrations 5%, 7.5%, 10%, 12.5% 15%, 17.5%, 20%, 22.5%, 25%, 27.5%, 30%, 32.5% and 35% NaCl. *H. halophila* was grown in 0.035 g, 0.2 g, 1g, 2g, 4g and 10g KCl and at different NaCl conc 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25%, 27.5%, 30%, 32.5% and 35% NaCl.

Glycine betaine measurements: 100 ml cultures of *H. halochloris* and *H. halophila* cells (high, medium and low salt) were harvested in the late exponential phase. Cells were washed using isotonic NaCl solutions. Cells are freeze-thawed at -4°C for 30 minutes. Cells were diluted with distilled water [0.1mg/ml]. Cells were incubated with lysozyme. 10% (w/v) of 0.2N perchloric acid was added and pH adjusted to 7.The resulting cell free extract was passed through the weakly cationic resin Amberlite CG-50 for chromatographic extraction of glycine betaine. The column was eluted with phosphate-citrate buffer pH 5.3. 0.5 ml of fractions were made up to 2 ml using 2 N HCl. 1 ml of reagent (10g iodine + 12.4g KI) to convert glycine betaine to its periodide derivative which has absorption maxima at 365 nm. The solution was shaken and placed on ice for 20 min. 10 ml of 1, 2 dichloroethane was added and thoroughly mixed. The absorbance of the organic layer (lower layer) is measured at 365 nm using Shimadzu spectrophotometer with round tube adapter and compared to with standard curve of pure glycine betaine between 10 mM to 2000 mM (Detail protocol in Chapter 6, Section 6.2.4).

Examination of mixed strategy for osmoprotection:

Cell growth: *H halophila* cells were grown in DSMZ medium with different salt concentrations (5% and 35% NaCl) and KCl concentration (0.05 to 10 g/L) till late exponential phase [OD_{660nm}], and harvested.

Determination of cellular K^+ and Cl^- content: For plasma emission spectrometry 20 ml of cell culture was centrifuged (3,750 rpm, 25 minutes). Cell pellets were suspended in isotonic NaCl and Ammonium sulfate solutions, again pelleted, and dried for 48 hours at 60°C. The dried pellets were divided in two halves. First half was used to measure potassium and sodium content using inductively coupled plasma emission spectrometry (Spectro Arcos). The second half was used for the colorimetric estimation of chloride using the Lachat 8000 Quick Chem flow injection analyzer. Molar concentrations of the ions are calculated using E. coli as a standard.

4.3 Results

Glycine betaine uptake:

In Chapter 3 it was experimentally verified that H. halophila accumulates KCl as it osmoprotectant. *H. halophila* also contains genes for the biosynthesis and uptake of glycine betaine (Chapter 2). Here we examined possible increase in cell growth when glycine betaine is added externally to *H. halophila* cells in grown medium standard (1g/L), and reduced (0.4 and 0.02 g/L) medium KCl concentrations. No difference was observed in the growth of *H. halophila* cells grown in standard KCl medium concentration at any glycine betaine concentration. However slight increase in

absorbance is observed in *H. halophila* cells grown in reduced (0.02 g/l) KCl at 20 mM glycine betaine (Figure 4.1).

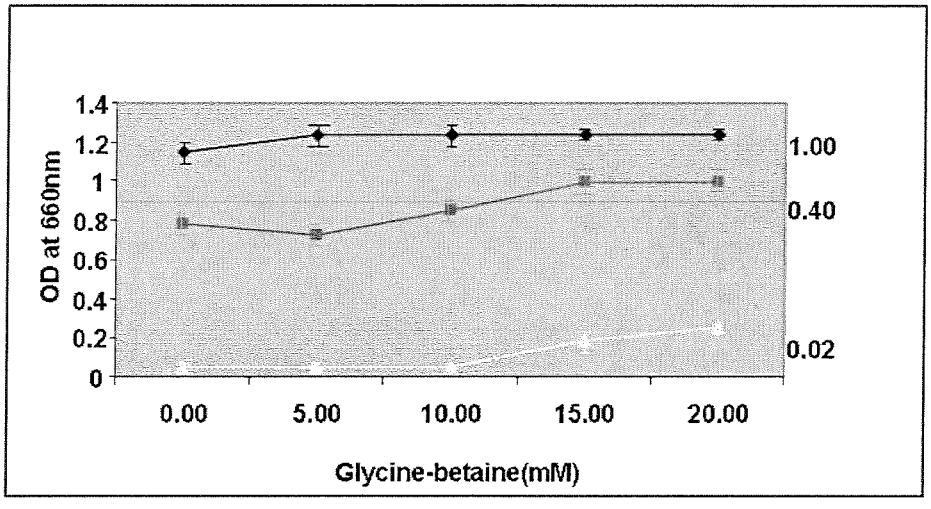


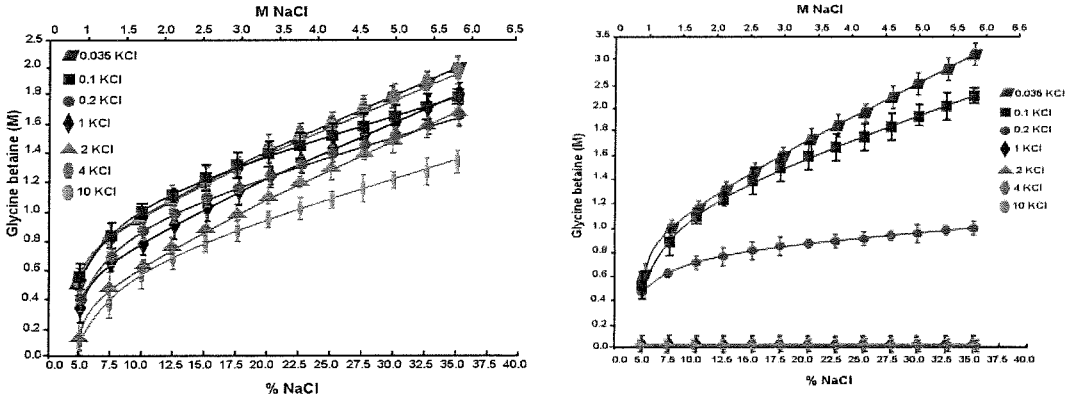
Figure 4.1: Uptake of glycine betaine by *H. halophila* .Blue line indicates *H. halophila* cells grown in 1g/L KCl, pink line indicates *H. halophila* cells grown in 0.4 g/L KCl and yellow line indicates *H. halophila* cells grown in 0.02 g/L KCl. The experiment was performed in triplos for two independent experiments for a total of 6 measurements per data point.

Intracellular glycine betaine concentrations:

As *H.halochloris*, a close relative of *H. halophila* employs glycine betaine as its osmoprotectant (23) and since H. halophila genome encodes genes for its biosynthesis and uptake *H. halophila* (Chapter 2) suggests accumulation of glycine betaine in *H. halophila.*

When intracellular concentrations of glycine betaine in both *H. halophila* and *H. halochloris* were examined under different NaCl and KCl medium concentrations, it was

observed that *H. halophila* indeed accumulates glycine betaine. However this occurred only when the cells were grown in media containing reduced KCl concentrations (0.2 to 0.035 g/l). When grown in standard (1 g/l) to high (2-10 g/l) KCl, *H. halophila* does not accumulate glycine betaine (Panel B, Figure 4.2). *H. halochloris* accumulates glycine betaine irrespective for medium KCl concentrations (Panel A, Figure 4.2).



A B

Figure 4.2: Cytoplasmic glycine betaine concentration of *H. halochloris* (panel A) and *H. halophile* (panel B). The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.

4.4 Discussion

Bioinformatic analysis (Chapter 2) indicates presence of genes for biosynthesis as well as uptake of glycine betaine in the *H. halophila* genome. *H. halochloris*, an extremely halophilic bacterium of closely related taxa accumulates glycine betaine pointed that *H. halophila* also might accumulate glycine betaine. *H. halophila* requires KCl as the main osmoprotectant when grown at 35% NaCl (Figure 3.6). *H. halophila* cells grown at 35% NaCl and KCl limiting conditions, the cells have lesser absorbance and take more time to attain high absorbance values. This indicates that there is slow recovery when cells are

grown in high salt and KCl limiting conditions (Figure 4.1). However addition of glycine betaine, in the high salt and KCl limiting growth medium stimulates *H. halophila* cell growth (Figure 4.1). This supports the hypothesis that *H. halophila* indeed has a functional glycine betaine uptake system.

The cytoplasmic concntration of glycine betaine in *H. halophila* and *H. halochloris* grown under various conditions was quantified. These bacteria show different pattern of accumulation of glycine betaine (Figure 4.2). In *H. halophila* glycine betaine is not accumulated at KCl concentartions equal or above 1 g/L while *H. halochloris* accumulates glycine betaine at all KCl concentrations under salt stress. Apparently *H. halophila* prefers potassium as its osmoprotectant when provided with KCl equal to or above 1 g/L (Figure 4.3).

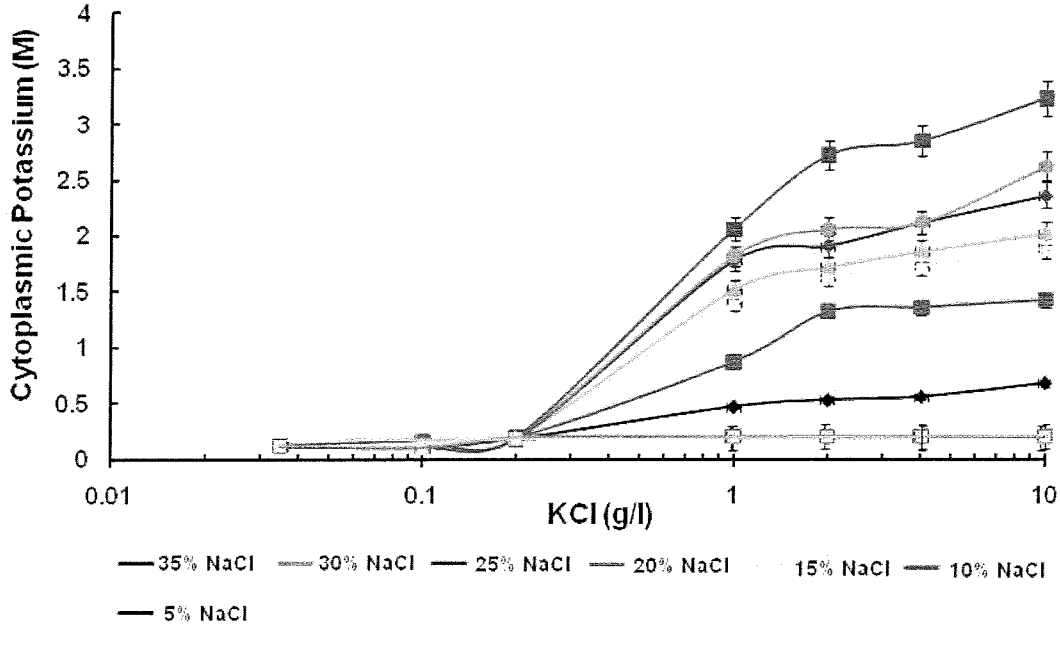


Figure 4.3: Cytoplasmic potassium of *H. halophila* (Solid symbols) and *H. halochloris* (Open symbols) at various KCl concentrations. The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.

At low medium KCl concentrations *H. halophila* no longer is able to use K^+ as its main osmoprotectant, and synthesizes glycine betaine. This switch in strategy occurs at 1g/l of potassium availiablity.

The possible ecologically relevance of this swtich was considered. A geochemical analysis of 6 Wadi Natrun lakes in which *H. halophila* is found, has been carried out (33). Potassium concentrations of the six Wadi Nantrun lakes were averaged and a gaussian curve calculated (Figure 4.4). As Figure 4.4 indicates, *H. halophila* switches strategy of halophilic adaptations around the concentrations at which potassium is available to it in nature. When potassium avaliablity is reduced, *H. halophila* either takes up glycine betaine from the external medium or biosynthesizes it. In the natural conditions, *H. halophila* would encounter fluctuating salinities (evaporation of water during summer and dilution of salts during rain). Having this switch mechanism gives *H. halophila* flexiblity to grow in broad range of NaCl concentrations which is not the case with other extreme halophilic organisms which only rely on accumulation of high salts in its cytoplasm as the osmoprotectant.

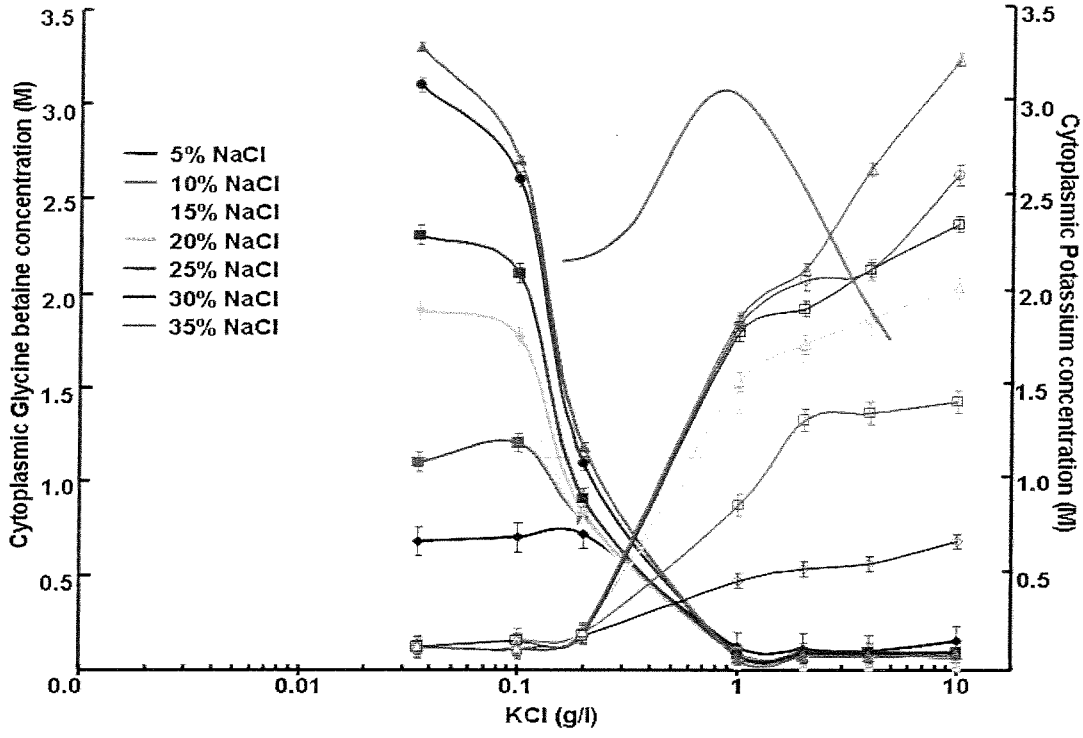


Figure 4.4: Intracellular potassium and glycine betaine concentration of *H. halophila* cells at various NaCl and KCl concentrations with a Gaussian distribution of K^+ concentrations observed for 6 Wadi Nantrun lakes. The experiment was performed in triplos for three independent experiments for a total of 9 measurements per data point.

4.4 Conclusion

The current understanding of the 'low-salt, organic solute-in' strategy halophilic adaptations is sole use of compatible solutes as the main osmoprotectant. This strategy does not require the organism to modify all of its proteins to be compatible with the presence of high salt concentrations.

H. halophila has an acidic proteome but when grown in low NaCl concentrations its cytoplasmic KCl concentrations are approximately same as *E. coli*. Additionally *H. halophila* also accumulates glycine betaine. The switch of osmoprotectants exhibited by *H. halophila* enables it to grow over broad range of NaCl concentrations. We propose

that this switch is ecologically relevant, as the average concentration of the Wadi Natrun lakes is same as the concentration at which *H. halophila* switches to 'Low-salt, osmolyte-in' strategy of osmoprotection.

CHAPTER FIVE

CONCLUSION

H. halophila breaks away from the current understanding of both, the 'High-salt-in' and 'Low-salt, organic solute-in' type of halophilic adaptations. It has an acidic proteome, but can function in absence of increased cytoplasmic KCl concentrations when cells are grown at low NaCl concentrations. The acidicity of the *H. halophila* proteome was demonstrated through calculated average pI of its predicted proteins and IEF of total cellular proteins. High cytoplasmic KCl concentrations were observed by electron microprobe analysis and quantified using plasma emission spectrometry. Despite the acidic property of the *H. halophila* proteome, its proteins apparently are not halophilic in nature like proteins of other extreme halophiles which employ 'High-salt-in' strategy since they donot require permanent presence of high cytoplasmic salts for their functionality. *H. halochloris*, a close taxomoic relative *H.halophila* does not accumulate KCl. This is matched by the broad distribution of pI values of its proteome as revealed by isoelectric focusing gel electrophoresis. Interestingly, *H. halochloris* uses the organic osmolyte glycine betaine as its main osmoprotectant

H. halophila can also accumulate glycine betaine and uptake from the medium through its uptake systems. This was demonstrated by checking the stimulation of cell growth by addition of glycine betaine to *H. halophila* cells grown under potassium limiting conditions and salt stress.

H. halophila does not confer with either of the two halophilic strategies. It uses an unusual mixed KCl/compatible solute strategy for osmoregulation, with an

osmoprotectant switch near 1 g/l KCl in growth medium. The average K^+ concentration of the Wadi Natrun lakes from which *H. halophila* was isolated is same as the concentration at which it switches to 'Low-salt, osmolyte-in' strategy of osmoprotection. Hence this switch in osmoprotection strategy might be ecologically relevant for survival of *H. halopila*.

CHAPTER SIX

APPENDIX

This section contains the following:

- Experiments which were carried out but were not included in the main dissertation.
- Detailed protocols are also presented.

6.1 Supplementary experiments

6.1.1 Effect of reduced KCl concentration on *H. halopila* growth

At reduced KCl concentrations *H. halophila* cells are forced to use glycine betaine as an osmoprotectant (Chapter 4). This requires a large amount of reducing equivalents from thiosulfate to fix CO_2 into glycine betaine. Thus, the reduction of growth (indicated by decrease in optical density) in media containing low KCl concentrations would be expected to be rescued by increased thiosulfate concentrations. This expectation was tested by growing *H. halophila* cells in low and high NaCl along with reduced and standard KCl concentration in media containing increasing amounts of sodium thiosulfate and recording the final OD_{660} and doubling time.

Methods

H. halophila cells were grown in DSMZ (without the yeast extracts) medium with different salt concentrations (5%-35% NaCl) and different KCl concentrations (0.1 and 1%) till late exponential phase [OD_{660nm}] in increasing concentrations (0.5 to 6 g/l) of Na_2S and harvested. Final OD_{660nm} was measured in Shimadzu spectrophotometer using the round tube adapter. Doubling time was calculated based on the OD_{660nm}.

Results

Reduced KCl slows down growth at all thiosulfate concentrations. Reduced KCl diminishes final OD at all thiosulfate concentrations. At 1 g/l KCl growth is essentially independent of thiosulfate concentrations ≥1g/l. However, at 0.2 or 0.05 g/l KCl both

doubling time and final OD are stimulated by increased thiosulfate at both low and high salts (Figure 6.1).

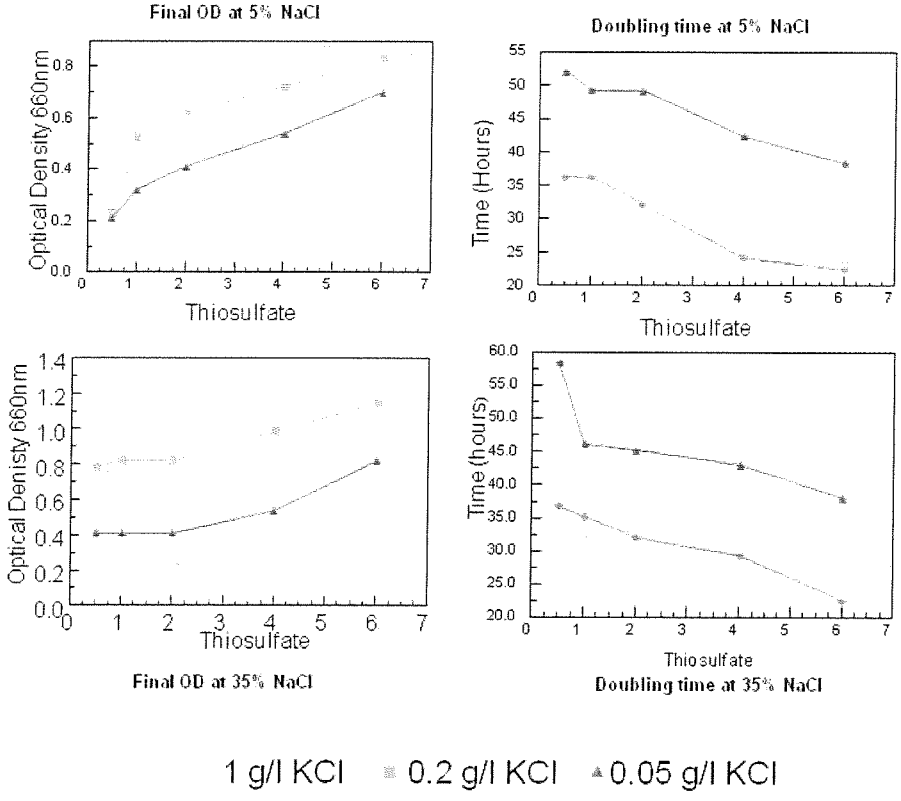


Figure 6.1: Final OD and doubling time of *H. halophila* at different NaCl, KCl and Thiosulfate concentrations. The experiment was performed in triplos for two independent experiments for a total of 6 measurements per data point.

Discussion and Conclusion

No conclusion could be made about role of increased concentration of thiosulfate in rescue of *H. halophila* cells grown at low KCl concentration. Final OD is increased and doubling time is reduced with increasing concentration of thiosulfate in both low and high salt medium.

6.1.2 Proteomic analysis of *H. halophila*

Studies on extremely halophilic organisms have been increased in the last decade since the genome sequencing of *Halobacteria* sp NRC-1 (49, 53). Understanding the haloadaptation mechanisms of halophilic bacteria may provide novel approaches to science and biotechnology. Unfortunately, very few genome sequences are available for these microorganisms (Chapter 1, section 1.5) and hence it is not easy to understand halophilic adaptation mechanisms of extremely halophilic bacteria since only limited genetic information is provided. Proteomics is an important method of gaining data about protein expression levels of these microorganisms. Proteome studies of *H. halophila* may give insight in some of the essential physiological processes in its haloadpation mechanisms.

Methods

H. halophila cells were grown in DSMZ (without the yeast extracts) medium with different salt concentrations (5%-35% NaCl) and different KCl concentrations (0.1 and 1%) till late exponential phase [OD_{660nm}] and harvested. The cells are washed with lysis buffer and sonicated on ice, three times for 10 seconds. Cells are centrifuged for 15 min at 15,000 rpm in a Microcentrifuge. The supernatant is dialyzed using Slide-a-Lyzer and lysis buffer for 24 hours. The lysis buffer is changed thrice. The volume of the sample is measured and adjusted with the lysis buffer. The sample is TCA precipitated using ice cold acetic acid and trichloroacetic acid. The pellet obtained is washed in Urea buffer. The supernatant is used for both SDS PAGE analysis and Mass spectrometry (Detailed protocol in Section 6.2.2 and 6.2.4).

Results

Good separation of proteins of *H. halophila* cells grown at different NaCl and KCl concentrations was obtained on SDS gels (Figure 6.2). However only 73 of the 2407 predicted proteins were identified in 6 data sets in 2 independent experiments.

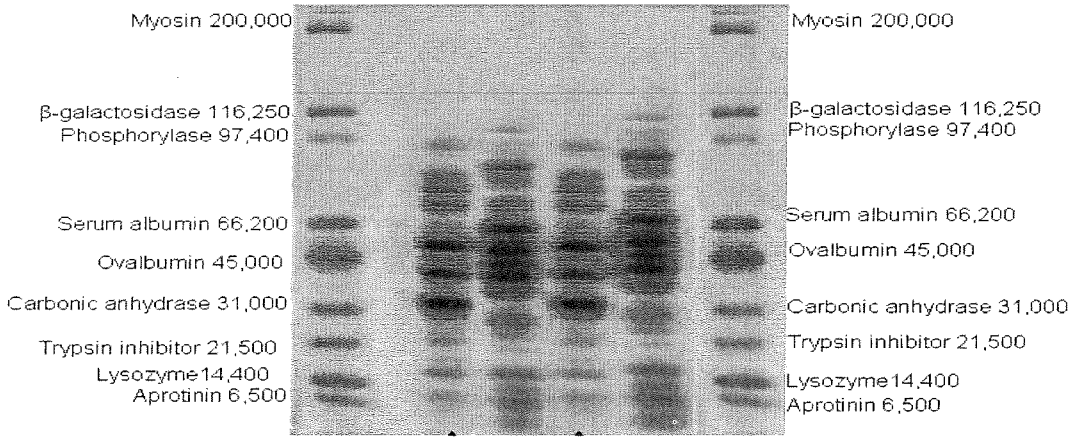


Figure 6.2: SDS-PAGE gel of TCA precipitate of *H. halophila* proteins grown at different NaCl and KCl concentrations. Lane 1 and 6-Molecular weight standard. Lane 2- Proteins of *H. halophila* grown at 5% NaCl and 1% KCl medium concentration. Lane 3- Proteins of *H. halophila* grown at 35% NaCl and 1% KCl concentration. Lane 4- Proteins of *H. halophila* grown at 5% NaCl and 1% KCl concentration. Lane 5- Proteins of *H. halophila* grown at 35% NaCl and 0.1% KCl concentration.

Discussion and Conclusion

No insight in the physiological processes of *H. halophila* osmoadaptation was obtained because small numbers of expressed proteins were identified in Mass spectrometry analysis.

6.2 Protocols

6.2.1 Protocol for IEF of *Halorhodospira halophila* proteins

Sample: *H. halophila, Halobacterium sp NRC-1, H. halochloris* cells and *E. coli* cells

Sample preparation:

1. *H. halophila, Halobacterium* sp-NRC-1, *H. halochloris* and *E. coli* cells are grown to exponential phase.
2. 10 ml of cells of OD value 1 at 660nm are centrifuged for 15 min at 30,000 rpm.
3. The pellet is re-suspended in 200 µl of 20mM Tris-buffer (pH-6.8).
4. Cells are micro-dialyzed.

   Dialysis: Cassette used- Slide-a-Lyzer

   Dialysis buffer- PBS buffer with pH 7.2

   Procedure:

   - Dialysis cassette is hydrated by soaking it in buffer for 30 sec
   - Add 200 µl sample in the cassette using a syringe
   - Sample is dialyzed in 50 ml PBS for 24 hours
   - Buffer solution is changed 3 times.
   - Sample is removed from the cassette and measured
5. 1 ml Sample buffer* is added to 0.5 ml of sample.

*Sample buffer preparation: 9M Urea

4% Triton

2% Mercaptoethanol

Adjust the pH to 9

Make the volume to 10ml

Electrophoresis using MINI Protean apparatus:

- Fill the lower tank with 0.1% phosphoric acid.
- Place the pre-cast IEF gel in the apparatus
- Fill the upper tank with 20mM NaOH [Note dislodge any air bubbles if formed]
- Load 20 µl prepared sample along with IEF marker
- Run at 500 V

6. Remove the gel from the apparatus carefully.
7. Wash the gel once with de-ionised water.
8. Stain the gel with Commasie brilliant blue R-250.
9. Destain the gel overnight.

6.2.2 Protocol for sample preparation for SDS PAGE

Sample preparation:

1. 18 ml of *Halorhodospira halophila* cells are harvested when they reach OD 0.8The cells are centrifuged at 3750 rpm for 20 min at 4^0C.
2. The supernatant is discarded and the pellet is re-suspended in 80-100 µl of 10mM Tris-buffer.
3. The sample is sonicated, making sure there is no bubbling.
4. The sonicated sample is dialyzed using a miro-dialysis chamber for 24 hours.10 mM Tris buffer is used at 4^0C, the buffer is changed after 10-12hours.
5. The volume of the sample increases (around 120-140 µl).
6. After sonication the sample is equally divided into 2 fractions.
7. First fraction (60 µl) is mixed with twice the volume of SDS reducing buffer (This will give the total protein profile of the cells).
8. The second fraction is micro-centrifuged at 10,000 rpm for 10 min.
9. The pellet and the supernatant are separated.
10. The pellet is re-suspended in 60 µl Tris buffer.
11. The supernatant measured and if necessary volume made up to 60 µl with Tris buffer.

12. 120 µl of SDS reducing buffer is added to both the supernatant and the pellet (This will give the profile of cytoplasmic and membrane proteins in the cells).

13. Heat the samples at 95^0C for 4 minutes.

14. 20 µl of each sample (total, cytoplasmic and membrane proteins) is loaded on the gel.

Materials used:

SDS reducing buffer*:

Deionized water	3.55 ml
0.5 M Tris HCl, pH 6.8	1.25 ml
Glycerol	2.5 ml
10% SDS	2 ml
0.5% Bromo phenol blue	0.2 ml
Total volume	9.5 ml

*50 µl of Mercaptoethanol is added to 950 µl of reducing buffer just before use

Electrode buffer: 1X

Tris base	3.03 g
Glycine	14.4 g
SDS	1 g

Gel formulations used: 4% stacking and 6%

6.2.3 Protocol for protein profiling of *H. halophila* cells grown at different NaCl and KCl concentrations

Materials and Methods:

Samples:

1. *H. halophila* cells grown in 5% NaCl and 1% KCl
2. *H. halophila* cells grown in 5% NaCl and 0.2% KCl
3. *H. halophila* cells grown in 35% NaCl and 1% KCl
4. *H. halophila* cells grown in 35% NaCl and 0.2% KCl

Sample preparation:

- All the 4 samples are grown in DMSZ medium with respective NaCl and KCl concentrations to exponential phase.
- Cells of OD value 1 at 660nm are centrifuged for 15 min at 30,000 rpm. The same volumes of cells of OD660 value 1 are used for all 4 samples.
- Cell pellets are suspended in 1 ml Lysis buffer
- Lysis buffer recipe: 40mM Tris pH-7.4

  0.5% TritonX-100

  0.3% SDS

- Resuspended cells are sonicated on ice 3X10 seconds. The power of the sonification is adjusted to be as high as possible but just below the foaming limit.

- Samples are centrifuged at 30,000 rpm for 15 minutes in Microcentrifuge to remove debris.

- Supernatants are micro-dialyzed.

  Dialysis: Cassette used- Slide-a-Lyzer

  Dialysis buffer- Lysis buffer pH 7.4

Procedure:

- Dialysis cassette is hydrated by soaking it in buffer for 30 sec
- Add 200 µl sample in the cassette using a syringe
- Sample is dialyzed in 50 ml Lysis buffer for 24 hours
- Buffer solution is changed 3 times.
- Sample is removed from the cassette and measured and adjusted to 5 ml using Lysis buffer.

TCA precipitation:

- The suspended cell extract, ice-cold acetic acid and trichloroacetic acid are mixed in following proportion: 1:8:1

- The mixture is precipitated at -20oC for 1 hour.

- The mixture is centrifuged at 11,500 rpm for 15 min at 4°C in microcentrifuge.
- Pellet is washed with ice-cold acetone by centrifugation at 11,500 rpm for 15 min at 4°C.
- The pellet is resuspended in 50µl Urea buffer and incubated at RT for 30 min with intermitant vortexing.

Urea buffer: 100 mM Tris-HCl pH 8.5

8 M Urea

- The resuspended samples are centrifuged at 14,000 rpm and supernatant is used for further analysis by SDS-PAGE and mass spectrometry.

- The resuspended samples are divided into 2 fractions. First fraction is used for SDS-PAGE other fraction is stored for mass spectrometry.

6.2.4 Protocol for intracellular glycine betaine measumement

Cultures used:

Sample: *H. halochloris* grown in 0.035g, 0.2g ,1g, 2g, 4g and 10g KCl and at different NaCl concentrations 5%, 7.5%, 10%, 12.5% 15%, 17.5%, 20%, 22.5%, 25%, 27.5%, 30%, 32.5% and 35% NaCl. *H. halophila* was grown in 0.035 g, 0.2 g, 1g, 2g, 4g and 10g KCl and at different NaCl conc 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 22.5%, 25%, 27.5%, 30%, 32.5% and 35% NaCl.

Method:

- 100 ml *H halochloris* and *H haophilal* cells (high and low salt) are harvested in the late exponential phase [OD660 nm: ~ 0.86 & 0.78 and 0.42 & 1.2 respectively]
- Cells are washed using isotonic NaCl solutions
- Cells are freeze-thawed
- Cells are diluted with distilled water [0.1mg/ml]
- Cells are incubated with lysozyme
- 10%(w/v) of 0.2N perchloric acid is added
- pH is adjusted to 7
- The protein free extract is passed through weakly cationic resin Amberlite CG-50
- Column is eluted with phosphate-citrate buffer pH 5.3

- 0.5 ml of fractions are made up to 2 ml using 2 N HCl
- 1 ml of reagent [10g iodine + 12.4g KI] is added
- Solution is shaken and placed on a ice bath for 20 min
- 10 ml 1,2 dichloroethane is added and thoroughly mixed
- Absorbance of the organic layer (lower layer) is measured at 365nm

Standard Curve:

- Make standard solutions of glycine betaine (Mol. wt 117.14) from 10 mM [1.17 mg/10ml H_2O] to 2M [23.4 mg/10ml H_2O]
- Aliqouts of 0.5 ml of standard solutions and make up the volume to 2 ml with 2 N HCl
- 1 ml of reagent [10g iodine + 12.4g KI] is added
- Solution is shaken and placed on a ice bath for 20 min
- 10 ml 1,2 dichloroethane is added and thoroughly mixed
- Absorbance of the organic layer (lower layer) is measured at 365nm

6.2.5 Raw data:

Cytoplasmic potassium concentrations (M) in *H.halophila* at various NaCl and KCl concentrations:
First 3 data sets were obtained from isotonic NaCl wash and the rest of the data sets were isotonic ammonium sulfate washes.

5% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.13	0.14	0.15	0.11	0.12	0.17	0.1	0.09	0.08	0.12	0.03
0.1	0.12	0.12	0.16	0.11	0.09	0.13	0.09	0.07	0.08	0.11	0.03
0.2	0.21	0.19	0.2	0.13	0.21	0.15	0.19	0.2	0.17	0.18	0.03
1	0.34	0.37	0.62	0.67	0.32	0.31	0.69	0.48	0.47	0.47	0.15
2	0.67	0.49	0.46	0.66	0.39	0.41	0.57	0.59	0.57	0.53	0.10
4	0.58	0.54	0.49	0.42	0.38	0.65	0.71	0.74	0.57	0.56	0.12
10	0.78	0.81	0.52	0.54	0.67	0.71	0.68	0.69	0.68	0.68	0.10

10% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.08	0.09	0.1	0.11	0.12	0.13	0.14	0.15	0.17	0.12	0.03
0.1	0.2	0.18	0.16	0.13	0.21	0.15	0.14	0.19	0.1	0.16	0.04
0.2	0.21	0.19	0.2	0.18	0.21	0.15	0.19	0.2	0.17	0.19	0.02
1	0.87	0.85	0.92	0.74	0.87	0.91	0.98	0.79	0.88	0.87	0.07
2	1.47	1.29	1.33	1.28	1.43	1.27	1.29	1.29	1.26	1.32	0.08
4	1.43	1.26	1.27	1.28	1.29	1.29	1.43	1.43	1.54	1.36	0.10
10	1.63	1.26	1.27	1.28	1.39	1.59	1.43	1.43	1.54	1.42	0.14

15% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.13	0.14	0.15	0.11	0.12	0.17	0.1	0.09	0.08	0.12	0.03
0.1	0.2	0.15	0.16	0.13	0.13	0.15	0.14	0.13	0.1	0.14	0.03
0.2	0.21	0.19	0.2	0.18	0.21	0.19	0.19	0.2	0.19	0.20	0.01
1	1.57	1.26	1.27	1.28	1.39	1.55	1.43	1.39	1.5	1.40	0.12
2	1.62	1.76	1.77	1.28	1.69	1.6	1.73	1.69	1.5	1.63	0.16
4	1.64	1.76	1.77	1.58	1.69	1.8	1.73	1.69	1.9	1.73	0.09
10	1.94	1.83	1.83	1.98	1.96	1.8	1.83	1.99	1.84	1.89	0.08

20% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.17	0.14	0.15	0.11	0.12	0.13	0.1	0.09	0.08	0.12	0.03
0.1	0.12	0.12	0.09	0.11	0.16	0.13	0.09	0.07	0.08	0.11	0.03
0.2	0.21	0.19	0.2	0.18	0.21	0.15	0.19	0.2	0.17	0.19	0.02
1	1.62	1.76	1.57	1.38	1.39	1.6	1.33	1.49	1.5	1.52	0.14
2	1.64	1.76	1.77	1.58	1.69	1.8	1.73	1.69	1.8	1.72	0.07
4	1.84	1.83	1.83	1.91	1.92	1.8	1.83	1.92	1.84	1.86	0.05
10	1.99	1.93	1.99	1.98	2.3	1.99	2.1	1.99	1.93	2.02	0.12

25% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.13	0.13	0.15	0.12	0.12	0.17	0.1	0.09	0.08	0.12	0.03
0.1	0.24	0.15	0.16	0.13	0.13	0.15	0.14	0.13	0.19	0.16	0.04
0.2	0.17	0.19	0.2	0.18	0.21	0.15	0.19	0.2	0.17	0.18	0.02
1	1.84	1.76	1.77	1.88	1.69	1.8	1.73	1.69	1.9	1.78	0.08
2	1.84	1.83	1.83	1.91	1.92	2.2	1.93	1.92	1.84	1.91	0.12
4	2.3	1.99	1.99	1.98	2.4	2	2.4	1.99	1.99	2.12	0.19
10	2.5	2.4	2.6	2.3	2.4	2	2.4	2.4	2.2	2.36	0.17

30% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.08	0.09	0.1	0.11	0.12	0.13	0.14	0.15	0.17	0.12	0.03
0.1	0.12	0.09	0.09	0.11	0.16	0.13	0.09	0.09	0.08	0.11	0.03
0.2	0.19	0.19	0.2	0.18	0.21	0.15	0.19	0.2	0.17	0.19	0.02
1	1.74	1.73	1.83	1.87	1.86	1.8	1.83	1.92	1.84	1.82	0.06
2	2.02	1.98	1.99	2.09	2.3	1.99	2.19	2.03	1.99	2.06	0.11
4	2.4	1.99	1.99	1.98	2.4	1.9	2.4	1.99	1.99	2.12	0.22
10	2.7	2.7	2.6	2.8	2.5	2.6	2.4	2.4	2.9	2.62	0.17

35% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.09	0.09	0.09	0.11	0.12	0.13	0.14	0.15	0.17	0.12	0.03
0.1	0.12	0.09	0.09	0.11	0.16	0.13	0.09	0.09	0.08	0.11	0.03
0.2	0.2	0.2	0.2	0.19	0.24	0.19	0.19	0.23	0.17	0.20	0.02
1	2.02	2.09	1.99	2.09	2.3	2.08	2.19	2.1	2.04	2.10	0.09
2	2.7	2.8	2.9	2.8	2.5	2.6	2.9	2.4	2.9	2.72	0.19
4	3.09	2.89	2.97	2.84	2.9	2.6	2.9	2.49	2.99	2.85	0.19
10	3.24	2.99	2.97	2.84	3.25	3.6	3.45	3.49	3.23	3.23	0.26

Cytoplasmic chloride concentrations (M) in *H.halophila* at various NaCl and KCl concentrations:
First 3 data sets were obtained from isotonic NaCl wash and the rest of the data sets were isotonic ammonium sulfate washes.

H.halophila											
NaCl	1	2	3	4	5	6	7	8	9	Average	SD
5	0.43	0.57	0.59	0.43	0.38	0.59	0.48	0.58	0.6	0.52	0.09
10	0.63	0.68	0.59	0.67	0.58	0.64	0.49	0.6	0.6	0.61	0.06
15	0.97	1.1	0.99	0.94	0.98	1.12	1.22	1.22	1.39	1.10	0.15
20	1.64	1.71	1.72	1.58	1.69	1.75	1.73	1.69	1.8	1.70	0.06
25	2.5	1.99	2.2	1.98	2.7	2.2	2.3	1.97	1.99	2.20	0.26
30	2.98	2.9	2.91	2.8	3.21	3.52	3.2	3.2	3.2	3.10	0.22
35	3.54	3.5	2.87	2.84	3.25	3.5	3.45	3.49	3.23	3.30	0.27

H.halochloris											
NaCl	1	2	3	4	5	6	7	8	9	Average	SD
5	0.34	0.3	0.37	0.33	0.32	0.31	0.32	0.4	0.32	0.33	0.03
10	0.34	0.37	0.42	0.43	0.32	0.31	0.39	0.41	0.37	0.37	0.04
15	0.58	0.57	0.49	0.42	0.45	0.65	0.71	0.74	0.57	0.58	0.11
20	0.67	0.49	0.46	0.66	0.39	0.41	0.57	0.59	0.52	0.53	0.10
25	0.34	0.37	0.62	0.67	0.32	0.31	0.69	0.48	0.47	0.47	0.15
30	0.39	0.37	0.62	0.67	0.32	0.31	0.69	0.48	0.47	0.48	0.15
35	0.44	0.57	0.62	0.62	0.32	0.31	0.49	0.48	0.47	0.48	0.11

H.salinarum											
NaCl	1	2	3	4	5	6	7	8	9	Average	SD
15	1.17	1.26	1.27	1.22	1.13	1.15	1.13	1.39	1.12	1.20	0.09
20	1.62	1.7	1.57	1.38	1.39	1.6	1.33	1.49	1.5	1.51	0.12
25	2.49	2.36	2.2	2.3	2.4	2	2.4	2.4	2.2	2.31	0.15
30	3.2	2.99	2.97	2.84	3.25	3.55	3.45	3.43	3.13	3.20	0.24
35	4.78	4.23	4.12	4.37	4.45	4.53	4.39	4.38	4.39	4.40	0.18

Cytoplasmic glycine betaine concentrations (M) in *H.halophila* at various NaCl and KCl concentrations:

5% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.78	0.81	0.52	0.54	0.67	0.71	0.68	0.69	0.68	0.68	0.10
0.1	0.69	0.68	0.79	0.67	0.78	0.64	0.69	0.68	0.69	0.70	0.05
0.2	0.72	0.74	0.82	0.67	0.78	0.67	0.71	0.68	0.69	0.72	0.05
1	0.08	0.09	0.1	0.11	0.12	0.13	0.14	0.15	0.17	0.12	0.03
2	0.12	0.1	0.09	0.11	0.16	0.13	0.09	0.09	0.08	0.11	0.03
4	0.1	0.12	0.09	0.1	0.16	0.13	0.09	0.07	0.08	0.10	0.03
10	0.2	0.15	0.16	0.13	0.13	0.15	0.14	0.13	0.19	0.15	0.03

10% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	0.97	1.12	0.99	0.94	0.98	1.1	1.22	1.22	1.39	1.10	0.15
0.1	1.17	1.26	1.27	1.22	1.13	1.15	1.13	1.39	1.12	1.20	0.09
0.2	0.77	0.83	0.72	0.74	0.85	0.71	0.78	0.79	0.86	0.78	0.05
1	0.1	0.1	0.09	0.09	0.11	0.12	0.09	0.07	0.08	0.09	0.02
2	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
4	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
10	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.00

15% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	1.6	1.72	1.53	1.38	1.39	1.6	1.33	1.49	1.5	1.50	0.12
0.1	1.57	1.26	1.27	1.28	1.39	1.55	1.43	1.39	1.5	1.40	0.12
0.2	0.75	0.85	0.73	0.73	0.85	0.71	0.78	0.79	0.86	0.78	0.06
1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
2	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
4	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
10	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00

20% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	1.94	1.85	1.89	1.98	1.96	1.8	1.83	1.99	1.84	1.90	0.07
0.1	1.64	1.76	1.77	1.78	1.79	1.8	1.73	1.69	1.9	1.76	0.07
0.2	0.87	0.85	0.72	0.74	0.87	0.81	0.98	0.79	0.88	0.83	0.08
1	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
2	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
4	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
10	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00

25% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	2.3	2.2	2.56	2.3	2.4	2	2.36	2.4	2.2	2.30	0.16
0.1	1.99	2.02	2.04	2.08	2.09	2.09	2.1	2.19	2.3	2.10	0.09
0.2	0.87	0.95	0.92	0.94	0.87	0.91	0.98	0.89	0.88	0.91	0.04
1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
2	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.00
4	0.1	0.1	0.09	0.09	0.11	0.12	0.09	0.07	0.08	0.09	0.02
10	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.00

30% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	3.29	2.91	2.97	2.84	3.25	3.15	3.15	3.23	3.13	3.10	0.16
0.1	2.3	2.65	2.6	2.71	2.7	2.6	2.4	2.6	2.81	2.60	0.16
0.2	1.31	1.22	1.22	1.12	1.1	0.99	0.98	0.97	0.94	1.09	0.13
1	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
2	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
4	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00
10	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00

35% NaCl											
KCl	1	2	3	4	5	6	7	8	9	Average	SD
0.035	2.84	2.87	3.23	3.25	3.45	3.49	3.5	3.5	3.54	3.30	0.27
0.1	2.5	2.7	2.6	2.77	2.97	2.6	2.4	2.9	2.9	2.70	0.20
0.2	1.17	1.2	1.21	1.22	1.13	1.15	1.13	1.29	1.12	1.18	0.06
1	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.00
2	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
4	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.00
10	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.00

REFERENCES

1. **Abd-el-Malek, Y., and S. G. Rizk.** 1963. Bacterial sulfate reduction and the development of salinity III. Experiments under natural conditions in the Wadi Natrun. Journal of Appllied Bacteriology **26:**20-26.

2. **Anton, J.** 2002. *Salinibacter ruber* gen, nov; a novel extremely halophilic member of bacteria from saltern crystalizer ponds. International Journal of Systematic Bacteriology **52:**7-22.

3. **Argüelles, J. C.** 2000. Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. Archives of Microbiology **174:**217-224.

4. **Avonce, N., A. Mendoza-Vargas, E. Morett, and G. Iturriaga.** 2006. Insights on the evolution of trehalose biosynthesis. BMC evolutionary biology **6:**109-119.

5. **B Kempf, E. P.** 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high osmolarity environments. Archives of Microbiology **170:**11-25.

6. **Baumgartner, J. G.** 1937. The salt limits and thermal stablity of a new species of anaerobic halophile Food Research **2:**321-329.

7. **Becking, B., and G. M. Lourens (ed.).** 1934. *Geobiologie of inleiding to de milieu selecteert* Netherlands.

8. **Becking, B., and G. M. Lourens.** 1928. Historical notes on salt and salt manufacture. Scientific Monthly **32:**321-329.

9. **Bell, W., W. Sun, S. Hohmann, S. Wera, A. Reinders, C. De Virgilio, A. Wiemken, and J. M. Thevelein.** 1998. Composition and Functional Analysis of the *Saccharomyces cerevisiae* Trehalose Synthase Complex. Journal of Biological Chemistry **273:**33311-33319.

10. **Boch, J., B. Kempf, R. Schmid, and E. Bremer.** 1996. Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the gbsAB genes. J. Bacteriol. **178:**5121-5129.

11. **Bonnete, F., C. Ebel, G. Zaccai, and H. Eisenberg.** 1993. Biophysical study of halophilic malate dehydrogenase in solution: revised subunit structure and solvent interactions of native and recombinant enzyme. Journal of the Chemical Society, Faraday Transactions **89:**2659-2666.

12. **Canovas, D.** 1998. Charaterisation of genes for the biosynthesis of ecotine in moderately halophilic bacterium *Halomonas eleongata* DSM 3043. Systematic and Applied Microbiology **21:**11-19.

13. **Caumette, P.** 1999. Ecology and physiology of phototrophic bacteria and sulfur reducing bacteria in marine salterns. Experimentia **49:**473-481.

14. **Cayol JL, O. B., Patel KC, Prensier G, Guezennec J, Garcia J-L.** 1994. Isolation and characterisation of *Halothermothrix orenii* gen. nov. sp. nov. a

halophilic, thermophilic fermentative strictly anaerobic bacterium. Int J System Bacteriol **44:**534-633.

15. **Christian, J. H. B.** 1962. Solute concentrations within the cells of halophilic and non-halophilic bacteria. Biochemi and Biophysical Acta **65:**506-508.

16. **DasSarma, S., S. DasSarma, M. Capes, and P. DasSarma.** 2010. HaloWeb: The haloarchael genome database, Saline Systems, vol. 6.

17. **De Smet, K. A. L., A. Weston, I. N. Brown, D. B. Young, and B. D. Robertson.** 2000. Three pathways for trehalose biosynthesis in mycobacteria. Microbiology **146:**199-208.

18. **Ebel, C., P. Faou, and B. Kernel (ed.).** 1999. Molecular interaction in extreme halophiles- the solvation-stabilization hypothesis of halophilic proteins. CRC Press, Boca Raton.

19. **Ebel, C., P. Faou, and B. Kernel.** 1999. Relative role of anions and cations in the stabilization of halophilic malate dehydrogenase. Biochemistry **38:**9039-9047.

20. **Epstein, W., and S. G. Schultz.** 1965. Cation Transport in *Escherichia coli*. The Journal of General Physiology **49:**221-234.

21. **Fukuchi, S., and K. Nishikawa.** 2001. Protein surface amino acid compositions distinctively differ between thermophilic and mesophilic bacteria. Journal of Molecular Biology **309:**835-843.

22. **Galinski, E. A., and H. Truper.** 1994. Microbial behavior in salt stressed ecosystems. FEMS Microbiology review **15:**14-23.

23. **Galinski, E. A., and H. G. Truper.** 1982. Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris*. FEMS Microbiology Letters **13:**357-360.

24. **Gibson, R. P., J. P. Turkenburg, S. J. Charnock, R. Lloyd, and G. J. Davies.** 2002. Insights into Trehalose Synthesis Provided by the Structure of the Retaining Glucosyltransferase OtsA. Chemistry & biology **9:**1337-1346.

25. **Grammann, K., A. Volke, and H. J. Kunte.** 2002. New Type of Osmoregulated Solute Transporter Identified in Halophilic Members of the Bacteria Domain: TRAP Transporter TeaABC Mediates Uptake of Ectoine and Hydroxyectoine in *Halomonas elongata* DSM 2581T. J. Bacteriol. **184:**3078-3085.

26. **Grant, W., R. T. Gemmel, and T. J. McGenity (ed.).** 1998. Microbial Life in Extreme Environments.

27. **Grant, W. D., and B. J. Tindall (ed.).** 1986. The alkaline saline environments. Academic Press, London.

28. **Griaever, H. M.** 1986. Biochemical and genetic characterisation of osmoregulatory trehalose synthesis in *E coli*. Journal of Bacteriology **170:**9-17.

29. **Gunde-Cimerman, N., J. Ramos., and A. Plemenitas.** 2009. Halotolerant and Halophilic fungi. Mycology Research **113:**1231-1241.

30. **Hof, T.** 1935. Investigations concerning bacterial life in strong brines. Rec. Trav. Bot. Neerl. **32:**92-173.

31. **Imhoff, J.** 2006. The Family Ectothiorhodospiraceae, p. 874-886. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The Prokaryotes. Springer New York.

32. **Imhoff, J. (ed.).** 1988. Halophilic phototrophic bacteria vol. 1. CRC Press, Boca Raton.

33. **Imhoff, J., H. G. Sahl, G. H. Soliman, and H. G. Truper.** 1979. The Wadi Natrum: chemical composition and microbial mass development in alkaline saline brines of entropic desert lakes. Journal of Geomicrobilogy **1:**219-234.

34. **Imhoff, J. F., F. Hashwa, and H. Truper.** 1978. Isolation of extremely halophilic phototrophic bacteria from the alkaline Wadi Natrun, Egypt. Archiv fuer Hydrobiologie **84:**381-388.

35. **Imhoff, J. F., and F. Rodriguez-Valera.** 1984. Betaine is the main compatible solute of halophilic eubacteria. J. Bacteriol. **160:**478-479.

36. **Imhoff, J. F., and J. Süling.** 1996. The phylogenetic relationship among *Ectothiorhodospiraceae*: a reevaluation of their taxonomy on the basis of 16S rDNA analyses. Archives of Microbiology **165:**106-113.

37. **Javor, B. (ed.).** 1989. Hypersaline Environments. Springer.

38. **Ji, C., and N. Kaplowitz.** 2003. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. Gastroenterology **124:**1488-1499.

39. **Kaasen, I., J. McDougall, and A. R. Strøm.** 1994. Analysis of the otsBA operon for osmoregulatory trehalose synthesis in *Escherichia coli* and homology of the OtsA and OtsB proteins to the yeast trehalose-6-phosphate synthase/phosphatase complex. Gene **145:**9-15.

40. **Kanner, B. I., and E. Racker.** 1975. Light dependant proton and rubidium translocation in membrane vesicles from *Halobacterium halobium*. Biochem. Biophys. Res. Commun. **64:**1054-1061.

41. **Kappes, R., B. Kempf, and E. Bremer.** 1996. Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J. Bacteriol. **178:**5071-5079.

42. **Kennedy, S. P., W. V. Ng, and S. L. Salzberg.** 2001. Understanding the adaptation of *Halobacterium* species NRC-1 to its extreme environment through computational analysis of its genome sequence. Genome research **11:**1641-1650.

43. **Lai, M. C., K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus.** 1991. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. J. Bacteriol. **173:**5352-5358.

44. **Lanyi, J. K.** 1974. Salt-dependant properties of proteins from extremely halophilic bacteria. Bacteriological Reviews **38:**272-290.

45. **Lewis, E.** 1982. The practical salinity scale and its antecedents. Marine Geodesy **5:**350-357.

46. **Lobyrena, L. B., et al.** 1991. Intracellular pool and tranport of aromatic amino acids in the *Halobacterium* sp NRC-1 cells. Microbiology **60:**149-152.

47. **Madern, D., et al.** 2000. Halophilic adaptation of enzymes. Extremophiles **64:**491-498.

48. **Martin, D.** 1999. Osmoadaptation in archaea. Applied Environmental Microbiology **65:**1815-1825.

49. **Mavromatis, K., N. Ivanova, I. Anderson, A. Lykidis, S. D. Hooper, H. Sun, V. Kunin, A. Lapidus, P. Hugenholtz, B. Patel, and N. C. Kyrpides.** 2009. Genome Analysis of the Anaerobic Thermohalophilic Bacterium *Halothermothrix orenii*. PLoS ONE **4:**4192-4203.

50. **Meseguer, I., M. Torreblanca, and T. Konishi.** 1995. Specific inhibition of the halobacterial Na+/H+ antiporter by halocin. Journal of Biological Chemistry **151:**530-536.

51. **Meury, J., and M. Kohiyama.** 1989. ATP is required for K+ active transport in archaebacterium *Halofarex volcanii*. Archives of Microbiology **19:**363-365.

52. **Mongodin, E. F., K. E. Nelson, S. Daugherty, and R. T. Deboy.** 2005. The genome of *Salinibacter ruber*: convergence and gene exchange among

hyperhalophilic bacteria and archaea. Proceedings of National Academy of Sciences **102:**18147-18152.

53. **Ng, W. V.** 2000. Genome sequence of *Halobacterium* sp NRC-1. Proceedings of the National Academy of Sciences **97:**6-23.

54. **Nyyssölä, A., J. Kerovuo, P. Kaukinen, N. von Weymarn, and T. Reinikainen.** 2000. Extreme Halophiles Synthesize Betaine from Glycine by Methylation. Journal of Biological Chemistry **275:**22196-22201.

55. **Nyyssölä, A., and M. Leisola.** 2001. *Actinopolyspora halophila*; has two separate pathways for betaine synthesis. Archives of Microbiology **176:**294-300.

56. **Oren, A.** 1999. Bioenergetic Aspects of Halophilism. Microbiol. Mol. Biol. Rev. **63:**334-348.

57. **Oren, A. (ed.).** 2002. Cellular Origin and Life in Extreme Habitat, vol. 5.

58. **Oren, A.** 1991. Dynamics of a bloom of halophilic archaea in the Dead Sea. Hydrobiologia **315:**149-158.

59. **Oren, A. (ed.).** 1999. Life at high salt concentrations.

60. **Oren, A.** 2008. Microbial life at high salt concentrations. Saline Systems **4.**

61. **Oren, A.** 1983. Population dynamics of halobacteria in the Dead Sea water column. Limnology and Oceanography **28:**1094-1103.

62. **Oren, A., and E. A. Galinski.** 2002. Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*. Extremophiles **6:**491-498.

63. **Oren, A., F. Larimer, P. Richardson, A. Lapidus, and L. N. Csonka.** 2005. How to be moderately halophilic with broad salt tolerance: clues from the genome of *Chromohalobacter salexigens*. Extremophiles **9:**275-279.

64. **Oren, A., and C. D. Litchfield.** 1998. Early salt production at the Dead Sea and the Mediterrean coast of the Holy Land.

65. **Pan, Y. T., J. D. Carroll, and A. D. Elbein.** 2002. Trehalose-phosphate synthase of *Mycobacterium tuberculosis*. European Journal of Biochemistry **269:**6091-6100.

66. **Perez-Fillol, and Rodriguez-Valera.** 1986. Potassium ion accumulation in cells of different halobacteria. Microbiologia SEM **2:**73-80.

67. **Peter, H., B. Weil, A. Burkovski, R. Kramer, and S. Morbach.** 1998. *Corynebacterium glutamicum* Is Equipped with Four Secondary Carriers for Compatible Solutes: Identification, Sequencing, and Characterization of the Proline/Ectoine Uptake System, ProP, and the Ectoine/Proline/Glycine Betaine Carrier, EctP. J. Bacteriol. **180:**6005-6012.

68. **Petter, H.** 1931. Over roode en andere bacterieen van gezouten visch. University of Utrecht.

69. **Pierce, G.** 1914. The behavior of certain microorganisms in brine. Carnegie Institution of Washington **193:**21.

70. **Reed, R. H.** 1984. Use and abuse of osmo-terminology. Plant Cell Environment **7:**165-170.

71. **Robert, M. F.** 2005. Organic compatible solutes of halotolerant and halophilic microrganisms. Saline Systems:5-18.

72. **Roberts, M. F.** 2005. Organic compatible solutes of halotolerant and halophilic micro-organisms. Saline Systems **1**.

73. **Ross, H. N. M., M. D. Collins, B. J. Tindall, and W. Grant.** 1981. A rapid method for the detection of archaebacterial lipids in halophilic bacteria. Journal of General Microbiology **123:**75-80.

74. **Saums, S., and e. al.** 2008. Regulation of osmoadaptation in the moderate halophile *Halobacillus halophilus*: chloride,glutamate and switching osmolyte strategies. Saline Systems **4:**1746-1748.

75. **Schmidt, K., and H. G. Trüper.** 1971. Carotenoid composition in the genus *Ectothiorhodospira*. Archives of Microbiology **80:**38-42.

76. **Schmidt, S., K. Pflüger, S. Kögl, R. Spanheimer, and V. Müller.** 2007. The salt-induced ABC transporter Ota of the methanogenic archaeon *Methanosarcina mazei* Gö1 is a glycine betaine transporter. FEMS Microbiology Letters **277:**44-49.

77. **Schubert, T., T. Maskow, D. Benndorf, H. Harms, and U. Breuer.** 2007. Continuous Synthesis and Excretion of the Compatible Solute Ectoine by a Transgenic, Nonhalophilic Bacterium. Appl. Environ. Microbiol. **73:**3343-3347.

78. **Shailaditya, D., and e. al.** 2001. Genome sequence of *Halobacterium* species NRC-1. Proceedings of National Academy of Sciences **97:**12176-12181.

79. **Shailaditya, D., and D. Priya (ed.).** 2006. Halophiles.

80. **Takaichi, S., T. Maoka, and S. Hanada.** 2001. Dihydroxylycopene diglucoside diesters, a novel class of carotenoids from the phototrophic purple sulfur bacteria *Halorhodospira abdelmalekii* and *Halorhodospira halochloris*. Archives of Microbiology **175:**167-171.

81. **Tschichholz, I., and H. G. Trüper.** 1990. Fate of compatible solutes during dilution stress in *Ectothiorhodospira halochloris*. FEMS Microbiology Letters **73:**181-185.

82. **Volcani, B.** 1944. The micro-organisms of the dead sea, Papers Collected to commemorate the 70th anniversary of Dr Chaim Weizman Collective Volume, Rehovoth.

83. **Volcani, E.** 1940. Studies of the microflora of the Dead Sea. The Hebrew University of Jerusalem.

84. **Vreeland, R. H., C. D. Litchfield, E. L. Martin, and E. Elliot.** 1980. *Halomonas elongata*, a new genus and species of extremely salt tolerant bacteria. International Journal of Systemic Bacteriology **30:**485-495.

85. **Walsby, A. E., v. R. J, and C. Y.** 1983. The biology of a new gas-vacuolate cyanobacterium *Dactyloccopsis salina* sp. nov. in Solar lake. Proceedings of Royal Society **B217:**417-447.

86. **Zaccai, G., F. Cendrin, Y. Haik, N. Borochov, and H. Eisenberg.** 1989. Stabilization of halophilic malate dehydrogenase. Journal of Molecular Biology **208:**491-500.

87. **Zaccai, G., and H. Eisenberg (ed.).** 1991. A model for the stabilization of halophilic protein. Springer-Verlag, Berlin.

88. **Zimmermann, U., G. Pilwat, and T. Günther.** 1973. Regulation of the intracellular potassium concentration in *Escherichia coli* B 525. Biochimica et Biophysica Acta (BBA) - Biomembranes **311:**442-451.

VITA

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Doctor of Philosophy

Thesis: A NOVEL STRATEGY FOR HALOPHILICITY IN THE PHOTOAUTOTROPHIC PROTEOBACTERIUM *HALORHODOSPIRA HALOPHILA*.

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Title of Study: A NOVEL STRATEGY FOR HALOPHILICITY IN THE PHOTOAUTOTROPHIC PROTEOBACTERIUM *HALORHODOSPIRA HALOPHILA*.

Pages in Study: 118 Candidate for the Degree of Doctor of Philosophy

Major Field: Microbiology and Molecular Genetics

Scope and Method of Study: Approximately 97% of all water on earth is present in saline oceans, saline lakes, inland seas, and saline groundwater. Additionally salt deposits occupy one quarter of the land on earth. Thus, saline and hypersaline environments are highly abundant and of great ecological significance. In addition, salinity is a major determinant for microbial community composition. Therefore, halophilic adaptations are of general biological interest. Extreme halophiles thrive in hypersaline environments by accumulating molar amounts of potassium chloride or compatible solutes in their cytoplasm. Here we studied the osmoprotection strategy of the extremely halophilic purple photosynthetic γ-proteobacteria *H. halophila* and *H. halochloris*. Genes involved in osmoprotection were studied by bioinformatic analysis of the *H. halophila* genome. Proteome acidicity, which is generally associated with the use of KCl as a major osmoprotectant, was studied through calculated average pI of the predicted proteins and isoelectric focusing gel electrophoresis of total cellular proteins. Cytoplasmic KCl and glycine betaine concentrations were quantified using plasma emission spectrometry and colorimetric assay. Electron microprobe elemental analysis was used to observe cytoplasmic KCl content.

Findings and Conclusions: The work described here demonstrates that *H. halophila* breaks away from the current understanding of halophilic adaptations. It has an acidic proteome, but can function in the absence of increased cytoplasmic KCl concentrations when cells are grown at low NaCl concentrations. *H. halochloris*, a close taxomoic relative of *H. halophila*, does not accumulate KCl, which indicates recent rapid evolution in osmoprotection strategy. This is matched by the broad distribution of pI values of its proteome as revealed by gel electrophoresis. Interestingly, *H. halochloris* uses the organic osmolyte glycine betaine as its main osmoprotectant. *H. halophila* can also perform glycine betaine biosynthesis and uptake from the medium through its uptake systems. This was demonstrated by checking the stimulation of cell growth by addition of glycine betaine to *H. halophila* cells grown under K^+ limiting conditions and salt stress. *H. halophila* does not confer with the two known halophilic strategies. It uses an unusual mixed KCl/compatible solute strategy for osmoregulation, with an osmoprotectant switch near 1 g/l KCl in growth medium. The average K^+ concentration of the Wadi Natrun lakes from which *H. halophila* was isolated is close to the concentration at which it switches to the 'Low-salt, osmolyte-in' strategy of osmoprotection. Hence we propose this switch in osmoprotection strategy is ecologically relevant for survival of *H. halopila*.

ADVISOR'S APPROVAL: Dr Wouter Hoff