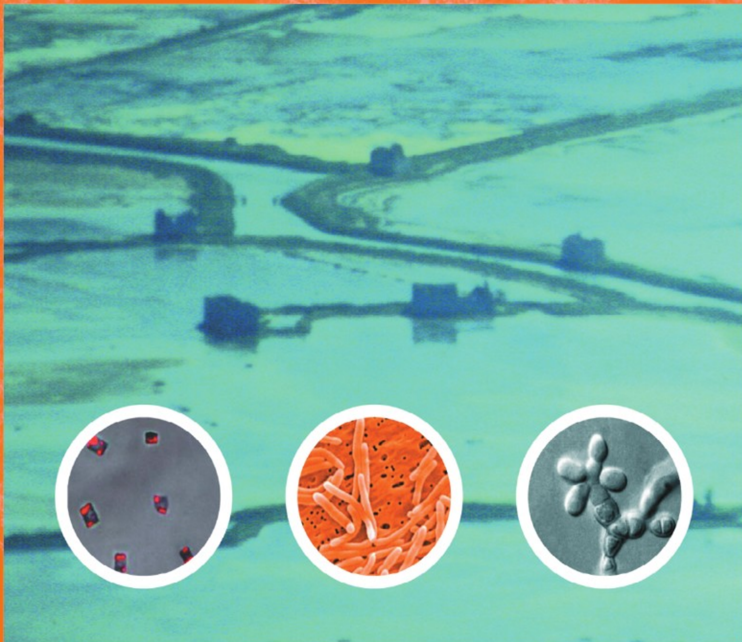
CELLULAR ORIGIN, LIFE IN EXTREME HABITATS AND ASTROBIOLOGY

Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya

Edited by
Nina Gunde-Cimerman, Aharon Oren
and Ana Plemenitaš



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ADAPTATION TO LIFE AT HIGH SALT CONCENTRATIONS
IN ARCHAEA, BACTERIA, AND EUKARYA

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 9

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Joseph Seckbach
The Hebrew University of Jerusalem, Israel

Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya

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FOREWORD

Salt is an essential requirement of life. Already from ancient times (e.g., see the books of the Bible) its importance in human life has been known. For example, salt symbolizes destruction (as in Sodom and Gomorra), but on the other hand it has been an ingredient of every sacrifice during the Holy Temple periods.

Microbial life in concentrated salt solutions has fascinated scientists since its discovery. Recently there have been several international meetings and books devoted entirely to halophiles. This book includes the proceedings of the "Halophiles 2004" conference held in Ljubljana, Slovenia, in September 2004 (www.uni-lj.si/~bfbhaloph/index.html). This meeting was attended by 120 participants from 25 countries. The editors have selected presentations given at the meeting for this volume, and have also invited a number of contributions from experts who had not been present in Ljubljana. This book complements "Halophilic Microorganisms", edited by A. Ventosa and published by Springer-Verlag (2004), "Halophilic Microorganism and their Environments" by A. Oren (2002), published by Kluwer Academic Publishers as volume 5 of "Cellular Origins, Life in Extreme Habitats and Astrobiology" (COLE), and "Microbiology and Biogeochemistry of Hypersaline Environments" edited by A. Oren, and published by CRC Press, Boca Raton (1999).

Salt-loving (halophilic) microorganisms grow in salt solutions above seawater salinity (~3.5% salt) up to saturation ranges (i.e., around 35% salt). High concentrations of salt occur in natural environments (e.g., in the Dead Sea and the Great Salt Lake, Utah) and in man-made hypersaline environments such as solar salterns. The research of halophiles covers all three domains of life, i.e., Archaea, Bacteria, and Eukarya.

This multi-author review has been edited by Professors Nina Gunde-Cimerman, Aharon Oren and Ana Plemenitaš. It is volume 9 of the COLE book series ["Cellular Origins, Life in Extreme Habitats and Astrobiology", edited by J. Seckbach, now published by Springer] (www.springeronline.com). It covers most recent research on halophiles in chapters contributed by experts in this field. I wish to thank the editors for suggesting the publication of this volume in the framework of the COLE series and for editing all the chapters of this book.

January 2005

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INTRODUCTION

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This book, entitled "Adaptations to life at high salt concentrations in Archaea, Bacteria and Eukarya", intends to provide an overview of current research in the field of halophilic microorganisms, with special emphasis on the diversity of life forms adapted to tolerate high salt concentrations and accordingly low water activities. Most of the chapters are based on lectures given by the participants of the international symposium "Halophiles 2004", held in Ljubljana, Slovenia, from 4-9 September 2004. To ensure a broader scope of the book we have invited a number of chapters from colleagues who had not been present at the Ljubljana symposium.

The first multi-author review written about halophilic microorganisms was, to the best of our knowledge, written in 1978 (Caplan and Ginzburg, 1978). Looking at the contents of that book, one discovers that no less than thirty-eight chapters were devoted solely or primarily to *Halobacterium salinarum* (= *H. halobium*, *H. cutirubrum*), four chapters dealt with other halophilic Archaea of the family Halobacteriaceae, three were on *Dunaliella*, and only eight chapters had as their topic other types of halophilic or halotolerant microorganisms, including one chapter that dealt with fungi. We now realize that the world of halophilic microorganisms is very diverse, and that life at high salt concentrations cannot be understood from the study of a single organism or group of organisms alone. The halophiles are as diverse as the environments they live in (see also Oren, 2002).

How large the diversity of halophilic microorganisms really is, is quickly recognized when one examines samples of water and sediment from saltern evaporation ponds in the microscope. The "halophilic safari" presented by Oren in this volume, which explores just one example of a hypersaline environment, shows the tremendous morphological variations found – variations so extensive that one sometimes wonders whether presence of high salt concentrations is indeed so stressful to the microbial world as is often assumed. Indeed, we find halophiles all over the phylogenetic tree, in each of the three domains of life, Archaea, Bacteria and Eukarya, and in different branches of each domain. Many of these still await isolation and characterization.

While the world of prokaryotic halophiles, Archaea as well as Bacteria, has been explored quite extensively in the past, halophilic and halotolerant Eukarya are still a relatively neglected group. A notable exception is the unicellular green alga *Dunaliella*,

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which is present worldwide in hypersaline lakes and is responsible for most of the primary production in aquatic high-salt environments, thereby supporting varied communities of heterotrophs. This book is published exactly hundred years after *Dunaliella* was described as a new species of algae (Teodoresco, 1905), and this fact is memorized in a chapter by Oren in this volume. The chapter by Premkumar and coworkers presents an overview of the most recent advances in the study of this intriguing organism.

The fungi form another group of eukaryotes that can grow at high salt concentrations. While it has been known for long that many fungi can tolerate low water activities, the realization that fungi are an integral part of saline and hypersaline ecosystems came only recently. One group, the black yeasts, even appears to have high salt aquatic systems as its primary habitat (Gunde-Cimerman et al., 2000). Following comprehensive surveys by de Hoog et al. and Gunde-Cimerman et al. of halophilic fungal life in temperate and cold environments, more in-depth explorations of fungal life at high salt are presented in the chapters by Ramos and by Plemenitaš and Gunde-Cimerman. The potential of the fungi to produce secondary metabolites, as described by Frisvad in this volume, makes the group especially interesting, also from the point of view of applied science. We hope that the contributions in this book will give the fungi, long neglected by the halophilic scientific community, the place they deserve. Another example of a neglected group of salt-loving or salt-tolerant organisms is the lichens, as convincingly shown in the chapter by Grube and Blaha.

Another group often ignored in studies of microbial diversity at high salt concentrations is the protozoa. Although their presence is easily revealed by microscopic examination (see e.g. the “halophilic safari” chapter by Oren in this volume), only very few studies have been devoted to the function that ciliate, flagellate and amoeboid protozoa have in hypersaline environments. That such protozoa may also play a quantitatively important role even in the most salty ecosystems was nicely shown in a study of saltern crystallizer ponds by Park et al. (2003). The chapters by Hauer and Rogerson and by Cho provide an overview of our current knowledge of these organisms. Viruses are also an integral part of the biota of the hypersaline environments. The chapter by Dyall-Smith et al. summarizes our present understanding of their diversity and their importance.

We are now in the era of genomics, and approaches of genomics and proteomics have not bypassed the halophiles. The first complete genome of a halophilic archaeon, *Halobacterium* strain NRC-1 (recently described as a strain of *Halobacterium salinarum*; Gruber et al., 2004) was published already five years ago (Ng et al., 2000), and the second genome sequence, that of the Dead Sea isolate *Haloarcula marismortui*, was released just a few weeks before this introduction was written (Baliga et al., 2004). To what extent the approach of comparative genomics can add to our understanding of halophilic microorganisms is clearly shown in the chapter by Berquist et al. Although no complete descriptions of genomes of halophilic members of the Bacteria have been published yet, intriguing previews of what we may expect in the future can already be found in the chapters on the genomes of the extremely halophilic *Salinibacter ruber* by Antón et al. and on the moderately halophilic versatile *Chromohalobacter salexigens* by Csonka et al.

However important the study of microbial genomes may be, it can only complement, but never replace physiological studies of the organisms themselves. In this respect,

those who study life at the highest salt concentrations are in a much better situation than many colleagues who work on "conventional" ecosystems. We now have a quite thorough understanding of the nature of the organisms growing there, and – most important – these organisms are now available in pure culture thanks to a few breakthroughs in recent years. *Salinibacter*, an extremely halophilic representative of the domain Bacteria, numerically probably the second-most important organism in saltern crystallizer ponds worldwide, was isolated and described by Antón et al. (2002). The number-one organism in this environment is the famous flat square archaeon first described by Walsby from a brine pool (Walsby, 1980). This intriguing microbe long resisted all attempts towards its isolation, but now two groups have independently succeeded the task (Burns et al., 2004a; Bolhuis et al., 2004). We are pleased that the present volume contains contributions from both groups (the chapters by Bolhuis and Dyall-Smith et al.). It was convincingly shown that also most other inhabitants of these salt-saturated environments can be cultivated when using the proper techniques as well as much patience (Burns et al., 2004b), so it may be expected that within a short time the combination of physiological studies, environmental genomics and genomic and proteomic studies of representative isolates will yield an excellent picture of the microbial communities present and the processes they perform.

Considerable progress is being made in the understanding of the physiology of halophilic microorganisms, in particular those processes that enable these organisms to cope with the high salt concentration in their surrounding medium. Careful regulation of the intracellular ionic concentrations is a prerequisite for the ability to live at high salt. Some of the mechanisms involved are presented in the chapters by Kunte and by Müller and Saum. A widespread strategy is to balance the high ionic concentration in the medium by *de novo* biosynthesis of by uptake of organic osmotic, "compatible" solutes. Recent advances in our understanding of the metabolism of these compounds, in part guided by information gained using genomic approaches, can be found in the contributions by Reshetnikov et al., Pflüger et al., and Da Costa and Empadinhas, each dealing with a different group of microorganisms. Halophiles are also convenient model organisms to study other aspects of microbial physiology such as gene regulation (Pfeifer et al. in this volume), the properties of salt-dependent enzymes (the chapter by Mizuki et al.), and protein translocation through membranes (Eichler et al. in this volume) – all chapters dealing with extreme halophiles belonging to the archaeal domain. Some halophiles also produce chemicals of considerable economic value, as described in the chapter by Quillaguamán and coworkers, and their exploitation may well lead to novel biotechnological processes. Halophiles have also considerable potential to degrade a variety of organic chemicals, including toxic ones, as documented on the level of the genome in the chapter by Csonka et al., culture and enzyme studies (presented by Ventosa et al., and polluted hypersaline environments (Patzelt in this volume).

Most studies that involve sampling outdoors come from the man-made environment of solar saltern evaporation and crystallizer ponds. The chapter by Pedrós-Alió gives a beautiful example of an in-depth multidisciplinary study of such a saltern environment. The study of halophilic microorganisms in natural hypersaline lakes is a relatively neglected aspect. This is understandable as salterns form a more convenient environment for sampling, and the salinity gradients present are relatively constant and to a large extent predicable and reproducible. The properties of natural lakes are often far from constant, and the impact of short term and long term changes in their salt concentration

and their limnological properties is in most cases poorly known. The Dead Sea as an ecosystem is now quite well understood. At present its conditions are too extreme to support growth of even the best salt-adapted microorganisms. A thorough understanding of the biology of this lake in the past and in the present is essential to predict the possible effects of planned future intervention in the lake's properties (Oren et al. in this volume). Unfortunately, little is known about the microbiology of the Great Salt Lake, Utah. After the pioneering studies by Fred Post in the 1970s, the study of the microbiota of this lake was almost completely abandoned. As a result, we have no record of the effects of the dramatic changes that have occurred in this environment in the past quarter of a century on the microbial communities. The situation, however, is now bound to improve, as finally the local scientific community has started to show interest in this unique environment and has discovered its scientific potential. As described in the chapter by Baxter and coworkes, this has led to the initiation of an interdisciplinary research program in which educational aspects are not neglected, so that the properties of the halophilic microorganisms inhabiting the lake are now brought to the attention of a broader public.

Techniques of environmental genomics are also now being applied to the study of natural communities of halophilic microorganisms. In fact, hypersaline environments, and in particular the most "salty" ones in which salt concentrations approach saturation, are an attractive test object for such studies as the diversity, though larger than assumed in the past, is still much smaller than in most "non-extreme" environments, and microbial community densities are high, making sampling and sample processing relatively simple. Some intriguing results based on environmental genomics methodology were presented at the Ljubljana symposium, and some of this can be found in the chapters by Litchfield et al. and Baxter et al.

A final reason for studying the halophiles is their relevance to exobiology. If life exists also elsewhere in the Universe, it may be expected to occur in environments of low water activity, and probably in the present of high ionic concentrations as well, as discussed in the chapters by Seckbach and Mancinelli. The apparent ability of at least some halophiles to survive within salt crystals for thousands of years and possibly even millions of years (see e.g. the chapter by Leuko et al.) makes these organisms especially intriguing. The understanding of halophilic life on Earth can therefore provide us with much relevant information when searching for life in outer space.

The editors want to express their gratitude to all chapter authors, whose cooperation enabled the production of the book within a short time after the Ljubljana meeting. In addition, we thank Prof. Joseph Seckbach, editor of the "Cellular Origins, Life in Extreme Habitats and Astrobiology" series, who gladly agreed to publish this book within the framework of this series. We are further grateful to the people of Springer Science, and in particular Ms. Claire van Heukelom, who has been extremely helpful during all stages of the preparation of the book.

Finally we would like to thank Mobitel d.d., the Slovenian mobile operator, for its generous support of the "Halophiles 2004" symposium in Ljubljana. Without its support, the symposium could not have taken place and this book would not have been written. Mobitel has recently "adopted" the ancient system of traditionally operated saltens of Sečovlje at the Slovenian coast as part of its involvement in environmental projects. Mobitel's support of the hypersaline microbiology is greatly appreciated. We also

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The 34 chapters that follow are as diverse as the world of halophilic microorganisms themselves, and they cover nearly all aspects of life at high salt concentrations. We hope you will enjoy the result!

Ljubljana and Jerusalem, January 2005

Nina Gunde-Cimerman, Aharon Oren and Ana Plemenitaš
at the salterns of Sečovlje, Slovenia

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Section I. The environments and their diversity

Microbial diversity of Great Salt Lake
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Halophiles and other extremophiles and their relevance to Martian and extraterrestrial environments
Joseph Seckbach

Halophiles: a terrestrial analog for life in brines on Mars – Halophiles on Mars
Rocco L. Mancinelli

Biodata of **Bonnie K. Baxter,** author of **"*Microbial Diversity of Great Salt Lake*"**

Bonnie K. Baxter is an Associate Professor of Biology at Westminster College in Salt Lake City, Utah where she studies DNA repair and photoprotection in halophiles with her undergraduate students. She has been drawn by Great Salt Lake since her arrival in the valley and is excited about the collaborative biodiversity studies reported here. Professor Baxter also does research in science teaching and learning and is an elected member of the Project Kaleidoscope Faculty for the 21st Century network, dedicated to reform in undergraduate science education. Professor Baxter obtained her Ph.D. in Genetics at the University of North Carolina, Chapel Hill, and did her post-doctoral research in the Department of Biochemistry and Biophysics at Washington State University.

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MICROBIAL DIVERSITY OF GREAT SALT LAKE

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Lake of paradoxes, in a country where water is life itself and land has little value without it, Great Salt Lake is an ironical joke of nature-water that itself is more desert than a desert.
Dale Morgan in "The Great Salt Lake"

1. The Dynamic and Dichotomous Great Salt Lake

Great Salt Lake is a salty oasis in the elevated desert of Utah, USA. It is a remnant of the immense prehistoric Lake Bonneville, which existed from about 32 to 14 thousand years ago (Sack, 1989). A significant body of water, Lake Bonneville covered about 20,000 square miles (52,000 square kilometers) of western Utah and smaller portions of eastern Nevada and southern Idaho. This ancient freshwater sea was formed by direct precipitation, rivers, streams, and melting glaciers flowing into the Great Basin, an enormous region of internal drainage in the western United States. Over time, flooding eroded a natural dam in Idaho and led to a lowered lake elevation. This, coupled with a warmer, drier climate, began the evaporation process that formed Great Salt Lake, whose current margins were set about 12,000 years ago.

Great Salt Lake is a terminal lake that is still subject to change. It is approximately 122 km long and 50 km wide with an average depth of 4.3 m, though its precise margins vary with seasonal precipitation and drought cycles. It is the second saltiest lake on Earth (Hassibe and Keck, 1993), and the fourth largest terminal lake (Stephens, 1997; Aldrich and Paul, 2002). The lake is surrounded by a desiccated salt playa, and this high elevation desert biome contributes to intense daytime solar radiation and significant seasonal temperature variation (–5 to 35°C).

Human intervention resulted in the split of Great Salt Lake into two isolated ecosystems. A railroad causeway built between 1955 and 1959 (Cannon and Cannon, 2002) effectively divided the lake into a North and South Arm with differing salinities (Fig. 1). Freshwater from snow melt in the Wasatch Mountain Range flows into the South Arm by way of three rivers, but the North Arm has

input from only one river, and only when the lake is at a high elevation. Currently, with the low elevation of Great Salt Lake due to drought, there is little input of fresh water into the North Arm, and it has grown twice as saline as the South Arm. In fact, the North Arm is fed by South Arm water, which flows northward from the breach in the causeway. When the lake elevation is low, the salinity of both arms is increased (Fig. 2). However, the geochemical composition remains constant in terms of the relative ratios of specific ions (Utah Geological Survey, unpublished data). For July, 2004, the South Arm ion concentrations were: Cl^- 95 g l^{-1}, Na^+ 59 g l^{-1}, SO_4^{2-} 13 g l^{-1}, Mg^{2+} 4.6 g l^{-1}, K^+ 3.4 g l^{-1}, and Ca^{2+} 0.26 g l^{-1}. Average North Arm ion concentrations were: Cl^- 175 g l^{-1}, Na^+ 101 g l^{-1}, SO_4^{2-} 22 g l^{-1}, Mg^{2+} 8.5 g l^{-1}, K^+ 6.9 g l^{-1}, and Ca^{2+} 0.28 g l^{-1}. Great Salt Lake is, therefore, considered to be a sodium chloride lake with an exceptionally high sulfate concentration, in contrast to the divalent-cation-rich Dead Sea (Post, 1977).

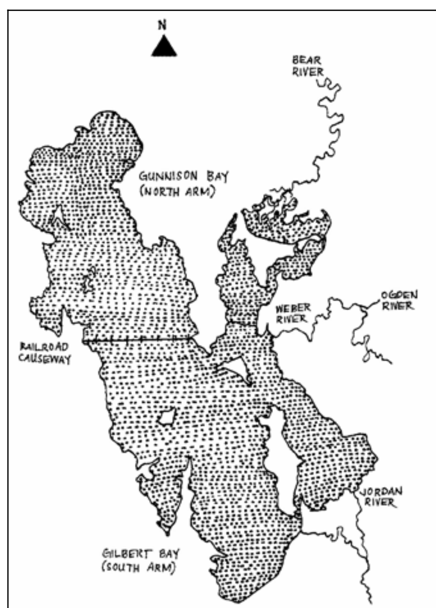

Figure 1. Map of Great Salt Lake indicating the position of the North and South Arms, the railroad causeway, and the inflowing rivers.

Evident in both the North and South Arms of Great Salt Lake, the shore margins swell and shrink in response to environmental conditions (Fig. 2). Since Great Salt Lake is a shallow lake (average depth 4.3 m), even modest changes in its elevation may have an enormous impact on the lake's area and volume. Since history has been recorded in the Salt Lake valley, fluctuations of the lake level

have varied over a range of 20 feet (6 m), from a low of 4,191.35 feet (1278 meters) above sea level in 1963, to a high of 4,211.85 feet (1284 m) in 1987 following significant precipitation between 1982 and 1987. At present, six years of drought have had the inverse effect as the lake is near the 1963 historic low. Thus, lake level is in constant flux, subjecting the flora and fauna to major changes in the salinity of their environment.

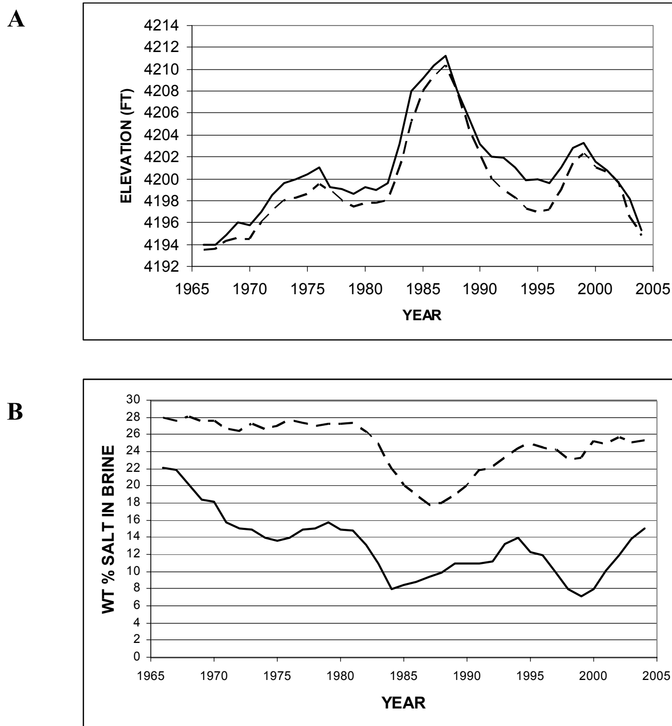

Figure 2. Historic record of Great Salt Lake elevation and salinity following construction of the causeway. Data (courtesy of W. Gwynn, Utah Geological Survey) were collected from the North (broken lines) and the South Arms (solid lines). Panel A shows the fluctuation of lake elevation while Panel B shows the corresponding changes in salinity.

Despite the extreme and dynamic conditions, Great Salt Lake organisms flourish in this ecosystem. Great Salt Lake is a stop-over for five million birds including 250 different bird species (Aldrich and Paul, 2002). They feed mostly on large populations of the main animal browsers of the lake: the brine shrimp, *Artemia franciscana*, and the brine flies, *Ephydra* spp., which are found in both arms of the lake. Algae discovered in

Great Salt Lake include at least seven species of green algae, one dinoflagellate and 17 diatom species. All of these species are restricted to the South Arm with the exception of *Dunaliella* spp. (Felix and Rushford, 1979). In addition to algae, protozoa as well as yeasts have also been reported from Great Salt Lake. *Porodon utahensis,* a single protozoan, and a saprophytic hyphomycete, *Cladosporium glycolicum*, have been reported in the South Arm (Post et al., 1983). Few reports are available on microbial mats from Great Salt Lake though they have been observed, and at least four species of cyanobacteria have been noted (Felix and Rushford, 1979).

This simple but extensive community adds to the complexity of the carbon and nitrogen pools in the lake, including the major glycerol contributions of the predominant alga *Dunaliella* spp. (Oren, 1993). Even the brine flies discard their larval cases in the lake waters, resulting in a high level of chitin input. Primary productivity studies have been reported to average 145 g C m^{-2} per year in the South Arm (Stephens and Gillespie, 1976). This production is limited by the grazing on the algae of *Artemia* and brine fly larvae (Hammer, 1981). The C:N ratio for the North Arm has been reported to be 5.5:1 (Post, 1981). As a result of the high productivity levels, Great Salt Lake should support a complex microbial community within its waters and sediments.

A major volume on Great Salt Lake appeared in 2002 (Gwynn, 2002), covering the geology, chemistry, industries, recreation, and even art. However, only five chapters of this 50-chapter monograph dealt with the biology of the lake with a single chapter on microbiology, focusing on sulfur cycling by anaerobic bacteria (Ingvorsen and Brandt, 2002). This reflects the state of microbiological studies on Great Salt Lake as the last published major investigation of the microbial diversity of the lake was in 1977 (Post, 1977). In 1981, Post published an extensive review of the work on the prokaryotes of the lake. At that time, the only members of the Halobacteriales identified were species of the genera *Halobacterium* and *Halococcus* (Post, 1981). Since then, the recognized diversity of prokaryotes generally and halophilic microbes specifically has increased dramatically (DasSarma and Arora, 2002; Oren, 2001, 2002). A number of publications have characterized single isolates from the lake since Post's study (Table 1). However, no comprehensive studies have been conducted examining the halophilic microbial community, its metabolic potential, and interactions between populations over both a temporal and spatial period.

2. Diversity of Halophilic Microorganisms in Great Salt Lake

Extreme halophiles flourish in the Great Salt Lake North Arm (which has ranged in salinity from 20-30% in the last five years, currently at ~30%), while moderate halophiles grow optimally in the South Arm (which has ranged from 9-15%, currently at ~15%). Therefore, Great Salt Lake presents a unique research opportunity, as there are two distinct microbial ecosystems that have evolved at one location. One would expect to find consortia specific to each arm of the lake. Most likely there are communities specific to sub-ecosystems within each arm as well, due to variables in the lake including thermal benthic activity and deep saturated brine pockets in the less saline South Arm.

TABLE 1. Available Great Salt Lake strains

Type Strain	Name	Location	Reference
Aerobic Compartment			
ATCC 700849 DSM 11805 JCM 10718	*Gracilibacillus halotolerans*	South Arm	Wainø et al., 1999
ATCC 700076 DSM 10405	*Halobacillus litoralis*	South Arm	Spring et al., 1996
ATCC 700077 DSM 10404	*Halobacillus trueperi*	South Arm	Spring et al., 1996
ATCC 49240 DSM 3051	*Halomonas variabilis*	North Arm	Fendrich, 1988 Franzmann and Tindall, 1990
DSM 12940 JCM 11049	*Halorhabdus utahensis*	South Arm	Wainø et al., 2000
	Haloterrigena turkmenica	North Arm	Ventosa et al., 1999
ATCC 49241 DSM 3050	*Pseudomonas halophila*	North Arm	Fendrich, 1988
Anaerobic Compartment			
ATCC 700426 DSM 11763	*Desulfocella halophila*	South Arm	Brandt et al., 1999
DSM 11383	*Desulfobacter halotolerans*	South Arm	Brandt and Ingvorsen, 1997
ATCC 33744 DSM 2228	*Halanaerobium praevalens*	South Arm	Zeikus et al., 1983
DSM 8275	*Halanaerobium alcaliphilum*	South Arm	Tsai et al., 1995
ATCC 35705	*Methanohalophilus mahii*	South Arm	Ingvorsen and Brandt, 2002

Post (1977) reported bacterial counts in the range of 10^7-10^8 per milliliter in the North Arm and less than 10^6 per milliliter in the South Arm, determined by direct microscopy. Comparison of these numbers to colonies growing on agar plates indicates that less than 10% of microbial cells visible by light microscopy in the North Arm of Great Salt Lake were culturable (Fendrich, 1988; Post, 1975, 1977; Baxter, unpublished). Since most of the work done on Great Salt Lake has involved traditional isolation, a large fraction of the indigenous microbial population has remained completely unstudied and its analysis will require non-cultivation approaches and/or modern cultivation techniques.

2.1. THE AEROBIC COMPARTMENT OF GREAT SALT LAKE

Unfortunately, the early cultivation of Great Salt Lake aerobes did not result in deposited strains, and only one original strain remains: Post's GSL-11 (now *Haloterrigena turkmenica*, Ventosa et al., 1999). In fact, the other strains reported in the 1970s have been lost (Post, personal communication). Table 1 displays the

available microorganisms that have been isolated from the water column and the sediments of Great Salt Lake. The location of the sampling site and references for each strain is shown.

There have been two major studies on the aerobic heterotrophic activities in Great Salt Lake. One study examined nitrogen cycling in microcosms (Post and Stube, 1988). Of the four compounds tested, only glutamate was found to stimulate bacterial production directly, increasing the numbers to > $5x10^8$ ml^{-1}. The other three nitrogen sources tested (ammonia, nitrate, and urea) were indirect stimulants, increasing algal productivity and subsequently releasing uncharacterized organic matter. Methane, ethane, ethene, and propane were increased in microcosms exposed to light but CO_2 levels were highest in the microcosms kept in the dark, presumably due to bacterial respiration.

Previously, an examination of hydrocarbon and glutamate degradation in the evaporation ponds of a saltern in Great Salt Lake (Ward and Brock, 1978) noted a decrease in hydrocarbon turnover with increasing salinity. Glutamate could still be degraded but more slowly at higher salinities. The investigators failed to cultivate bacteria from the natural oil seeps at Rozel Point (North Arm), although they noted that the phytanes were removed in enrichment cultures. Degradation of other organics in both the North and South Arms of Great Salt Lake was measured (Fendrich and Schink, 1988). Conversion of glucose, glycerol, and acetate to CO_2 was much slower in the North Arm than in the South Arm.

2.2. THE ANAEROBIC COMPARTMENT OF GREAT SALT LAKE

A large variety of facultative and strictly anaerobic Bacteria and Archaea inhabit the bottom layers of microbial mat communities and sediment in hypersaline lakes, and Great Salt Lake is no different. These include fermentative bacteria, homoacetogenic bacteria, sulfate-reducing bacteria, and methanogenic Archaea. Only a few anaerobic organisms are available from culture collections, and all were isolated from the more accessible but less saline South Arm (Table 1). *Halanaerobium praevalens,* which grows in 30% NaCl fermenting a range of carbohydrates, amino acids and glycerol, is among the fermentative species isolated from Great Salt Lake. Among the terminal members of anaerobic consortia that utilize fermentation products, several sulfate-reducers have been identified from the lake including *Desulfobacter halotolerans* (Brandt and Ingvorsen, 1997) and *Desulfocella halophila* (Brandt et al., 1999). Biogenic methanogenesis from Great Salt Lake is reported to result from the methylotrophic substrates methylmercaptan, methanol or methionine rather than carbon dioxide and hydrogen, or acetate. Several moderately halophilic methylotrophic methanogens have been described including *Methanohalophilus mahii* isolated from Great Salt Lake (Ingvorsen and Brandt, 2002). Although bacterial sulfate reduction is the predominant terminal degradative process in sulfate-rich Great Salt Lake, methanogenesis of fermentative products such as hydrogen and acetate has been detected, albeit at significantly lower rates (Lupton et al., 1984; Phelps and Zeikus, 1980; Zeikus et al. 1983). However, the microorganisms that catalyze this process have not yet been described. It is possible that as in marine environments, methanogenesis may have a more predominant role in niches where sulfate has

been depleted such as lower sediment depths and in elevated portions of near-shore sediments.

Anaerobic metabolism in the sediments has received only slightly more attention than that of aerobes. No utilization of acetate or butyrate by sulfate reducers has been reported at salinities greater than 13% leading to incomplete oxidation of fermentation intermediates such as acetate and their accumulation in the sediments. Furthermore, the methanogens that have been studied thus far use methylated compounds only, which suggests an incomplete sulfate reduction resulting in the accumulation of acetate in the sediments, and those methanogens inhabiting hypersaline environments such as the North Arm of Great Salt Lake are strict methylotrophs (Ollivier et al., 1994, 2000).

Additional work on the sulfate reducers found significantly reduced rates of sulfate reduction in the North Arm versus the South Arm of Great Salt Lake (Brandt et al., 2001). The rates for slurries from both the North and South Arm sediments were increased when the salinity was artificially reduced from the *in situ* salt concentration with optima at 12% and 5%, respectively. The numbers of cells determined by the tracer-most probable number technique was also reduced by approximately an order of magnitude in the North Arm compared to the South Arm.

Although there have been reports on organic carbon input into Great Salt Lake from sources such as phototrophic bacteria and algae (Javor, 1989), there is little information on the fate of this carbon. As Great Salt Lake is highly productive, and hypersaline waters have a reduced solubility for oxygen, the complementary activities of both aerobic and anaerobic microorganisms would be required for complete mineralization of organic materials.

3. The Great Salt Lake Consortium: Preliminary Study

Clearly, the viability of the Great Salt Lake ecosystem is dependent upon metabolic activities of a diverse community of halophilic microorganisms. With the advent of molecular tools for studying microbial populations *in situ,* Great Salt Lake is currently well-poised for a renewed comprehensive study on the microbial communities that drive this unique ecosystem. We have begun such a study, an enormous undertaking for a vast lake with a great number of sub-ecosytems. The “Great Salt Lake Consortium,” a team of scientists (including the authors of this manuscript), working with partners in the Utah State government and local industry, are pursuing an exploration of the lake with modern tools and novel approaches. Our aim is not only to survey the members of the aerobic and anaerobic consortia, but to build an understanding of the diverse and dynamic microbial communities of the lake. To this end we have committed to a comprehensive long-term study. Our preliminary work will be discussed below as well as our educational efforts.

We recently sampled brine and sediment at a site in both the North and South Arms of Great Salt Lake and conducted preliminary microscopic, molecular, and metagenomic analysis. Sampling at the North Arm, we recorded salinities between 30% and 32% while the South Arm gave readings between 15% and 16%. For

preliminary work, our efforts have been mainly concentrated at a single hypersaline North Arm site, at Rozel Point, near the unique eco-sculpture, “Spiral Jetty” (Fig. 3).

Figure 3. Sampling site by the art work “Spiral Jetty” off the North Shore of Great Salt Lake

3.1. MICROSCOPIC ANALYSIS OF GREAT SALT LAKE BRINE AND CULTIVATION

Previous microscopic examination of the microbial community in the North Arm indicated that more than 90% of the cells that are visible cannot be cultured, and the South Arm, with lower salinity, is likely to harbor even greater complexity (Fendrich, 1988; Post, 1975, 1977). This was confirmed with recent studies on the lake at its current elevation (Baxter, unpublished). Bright field, dark field and electron microscopy of North Arm brine indicated a potential for enormous diversity of the microbial communities of Great Salt Lake (EM shown in Fig. 4). Various morphologies of prokaryotic cells are present as well as several types of halophages. The cell morphotype distribution (June, 2004) is dominated by spheres (45%) with others represented at lower numbers in the population; squares (23%), rods (15%), triangles/pyramids (10%), and curved rods (7%) (Fig. 4). These findings in this natural lake are in contrast to reports in the literature on crystallizer basins that the enigmatic square archaeon is the predominant member of some of these hypersaline communities (Antón et al., 1999; Casamayor et al., 2002; Oren, 1999). Immediate culturing of aerobes from North Arm lake samples has resulted in dozens of isolates from the water column (Litchfield and Baxter, unpublished). Genomic fingerprinting by ATCC using the technology of the Diversilab system (Bacterial Barcodes), of several of these isolates indicates that these are novel, undeposited species. For example, one (rod-shaped) isolate is

distantly related to the *Halorubrum* genus, while the second (spherical) isolate is 88% similar to *Halococcus morrhuae*. This taxonomic work is ongoing.

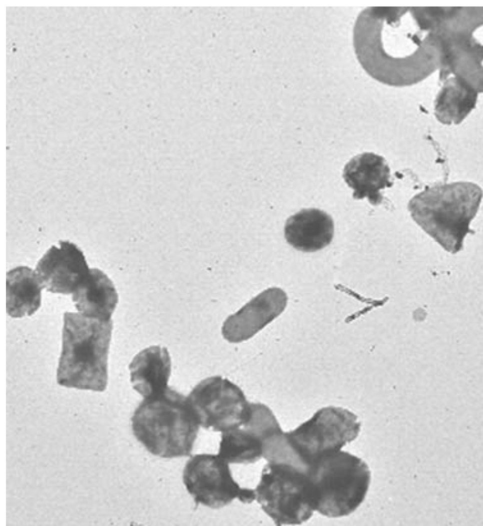

Figure 4. Electron micrograph of North Arm Great Salt Lake microbes. Great Salt Lake brine was centrifuged, and the cells were concentrated. The cell suspension was applied to a thin carbon foil for 5 minutes and then stained with 20 succesive drops of 2% uranyl acetate in water with a final wash with one drop of water. The samples were imaged in a Philips/FEI Tecnai 12 TEM at 60 KV and the images recorded on a 4kx4k GATAN CCD camera.

3.2. EVIDENCE OF METHANOGENESIS IN NORTH ARM SEDIMENT

Anaerobic enrichments from Great Salt Lake North Arm brine exhibited methanogenic activity from starch (Fig. 5). In addition, sub-enrichments with the methanogenic substrates hydrogen, acetate or trimethylamine yielded modest levels of methane from all three substrates (not shown). Although the methylotrophic methanogen *Methanohalophilus mahii* was previously isolated from Great Salt Lake, this is the first evidence for a complete methanogenic consortium consisting of polymer fermenting bacteria and hydrogen and acetate-utilizing methanogenic Archaea from this hypersaline environment. The detection of sulfide in these enrichments suggests that polymer degrading sulfidogenic consortia are also present. Microbial isolation and amplified rDNA restriction analysis (ARDRA) analyses of these cultures are in progress to determine the diversity of anaerobic consortia in Great Salt Lake sediments.

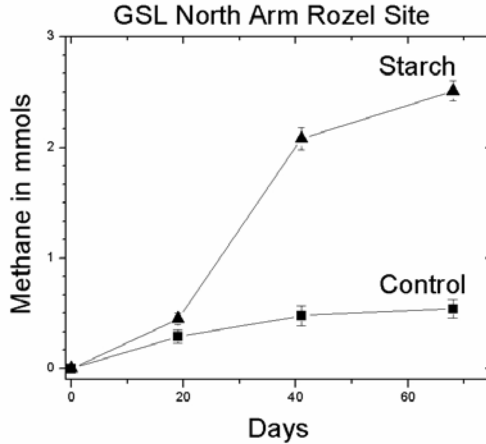

Figure 5. Methanogenesis in North Arm Great Salt Lake sediment. Anaerobic consortia of bacteria were enriched by inoculating sediment obtained from selected sites in the North Arm of Great Salt Lake into medium prepared anaerobically in 25 ml Balch-style anaerobe tubes inside of a COY anaerobic glove box. The medium consisted of basal nutrient salts and saturated sea salts to which 1% w/v soluble starch was added. The vials were sealed with N_2-CO_2 (4:1) in the headspace using butyl stoppers secured by aluminum crimp seals. Microcosms were incubated at ambient temperatures in the dark and periodically assayed for methane with a gas chromatograph equipped with a flame ionization detector as previously described (Sowers, 1995).

3.3. FINGERPRINTING OF NORTH ARM MICROBIAL POPULATIONS

For fingerprinting analysis, DNA was extracted from microbial cell pellets from seasonally sampled North Arm brine after filtration for removal of brine shrimp and their cysts. After PCR amplification of 16S rDNA, fingerprinting was conducted by the amplicon length heterogeneity (LH) method (Suzuki et al., 1998). This procedure has previously been found to be effective for studies of hypersaline environments (Litchfield and Gillevet, 2002). Primers specific to the haloarchaea and universal bacterial primers for 16S were tested in the aqueous extracts, and the amplified fragments of various lengths were quantified. The results show, based on the heterogeneity of amplicons, that there was an apparent five-fold greater diversity of Archaea compared to Bacteria in the North Arm (Table 2). Modest changes in the percentages of the various amplicons were observed in a seasonal comparison. However, it should be noted that multiple contigs can exist within each of the amplicons, and also each amplicon can represent numerous genera. These results suggest that a high diversity of halophiles exists in the North Arm of Great Salt Lake and seasonal cycling of the halophilic community may occur in the water at Rozel Point. We are currently in the process of cloning and sequencing these amplicons. Preliminary work in the South Arm indicates that a greater diversity of Bacteria is present in the North Arm compared to Archaea, six Bacteria

amplicons versa one in the North Arm (not shown). Future work will apply the same approach to other sampled sites and the anaerobic compartment.

TABLE 2. 16S rDNA length heterogeneity analysis for Great Salt Lake North Arm aqueous extracts.

	Archaeal 16S domain					Bacterial 16S domain
Length of amplicon (bp)	510.8	512.91	513.76	514.87	515.96	513.76
	Percentage of amplicons for each length domain by sampling date					
June 2003	53.5	8.75	8	7.6	23.2	100
October 2003	58.7	8.00	7.3	7.3	19	100
June 2004	48	7	8	10	27	NR

*NR = not reported

3.4. THE GREAT SALT LAKE ENVIRONMENTAL GENOME PROJECT

From North Arm brine collected in May of 2003, DNA was extracted using established methods for brine and sediment samples (DasSarma et al., 1995; Purdy et al., 1996). A large BAC/fosmid library with 2,000 Mbp of cloned DNA was constructed from the extracted DNA. This represents 500 complete genomes of 4 Mbp size, and indicates a detection limit of only 0.2% of species present. Preliminary end-sequencing of a representative number of clones and a BLAST comparison indicated that 20% of the clones show homology to haloarchaea, 25% to Bacteria, and 55% are novel sequences without hits to any sequences in the NCBI database (DasSarma et al., unpublished). A large-scale sequence analysis of the clones is currently underway. These preliminary results indicate that many novel species of Bacteria and Archaea are present in Great Salt Lake and are accessible using modern molecular and genomic approaches.

4. Great Salt Lake Halophiles in Education

Great Salt Lake is unique and compelling. At Westminster College in Salt Lake City, the lake has provided a backdrop for a number of educational programs. We have employed the Great Salt Lake ecosystem as a scientific model in training undergraduate students. For those students who are science majors who are planning to apply for graduate or professional school, undergraduate research opportunities are offered. Our curriculum in the sciences is by design interdisciplinary. In fact, multiple faculty are researching various aspects of Great Salt Lake. As a result, a Chemistry student doing analytical chemistry on selenium contamination in the lake may team up with Biology students working on halophile populations. This interdisciplinary approach within the single theme of Great Salt Lake brings these future scientists and physicians to science as it is practiced, where disciplinary boundaries are crossed. The backbone of this program can

be seen at the following website, which won two national awards in 2004: http://www.westminstercollege.edu/gsl/.

Our responsibility to teaching the skills of doing science may be even greater with undergraduates who are studying to be schoolteachers. The involvement of future teachers in research means that they incorporate process skills and the joy of discovery in their pedagogical methods. The *Great Salt Lake Project* was funded by the National Science Foundation to assist Westminster College in training future teachers in inquiry-based methodologies (Jenkins et al., 2001). Using the unique hypersaline ecosystem as a playground for research, students in the course designed experiments, carried them out, and interpreted data (Fig. 6). The instructors, Baxter and a Professor in the School of Education, facilitated the process and supplied resources. Following the Great Salt Lake research project, the students designed curricula, inspired by their project, for use by teachers in the field. These teaching lessons and a description of the Great Salt Lake program can be found at the website: http://www.westminstercollege.edu/education_gslp/.

Figure 6. Westminster College science-education students collect samples for experiments on South Arm halophile populations. These students developed their research project into lessons for school age children.

The convergence of scientific interest, modern technology, and educational opportunities has brought Great Salt Lake out of obscurity and into the limelight. The studies of the Great Salt Lake Consortium will change the current perception of Great Salt Lake as a simple ecosystem by establishing the complexity at the prokaryotic level. This research and outreach will build a deeper understanding of the ecology of Great Salt Lake, a unique and remarkable terminal lake.

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MICROBIAL COMMUNITIES IN THE DEAD SEA – PAST, PRESENT AND FUTURE

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1. Introduction

During the World Summit on Sustainable Development held in Johannesburg in September 2002, the State of Israel and the Hashemite Kingdom of Jordan signed an agreement in which the two countries announced their mutual commitment to construct a canal or a pipeline between the Gulf of Aqaba (Red Sea) and the Dead Sea. This agreement extended earlier proposals for the construction of a water carrier that had been discussed since the peace treaty between Israel and Jordan was signed in 1994.

The joint interest in the construction of the 'Peace Conduit' is based on a number of considerations (Gavrieli and Oren, 2004; Gavrieli et al., 2005):

1. As a result of the diversion of fresh water from the watershed area of the Dead Sea, the water balance of the lake is strongly negative. This has caused a rapid decrease in the water level and a deterioration of the infrastructure in the area. Introduction of massive amounts of seawater from the Gulf of Aqaba through the Aravah Valley may counteract these negative effects, and should enable the maintenance of the unique scenic beauty of the area. Stabilization of the now rapidly receding shoreline will allow development of tourist facilities and other projects.
2. Part of the seawater to be brought to the Dead Sea can be desalinated, and thus the major water shortage crisis in the area may be relieved. The more than 400 m difference in elevation between the Red Sea and the Dead Sea can provide energy for seawater desalination by reverse osmosis. Around 800 million cubic meters of fresh water could thus be generated annually (Harza JRV Group, 1996). Earlier schemes to connect the Dead Sea with the Mediterranean Sea proposed to exploit the difference in elevation for the generation of hydroelectric energy were examined but not carried out (Ne'eman and Shul, 1983).

The implementation of the 'Peace Conduit' will involve two stages. The first is a filling stage in which the inflow of seawater and/or concentrated brine formed in the course of the reverse osmosis desalination process will exceed evaporation. After the

lake's shoreline will have been restored to a desired level, the inflow of water should be regulated to balance evaporation, thus stabilizing the lake.

To the microbiologist the proposed 'Peace Conduit' presents an experiment in halophile microbial ecology on the grandest scale. It implies the manipulation of the physical and chemical conditions in a large lake that contains 130 cubic kilometers of brine. The Dead Sea has unique biological properties, and it is therefore essential that the possible implications of the implementation of the 'Peace Conduit' on the biology of the lake will be clear before the construction of the pipeline will be initiated.

The information that has accumulated on the biology of the Dead Sea provides us with many clues of what kinds of biological effects may be expected following dilution of its waters and other changes that will occur as a result of massive inflow of seawater. Simulation experiments are being performed, both on a laboratory scale and under field conditions, to improve our ability to predict the possible effects of the water carrier on the Dead Sea environment. This chapter provides an overview of our present understanding of the microbiology of the lake, and reviews some recent simulation experiments in an attempt to predict the future biological properties of the lake.

2. Chemical and Physical Properties of the Dead Sea

The Dead Sea is a terminal desert lake. Its water level is the lowest exposed surface on Earth and is currently 417 m below sea level. At its deepest place, the lake is over 300 meters deep. Its main source of water input was the river Jordan. With a total dissolved salt content of about 342 g l^{-1}, the Dead Sea is one of the most hypersaline environments on Earth. The unique ionic composition of its waters is dominated by divalent cations. Typical analyses performed in 2002 yielded 1.96 M Mg^{2+}, 1.49 M Na^{+}, 0.46 M Ca^{2+}, and 0.21 M K^{+}. Cl^{-} is the main counterion (99% of the anion sum), the remainder being mostly Br^{-}. Concentrations of sulfate and bicarbonate are low. The density of the brine is about 1.237 g cm^{-3}, and its pH is about 5.9.

The ionic composition has been subject to dramatic changes in recent years. As a result of the decrease of the water level and the concomitant increase in salinity of the upper water layers, the brines became supersaturated with respect to NaCl (halite). Massive amounts of NaCl have precipitated to the bottom as halite crystals (Gavrieli, 1997), leading to a relative increase in the abundance of divalent cations (Fig. 1E). The industrial activities of the Dead Sea Works Ltd. (Israel) and the Arab Potash Co. (Jordan) have also contributed to this effect. Dead Sea water is evaporated in shallow ponds near the southern region of the lake for the production of potash (KCl), bromine, metallic magnesium, and other minerals. Large amounts of sodium chloride precipitate in these evaporation ponds. The Mg and Ca-rich end brines are returned to the Dead Sea.

Biologically available nitrogen is abundantly found in Dead Sea water. Nitrate concentrations are low, but ammonium ion concentrations as high as 5.9 mg l^{-1} (1960) to 8.9 mg l^{-1} (1991) were reported. This ammonium is probably derived from the Jordan River, from springs and floods along the lake shore, and from diffusion from the bottom sediments. Phosphorus, however, is in short supply. Levels of dissolved phosphorus of about 35 µg PO_4^{3-}-P l^{-1} were measured, with particulate phosphorus being found at 30-50 µg l^{-1}. The sediments were suggested to contribute about half of the phosphate input to

the Dead Sea water column, the remainder being derived from the Jordan River and flood waters (Nissenbaum et al., 1990; Stiller and Nissenbaum, 1999).

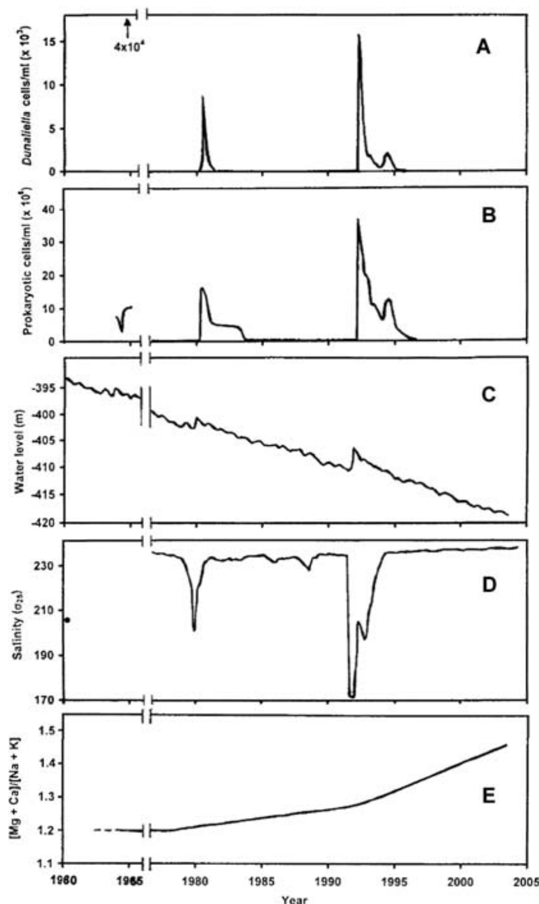

Figure 1. Population density of the unicellular green alga *Dunaliella* (A) and the community density of prokaryotes (B) in the upper meters of the Dead Sea water column, 1963-2004, the water level of the Dead Sea (C), the salinity of the upper water layer (expressed as σ_{25} - the density excess in kg m^{-3} to the standard reference density of 1000 kg m^{-3}) (D), and the molar ratio of divalent and monovalent cations ($[Mg^{2+} + Ca^{2+}]/[Na^{+} + K^{+}]$) of the Dead Sea water (E). Data were derived from Beyth, 1980; Kaplan and Friedmann, 1970; Oren, 2000, and from additional sources.

When the first in-depth limnological study of the Dead Sea was conducted in 1959-1960, the lake was stratified (meromictic), with a 40 m deep upper water layer with around 300 g l^{-1} salt floating on top of a denser lower water mass (332 g l^{-1} salt from a

depth of about 80 m down to the bottom, density 1.23 g cm^{-3}). The lower water mass was anoxic and contained sulfide (Neev and Emery, 1967). As a result of massive hydrological changes this picture has changd dramatically. The water balance of the Dead Sea has been negative since the middle of the 20th century, and between 1960 and present the water level has decreased by more than 20 meters (Fig. 1C). This decrease was caused mainly by human activities with only relatively minor effect of climatic changes, if at all. Fresh water from the drainage basin is increasingly being used for agricultural and domestic use, and water flow though the Jordan River has decreased from an estimated average of $1.1\text{-}1.3\times10^9$ m^3 per year in the beginning of the 20th century to less than 0.25×10^9 m^3 per year in recent years (Al-Weshah, 2000; Gavrieli et al., 1999; Gertman and Hecht, 2002; Oren and Gavrieli, 2002; Salameh and El-Naser, 1999, 2000).

The salinity of the upper water layers increased concomitanty, causing a weakining of the pycnocline that had existed during meromixis. This resulted in an overturn of the water column in February 1979 (Beyth, 1980; Steinhorn et al., 1979). Since that time, periods of holomictic and meromictic regimes have alternated. In the holomictic state (1982-1991 and 1995-present), spring warming leads to the development of an upper water layer, but as a result of summer evaporation (with temperatures of the upper water layers reaching 35-36°C), followed by cooling in winter and weakening of the summer thermocline, the density of the upper layer finally equals that of the colder (22-23°C) lower layer, and stratification is destroyed around November-December (Anati, 1997; Anati and Stiller, 1991; Anati et al., 1987; Gertman and Hecht, 2002). This holomictic regime has been interrupted by two meromictic episodes: from 1979-1982 and from 1992-1995. Unusually rainy winters added amounts of fresh water so large that evaporation during the following summer did not abolish the resulting strong pycnocline (Anati and Stiller, 1991). The winter of 1979-1980 saw a rise in surface level of 1.48 m, and an even more dramatic rise of 1.83 m occurred in the winter of 1991-1992, when approximately 1,500 million m^3 of fresh water entered the lake. The upper 5 m of the water column became diluted to about 70% of their previous salinity (density as low as 1.164 kg l^{-1}) (Beyth et al., 1993; see also Fig. 1C,D).

The drop in Dead Sea water level will undoubtedly continue in the coming years, but the lake is not expected to dry up completely. Modelling attempts have shown that under the current conditions a stable water level will probably be reached in about 400 years at a surface level of about -500 m. No further drop will occur as the hygroscopic properties of the brine (~380 g salt l^{-1}, density 1.27 g cm^{-3}) will prevent further evaporation (Gavrieli et al., 1999; Krumgalz et al., 2000; Yechieli et al., 1998).

3. The Microbiology of the Dead Sea – Past and Present

Since Elazari-Volcani first showed the presence of an indigenous microbial community in the Dead Sea more than seventy years ago (Elazari-Volcani, 1940; Volcani, 1944; Wilkansky, 1936), a large number of halophilic Archaea, Bacteria and Eukarya have been isolated from the lake. These include autotrophs as well as heterotrophs, and aerobes as well as anaerobes. In-depth information about these isolates can be found in earlier reviews (Nissenbaum, 1975; Oren, 1988, 1997, 1999, 2000).

It is to be regretted that we have so few quantitative data on the biota of the Dead Sea in the past. Systemtatic monitoring of the communities of unicellar algae and prokaryotes in the Dead Sea has started only in 1980, and before that time we only have a few measurements from the period December 1963 - November 1964, when the Dead Sea was still in its meromictic state. Numbers of up to 40,000 *Dunaliella* cells ml^{-1} were reported in surface water in 1964 (sampling date not specified) – the highest numbers recorded anytime in the Dead Sea. Microscopic counts of prokaryotes (probably dominated by red halophilic Archaea) in surface water varied between $2.3x10^6$ and $8.9x10^6$ cells ml^{-1} (Fig. 1B). At 100 m depth, numbers were about an order of magnitude lower (Kaplan and Friedmann, 1970).

Analysis of the wealth of data on algal and prokaryote community densities from 1980 onwards, including vertical profiles of the distribution of the cells, has enabled a good understanding of the community dynamics of the biota in the lake, and the behavior of the communities can now be understood on the basis of the changes in the physical structure of the water column described above. *Dunaliella* is the sole primary producer in the system. We twice witnessed a massive bloom of the alga: in 1980, reaching up to 8,800 cells ml^{-1} (Oren and Shilo, 1982), and once more in 1992, with up to $1.5x10^4$ cells ml^{-1} (Oren, 1993, 1999; Oren et al., 1995b). The timing of these blooms was in both cases correlated with more than usually rainy winters, in which large amounts of floodwater diluted the upper water layers sufficiently to terminate the preceding holomictic regime and establish a few years of meromixis. Simulation experiments in the laboratory have shown that undiluted Dead Sea water (density 1.23 g cm^{-3} or higher) is too saline for *Dunaliella* to develop. Algal growth is possibly only when the water becomes diluted to a density of 1.21 g cm^{-3} or less, i.e., a dilution with 10 percent fresh water at least. The population density reached by the alga then depends on the amount of phosphate available, as inorganic phosphate is the nutrient that limits algal development in the Dead Sea (Oren and Shilo, 1985). During the 1980 and the 1992 blooms the algae were found all over the lake. Analysis of LANDSAT images acquired at the time the 1992 bloom developed suggested that the bloom started in the shallow areas around the lake, to spread afterwards over the entire lake surface. Germination of the cysts in the shallow sediments that had become exposed to the diluted waters above the pycnocline explains the pattern observed (Oren and Ben-Yosef, 1997). Both during the 1980 summer bloom and the 1992 spring bloom the algal population was distributed evenly at all depths of the mixed layer above the pycnocline. However, in the autumn of 1992 cells started to accumulate at the bottom of the mixed zone near the pycnocline at a depth of 8 m and more, where light intensities were very low and salinity supraoptimal (Oren et al., 1995b). No full explanation has yet been obtained for the phenomenon, but it may be related to a severe depletion of essential nutrients (phosphate?) in the upper water layers. With the renewed overturn and the reestablishment of meromictic conditions *Dunaliella* disappeared from the water column, and no algal cells were observed in the Dead Sea water column from 1983-1991 and from 1996 onwards.

Development of *Dunaliella* blooms was followed by dense blooms of halophilic Archaea, coloring the Dead Sea brines red by their C_{50} carotenoid pigments (α-bacterioruberin and derivatives), and possibly by retinal pigments as well (Oren and Shilo, 1981). These Archaea developed at the expense of organic material produced by the algae. It may be expected that glycerol, produced by *Dunaliella* as an osmotic

solute, was one of the main substrates available to the Archaea. Up to 1.9×10^6 prokarotic cells per ml were reported in Dead Sea surface water in 1980 (Oren 1983c), and the 1992 algal bloom enabled an even more extensive development – up to 3.5×10^6 cells ml^{-1} (Oren, 1993; Oren and Gurevich, 1995). Analysis of the polar lipids extracted from the microbial bloom that developed in the lake in 1992 showed presence of the sulfated diglycosyl diether lipid S-DGD-1 as the sole major glycolipid, together with diether derivatives of phosphatidylglycerol and the methyl ester of phosphatidylglycerophosphate. The diether derivative of phosphatidylglycerosulfate was absent (Oren and Gurevich, 1993). Such a composition is characteristic of representatives of the genera *Haloferax* and *Halobaculum*. The blooms of prokaryotes were confined to the upper meters of the water column above the pycnocline, and their vertical distribution proved to be a sensitive tracer of stratification and mixing. During the holomictic periods the numbers of microscopically recognizable prokaryotic cells in the surface waters were below 5×10^5 ml^{-1}.

A survey of the Dead Sea bottom with a small manned submersible in November 1999 (see http://www.biblemysteries.com) showed a number of sites with patches of reddish material that cover the halite crust that had accumulated from the precipitated NaCl. These may be sites with underwater springs or seeps where salinity is locally reduced. Whether the red color of these patches is due to the presence of halophilic Archaea or some other type of pigmented microorganisms can only be ascertained when samples of this reddish material will be recovered in the future and studied.

Although a number of non-pigmented aerobic heterotrophic members of the Bacteria have been isolated from the Dead Sea, there are no indications that these Bacteria make a major contribution to the heterotrophic activity in the lake's water column. The same can be said about filamentous fungi (Eukarya). The first filamentous fungi were isolated from the lake only a few years ago (Buchalo et al., 1998), and many fungal species have since been identified in water samples (Kis-Papo et al., 2001, 2003). To what extent those fungi isolated from the Dead Sea are present as vegetative hyphae and may contribute to the heterotrophic activity in the lake remains to be determined. Obligatory anaerobic members of the Bacteria (order Halanaerobiales) have been cultured from the bottom of the lake (Oren, 1983b; Oren et al., 1984, 1987), and these may play an important role in anaerobic degradation processes in the sediments.

We know little about the factors responsible for the observed declines in the numbers of prokaryotes in the Dead Sea. Ciliate and amoeboid protozoa have been cultivated from Dead Sea water samples in the 1940s (Volcani, 1944), but their presence could not be ascertained in the past decades. It is well possible that bacteriophages may regulate the dynamics of the prokaryote communities in the lake. Electron microscopic examination of water samples collected in 1994-1995, at the time of the decline of the archaeal bloom that had developed in 1992, showed large numbers of virus-like particles to be present in the water. Numbers of virus-like particles were on the average an order of magnitue higher than the numbers of prokaryotic cells. Many of these particles had a spindle-shaped morphology, similar to many known phages that attack Archaea. Aggregates of virus-like particles were also observed, suggesting burst events of a bacterium with release of mature bacteriophages (Oren et al., 1997).

The overall picture that emerges from the more than twenty years of monitoring the microbial communities is that conditions in undiluted Dead Sea water are too harsh for even the best adapted organisms. The water activity of the Dead Sea brines, calculated

at 0.67 for 1979 (Krumgalz and Millero, 1982) and probably even lower today, is close to the lowest ever shown to support life. The high concentrations of magnesium and calcium are inhibitory to life, even when some Dead Sea Archaea such as *Haloferax volcanii* and *Halobaculum gomorrense* are highly magnesium-tolerant (Mullakhanbhai and Larsen, 1975; Oren, 1983a; Oren et al., 1995a), and the relatively low pH of the brine is not favored by most known halophiles.

4. Predicting the Future Microbiology of the Dead Sea

It may be expected that in the future microbial blooms will continue to develop in the Dead Sea whenever abundant rainfall in its drainage basin will lead to the formation of a sufficiently diluted upper water layer.

Implementation of the 'Peace Conduit' plan, which implies the addition of large amounts of seawater or its concentrate formed as the effluent of the planned reverse osmosis water desalination plant, will most probably lead to renewed meromixis - at least during the filling stage until stabilization will be reached. This undoubtedly will have a great impact on the biological properties of the Dead Sea. Future implementation of the plans to construct the 'Peace Conduit' therefore requires carefull planning based on an in-depth understanding of all possible positive and negative effects.

Large-scale outdoor simulation experiments were first performed in the early 1980s at an experimental facility at the northern shore of the Dead Sea (Oren and Shilo, 1985). Since 2002, simulation experiments are carried out in Sedom as part of an effort by the Geological Survey of Israel - the Israel Ministry of National Infrastructures to formulate a dynamic limnological model for the Dead Sea that will attempt to model the mixing of seawater in Dead Sea brine. The Dead Sea Works Ltd. provides the lake-front site, the infrastructure, manpower for on-site maintenance and sampling, and part of the chemical analyses (perfomed at TAMI-IMI, Haifa). The experimental setup consists of 1 m^3 plastic tanks filled with 900 liter of mixtures of Dead Sea and Gulf of Aqaba water, supplemented with different additives: phosphate, inocula of *Dunaliella* and halophilic Archaea, and other treatments as desired. Biological parameters such as numbers of algae and prokaryotes that develop in the tanks, pigment concentrations and others, are regularly monitored over times of months and years. Experiments in these experimental ponds have confirmed the effect of dilution of Dead Sea water and the availability of phosphate on the development of blooms of algae and red Archaea in Dead Sea - Red Sea water mixtures (Oren et al., 2004). Dilution of Dead Sea water with 15-30% Red Sea water gave rise to dense communities of *Dunaliella* and halophilic Archaea, imparting a brown-reddish color to the brine. The algal and archaeal densities reached exceeded any biological blooms that had been witnessed thus far in the lake, with algal counts up to 8×10^4 cells ml^{-1} and prokaryote counts up to 10^8 cells ml^{-1} being achieved under proper conditions (70% Dead Sea water, 10 µM phosphate). Microscopically the microorganisms resembled those that had developed in the Dead Sea during the 1980 and the 1992 blooms (Fig. 2A,B), and the polar lipids of the blooms induced in the ponds were the same types found in the lake in 1992 (Oren et al., 2004). After the initial rise in community density, periods of decline were sometimes observed, but overall we can conclude that blooms may be sustained in the outdoor ponds for periods of two years at least. Prolonged microbial blooming is clearly not a desired outcome of the 'Peace

Conduit' as it implies a major change in the ecology of the Dead Sea, the scenery around the lake and its attractiveness. Moreover, presence of dense microbial communities in the lake may influence the operation of the 'Peace Conduit' itself, e.g. by modifying surface water turbidity, thereby affecting evaporation.

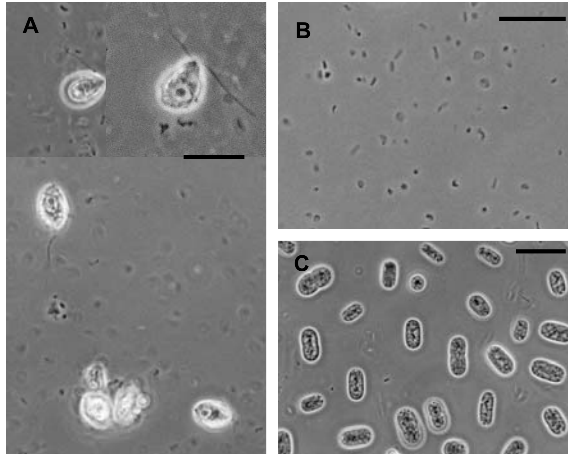

Figure 2. Unicellular green algae (*Dunaliella*) (A) and prokaryotes (B) developing in a simulation pond with 80% Dead Sea water – 20% Red Sea water, and unicellular cyanobacteria (*Aphanothece*/*Cyanothece* type) developing in a pond with 50% Dead Sea water – 50% Red Sea water (C). The bars represent 10 µm.

When experimental ponds were filled with mixtures of 50% Dead Sea water and 50% Red Sea water, the initial bloom of *Dunaliella* was replaced after a number of weeks by an *Aphanothece*/*Cyanothece*-like unicellular cyanobacterium (Fig. 2C). Such halophilic unicellular cyanobacteria were known from saline springs in the Dead Sea area, but have never yet been reported from the lake itself. The organism developed in large masses that floated near the surface of the brine, as large amounts of gas vesicles provided the cells with buoyancy. Laboratory experiments showed that this cyanobacterium developed optimally in mixtures containing 40-50% Dead Sea water, and was unable to grow above 55% Dead Sea water.

Another parameter examined was the effect of antiscalant compounds on microbial development. Antiscalants are routinely added to water subjected to reverse osmosis desalination to protect the expensive membranes and prevent their clogging by insoluble carbonates and sulfates. Many antiscalant compounds in use are based on polyphosphates. When released into the Dead Sea with the desalination reject brines, these compounds may degrade to yield orthophosphate which may trigger mass blooms of *Dunaliella*. Indeed we have shown that certain commercial antiscalant preparations tend to be as active as inorganic phosphate in stimulating algal development. These experiments are still in progress.

Another aspect of the biology of the Dead Sea to be taken into account is the possibility that anaerobic conditions will once more develop in the lower water mass similar to the pre-1979 situation (Neev and Emery, 1967) when a long-term meromictic period will become established, leading to consumption of the dissolved oxygen through the oxidation of the organic matter that will sink from the upper layer. In spite of many efforts in the past we still have no in-depth information on the source of the sulfide that was present at the time and on the dynamics of bacterial sulfate reduction in the Dead Sea sediments, if this process is active there at all. The isotopic signature of the sulfide pointed to its formation from sulfate in a dissimilatory process (Gavrieli et al., 2001; Nissenbaum and Kaplan, 1976). Attempts to assess the sulfate reduction rates in Dead Sea sediments by measuring formation of $H_2{}^{35}S$ from $^{35}SO_4{}^{2-}$ yielded exceedingly low rates, close to the detection limit of the method. We have had some success in our attempts to enrich for halophilic sulfate reducing bacteria using anaerobic Dead Sea sediments as inoculum, and some indications for the presence of endospore-forming *Desulfotomaculum*-like bacteria have been obtained. However, attempts to isolate these bacteria in pure culture have failed thus far. The presence of an anoxic, sulfide-containing lower water mass will not have any direct environmental impact on the surrounding of the Dead Sea. However, it may have a major impact on the potash industries because these would prefer to pump concentrated lower water mass brine rather than the upper water layers diluted with Red Sea water. The use of corrosive and disagreeably smelling sulfide-containing brine may cause both operational and environmental problems (Gavrieli et al., 2005). Laboratory experiments to assess the possibility that anaerobic conditions will be formed and sulfide will accumulate in the lower water mass following implementation of the 'Peace Conduit' are ongoing.

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MICROSCOPIC EXAMINATION OF MICROBIAL COMMUNITIES ALONG A SALINITY GRADIENT IN SALTERN EVAPORATION PONDS: A ‘HALOPHILIC SAFARI’

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1. Introduction

Halophilic microorganisms form a tremendously diverse group. Within the phylogenetic tree of life, halophilic and highly halotolerant microorganisms are found within each of the three domains: Archaea, Bacteria and Eukarya. The halophiles are no less diverse at the level of their physiology, biochemistry, molecular biology, and genetics. An aspect that is little known, even to many scientists who study life at high salt concentrations, is the surprising morphological diversity displayed by these halophiles in their natural environments. Only relatively few have spent time examining water or sediment samples from hypersaline environments in the microscope. This is to be regretted, as high-salt habitats are among the most gratifying objects for microscopic examination (Javor, 1989; Oren, 2002).

Here I show some of the esthetic aspects of halophilic microbial life by presenting pictures taken during a ‘halophilic safari’ along the salt gradient in the evaporation and crystallizer ponds of the solar salterns in Eilat, Israel, with a few contributions from salterns located elsewhere. Salterns are by no means the only habitats in which halophilic microorganisms are found, but for the microscopist they are one of the most rewarding ones, both because of the varied shapes of the organisms inhabiting them and because of the high densities at which many of these organisms are present. In addition, the saltern ecosystem encompasses environments from seawater salinity to halite saturation and beyond, and it thus provides a habitat for microorganisms adapted to different salt concentrations, all within a small and easily accessible area. Such solar salt works have rightfully been considered as natural laboratories for microbiological and geochemical investigations (Schneider and Herrmann, 1979).

2. The Saltern Environment

The first evaporation ponds of multi-pond salterns have salinities close to that of the seawater used as the raw material for the production of salt. These ponds are typically inhabited by macroalgae as well as by a varied community of photosynthetic microorganisms including benthic cyanobacteria and diatoms, as well as by different

kinds of protozoa, types of marine bacteria, as well as some higher organisms: macroalgae, crustaceans, and some other higher animals (Javor, 1989). Ponds in which the salt concentration has increased to twice that of seawater generally contain thick benthic microbial mats dominated by unicellular and filamentous cyanobacteria including *Aphanothece* (*Halothece*), *Oscillatoria*, *Halospirulina*, and others (Clavero et al., 1994; Giani et al., 1989; Javor, 1989; Margheri et al., 1987; Oren, 2000a; Schneider and Herrmann, 1979; Taher et al., 1995; Thomas and Geisler, 1982). Purple sulfur bacteria are found below the cyanobacterial layer. In those ponds in which the salinity has increased to over 3-4 times that of seawater, massive amounts of gypsum ($CaSO_4.2H_2O$) precipitate. The gypsum crusts that accumulate on the bottom of these ponds often show beautifully colored layers of phototrophic microbial communities with different types of cyanobacteria and purple sulfur bacteria (Caumette et al., 1994; Cornée, 1984; Oren, 2000b; Oren et al., 1995; Sørensen et al., 2004) (Fig. 1). This stratification of the communities within this crust is determined by the vertical gradients of light, oxygen, and sulfide.

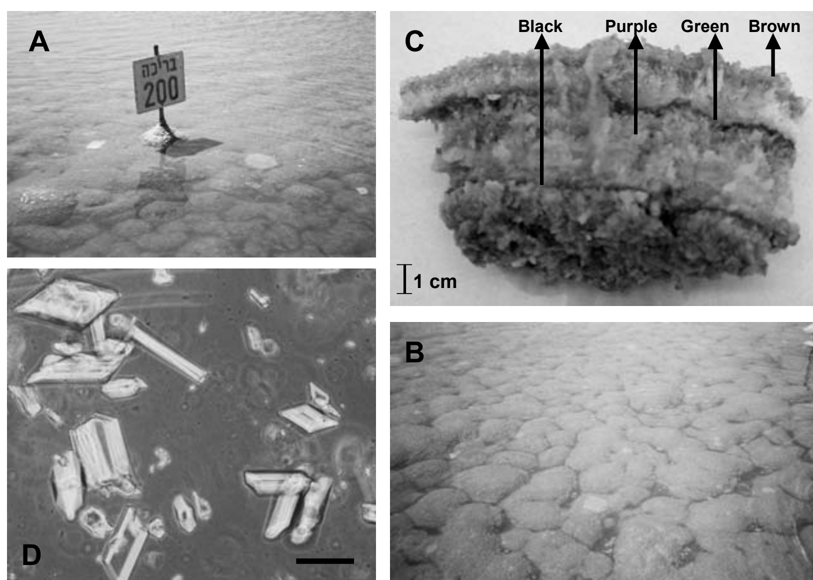

Figure 1. Benthic gypsum deposits in an evaporation pond of the Eilat salterns below a brine of 19.3% salt: photographs of the surface of the crust (A, B) colored brown-orange by dense communities of unicellular *Halothece*-type cyanobacteria (see Fig. 4A-C), a section through the crust (C; photograph courtesy of Tomer Beeri), showing colored bands of unicellular brown-orange (1) and filamentous green cyanobacteria (2), and purple sulfur bacteria (3), and a microscopic view of gypsum crystals from this layer (D; bar = 50 μm).

The crystallizer ponds in which NaCl precipitates in the form of halite crystals represent the last stage in the evaporation process. In these ponds the most conspicuous microbial communities are not found in the form of benthic mats as in the lower salinity ponds, but in the form of dense planktonic populations. The brine of the NaCl production ponds is generally colored red as a result of the presence of dense communities of halophilic Archaea of the family *Halobacteriaceae*, as well as β-carotene-rich unicellular green algae (*Dunaliella salina*) (Javor, 1983; Oren, 2002). Pigmented representatives of the domain Bacteria (the genus *Salinibacter*) may also contribute to the red color of the water (Antón et al., 2002; Oren and Rodríguez-Valera, 2001).

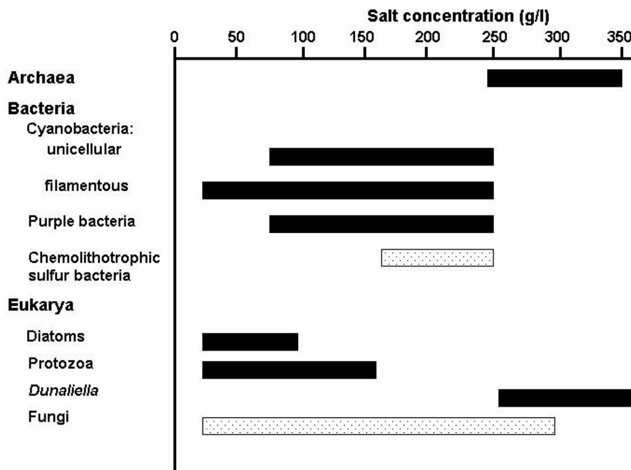

Figure 2. The distribution of the major microscopically distinct types of microorganisms along the salinity gradient in saltern ponds.

The above-described sequence of biological phenomena, as summarized in Fig. 2, is found in solar salt production plants worldwide. Minor variations may occur, caused for example by differences in the nutrient status of the ponds (Javor, 1983) and also by the source of the saline water used to feed the evaporation ponds. Thus, the Eilat saltern that served as the main object of our studies is nowadays mainly fed by reject brine from the reverse osmosis water desalination plant that supplies drinking water to the city of Eilat.

The dense microbial communities that develop in the salterns are not only of scientific interest, as their presence is important for the proper operation of the salt works. The benthic microbial mats in the low- and medium salinity ponds effectively seal the bottom of the ponds, preventing leakage of brine. Both the benthic cyanobacterial mats and the red communities of *Dunaliella*, Archaea and Bacteria in the crystallizer ponds absorb light and thereby raise the water temperature, leading to higher evaporation rates and increased salt production (Davis, 1974; Javor, 1989). In addition,

some of the halophilic microorganisms found in these salterns have considerable potential in biotechnology (Margheri et al., 1987; Oren, 2002).

3. Archaea

Microscopic examination of the red crystallizer brines of the Eilat saltern shows an abundance of flat, square to rectangular Archaea (Fig. 3). Together with the orange *Dunaliella salina* cells (see section 5.2) they are the most conspicuous components of the biota of these ponds. Small crystals of celestite ($SrSO_4.2H_2O$) are seen as well (Fig. 3A, 3B). Celestite precipitates at about the same time as halite.

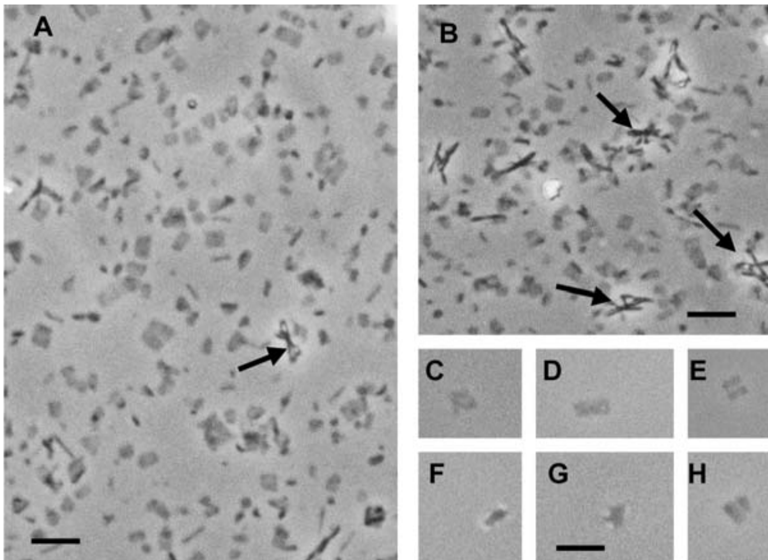

Figure 3. Square, rectangular and trapezoid shaped halophilic Archaea from an NaCl-saturated crystallizer pond of the salterns of Eilat. Cells in panel A and B were concentrated by centrifugation, causing collapse of the gas vesicles. Numerous crystals of celestite ($SrSO_4.2H_2O$) are visible especially in panel B (arrows). Panels C-H show single cells of square halophilic Archaea not subjected to centrifugation. Refractile gas vesicles are visible in panel C, F, and G. Panel E and H show dividing cells. Scale bar = 10 µm.

The flat square Archaea were first described by Walsby (1980) from a brine pool in Sinai, Egypt. After their biological nature was recognized, it rapidly appeared that they are abundant in saltern crystallizer ponds worldwide. The organism contains refractile gas vesicles (visible in Fig. 3C, 3F, and 3G, not visible in Fig. 3A and 3B as these show cells concentrated by high-speed centrifugation, which had caused the collapse of the

gas vesicles). A number of electron microscopic studies have been devoted to the characterization of these fascinating organisms (Kessel and Cohen, 1982; Parkes and Walsby, 1981; Stoeckenius, 1981). Our understanding of these unusually shaped prokaryotes has been reviewed a few years ago (Oren, 1999).

The square Archaea belong to the *Halobacteriaceae*, and are phylogenetically remotely related to the genus *Haloferax*. Until recently no cultures were available, and therefore little was known about the physiology of the organism. However, the square archaeon has now been brought into culture (Bolhuis et al., 2004; Burns, 2004), and it may therefore be expected that we will soon learn much more about this fascinating organism.

Other species of the family *Halobacteriaceae* have been isolated from saltern ponds worldwide (Oren, 2001). Numerically these are less abundant than the square archaeon, and they are less distinctive morphologically, so that they cannot be easily recognized in micrographs of the crystallizer pond community.

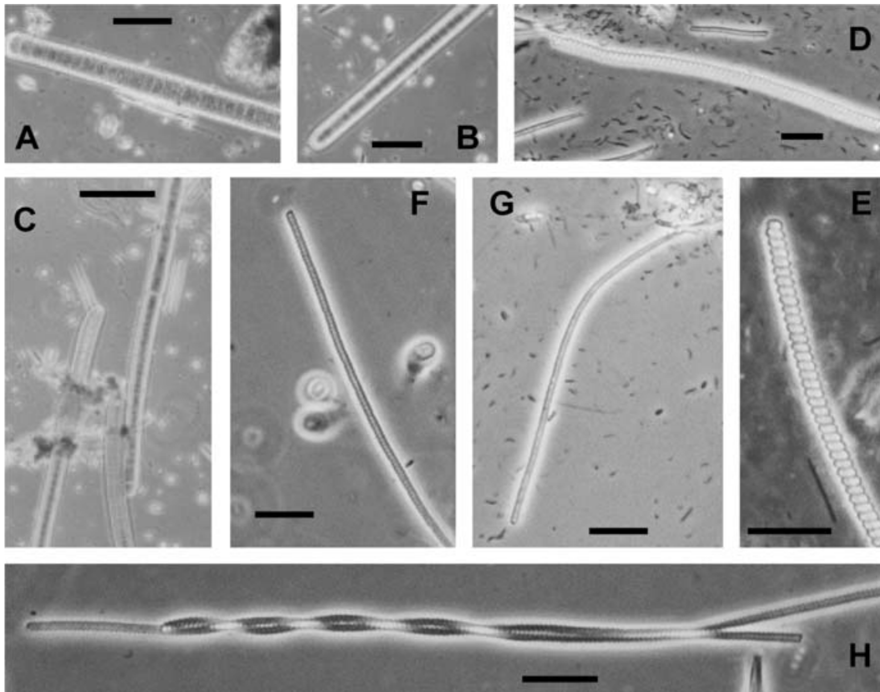

Figure 4. Filamentous benthic cyanobacteria from low-salinity concentrating ponds of the Eilat saltern: large *Oscillatoria* filaments from a pond containing approximately 4% salt (A-C), *Halospirulina tapeticola* from a pond with about 8% salt (D,E), and another type of *Halospirulina* with very narrow filaments from the same pond (F-H). Scale bars represent 50 μm (A-C) or 20 μm (D-H).

4. Bacteria

In many of the recognized phyla within the domain Bacteria we find halophilic or halotolerant representatives. Certain types of halophilic Bacteria are found at high densities in the saltern ecosystem, and many of these have conspicuous morphologies so that they can easily been recognized microscopically. Two groups, the cyanobacteria and the purple sulfur bacteria, are especially prominent because the colors they impart to the sediments.

4.1. CYANOBACTERIA

Cyanobacteria are the main components of the soft gelatinous benthic microbial mats that cover the lower salinity evaporation ponds, and their presence is also conspicuous in the gypsum crusts on the bottom of the ponds of intermediate salinity (see also Fig. 1).

Figure 4 shows a variety of filamentous cyanobacteria present in the benthic microbial mats that cover the bottom of the lower salinity evaporation ponds of the Eilat saltern. Several morphological types can be seen in these mats: thick filaments of *Oscillatoria* sp. (Fig. 4A-C) and tightly wound spirals of *Halospirulina* or *Spirulina* spp. of different filament diameter (Fig. 4D-H). Similar spirally wound filaments and other types of filamentous cyanobacteria have been recorded from salterns in Spain, France, and elsewhere (Clavero et al., 1994; Giani et al., 1989; Nübel et al., 2000; Thomas and Geisler, 1982).

The gypsum crust that develops on the bottom of the intermediate salinity ponds contains brightly colored layers, of which the upper brown-orange layer and the green layer below derive their coloration from the dense communities of cyanobacteria inhabiting them. The upper 0.5-2 cm thick brown layer is populated by carotenoid-rich unicellular cyanobacteria (*Halothece*-*Aphanothece* type) (Fig. 5A-C). These cells have a low chlorophyll and phycocyanin content. A *Phormidium*-type filamentous cyanobacterium is the dominant organism in the green layer (Fig. 5D-F). An olive-green layer is sometimes found below the red-purple layer of phototrophic sulfur bacteria (see section 4.2). Slender filamentous, yet unidentified microorganisms are found in this layer (Fig. 5G-H). The biology of the Eilat gypsum crust has been documented before (Oren, 2000a, 2000b; Oren et al., 1995; Sørensen et al., 2004). Similar layered cyanobacterial communities have been described from gypsum deposits in Spanish and French salterns (Caumette et al., 1994; Cornée, 1984; Thomas, 1984) and from a mixed gypsum-halite crust from salt pools of Baja California, Mexico (Rothschild et al., 1994).

4.2. PHOTOSYNTHETIC PURPLE BACTERIA

Red-purple layers of photosynthetic sulfur bacteria are often found below the cyanobacterial layers in the benthic microbial mats that develop in saltern evaporation ponds. The organisms responsible for the red color belong to genera such as *Halochromatium*, *Thiohalococcus* and *Ectothiorhodospira* or *Halorhodospira*, all members of the Proteobacteria branch of the domain Bacteria. These are anaerobes that obtain their energy from light, especially from the near-infrared radiation (800-900 nm) that is not absorbed by the cyanobacterial layers above. Sulfide, produced by dissimilatory sulfate reduction in the layers below, serves as electron donor for the

photoautotrophic fixation of CO_2. Such red layers have been reported from salterns in France, Spain, Slovenia, and elsewhere (Caumette et al., 1994; Cornée, 1984; Schneider and Herrmann, 1979).

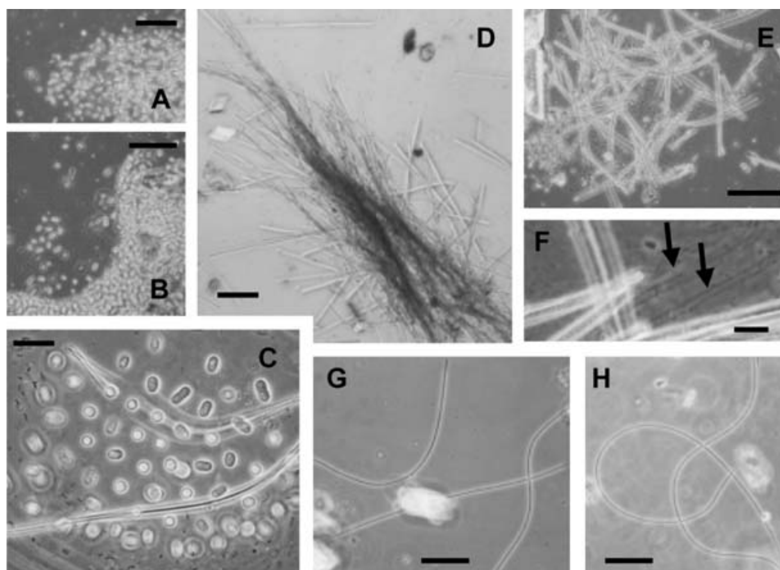

Figure 5. Unicellular *Halothece* (*Aphanothece*)-like cells from the upper brown layer of a gypsum crust found on the bottom of a saltern crystallizer pond with 19.3% salt (A-C), *Phormidium*-type filamentous cyanobacteria from the green layer in this crust (D-F) (compare also Fig. 1C), with empty sheaths seen in panel F; arrows), and unidentified slender cyanobacteria (?) from an olive-green layer below the red-purple layer (G,H). The bars represent 100 μm (A,B,D), 50 μm (E,G) or 20 μm (C,F).

In the Eilat salterns, patches of red *Halochromatium*-type cells are often found below the cyanobacterial layer in the low-salinity evaporation ponds. A prominent red layer is present in the gypsum crust in the ponds of intermediate salinity (Oren et al., 1995; Sørensen et al., 2004; see also Fig. 1C). These layers contain mainly ovoid cells morphologically resembling *Halochromatium* (Fig. 6A-D). Intracellular sulfur granules, formed by partial oxidation of sulfide, are often seen within the cells (Fig. 6D). *Halorhodospira*-like cells have also been reported to occur in the Eilat gypsum crust (Oren et al., 1995).

4.3. CHEMOLITHOTROPHIC SULFUR BACTERIA

Spherical bacteria with intracellular granules, apparently of elemental sulfur, are associated with the green layer of the Eilat gypsum crust, or are found in a whitish layer just below (Fig. 6E and 6F). They may represent a yet-uncultured type of

chemolithotrophic sulfur oxidizing bacteria, and as such ('*Achromatium*'-like cells) they were described earlier (Oren et al., 1995). A chemoautotrophic way of life is suggested for these organisms, as no prominent coloration is found in accumulations of these cells. However, presence of photosynthetic pigments and the possibility of a phototrophic way of life cannot be strictly excluded.

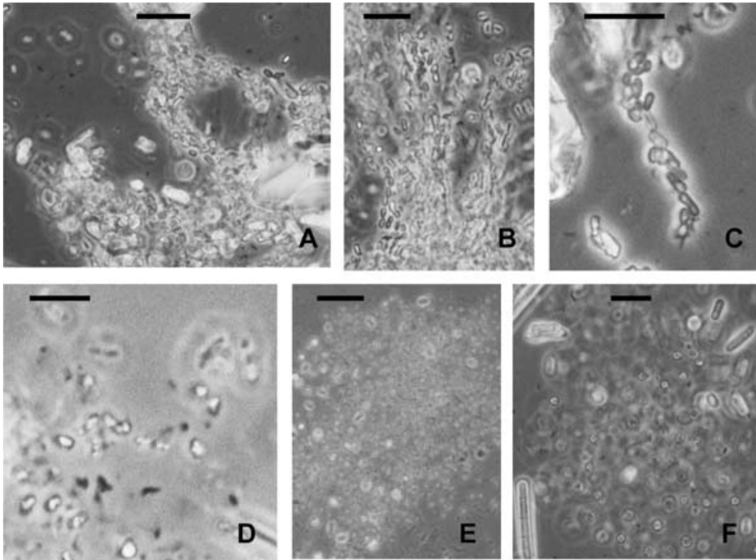

Figure 6. Red *Halochromatium*-type photosynthetic sulfur bacteria from the purple layer of the Eilat saltern gypsum crust in a pond of 19.3% salt (A-D); see also Fig. 1C. Intracellular sulfur granules are visible in panel D. Panels E and F show small spherical bacteria with intracellular granules, apparently of elemental sulfur, associated with the green layer of the Eilat gypsum crust. These yet unidentified and uncultured organisms may be chemolithotrophic sulfur oxidizing bacteria, but presence of photosynthetic pigments and the possibility of phototrophic life cannot be excluded. The bars represent 20 µm (A-C,F), 10 µm (D) or 20 µm (F).

4.4. HETEROTROPHIC BACTERIA

A variety of heterotrophic bacteria, both benthic and pelagic, are associated with the autotrophic communities in the saltern evaporation ponds, and these live at the expense of organic material produced by the photosynthetic microbial communities present. Morphologically these organisms are generally little distinctive, but their presence is conspicuous especially where large amounts of organic material accumulate, for example in the form of the polysaccharide slime excreted by the unicellular cyanobacteria in the upper layer of the gypsum crust in the Eilat saltern (Fig. 7).

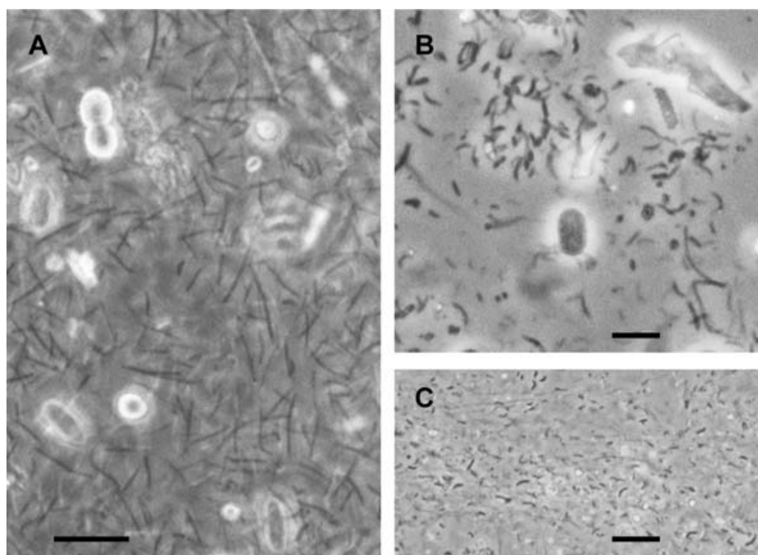

Figure 7. Different morphological types of probably heterotrophic bacteria found within the polysaccharide matrix excreted by the unicellular cyanobacteria in the upper brown-orange layer of the Eilat saltern gypsum crust below brine of 19.3% salt (A-C). The bars represent 20 µm (A,C) or 10 µm (B).

Representatives of the domain Bacteria also occur at the highest salinity in the crystallizer ponds. The halophilic Archaea described in section 3 (Fig. 3) share their habitat with *Salinibacter*, a recently discovered member of the Bacteria (*Cytophaga*/*Flavobacterium* branch). *Salinibacter* is a red pigmented heterotrophic bacterium that physiologically resembles the halophilic Archaea to a large extent. In Spanish saltern crystallizer ponds it may represent up to 5-25% of the total prokaryotic community in the brines (Antón et al., 1999, 2002; Oren and Rodríguez-Valera, 2001; Oren et al., 2004). In the Eilat salterns *Salinibacter* is present as well, but it is less is abundant (Elevi and Oren, 2004).

5. Eukarya

Generally spoken the biota of the saltern evaporation ponds are dominated by prokaryotes. Eukaryotic microorganisms (and macroorganisms as well) still abound in the first concentrating ponds, whose salinity does not greatly differ from that of seawater. When conditions become hypersaline, eukaryotes become rarer. A few types of diatoms and of protozoa are found along with the cyanobacteria and other prokaryotic organisms in the microbial mats that cover the bottom of the evaporation ponds along the salinity gradient, but they are present in low numbers. However, when halite

saturation is reached in the crystallizer ponds, a eukaryotic unicellular alga, *Dunaliella salina*, becomes one of the dominant components of the biota, and is responsible for most or all of the primary productivity in the salt-saturated brines.

5.1. DIATOMS

Diatoms are found in the Eilat saltern ponds especially in the initial stages of evaporation (Fig. 8A-D). They become rarer with the increasing salinity of the ponds, but some diatoms can be found at salt concentrations up to about 20% (Fig. 8E-G).

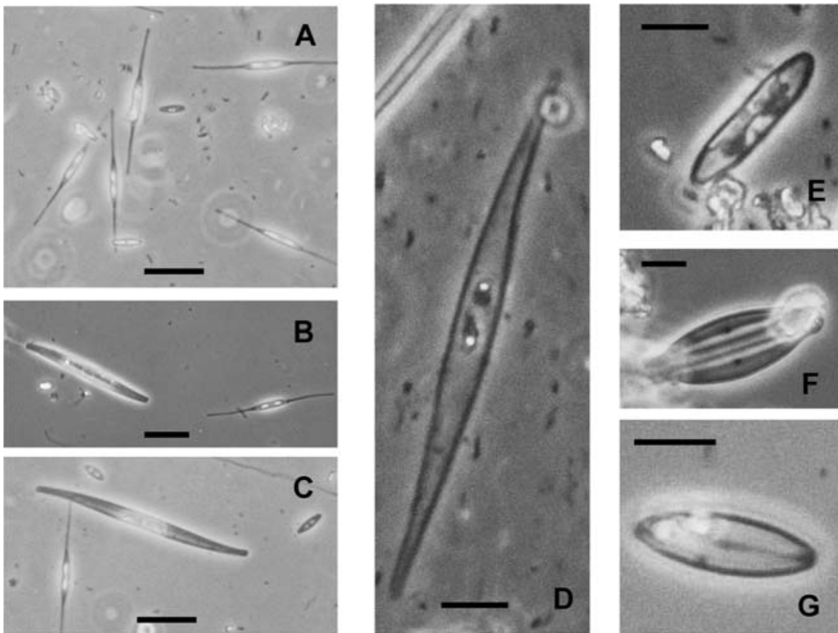

Figure 8. Different types of diatoms encountered in the Eilat salterns in ponds with 4% (A-C), 8% (D), and 19.3% salt (E-G). They can tentatively be identified as *Nitzschia* sp. (A-C), *Gyrosigma* or *Pleurosigma* sp. (B-D), *Navicula* sp. (E,G), and *Amphora* sp. (F). The bars represent 20 μm (A-C) or 10 μm (D-G).

Detailed studies have been made of the taxonomic distribution of diatoms in saltern ponds of different salinities worldwide, including Spain, France, Italy, Mexico, and South Africa (Campbell and Davis, 2000; Clavero et al., 1994, 2000; Javor, 1989; Margheri et al., 1987; Noël, 1982, 1984; Rincé and Robert, 1983). Among the genera most widespread in salterns are *Nitzschia*, *Amphora*, *Navicula*, *Entomoneis*, and *Pleurosigma*. It has been suggested that diatoms may be useful indicators to monitor the physical and chemical status of saltern evaporation ponds (Campbell and Davis, 2000).

5.2. THE GREEN ALGA *DUNALIELLA*

Dunaliella salina (*Chlorophyceae*, *Volvocales*) is a characteristic inhabitant of saltern crystallizer ponds. Microscopic examination of the brines shows red carotenoid-rich flagellated cells (Fig. 9). These contribute to the coloration of the water, together with the halophilic Archaea and possibly with Bacteria of the genus *Salinibacter* (see sections 3 and 4.4). Biotechnological operations have been set up in several places in the world to exploit the alga for the production of β-carotene (Oren, 2002).

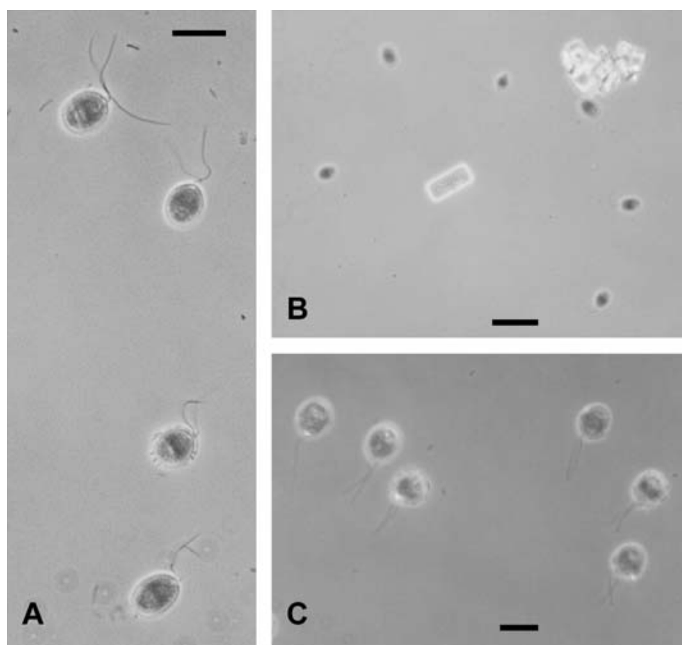

Figure 9. Red-orange cells of *Dunaliella salina* from the NaCl-saturated crystallizer ponds of the Eilat salterns. Small halite crystals are seen in panel B. The bars represent 20 μm (A,C) or 50 μm (B).

5.3. PROTOZOA

The cyanobacteria-dominated mats on the bottom of the lower and intermediate salinity evaporation ponds are grazed by different types of ciliate, flagellate and amoeboid protozoa. Some are found at salt concentrations as high as 20%. Figure 10 shows a selection of types of protozoa found in the Eilat salterns. Occurrence of protozoa at high salt concentrations has been documented in the past (Javor, 1989; Oren, 1989; Post et al., 1983), but little is know about the biology of these organisms.

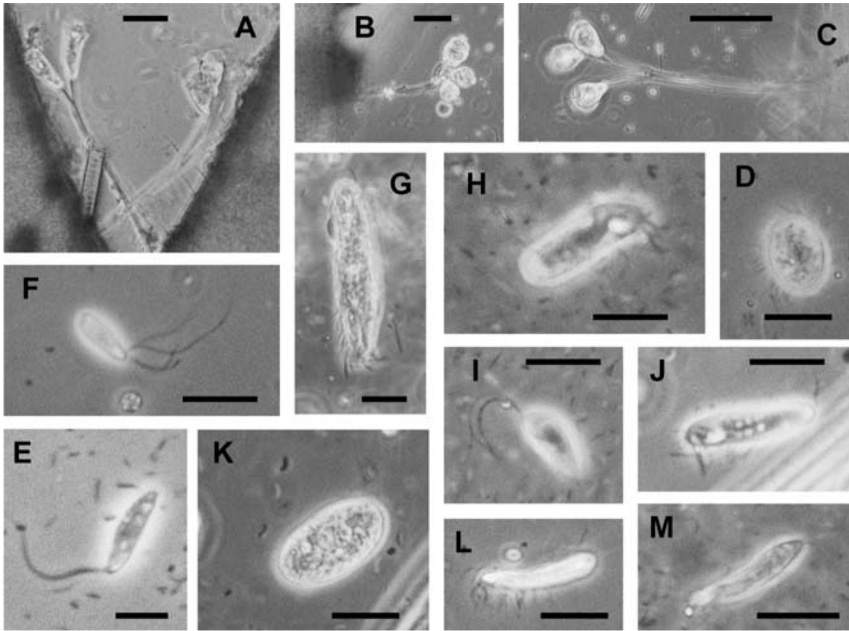

Figure 10. Different types of ciliate and flagellate protozoa encountered in the Eilat salterns in ponds with 4% (A-D), 8% (E), and 19.3% salt (F-M). The sessile stalked ciliate protozoa shown in panels A-C attached to leaves of *Zostera* (?) probably belong to the genus *Zoothamnium*. The bars represent 50 µm (A-C), 20 µm (D,F-H,J-M), or 10 µm (E,I).

5.4. FUNGI

Direct microscopic examination of saltern brine and sediment samples does not show conspicuous communities of yeasts and filamentous fungi to be present. However, it is now well established that halophilic yeasts and fungi are present in the saltern ecosystem (Gunde-Cimerman et al., 2000; Zalar et al., 1999a, 1999b). Figure 11 shows micrographs of representative isolates obtained from the Sečovlje salterns on the Adriatic coast at the border between Slovenia and Croatia.

6. Epilogue

Although microorganisms, and especially the prokaryotic types, show only a limited morphological diversity, a microscopic examination of microbial communities in their natural environment can be an esthetically highly rewarding experience. The beauty of the marine microflora was convincingly shown by Sieburth (1975, 1979) in his two books of electron micrographs of marine microorganisms. The hypersaline system presented by the salterns, with their series of evaporation ponds of increasing salinity –

each harboring its characteristic microbial communities, presents us with an even greater morphological variety, which is no less fascinating than that in the marine environment.

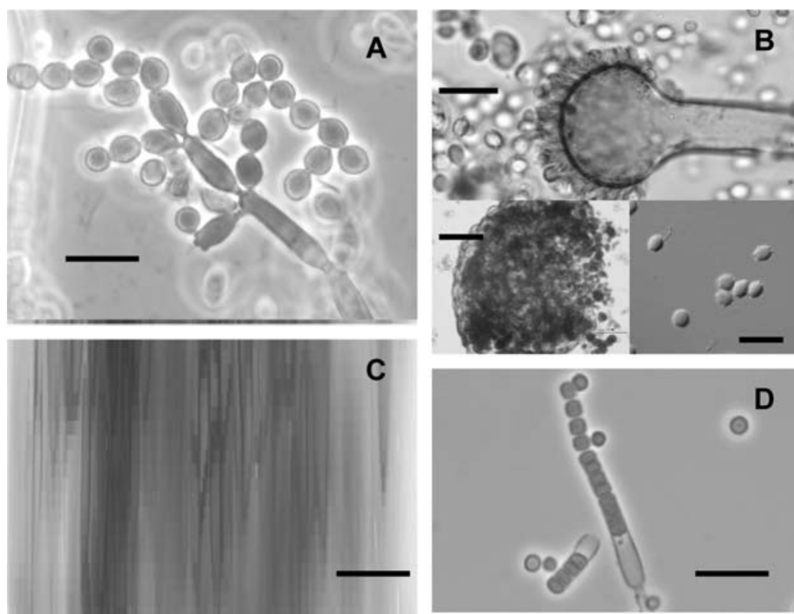

Figure 11. Halophilic fungi isolated from Mediterranean salterns: conidia and conidiophore of *Cladosporium halotolerans* (A) (The bar represents 10 µm), conidiophore with conidia, cleistothecium, and ascospores of *Eurotium amstelodami* (anamorph: *Aspergillus hollandicus*) (B) (The bars represent 10, 25, and 10 µm, respectively), endoconidia in hyphae of *Phaeotheca triangularis* (C) (The bar represents 20 µm), and conidia of *Wallemia sebi* (D) (The bar represents 10 µm). Photographs by P. Zalar and L. Butinar.

The survey presented here should in no way be considered as a complete inventory of the microbiota of the saltern system. It only shows the morphologically most conspicuous organisms, and many less conspicuous organisms that may play an important role in the functioning of the biological system of the salterns thus remain undetected. Although there is a large extent of similarity between the biological properties of salterns worldwide, local variations do exist, and a microscopical study of the planktonic and benthic microflora of saltern ponds from other geographic locations will undoubtedly show different features and yield additional types of microorganisms not prominent in the Eilat salterns described here.

Each of the microorganisms shown in the photographs presented above is interesting in its own right, and deserves in-depth studies of its mode of life and its function in the saltern ecosystem. Many of these organisms are yet to be isolated in culture and studied in the laboratory. Such studies will add much to our understanding of the biology of the

salterns and the functioning of microorganisms at high salt concentrations. Meanwhile, microscopic examination of the organisms in their natural environment remains a rewarding pastime, not only scientifically but from an esthetic point of view as well.

Acknowledgments

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THE MICROBIAL DIVERSITY OF A SOLAR SALTERN ON SAN FRANCISCO BAY

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1. Introduction

In the last few years, there have been a number of papers describing various aspects of the halophilic bacteria. A review by Ventosa et al. (1998) summarized our understanding of the distributions, taxonomy, and activities of halophilic Bacteria. This review article also discussed the biochemistry of these bacteria including what was known up to then of the organic osmolytes, enzymes, cell envelopes, and genetics. The halophilic Bacteria encompass both Gram-positive and Gram-negative microbes and are found in over 50 genera. Additionally, the halophilic Bacteria can be found over a very broad range of salinities including saturation or near saturation. This includes some members of the genus *Halomonas* (Vreeland et al., 1980) and *Salinibacter ruber* (Antón et al., 2002).

In 2002, Oren published a review of the molecular ecology of the halophilic Bacteria and Archaea. This review covered the fluorescent in situ hybridization technique (FISH), and the use of polar lipids, radioactive thymidine or leucine uptake studies in various salterns especially in Eilat, Israel and the Dead Sea. Additionally, the halophilic microbes from Antarctic lakes and alkaline lakes in Africa were also described (Oren, 2002a). Oren (2002b) also described the microbial communities in solar salterns noting the mats that form in these environments contain cyanobacteria as well as sulfur bacteria and anaerobic bacteria along with various algae. He described the isolation of halophilic Bacteria and Archaea from salterns, the pigments found in the hypersaline waters due to the alga *Dunaliella* and the halophilic Archaea and *Salinibacter*, and the various lipids associated with the microbial communities. He also described the experiments using radiolabeled thymidine uptake to estimate growth rates and noted that in the less saline ponds the growth rate was faster than in the crystallizers, 1-12 days vs. 6-22.6 days, respectively (Oren, 2002b).

An update of the various methodologies used to investigate microbial communities and their metabolic activities in hypersaline environments was published by Litchfield in 2004. This review included more recent studies looking at stable carbon isotope ratios to determine sources of carbon used by the halophilic bacteria. Also the uses of various Polymerase Chain Reaction (PCR) based techniques such as amplicon length heterogeneity (ALH), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length (T-RFLP) and cloning to analyze the total microbial

community. In most cases, investigators have used primers specific for Archaea as well as the universal bacterial primers.

Because of the number of comprehensive recent reviews of microbes in hypersaline environments and salterns, this review will concentrate on one specific solar saltern - the Cargill Solar Salt Plant in Newark, California, U.S.A. - and will describe the physical and chemical information available on the saltern and the source waters for the saltern, San Francisco Bay.

1.1. SAN FRANCISCO BAY

For over 150 years salt has been produced commercially on and around San Francisco Bay, and it is likely that Native American Indians were obtaining salt before that. San Francisco Bay is fed by water from the Pacific Ocean as well several rivers which flow into the Bay, especially the Sacramento and San Joaquin rivers which in earlier times brought silt and a few nutrients into the Bay. Today these rivers and the other smaller streams and the Bay itself serve many industries and communities so that the waters are now eutrophic and provide a rich source of nutrients to the only remaining solar saltern on the Bay, the Cargill Solar Salt Plant in Newark, California.

The Bay is really two entities - a Northern part which extends from the Golden Gate Bridge to the San Joaquin River and the Southern Bay which is more influenced by tidal fluctuations and which feeds the Cargill Solar Salt Plant. Rainfall, mostly during the winter months, causes strong estuarine circulation patterns that interact with the tidal patterns so that sediments are resuspended, and the Bay is well mixed on a generally diurnal basis (Conomos et al., 1985). The Sacramento River also flows into the Northern Bay.

To illustrate the pollution levels in the Bay, as early as 1979 there was concern about the levels of copper, silver, and zinc flowing into the Bay and their contamination of tellenid clams (Luoma and Cain, 1979). There is extensive industry, both marine and land-based, as well as community development all along the Bay both of which contribute to the pollution of the Bay. Thus the waters that flow into the solar saltern are rich in nutrients and, depending on the season, may be somewhat brackish. Both of these factors vary spatially and temporally throughout the Bay.

The main driving force in the estuary is the phytoplankton which provides the primary source of particulate organic matter throughout both portions of the Bay. In addition, freshwater algae are important to the clams and other animals in the northern Bay (Canuel et al., 1995). The cyanobacterium *Synechococcus* was found by Ning et al. (2000) to comprise up to 15% of the total phytoplankton biomass during nonbloom periods but less than 2% of the chlorophyll during algal blooms indicating that an extremely diverse phytoplankton community also exists during blooms. They also noted that the numbers of this cyanobacterium increased as the nutrient levels decreased, indicating that there was more of this bacterium in the Southern Bay region than in the Northern Bay. The other major microbiological study of the Bay was conducted by Murrell et al. (1999) who studied the impact of particles on the microbial populations. They noted that as the freshwater inflow increased there were sharp decreases in the bacterial biomass and activity. They found increased aminopeptidase activity with increasing salinity which was the opposite for β-galactosidase activity. They also found that particles were a major site for bacterial attachment and activity.

1.2. THE CARGILL SOLAR SALTERN

The Cargill Solar Salt Plant is the latest saltern on San Francisco Bay. The Cargill Company bought the former Leslie Salt Company's saltern in September 1978 (R. Douglas, personal communication). The first commercial salt was produced in 1854 by Captain John Johnson, and until 1862, everything that could be produced was shipped to Virginia City to be used in the silver mines there. At one time there were 36 salt-producers on the Alameda, San Mateo, and Santa Clara counties around the southern marshy areas bordering the Bay, but these were consolidated by the Leslie Salt Company in 1936 (Ver Planck, 1958). Thus salt has been produced for several centuries for either local consumption by the original Ohlone inhabitants, the later Mexican and Indian communities, or commercially starting in the nineteenth century.

Regardless of this history, there are efforts to "restore" these salty marshes to their natural condition. This has prompted an extensive study of the macroflora and fauna and zooplankton in selected portions of the saltern to try to determine the ecological impact such changes might cause. In a preliminary report available on line, Takekawa et al. (2000) noted 62 different macro-invertebrates in the lower density pond with some appearing throughout the sampling period and others restricted to the summer months, while, as expected, the diversity decreased with increasing salinity until only 8 were found in the highest density pond studied. Similarly, fish were found only in the lower salinity ponds but a total of 280,241 birds covering 65 species was counted in all of the ponds sampled though the highest density pond had the fewest bird citings recorded. The crystallizers were not sampled nor was there any attempt to examine the algal or bacterial community.

2. Microbiology of the Cargill Solar Salt Plant

2.1. EARLY STUDIES

The only major published work on the algal community of this diverse environment was completed by Carpelan in 1953. At that time the saltern was owned by the Leslie Salt Company and the area studied was the Alviso salt ponds which are on the south side of the Bay, across the Bay from the main Cargill saltern. Today, the Alviso salt pans are fed by highly concentrated brine from the northern saltern through an under Bay pipeline (R. Douglas, personal communication). At the time of Carpelan's study, the Alviso ponds were fed directly from the Bay similar to the area on the northern side of the Bay. Thus it is appropriate to describe the data obtained from the Carpelan study.

Initial salinities ranged from approximately 2% to approximately 9% in the first six ponds that were studied. The pH range was up to pH 9.8 at the inlet to pH 8 at the outlet to Pond 6. These pH values changed during the day as well as monthly indicating that the great variability in this value was dependent on the nutrient loading of the inlet water and its availability to algal and bacterial metabolism. Similarly, oxygen values ranged from approximately 2 ml l^{-1} to 9 ml l^{-1}, again depending on the time of day the measurements were taken, the location within the pond, and the month. While nitrate and phosphate were generally low except at the outlet to Pond 6, the ammonia concentrations were usually 10 to 20 times higher at 20-30 μg atoms l^{-1} (Carpelan,

1953).

Carpelan (1953) noted that *Enteromorpha* sp. and *Rhizoclonium* sp. were the major macroscopic algae in the lower salinity ponds in the summer and fall, while *Stichococcus bacillaris* Nägeli was found only in the spring. He also noted many macro-invertebrates in the lower density ponds as well as several fish and many birds. Other organisms noted were the brine shrimp *Artemia* and the alga *Dunaliella* along with numerous diatoms and other algae, depending on the salinity and season. There was no discussion of the bacterial or fungal community in these ponds though a discussion of their potential activities was included (Carpelan, 1953).

Klug et al. (1985) also examined the San Francisco salterns looking at sulfate reduction and the carbon cycle in this system. Again, the studies were conducted on the Alviso salt ponds. They examined four ponds containing between 3 and 25% salt. With hand-held augers, they obtained sediment cores for analysis of the sulfate, sulfide, chloride, and volatile fatty acids. They also tested the percent moisture and percent organic matter. In general, the sulfate/chloride ratio decreased with depth. This ratio was considered a better indicator of biological sulfate reduction than the sulfide pool because sulfide can react abiologically with various cations and precipitate. Sulfate reduction was maximal in the upper centimeters of the sediments and organic matter and volatile fatty acids increased with increasing salinity. The rate of sulfate reduction in the hypersaline ponds was similar to other evaporite environments, but slower than in the 3.3% salt pans where the rate was 2 to 50 times higher than reported for temperate salt marshes. There was no reported attempt to cultivate any of the sulfate reducers or other anaerobic microbes.

2.2. MORE RECENT INVESTIGATIONS

Although there have been several long term microbiological studies on the salterns in Spain (for example Rodriguez-Valera, 1988; Rodriguez-Valera et al., 1981, 1985; Ventosa et al., 1982), this has not generally been true for salterns in the United States. The Western Salt Company in Chula Vista, California was studied by Javor in 1982-83 (Javor, 1983, 1989). The results indicated that there were more bacteria in the nutrient-rich Western Salt Co. waters than in the more oligotrophic saltern Exportadora del Sal in Mexico. Thus there were higher numbers of bacteria and halobacteria in the crystallizer ponds and higher concentrations of ammonia than found in other more oligotrophic salterns.

Starting in 1993, a multi-seasonal study of the microorganisms in the Cargill saltern was initiated. The intent was to select representative or microbiologically important transitional zones based on earlier studies on the solar saltern in Bonaire, Netherlands Antilles (Litchfield, 1977). In that earlier study it was noted that there were three major ecological zones in the Bonaire saltern comprising the inlet area, the area where the salinity reached 7-25% salt, and finally the crystallizers (Vreeland, 1976). In the study of the Cargill saltern, the inlet waters (2-4% salt), ponds containing 6-10%, 10-15 %, 15-25% salt, the crystallizers, and ponds with 26-28% salt were sampled over a five-year period. This study was designed to use traditional cultivation techniques along with molecular analyses of the uncultivated community and analysis of biochemical markers such as the total lipid and metabolic potential of the saltern communities.

For the cultivation procedures, a modified casamino acid medium (MCAT) was used

along with R2A (Difco). This latter medium has lower amounts of casamino acids plus peptone (0.5 g l^{-1}), and includes dibasic potassium phosphate (0.3 g l^{-1}), dextrose (0.5 g l^{-1}), soluble starch (0.5 g l^{-1}), and pyruvate (0.3 g l^{-1}) which are not in the MCAT medium. It was modified for halophile cultivation by the addition of 20 g l^{-1} $MgSO_4.7H_2O$ (MR2A). The MCAT contains per liter: casamino acids (7.5 g), sodium citrate (3 g), yeast extract (0.5 g), and $MgSO_4.7H_2O$ (20 g). In all cases the pH was adjusted to 7.2-7.5, and the appropriate amount of solar salt was added on a weight:volume basis.

In almost all cases the MR2A medium resulted in higher numbers of both total bacterial enumerations and the total number of pigmented colonies (Litchfield et al., 1999). The range for the total colony-forming units (TCFU) was from 10^4 to $5x10^7$ for the inlet to $5x10^4$ to $8x10^7$ for the higher density pans and crystallizers (Litchfield et al., 1999).

Lipids were extracted from aliquots of pellets obtained by centrifuging 3-4 l of the samples as described in Litchfield et al. (2000). Both pigments and total lipid contents were analyzed by spectrophotometric scans or thin-layer chromatography, respectively.

Lipid distributions can be summarized by the following comments. The spectral scans showed that β-carotene, chlorophylls *a* and *b*, algal carotenoids, and bacterioruberins were present. There were marked seasonal differences, especially in the inlet and 10-12% salt pans. The greatest amount and most complex assortment of pigments were found in the inlet samples. The data were only semiquantitative due to the fact that the biomass varied between samples and seasonally even though the same amounts of water were centrifuged and extracted each time. The total lipids extracted from the whole community pellets also showed both seasonal and salt-pan differences. Almost all samples contained an orcinol positive spot running ahead of phosphatidyl glycerol, several other orcinol positive spots (indicating glycolipids), and methyl phosphatidyl glycerol phosphate were found in pans containing greater that 15% salt indicating the presence of haloarchaea. Frequently in the higher salinity pans phosphatidylglycerosulfate and a sulfated diglycosyl diether lipid were also identified, again indicative of the presence of haloarchaea (Litchfield et al., 2000).

In an attempt to understand the metabolic potential of the whole community, the investigators used the BIOLOG system to test for the metabolism of 95 different carbon sources (Litchfield et al., 2001). Early on they discovered that high salt concentrations interfered with the reproducibility of the assays, so their data were limited to those pans containing less than 15% salt. Of the 95 different carbon sources including amino acids, sugars, tweens, organic acids, nucleic acids, there were nine substrates which were utilized 85% of the time: L-alanine, L-asparagine, L-proline, citric acid, D-glucosaminic acid, D-glucose, maltose, sucrose, and D-trehalose. These data were analyzed by simple matching coefficient and by principle component analysis. Both tests gave essentially the same result with the lower salinity pans and the inlet comprising one large cluster that encompasses 7 smaller clusters (data not shown). The higher salinity pans formed another less heterogeneous cluster.

The results from all of these studies on the cultivated colonies, the lipid patterns, and the metabolic potential confirmed each other. Basically, there was extensive seasonal variability at the Cargill solar salt works which seemed to correlate with the seasonal rainfall patterns. The question to be answered next was whether this seasonal variability could also be observed in the total microbial community structure.

More recently the research has centered on the biodiversity and complexity of the total microbial community. Molecular fingerprinting was accomplished using the amplicon length heterogeneity procedure described by Suzuki et al. (1998) with details of its application to halophiles in Litchfield and Gillevet (2002). Aliquots of the pellets used in the lipid analyses were subjected to extraction of the 16S rRNA gene using the Bio101 FastDNA Spin Kit for Soil (Qbiogen Inc., Vista, California). The resulting 16S rRNA gene was amplified using a set of the universal bacterial primers 27F and 355R (Lane, 1991) or a set of consensus haloarchaea primers 1HKF and H305R (5'-ATTCCGGTTGATCCTGCCGG-3' and 5'-GTTACCCCACCGTCTACCT-3', respectively) (Litchfield and Gillevet, 2002). These products were cloned and sequenced using an ABI 377 DNA sequencer for the Bacteria and a Spectrumedix 96-capillary Sequencer for the Archaea. For comparison purposes, pure cultures obtained from the plate enumerations were also sequenced to determine whether they were accounted for in the whole community analyses. For fingerprinting, the same primers were used, but the 5' ends of the forward primers were labeled with the fluorochrome 6-carboxyfluorescein (6-FAM). Replicate extractions were analyzed to demonstrate the reproducibility of the method and peaks appearing in two of the three replicates and accounting for greater than 1% of the amplicons were included in the analyses.

For the Bacteria the emphasis was on the 10-12% salt pans (8A) because they showed the greatest number of amplicons when sampled in December and again the following June. For the Archaea, the emphasis was on pan 8A (10-12% salt) along with pans 10A and 26 both of which contained about 26% salt. The numbers and lengths of the amplicons from the fingerprints are shown in Table 1. These data indicate that there were significant changes in the total community diversity between the December and June sampling dates. When the bacterial clones were sequenced, six major groupings for the Bacteria appeared and confirmed the seasonal impact on the microbial diversity (data not shown). Finally, despite obtaining partial sequences for over 80 pure cultures only an occasional strain appeared to match sequences from the clone library.

The amplicon length heterogeneity data indicate that the feeder ponds 10A and 26 had the greatest microbial diversity with 10 peaks in December but only five in June for pan 10A. Pan 26 resulted in five peaks in December and nine peaks in June. There was much less diversity over the time period in pan 8A. When the sequence data from the clones was clustered using Neighbor Joining, the archaeal-based clones resulted in distinctly different clusters for 8A and 26 while pan 10A contained a more uniform distribution of the clones independent of the season. In both December and June, the major strains in 10A had amplicons that were at 240 base pairs and contained 27 and 21 clones out of the 72 and 53 sequenced clones, respectively. However, for pan 8A, the major amplicon size for December was 244 base pairs while for June it was 246 with each amplicon containing 65 (out of 82) or 67 (out of 77) of the clones. A similar pattern was observed in pan 26 where in December the majority of the clones were found in the amplicon sizes 241 or 244 base pairs while in the June sample the amplicons were spread almost evenly over 10 sets of base pairs.

Thus all of the metabolic and molecular analyses demonstrate that the Cargill solar saltern contains a large and diverse microbial community with a broad range of metabolic activities. The importance of these findings is that it has been demonstrated that the metabolic activities, metabolic markers such as bulk lipid analyses, and that specific types of microorganisms vary both seasonally and within the higher salt

concentrations.

Although solar salterns have been known for hundreds, if not thousands, of years our understanding of the community complexity is very limited. Only recently an entirely new genus of halophilic Bacteria, the genus *Salinibacter*, was discovered in the Santa Pola salterns that have been extensively studied by Rodríguez-Valera and his group since the late 1970s and 1980s (Antón et al., 2000). Thus, it is not unexpected to find that the Cargill Solar Salt Plant contains microbes with great metabolic diversity but their isolation is limited by traditional cultivation techniques. The challenge now is to determine which members of the community are metabolically active and to obtain them in pure culture in order to increase our understanding of the microbial diversity in solar salterns worldwide.

TABLE 1. Percent of each amplicon during two sampling trips (December 1997, June 1998).

Sampling Site and Date								
	Bacteria							
		Amplicon length	347	349	350	351	352	354
8A – 12/1997			22	-	46	-	18	13
8A – 6/1998			17	35	-	14	35	-
10A – 12/1997			14	14	-	8	7	56
10A – 6/1998			-	-	-	-	-	100
26 – 12/1997			-	-	-	2	-	98
26 – 6/1998			-	-	10	4	19	56
	Archaea							
		Amplicon length	239	241	245	246	248	249
8A – 12/1997					100			
8A – 6/1998			-	85.8	14.2	-	-	-
10A – 12/1997			-	34.9	48.3	11	3.6	1.9
10A – 6/1998			-	36	49.1	10.3	-	1.9
26 – 12/1997			-	43.1	44.5	12.3	-	-
26 – 6/1998			4.5	49.6	38	7.9	-	-

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DIVERSITY OF MICROBIAL COMMUNITIES: THE CASE OF SOLAR SALTERNS

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1. Questions

The diversity of microbial communities is a very elusive reality (Pedrós-Alió 1993). As microbial ecologists we assume there is a certain number of species in an ecosystem and that each one of those species is represented by a certain number of individuals. If we could only identify all those little balls and rods under the microscope and assign each one to the appropriate species then, we think, we would know the diversity of that microbial community. The number of species would tell us the richness. An index such as Shannon's would tell as the diversity, and another index such as the equitability index would provide an estimate of evenness. These indexes could be calculated for different communities and their diversities compared. Unfortunately, there are several problems when attempting to do such a calculation.

The first problem, of course, is the difficulty in delineating what a microbial species is. However, this is not easy for any living being. There are many definitions of species for large animals and plants for example (Rosselló-Mora and Amann, 2001) and it is very difficult to delineate some species even with the well-studied birds and mammals. Fortunately, diversity can be calculated with other units besides species. All that is needed is to group individuals into non-overlapping classes according to a consistent classification criterion (Kolasa and Biesiadka, 1984; Margalef, 1974). Several descriptors that fulfill these requirements (and are appropriate for microbes) are the number of distinct populations in a flow cytometry graph, the number of DNA bands in a DGGE gel, or the number of clones in a DNA clone library. These units are operational taxonomic units (OTU).

A second problem concerns the sample size. In a forest, for example, the number of tree species found increases as the area of forest sampled increases. Eventually, the number of species stabilizes, when the area sampled is enough to accommodate all the tree species in that ecosystem. In fact, the shape of the species vs. sample size curve is very informative about the structure of the community. If we were to increase the sample size much further we would find a size where the number of new species would increase again. This is the result of having a sampling area larger than an ecosystem. If we were sampling a Mediterranean oak forest, and we were to keep increasing the sample area, we would eventually include a portion of a pine forest growing next to the oak forest. Obviously the number of new species would suddenly start to increase. What is the size

of an aquatic ecosystem for microorganisms? If we accept the structure of the marine environment proposed by Azam (1998), where a maze of organic carbon polymers surrounds and sustains individual algal and bacterial cells, the size of an ecosystem must be very small indeed. However, one could argue that all these microorganisms are periodically mixed with adjacent portions of the water and thus, the whole ocean might be the ecosystem. So, do we have to sample one microliter or one million liters? The fact is that whenever we sample microorganisms for diversity studies, we take an enormous number of individuals. In a recent study of the Sargasso Sea, for example, Venter et al. (2004) filtered 200 liters of seawater. There are around 10^{11} prokaryotic cells in this volume of water. If one were to include as many trees in the study of the diversity of a forest, the area involved would be two million square kilometers, or approximately the area of Mexico. Obviously, the kind of relationships between diversity we are looking at may be completely different from those we are used to with large animals and plants. Think for example of how many microbial species are there represented by only one cell in the 10^{11} cells assemblage (or represented by 100 or 1000 cells for that mater). Do they mean anything for the ecosystem? Do they still have the potential to grow and become significant members of the community? Can we detect them with the available techniques?

I think this question can be framed within the idea proposed by Ramon Margalef several years ago (Margalef, 1994). He proposed to use two words: biodiversity and diversity. Biodiversity would be the total non-redundant genetic information in an ecosystem, while diversity would be the collection of components which are active and abundant at one particular time and place. Using a literary metaphor, the biodiversity of a given language would be all the words in a dictionary, while diversity would include only those words used in a particular book. The dictionary contains many words that are seldom used. Many may have become obsolete. But, as long as they remain in the dictionary, there is the potential for a writer to use them in his or her latest book. In this sense the rare prokaryotes in the Sargasso Sea 200-liter sample would be part of the biodiversity of the Sargasso Sea, but presumably would be excluded from the diversity at the time of sampling. This provides a pragmatic approach to the microbial diversity of an ecosystem. Our techniques do not need to detect the extremely rare microbes in a sample. We can define the number of units found with our favorite technique as the diversity of the sample.

Now we only have to decide which questions are important and what techniques can be used to address them. Several years ago, James Tiedje proposed that the following questions needed to be answered (Tiedje, 1994):

- An order-of-magnitude estimate of the number of microbial species in different environments. That is, an estimate of microbial species richness.
- An assessment of how different or how similar in microbial species composition are different environments. This is related to the question of whether microbial species are cosmopolitan or not.
- A better understanding of whether the species commonly isolated in pure cultures are actually those important in nature.
- An answer to the question of whether microbial communities have high or low evenness or, in other words, are microbial communities dominated by one or very few species of each functional group or are there many species of approximately equal biomass?

- Does microbial diversity change in parallel to the diversity of larger organisms? Do predictions about diversity from general ecological theory apply to microbial communities?

We decided to analyze these questions by simultaneously applying as many different techniques as possible to the same model system: the solar salterns.

2. The MIDAS workshop

The biology of saltern ponds has been reviewed several times (Javor, 1989; Larsen, 1980; Oren, 1993, 1994; Pedrós-Alió, 2004; Rodríguez-Valera, 1988). Different aspects of the molecular ecology of salterns have been reviewed by Oren (2002), Litchfield et al. (1999), and Rodríguez-Valera et al. (1999). The latter study was the starting point for a major effort to use the salterns as a model system where many different molecular techniques could be compared. The hypothesis was that microbial diversity in solar salterns is better understood than in most other ecosystems. Therefore, applying several techniques simultaneously should reveal similarities and inconsistencies among them. Further, it would be possible to explore the questions about diversity of microorganisms posed by Tiedje (1994).

During the European Union project MIDAS (MIcrobial Diversity in Aquatic Systems) we carried out a detailed study in Santa Pola salterns in Alicante, Spain. Twenty researchers from the Universidad Miguel Hernández in Alicante, the Plymouth Marine Laboratory in Great Britain, the National Environmental Research Institute and DHI-Water and Environment in Denmark, the University of Bergen in Norway, and the Institut de Ciències del Mar (CSIC) from Barcelona, Spain spent two weeks studying the salterns intensively. The main objective of the workshop was to determine the diversity of the microorganisms along the gradient with as many different conventional and molecular techniques as possible. Results have been published in a number of papers (Benlloch et al., 2002; Casamayor et al., 2002a; Estrada et al., 2004; Øvreås et al., 2003; Sandaa et al., 2003). The second objective was to analyze the microbial food web along the gradient and this aspect was reviewed recently (Pedrós-Alió, 2004).

3. Methods

Several techniques were used in the salterns to determine diversity of microorganisms: inverted microscopy, flow cytometry, pigment composition through HPLC and molecular techniques were used for photorophs, while molecular techniques and culturing were used for heterotrophs. There has been a proliferation of molecular techniques in the last decade and a summary is presented in Fig. 1. The objectives of such techniques are to compare communities, to identify the members of a community, and to quantify the abundance of some or all of the members in the community. Each technique addresses one or more of these three objectives. Moreover, each technique has some advantages and problems. Thus, the most appropriate technique for the purpose of the researcher must be carefully chosen in each case and no technique can be

claimed to be superior to others in all aspects. The processes shown in Fig. 1 can be divided in three parts: concentration, separation, and identification.

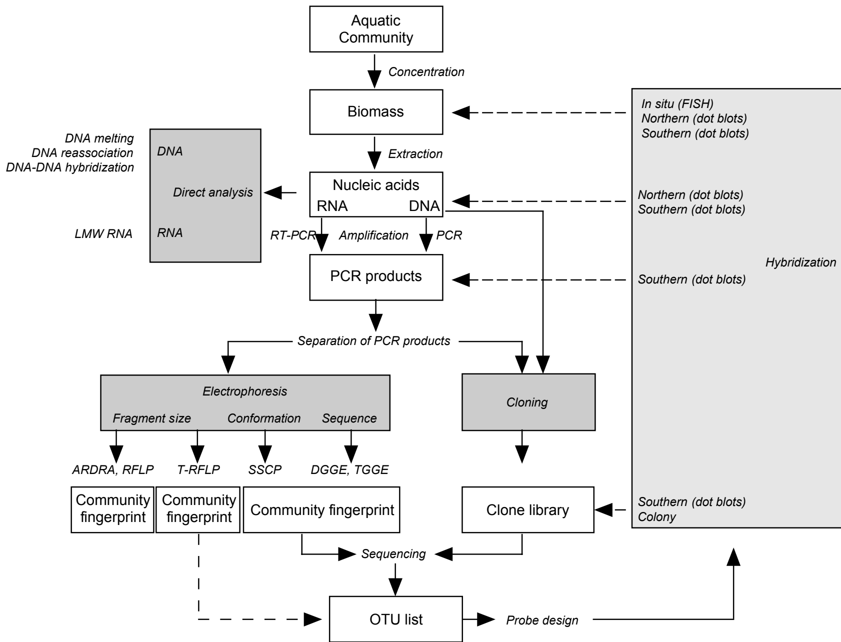

Figure 1. Scheme of approaches to the molecular ecology of microorganisms.

3.1. CONCENTRATION

The first part attempts to concentrate the target sequences so that they can be detected and studied. Usually this involves a filtration step in which the microbial biomass from a community is concentrated on a filter. The filter size determines the kind of microorganisms that will be collected. In principle this filtration step is not selective. That is, it does not collect some microorganisms with preference to others and, therefore, the biomass on the filter is a faithful reflection of the organisms present in the community and of their respective abundance. The next step consists of the extraction of the nucleic acids from the biomass. Several different procedures exist and there is potential for bias against or for some microorganisms. For example, some bacteria may be especially resistant to cell breakage and their DNA will be extracted with a lower efficiency than that of other bacteria. This step, therefore, requires checking. In MIDAS four different laboratories carried out extractions with different protocols from the same samples and the resulting DGGEs were identical (see Fig. 2 in Casamayor et al., 2002a).

If everything works properly the nucleic acids in the extract will be a faithful reflection of all the genomes in the community. This collection of all the genomes from a microbial community has recently been termed the "metagenome." This can be analyzed directly (see below) or subject to an additional concentration step: the polymerase chain reaction (PCR) to obtain many copies of the target sequences. This is a marvelous technique that literally allows picking the needle in the haystack. However, it is subject to many caveats. The efficiency and selectivity of the PCR is highly dependent on the primers used. Some primers may no be as universal as one might want. Or, perhaps they amplify better the DNA of some organisms than others, thus biasing the quantitative results of the amplification. Finally the primers may completely miss some organisms we might be interested in. For these reasons, results from PCR have to be checked in some independent way, such as microscopy or quantitative hybridization.

In summary, concentration techniques up to DNA extraction tend to be non selective but are not very powerful. PCR, on the other hand, may be selective but is extremely powerful.

3.2. SEPARATION

The second process is the separation of the targeted sequences. If we analyze the nucleic acids extract, we have a mixture of all the genes in the community. If we analyze the PCR products we have the selected genes belonging to all the "species" in the community. In either case, these sequences need to be separated from each other in order to analyze them. There are essentially two strategies to do this: cloning and electrophoretic separation of the PCR products. In cloning, the DNA fragments are incorporated into a suitable bacterial cell. This cell will be able to reproduce and make as many copies from the cloned sequence it carries as desired. In principle each PCR fragment will be incorporated into a single bacterium. When these bacteria are grown in a Petri dish, each cell will multiply producing a colony. This colony is a clone of the initial cell and the collection of clones is the clone library. It can be stored and retrieved at will for many years, so that one can always go back to the library to study some additional factor. Each clone can be sequenced and the sequences can be compared to those in the available databases to try to identify the organism responsible for the sequences. We used this approach in MIDAS (Benlloch et al., 2002). In addition, specific clone libraries from the crystallizer ponds had been published earlier (Benlloch et al., 1996, 2001).

The alternative to cloning is to separate the PCR products by electrophoresis. There are several ways to do this (Fig. 1). All these techniques produce a "fingerprint" of the community analyzed. In theory, each microorganism in the community should produce a characteristic band in a gel after electrophoresis. Thus, the fingerprint is a consequence of the microorganisms present in the metagenome. In reality each technique has several drawbacks and the ideal situation is seldom achieved. In general, however, the fingerprints are representative of a community: the same community will produce the same fingerprint if the treatment is the same. Thus, the fingerprints can be used to compare communities with each other. Several of these techniques were used in MIDAS: DGGE, RISA (Casamayor et al., 2002a), T-RFLP (Casamayor et al. 2002a,

Øvreås et al., 2003) and others had been used in the past such as RFLP (Martínez-Murcia et al., 1995) and LMW RNA (Casamayor et al., 2000). Since the genes amplified depend strongly on the primers, we used different combinations of primers to test how similar the results would be and to maximize the diversity retrieved from the environment (Fig. 2).

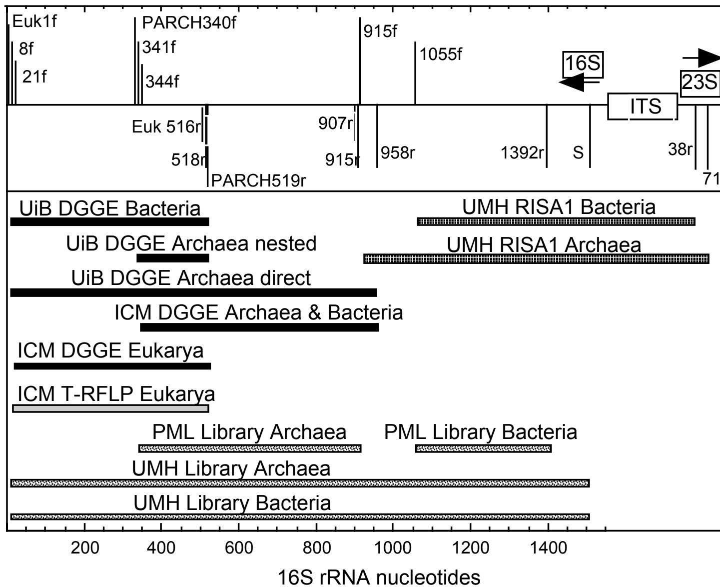

Figure 2. Upper panel shows the forward and reverse primers used in MIDAS. The lower panel shows the location and length of the corresponding PCR products used by different laboratories and techniques.

Since PCR is known to have some biases and problems, is there an alternative way to analyze the metagenome without the use of this technique? There certainly is. In Fig. 1 I show two alternatives. In one option, the environmental nucleic acids are used directly to analyze melting properties or to do hybridizations with nucleic acids from other samples in order to compare them. The prize one pays for not using PCR is the lesser resolving power of these approaches. In MIDAS we used DNA melting profiles and reassociation kinetics to explore this approach (Øvreås et al., 2003). In addition, we had used the low molecular RNA technique in a preliminary study of solar salterns (Casamayor et al., 2000).

Finally, one can clone the metagenome directly using what is termed a "shotgun" approach. The result is a huge library with tens or hundreds of thousands of clones. This is an extremely powerful approach, since all the genes from the community end up

in a library accessible for analysis. Here the prize is paid in terms of cost and time for screening and sequencing these clones. In fact, this approach has been used very few times in microbial ecology (Schmidt et al., 1991). Only recently are the necessary screening and sequencing capabilities becoming adequate to study such large libraries (Venter et al., 2004).

3.3. IDENTIFICATION

Both cloning and some of the fingerprinting techniques allow a more refined analysis. For example, T-RFLP results in a series of fragments of different sizes that produce separate peaks after electrophoresis. These peaks can be compared to a database and, in principle, the organism responsible for each peak can be identified. This is frequently not the case, because several organisms may produce the same peak. Also, new organisms (absent form the data bases) will not be identified. In SSCP, DGGE and TGGE bands can be cut and sequenced. The sequences can be compared to databases. In this case, each organism will produce a unique sequence and new sequences will appear as separate branches in phylogenetic trees. These approaches are, therefore, much more powerful. However, they also have their own limitations. The length of the sequence for example, is limited to about 600 bp in DGGE and TGGE. This may be insufficient for proper phylogenetic analysis.

In the case of clone libraries, the objective is to sequence as many clones as necessary to have a representative picture of the microbial diversity in the community. The more clones are analyzed, the more new OTUs appear. In principle, the curve of OTUs against clones analyzed should eventually reach an asymptote (the total number of OTUs in the library). In aquatic ecosystems, the total number of OTUs has been estimated to be around 80 on average, and this number could potentially be detected after sequencing slightly over 100 clones (Kemp and Aller, 2003). Libraries from the salterns were much smaller (number of clones between 9 and 31), however, the coverage was quite high (between 70 and 80%), suggesting that the total number of OTUs was not excessively large (Benlloch et al., 2002).

The end result of the analysis is a list of the sequences retrieved from the original metagenome. Thus, we are now in a position to develop probes against these sequences and use them in quantitative hybridizations. This step will provide stronger proof that the sequence was not an artifact. Further, under ideal conditions it will allow quantification of the different microorganisms in the sample. As shown in Fig. 1, hybridization can be done against DNA (Southern hybridization) or against RNA (Northern hybridization). Further, it can be done against the nucleic acids, against the clone colonies or against the whole cells themselves (fluorescent in situ hybridization, FISH). In MIDAS we did not carry out this final step, except for FISH with probes against *Salinibacter* (Antón et al., 2000). In addition, FISH with probes against the square arcahea (“Haloquadratum”) had also been conducted in a previous study of the crystallizers by Antón et al. (1999).

4. Results

4.1. RICHNESS

Microscopy is the technique of choice for the morphologically recognizable microorganisms. These can be reliably counted. In the past we had used Utermöhl's technique to count the number morphotypes in two salterns, Santa Pola and Trinitat (Pedrós-Alió et al., 2000a). In MIDAS we limited the counts from this technique to the phototrophic microorganisms (Estrada et al., 2004). However, many small eukaryotes and most prokaryotes cannot be discriminated by microscopy. Therefore we resorted to molecular techniques.

It has been estimated that microbial phylotypes constituting between 0.5 and 1% of all the cells in a particular ecosystem will produce a visible band in a DGGE gel (Muyzer et al., 1993; Casamayor et al., 2001). Therefore, the number of bands in a DGGE provides an estimate of the abundant members of the community. DGGEs were run for Eukarya, Bacteria and Archaea separately along the salinity gradient (See Figs. 3 and 4 in Casamayor et al., 2002a).

The number of different OTUs in clone libraries provides a second estimate of richness. The number of different OTUs increases as one analyzes more clones from the library. Eventually, the curve of OTUs vs. clones should approach an asymptote. This asymptote, however, may not be easy to reach. For example, more than 1000 clones may have to be sequenced with the consequent cost in time and expense. There are different statistical methods to estimate this asymptote when the library has a lower number of clones. For example Good's coverage index or Chao 1 indicator (see a review in Kemp and Aller, 2003). In the Santa Pola salterns, previous experience suggested that a small number of clones would be sufficient. Thus, Benlloch et al. (1996) sequenced six bacterial clones from crystallizer CR30 and found that all belonged to the same alpha-proteobacterial cluster. Likewise, all the 12 archaeal clones sequenced were very closely related to one single phylotype that has later been named "Haloquadratum walsbyi". In MIDAS we constructed two sets of libraries (with different primers) from three different salinities: 8, 22 and 32% (Benlloch et al., 2002).

These techniques will now be used to obtain an order of magnitude estimate of the microbial richness across the salinity gradient. We will discuss Eukarya, Bacteria and Archaea separately. When appropriate we will also use results from other techniques such as FISH (Antón et al., 1999, 2000), T-RFLP or DNA melting and reassociation kinetics (Øvreås et al., 2003).

4.1.1. Eukarya

Figure 3 shows the number of eukaryal OTUs obtained with different techniques across the salinity gradient. The number of DGGE bands was around 30 at the lower salinity ponds. Between 8 and 15% salinity, the number of bands decreased abruptly and remained at around 10 bands through the rest of the gradient. The empty squares show the number of OTUs of phototrophic eukaryotes determined by light microscopy. There was also a decreasing trend, although it was not completely parallel to that of DGGE bands. At low and high salinities, the number of OTUs was lower than the number of DGGE bands. At intermediate salinities, the two numbers were similar. In principle,

the number of phototrophic OTUs should be lower than, or equal to, the number of DGGE bands, since the latter include both phototrophs and heterotrophs. Thus, the two estimates are reasonably consistent with each other despite the completely different techniques used.

The filled squares show the total number of OTUs (photo- and heterotrophic) obtained by inverted microscopy in Santa Pola in a previous study (Pedrós-Alió et al., 2000a). The coincidence between DGGE bands in 1999 and OTUs in 1993 is striking up to 30% salinity, when the former are much higher than the latter. A similar OTU count for another saltern (Trinitat) carried out in 1994, also shows an extremely similar range of values and decrease pattern across the gradient. Thus, it seems that an estimate of 30 OTUs at low salinities and 10 at intermediate salinities is quite robust.

At the highest salinities (corresponding to the crystallizers) there was a discrepancy between DGGE bands and all microscopic counts. We think there is a problem with DGGE at this end of the spectrum (see below) and will consider the 2 to 3 microscopic OTUs as our best estimate for the crystallizers.

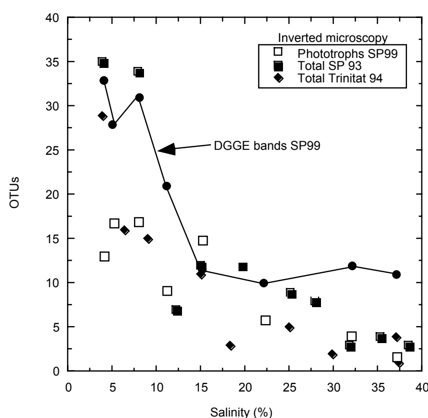

Figure 3. Number of eukaryotic OTUs across the salinity gradient. Filled symbols show number of DGGE bands. Squares and diamonds show microscopic counts.

4.1.2. Bacteria

Figure 4A shows the number of bacterial OTUs obtained with different techniques across the salinity gradient. Microscopy is not useful in the case of bacteria, and all approaches had to be molecular. Two types of DGGE were run: with bacterial universal primers (filled symbols, Casamayor et al., 2002a) and with primers biased in favor of oxygenic phototrophs (empty symbols, Estrada et al., 2004). These latter primers will mainly amplify sequences from phototrophic bacteria and chloroplasts and, therefore, cannot be compared directly to the DGGE with universal primers. The number of DGGE bands oscillated up and down between 9 and 15. Altogether it was surprisingly constant.

This number could be compared with the number of OTUs from the three sets of clone libraries (Fig. 4A). Clone libraries obtained with primer set 1055f-1392r are labeled as number 1 and clone libraries obtained with primer set 8f-1392r are labeled number 2. Between 10 and 31 clones were examined for each library and the number of OTUs is shown by the lowest edge of the vertical bars. Using the distribution of clones and OTUs, a coverage value was calculated and this coverage was then used to estimate the total number of OTUs in the sample (shown by the change of color in the vertical bars). Finally, the Chao 1 indicator was calculated giving another estimate of the OTU richness in the libraries (shown by the upper edge of the vertical bars). Since the number of clones examined was moderate, I have also added the lower and upper estimates for the two libraries combined (1+2 in Fig. 4). Regardless of the estimate used, there are two clear conclusions. First, the OTU richness did not change significantly along the gradient. And second, the number of OTUs from clone libraries was rather similar to the number of OTUs from DGGE. Thus, we will accept that the richness of bacteria was around 10 OTUs throughout the gradient.

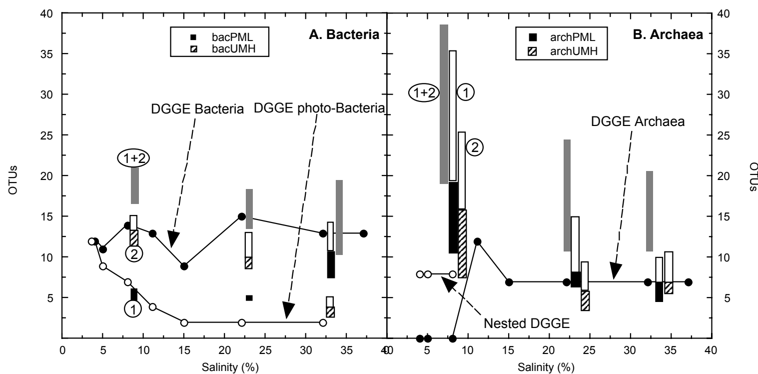

Figure 4. Number of OTUs across the salinity gradient. 1 and 2 are two sets of libraries while 1+2 is the addition of both (see text).

4.1.3. Archaea

Figure 4B shows the number of archaeal OTUs obtained with different techniques across the salinity gradient. Similar approaches than for Bacteria were used. The number of DGGE bands was zero for the lowest salinity ponds. Then it increased to 12 at 11% salinity and stabilized at 7 for the higher salinity ponds. In order to check whether the absence of bands at lower salinities was a consequence of the detection level of the technique, a nested PCR was performed. First the primer set 21f-958r was used to produce an amplicon that was subjected to a second PCR with the primer set PARCH340f and PARCH519r (see Fig. 2). The amplification products of this second reaction were run in another DGGE. The results are shown in Fig. 4B as empty circles. Thus, the OTU richness for Archaea was similar across the gradient. However, the very

low abundance of these Archaea at lower salinities combined with the large abundance of other microorganisms had put them below the detection level of conventional DGGE.

Results form the clone libraries are shown with the same conventions as for Bacteria. In the case of Archaea, the number of OTUs was higher at 8% than at 22 or 32%. This was surprising, since the abundance of Archaea is known to increase with salinity (Antón et al., 1999; Oren, 1993; Pedrós-Alió et al., 2000a; Rodríguez-Valera, 1988). Thus, OTU richness was inversely proportional to abundance. It is also interesting to see that the lower estimates of richness from clone libraries were similar to those from DGGE, while the higher estimates were three or four times higher. As mentioned earlier, DGGE will retrieve only those members of the community that constitute more than 0.5% of the total number of microorganisms. Clone libraries, on the other hand, can be made as large as desired so that, in principle, more OTUs can be retrieved with this technique than with DGGE. Results from Santa Pola are consistent with these differences between techniques. However, they suggest that the difference between the two was not large. In summary, the number of archaeal OTUs was approximately between 10 and 20 throughout the gradient, with a tendency for larger values at the lower salinity range.

4.1.4. Viruses

Diversity of viruses has escaped analysis until very recently. The problem is that viruses are obligate parasites and in order to identify them one needs to first culture their hosts. Since most host microorganisms from natural environments are difficult to isolate, the task of studying viral diversity was impossible. The use of PCR techniques is not appropriate either, because the genome of viruses is highly varibale. There are no universally conserved regions to use as templates for amplification. Thus, characterization of viruses could be done only through time-consuming electron microscopy. Guixa-Boixareu et al. (1996) examined the viruses in la Trinitat and Santa Pola salterns in this way and found a change in the relative proportions of different morphologies as salinity increased. These authors also found a peculiar lemon-shaped virus that seemed associated to the "Haloquadratum" cells and could detect viral infection in these square archaeon.

In the last decade, the use of pulse field gel electrophoresis (PFGE) has changed the situation. In this technique the whole genome or a few large fragments can be separated on a gel, so that the number of bands is related to the number of viral OTUs (Wommack et al., 1999). Díez et al. (2000) demonstrated that this technique could be applied to the hypersaline salterns and found reduced number of bands (between 1 and 8) with respect to those in marine environments (between 7 and 16, Wommack et al., 1999). During MIDAS, Sandaa et al. (2003) carried out a more detailed analysis of the whole salinity gradient through PFGE. They found that the number of OTUs varied between 4 and 12. It was highest at 11% salinity and decreased towards the crystallizers (4). Interestingly, viruses with relatively small genome sizes were important in the highest salinity ponds but absent from the lower salinity ponds. When cluster analysis was carried out, the band patterns grouped together into two clusters: one for salinities lower than 15 and a second for salinities higher than 22%. This was exactly what happened with the cluster analyses for microorganisms (Casamayor et al., 2002), showing the expected parallel changes between viruses and their putative hosts. As is the case for most microbial

ecolgy questions, the ongoing application of a metagenomic approach will provide a wealth of new information about virus diversity in the salterns.

4.1.5. The case of crystallizer ponds

The crystallizers offer a very especial ecosystem that has been studied for decades from different perspectives. Microscopically, samples from the crystallizers present a fascinating but not very diverse set of prokaryotes. Most prokaryotic cells in these ponds are square cells or curved rods. There are also some smaller cells and others with irregular morphologies. The two main morphotypes have now been identified molecularly and they have also been isolated in pure culture. The curved rod has been described as *Salinibacter ruber* (Antón et al., 2002) and the square cell as "Haloquadratum walsbyi" (Bolhuis et al., 2004; Burns et al., 2004). Using fluorescence labeled probes against these two microbes, Antón and colleagues showed that around 70% of the prokaryotic cells in crystallizers hybridized with the "Haloquadratum" probe (Antón et al., 1999) and around 15-20% with the *Salinibacter* probe (Antón et al., 2000). Thus, these two microorganisms together account for 90% of the prokaryotes in the crystallizers. Is this consistent with the remaining molecular evidence?

We have seen that clone libraries suggested between 3 and 18 OTUs for Bacteria and between 5 and 20 for Archaea. In both cases the lower number is the number of OTUs found and the higher number is the estimated richness. The numbers of bands in DGGE gels suggested similar numbers. This indicates that most of these OTUs would be found in very low abundance, so that together they would add up to the 10% of prokaryotes not accounted for by "Haloquadratum" and *Salinibacter*.

There is one intriguing fact. The number of OTUs in the crystallizers was similar to that in lower salinity ponds. In lower salinity ponds, OTUs were distributed through many different phyla (for example bacteria affiliated to alpha-, beta-, gamma-, and epsilon-proteobacteria, Bacteroidetes, and High GC Gram-positive bacteria in the 5% salinity clone library; Benlloch et al., 2002). Moreover, even within these phyla, most OTUs were fairly distant from each other. In the crystallizers the situation was very different. Only members of the Bacteroidetes group were retrieved among the Bacteria and 8 out of 10 OTUs affiliated with *Salinibacter*. For Archaea the situation was similar. In the 8% library there were distantly related unclassified euryarchaeota, Rice cluster V, Group III Archaea and Halobacteriaceae. In the crystallizer four OTUs affiliated with "Haloquadratum" and six others with different species of Halobacteriaceae. Thus, richness was similar between the different ponds, but at lower salinity diversity was at the level of phyla while at the crystallizers it was found at the level of species and genera.

In this scenario, the 70% of prokaryotes hybridizing with "Haloquadratum" probes would be distributed among different strains or species. The same would be true for the 20% hybridizing with *Salinibacter*. In fact, Antón et al. (2000) used probes for two different *Salinibacter* strains and found they accounted for different proportions of the total *Salinibacter* count.

The remaining 10% of prokaryotic cells must accommodate everything else. This includes all the Halobacteriaceae isolated from the crystallizers through the years. Thus, Benlloch et al. (2002) isolated from the crystallizer five Halobacteriaceae and three gamma-proteobacterial cultures that were not related to any of the sequences retrieved

by molecular methods. In this 10% we must also consider the cyanobacteria that were detected by light microscopy and by DGGE (Estrada et al., 2004). When the single cyanobacterial band obtained from the crystallizer sample was cut and sequenced it turned out to be 99% similar to *Euhalothece* spp., a halophilic cyanobacterium. These extremely halophilic cyanobacteria certainly deserve interest. Finally, the number of low abundance unknown prokaryotes may be very large. In this sense a metagenomic approach may provide many surprises (see last section).

The analysis of reassociation kinetics of the community DNA suggested that the metagenome had a complexity equivalent to 7 (22% pond), 13 (32% pond) and 4 (37% pond) times the complexity of the *Escherichia coli* genome (Øvreås et al. 2003). Using T-RFLP two *Salinibacter* and one archaeal OTU were obtained from the crystallizer (Øvreås et al. 2003). In addition, the base composition profiles obtained from DNA denaturation curves suggested the presence of two dominant populations in the crystallizer: the predominant one had a %G+C of 42-50 and the less abundant one 60-65% (Øvreås et al. 2003). These two populations probably correspond to *Salinibacter* and Haloquadratum. In effect, the %G+C content of *Salinibacter* strains has been shown to vary between 66.3 and 67.7% (Antón et al., 2002) and thus, the less abundant population probably corresponds to *Salinibacter*. The %G+C of "Haloquadratum" has not been published yet, but probably it will match that of the main population. Thus all techniques (including DNA melting and reassociation which do not depend on PCR, Fig. 1) point towards an assemblage formed by one main archaeal OTU ("Haloquadratum"), two *Salinibacter* OTUs and perhaps some other populations forming smaller percentages of the assemblage. The latter would be responsible for the complexity of 4 *E. coli* in the DNA reassociation analysis, the extra bands in DGGE, and the more rare clones in clone libraries.

4.2. SIMILARITY BETWEEN COMMUNITIES AND COSMOPOLITANISM

Fingerprinting techniques are very well suited to compare microbial communities among them. They allow processing of many samples with a reasonable investment of time and money and they provide reliable fingerprints of each community. In the salterns fingerprints have been taken with an array of different techniques: RFLP (Martínez-Murcia et al., 1995), ARDRA (Rodríguez-Valera et al., 1999), LMW RNA (Casamayor et al., 2000). In the MIDAS workshop several techniques were used simultaneously. Moreover, some of the techniques were carried out by more than one laboratory each with its own protocol. Thus, three DGGE, one RISA and one T-RFLP protocols were used for bacterial fingerprints, two DGGE and one RISA for Archaea, and one DGGE and one T-RFLP for Eukarya (Casamayor et al., 2002a). Despite the different concentration and DNA extraction procedures, despite the different sets of primers (Fig. 2) and the different techniques (Fig. 1) used, all produced the same general pattern: assemblages formed two main clusters, one with salinities up to 15% and another one with salinities above 22%. As mentioned earlier, the same clustering of samples was found for viruses with PFGE (Sandaa et al., 2003). This indicated that fingerprinting methods are quite robust to detect similarities and differences among communities.

The answer to the first question was that assemblages changed progressively along the salinity gradient but that at a certain salinity (around 20%) there was an abrupt and

major change in the composition of the microbial community. This is consistent with what is known about the physiology of microorganisms living at different salinities. At lower salinities, most microbes are halotolerant while at higher salinities most of them are halophilic (Rodríguez-Valera, 1988).

The second question concerns whether microbial species are cosmopolitan or endemic. This is very difficult to address because first one has to agree on what is a species. And second, if a given species is not found in a suitable place, it could always be argued that it had not been searched for carefully enough. Showing that a given microbe is absent from an ecosystem is nearly impossible. At any rate, here the relevant experiment would be to select a well-defined ecosystem and compare its microbes in different areas of the world. *Salinibacter* and "Haloquadratum" are extremely well suited for this kind of study. However, the MIDAS workshop was restricted to one saltern and thus, this problem was beyond its scope.

4.3. CULTURABILITY

Isolates show that richness is higher than that revealed by microscopy and molecular techniques. For the past thirty years at least, new microorganisms have been isolated from salterns and particularly from the crystallizers (see for example review in Oren 1993). In all cases these microorganisms could not be found to be abundant in the natural system. In the culturing attempt carried out during MIDAS, Benlloch et al. (2002) isolated twenty bacterial and archaeal cultures but only one was relatively close to sequence retrieved by cloning. Likewise, Benlloch et al. (2001) isolated over 30 cultures both in solid and liquid media. Most isolates were Halobacteriaceae of the genera *Halorubrum* and *Haloarcula*. However, none of these sequences could be retrieved from the same environmental sample directly. This is just one more example of the known difficulties in culturing the microorganisms that are abundant in nature. However, this does not mean that the latter are non culturable. The examples of *Salinibacter ruber* and "Haloquadratum walsbyi" show that abundant prokaryotes can be isolated in pure culture. In both examples, the key question was realizing that they were present. This was achieved by molecular methods, FISH in the case of *Salinibacter* and PCR and cloning in the case of "Haloquadratum". Once equipped with their 16S rRNA sequences, microbiologists could look for different strategies to enrich and eventually isolate them. In the case of "Haloquadratum" some strains seem to grow better in the presence of other bacteria, either *Salinibacter* or *Pseudomonas halophila*, and this may have caused difficulties in the past (Bolhuis et al., 2004). In the case of *Salinibacter* the problem was that its colonies were very similarly colored to those of Haloarchaea. Only when its presence was realized, could these colonies be distinguished (Antón et al., 2002).

In conclusion, the sequences retrieved by molecular methods provide the first steps in identifying and searching for the abundant microorganisms in nature. Then, culturing methods have to be modified to enrich them. In this step probes can be used to track the enrichments that are more promising. Eventually, the right conditions can be found. As pointed out by F. Rodríguez-Valera (personal communication), most culturing methods were developed more than a century ago to isolate human pathogens. It is no wonder

than environmentally relevant microorganisms are difficult to cultivate with such methods.

4.4. EVENNESS

The salinity gradient provides examples of both extremes. At the low salinities, a number of different OTUs seem to be present in relatively similar abundance. This is based on the band intensities in the DGGE, which is not a very good way of quantifying the microorganisms in situ. But at least it provides a first approximation to the question. At the opposite end of the gradient, the community clearly has a low evenness, since two or three organisms make up 70% of the cells. So the conclusion is that in this aspect, microbial communities are not different from those of animals and plants: both low and high evenness can be found in different ecosytems.

4.5. PATTERNS OF DIVERSITY

The salinity gradient provides an analog of other types of gradients from mild to extreme conditions such as moving up a mountain for birds or plants. In this sense, the diversity of the salterns follows a trend parallel to that of other living beings: as conditions become more extreme, both richness and evenness decrease. This is quite obvious from both the fingerprinting and the clone library analysis. Estrada et al. (2004) compared how different diversity indexes changed along the gradient. They chose phototrophic microorganisms and determined their diversity with independent techniques: inverted microscopy, HPLC pigment analysis, flow cytometry, and DGGE. In all cases there was a trend to lower diversity indices as salinity increased. In this respect, the salterns microbial communities behave in a similar way to those of animals and plants.

Microbial communities, however, present an interesting peculiarity. In the crystallizers richness is low and evenness is high. But, as presented earlier, each OTU in the crystallizer is actually a cluster of sequences that are very closely related to each other (above 98-99% similarity in the 16S rDNA). This microdiversity has been observed in other environments (Casamayor et al., 2002b; Fuhrman and Campbell, 1998) and seems to be a characteristic of microbial populations. Whether this microdiversity is ecologically relevant is unknown (Acinas et al., 2004). Again, the inhabitants of the crystallizers seem ideal candidates to analyze this question.

5. Perspectives

The advent of genomics has revolutionized most areas of the biological sciences. For one thing, biology has transformed itself from a data-poor to a data-rich science (Vukmirovic and Tilghman, 2000). This means that previously most effort had to be devoted to collect data experimentally. Now, sequence data become available at a very high speed. The challenge is not to get this kind of data anymore, but to use the data in the most efficient way.

In microbial ecology there are two of the different genomics approaches that are already very successful. One is the sequencing of the complete genomes of

environmentally relevant microorganisms. This provides a very large amount of information on the genes present, the potential metabolic pathways, adaptations to nutrient limitations and so on. A beautiful example of the usefulness of this approach was the publications of the genomes of four marine cyanobacteria in 2003 (Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003). Another approach is the sequencing of the metagenome of particular environments. This has already been done for relatively simple ecosystems such as an acid mine drainage (Tyson et al., 2004) and for relatively complex ecosystems such as a soil (Rondon et al., 2000) and Sargasso Sea waters (Venter et al., 2004). The advantage of genomic approaches is that all the diversity of genes present in nature becomes amenable to study. Obviously the salterns are an excellent model system for genomic approaches. The genomes of different *Salinibacter* and "Haloquadratum" strains have already been completed or will be sequenced shortly. On the other hand, attempts to sequence the metagenome from crystallizers are also ongoing. In my opinion the situation we had before genomics was like an explorer trying to describe a nocturnal landscape. With the help of a spotlight the explorer crisscrossed the landscape carefully recording what he/she saw in the few meters illuminated by the spotlight and tryed to put all this information together in a mental map of the landscape. With the advent of genomics, the sun has come out and now the whole landscape is visible. What an opportunity and what a challenge!

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Biodata of **Helga Stan-Lotter**, author of ***"Isolation of Viable Haloarchaea from Ancient Salt Deposits and Application of Fluorescent Stains for In Situ Detection of Halophiles in Hypersaline Environmental Samples and Model Fluid Inclusions"***

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ISOLATION OF VIABLE HALOARCHAEA FROM ANCIENT SALT DEPOSITS AND APPLICATION OF FLUORESCENT STAINS FOR *IN SITU* DETECTION OF HALOPHILES IN HYPERSALINE ENVIRONMENTAL SAMPLES AND MODEL FLUID INCLUSIONS

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1. Introduction

From alpine rock salt deposits of Permian and Triassic age viable, extremely halophilic Archaea (haloarchaea) have been isolated and some of them were described as novel species (Denner et al., 1994; Gruber et al., 2004; Stan-Lotter et al., 1999, 2002). Amplification of 16S rRNA genes, using the polymerase chain reaction with bacterial and archaeal primers, indicated evidence for the presence of a large haloarchaeal community in the salt deposits (Radax et al., 2001).These results suggested the possibility of microbial long term survival under conditions of desiccation, although it is enigmatic which mechanisms haloarchaea might use, since they are not known to produce resting stages such as spores (Grant et al., 1998; McGenity et al., 2000). The apparent longevity of the haloarchaeal isolates in dry salty environments is of interest for astrobiological studies and the search for extraterrestrial life. On Earth, microorganisms were the first life forms to emerge and were present perhaps as early as 3.8 billion years ago (Schidlowski, 1988, 2002). Halite has been detected in meteorites, some of which stem from Mars (Gooding, 1992; Treiman et al., 2000; Zolensky et al., 1999). The recent data from the Martian rovers Spirit (http://www.msnbc.msn.com/id/5166705/) and Opportunity (http://www.missionspace.info/news/merupdate/saltwater.html) suggested the formation of some Martian deposits from concentrated salt water. If Mars and Earth had a similar geological past (Nisbet and Sleep, 2001; Schidlowski, 2002), then microbial life, or the remnants of it, could still be present on Mars.

The search for life in the solar system and beyond is a goal of several space agencies in the 21st century (Foing, 2002). The development of suitable methodology for life

detection is a mandatory prerequisite for these plans. Identification of potential microbial forms within presumed sediments or perhaps also fluids will have to be perfected prior to deployment of probes and also for future return samples; therefore, sensitive *in situ* methods will be of great interest. Staining with fluorescent dyes produces intense signals which allow detection of single microbial cells, and might thus be considered for the use in the search for life in extraterrestrial samples or environments.

In this contribution, an overview of haloarchaeal isolates from alpine Permo-Triassic salt and their classification is presented, and the potential of the LIVE/DEAD kit and DAPI stain for the detection of microorganisms in hypersaline environmental samples and in fluid inclusions is discussed, in view of astrobiological studies.

2. Haloarchaea from Permo-Triassic Rock Salt

Halococcus salifodinae DSM 8989^T, strain BIp was the first isolate from an alpine salt deposit which was formally described as a novel species (Denner et al., 1994). This strain was isolated from a dry rock salt sample obtained from the salt mine near Bad Ischl, Austria, from a depth of approximately 650 m below surface. Based on the stratigraphic position of the horizon, palynological findings and sulfur isotope studies (see Radax et al., 2001, and references therein), the salt is believed to have been deposited during the Permian and early Triassic periods (213 to 286 million years ago). The cells of *Hcc. salifodinae* grow in tetrads which are often arranged in larger clusters (Fig. 1).

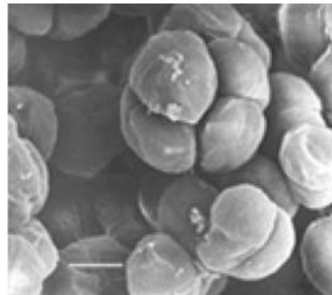

Figure 1. Scanning electron micrograph of *Halococcus salifodinae* BIp DSM 8989^T. Bar, 0.5 µm. Photograph taken by G. Wanner.

Since the species *Hcc. salifodinae* included initially only one strain, additional rock salt samples from the same site were investigated and several halococcal isolates, strains N1 to N7 and H1 to H4, were obtained and cultured (Stan-Lotter et al., 1999). Moreover, two independently isolated strains, Br3 from solution-mined brine in

England and BG2/2 from a salt bore core of Berchtesgaden, Germany, were found to resemble *Hcc. salifodinae* in morphology (Fig. 2). All strains were analyzed in detail according to recommended minimal standards for the description of Halobacteriaceae (Oren et al., 1997). They were found to possess identical 16S rRNA gene sequences, similar G+C contents (62-63 mol%), similar composition and relative abundances of polar lipids, antibiotic susceptibility, enzymatic activities and Fourier-transform infrared spectra. Therefore, all isolates were assigned to the same species and deposited as *Hcc. salifodinae* DSM13046 (Br3), DSM 13045 (BG 2/2), DSM 13070 (N1) and DSM 13071 (H2), respectively (Stan-Lotter et al., 1999).

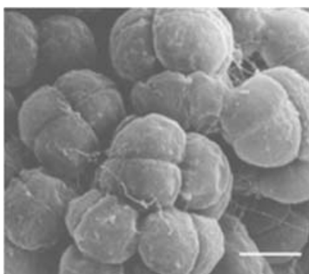

Figure 2. Scanning electron micrograph of *Halococcus salifodinae* Br3 DSM 13046. Bar, 0.5 µm. Photograph taken by G. Wanner.

The data demonstrated that in geographically separated halite deposits of similar geological age identical species of halococci are present, and that *Hcc. salifodinae* strains were re-isolated from the same site eight years after the first rock salt samples had been taken. These results supported the notion that the halophilic isolates from subterranean salt deposits may be the remnants of populations which inhabited ancient hypersaline seas. A second novel species was isolated from rock salt pieces which were obtained three days after blasting operations from the same salt mine. This strain differed significantly, according to its 16S rRNA gene sequence and chemotaxonomic characteristics, from known strains belonging to the genus *Halococcus* and was named *Hcc. dombrowskii* DSM 14522^T (Stan-Lotter et al., 2002). Cells of *Hcc. dombrowskii* grew in smaller aggregates which consisted of 4-8 cells (Fig. 3).

Both *Halococcus* strains have not yet been isolated from a surface location. A recent isolate from a deep drilling bore core of the alpine salt mine near Altaussee was identified as a novel species of the genus *Halobacterium* and named *Hbt. noricense* DSM 15987^T (Gruber et al., 2004). The 16S rRNA gene of *Hbt. noricense* had more than 97% similarity to that of the sequenced *Halobacterium* species, strain NRC-1 (Ng et al., 2000); other properties were sufficiently different to justify classification into a separate species. *Hbt. noricense* is the first rod-shaped haloarchaeal isolate from alpine rock salt; the cells were on average smaller than those of known *Halobacterium* species, e.g. *Hbt. salinarum* NRC-1 (see Fig. 4).

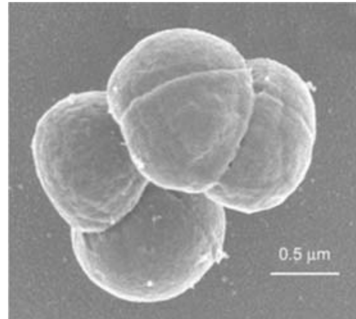

Figure 3. Scanning electron micrograph of *Halococcus dombrowskii* DSM 14522^T. Photograph taken by C. Frethem.

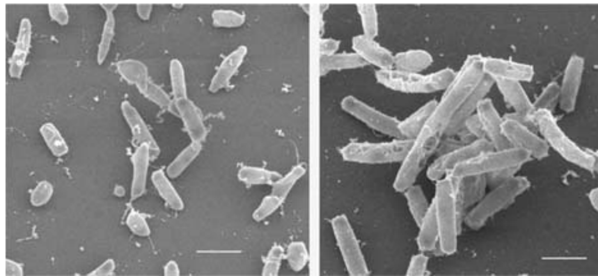

Figure 4. Scanning electron micrograph of *Halobacterium noricense* DSM 15987^T (left panel) and *Halobacterium salinarum* NRC-1 (right panel). Bars, 1 µm. Photographs taken by C. Frethem.

3. The Culture-Independent Approach

Although there were several reports of halophilic microbial isolates from halite deposits, only a few strains have been studied in detail, and as yet only eight (five independently isolated strains of *Halococcus salifodinae*, one strain of *Halococcus dombrowskii,* one strain of *Halobacterium noricense*; see above), including *Halosimplex carlbadense,* a strain from the Salado formation in New Mexico (Vreeland et al., 2002), were deposited in publicly available culture collections. The knowledge of the prokaryotic content of rock salt is still quite fragmentary, mainly because the classical procedures of microbial enrichment suffer from the notorious phenomenon of the "great plate count anomaly" (Staley and Konopka, 1985), since only a fraction of the existing community can be cultured in the currently used types of nutrient media (Amann et al., 1995). By using

extraction of DNA, following filtration of dissolved rock salt, and amplification of genes by polymerase chain reaction (PCR), we had previously obtained evidence for the presence of numerous novel haloarchaeal 16S rRNA genes in Permo-Triassic salt, some of which were similar to those of known haloarchaea, and some were corresponding to cultured haloarchaeal isolates from ancient salt deposits in other parts of Europe (Radax et al., 2001). Another DNA extraction revealed at least 12 different 16S rRNA gene species, which were designated phylotypes BI1 - BI12 (Stan-Lotter et al., 2003). None of the phylotypes was identical to sequences from databases. Phylotype BI8 corresponded to halococcal isolates from alpine rock salt, showing 99.6 % sequence similarity to the 16S rRNA gene. Seven phylotypes represented novel gene species, which were less than 97% similar to known sequences. Three phylotypes possessed 97-98% similarity to their closest cultured or uncultured relatives. Currently, similarities of 97% or less in 16S rRNA gene sequences are considered to indicate the delineation of a species (Stackebrandt and Goebel, 1994).

4. Fluorescent Staining of Microorganisms

For the direct enumeration of microorganisms in environmental samples, such as soil, marine and fresh waters, fluorescent dyes, e.g. acridine orange, 4',6-diamidino-2-phenylindole (DAPI) or others, are used in connection with epifluorescence microscopy and flow cytometry (for reviews see Hobbie et al., 1977; Kepner and Pratt, 1994). The LIVE/DEAD® *Bac*Light bacterial viability kit (referred to as LIVE/DEAD kit), which was developed by Molecular Probes (USA), proved especially useful, since it allows not only the estimation of total numbers of bacteria, but provides also an indication about the fraction of culturable and thus active bacteria in mixed populations, as opposed to non-culturable and supposedly "dead" bacteria. It shows excellent correlation with data obtained by other fluorescent dye-based methods (Gasol et al., 1999; Janssen et al., 2002) and has therefore found wide applications in monitoring the viability of bacteria in food and water samples (e.g. Boulos et al., 1999; Bunthof et al., 2001; Ramalho et al., 2001), or for biofilm (Strathmann et al., 2000) and soil research (Janssen et al., 2002). The LIVE/DEAD kit was developed for use with mesophilic microorganisms and moderate environmental conditions. We showed recently (Leuko et al., 2004) that it can also be used to stain microorganisms in extreme physicochemical surroundings such as high ionic strength, high and low pH, or high temperature. The LIVE/DEAD kit consists of the nucleic acid stains SYTO 9 and propidium iodide, which have emission maxima of 500 nm and 635 nm, respectively. Whereas SYTO 9 penetrates most membranes freely and has moderate affinity to nucleic acids, propidium iodide is highly charged and therefore normally cell-impermeant, but it will penetrate damaged membranes; since it possesses a high affinity for nucleic acids, it can displace weaker bound dyes, such as SYTO 9 (Haugland, 2002). This is the basis for the simultaneous application of both dyes for viability staining - viable cells with an intact membrane exhibit green fluorescence, whereas dead cells, due to a compromised membrane, show strong red fluorescence.

5. Staining of Haloarchaea with the LIVE/DEAD Kit and DAPI

5.1. ASSESSING THE RELIABILITY OF THE LIVE/DEAD KIT WITH *HALOBACTERIUM* AND *HALOCOCCUS* SPECIES

The haloarchaeal genera *Halococcus* and *Natronococcus* (Grant et al., 2001) possess thick cell envelopes which consist of a heteropolysaccharide (Steber and Schleifer, 1975; Niemetz et al., 1997); in contrast, the genera *Halobacterium*, *Haloferax*, *Haloarcula*, or *Natronomonas* are surrounded by a cytoplasmic membrane and an S-layer made of proteins or glycoproteins (Kandler and König, 1993). This difference in envelope composition is one of the characteristics for differentiation between halococci and non-coccoid haloarchaea; the latter will lyse within an hour or less by placement in distilled water, in contrast to halococci, which will keep their morphological features (Grant et al., 2001).

When cells of *Hbt. salinarum* NRC-1 had been placed in distilled water for 5 min prior to staining with the LIVE/DEAD kit, cell morphology was generally still recognizable, but only red fluorescence was observed (color plate, Fig. 5, right panel), which indicated non-viable cells. Determination of colony forming units (CFU) corroborated this assessment, since no colonies were detected, following incubation of plates for up to 4 weeks. Longer exposure of *Hbt. salinarum* NRC-1 to water (> 5 min) caused complete disintegration of cells which manifested itself as the appearance of string-like structures and debris, both of red fluorescence.

When cells of *Hcc. dombrowskii* DSM 14522^T were exposed for 30 or 60 min to distilled water, only a fraction of about 15-17% were apparently killed, as indicated by red fluorescence (a total of 750 cells were counted); the majority of cells was viable, following this treatment, as indicated by their green fluorescence (color plate, Fig. 6) and by growth on agar plates.

The results suggested that the membranes of non-coccoid haloarchaea became permeable by exposure to water within minutes, and that the resulting non-viability was readily apparent by the changing fluorescence of cells stained with the LIVE/DEAD kit; in contrast, cells of *Hcc. dombrowskii* DSM 14522^T were rather resistant to the action of water. If this was due to a different membrane composition of *Halococcus* cells, limited access of water to the membranes or other factors is not known. The LIVE/DEAD kit thus allowed a rapid evaluation of the status of haloarchaeal membranes and implicitly, viability of cells, following exposure to lethal treatments, as for instance low salt stress for haloarchaea.

5.2. DAPI STAINING OF HALOARCHAEA AND COMBINATION WITH THE LIVE/DEAD KIT

Staining with DAPI has been used for the enumeration of Bacteria (Porter and Feig, 1980) and some Archaea, predominantly methanogens (Lanoil et al., 2001), although at least one report cites enumeration of haloarchaea in crystallizer ponds (Antón et al., 1999). The classical procedures usually involve a fixing step prior to staining, to increase permeability for the stain. We used fixing with 60% ethanol (final concentration) for *Halococcus* species; color plate, Fig. 7 (left and middle panel) shows

cells of *Hcc. salifodinae* N1 (DSM 13070) and *Hcc. dombrowskii* DSM 14522^T, following staining with DAPI. Besides the morphological features of the cells, the nucleoids inside the cytoplasm could easily be discerned (color plate, Fig. 7). Similar staining was achieved with *Hcc. salifodinae* BIp, *Hcc. saccharolyticus* and *Hcc. morrhuae* (data not shown). The fixing procedure for non-coccoid haloarchaea was carried out without ethanol, since those cells would lose their shape following this treatment; instead, *Halobacterium* cells were embedded in agarose and fixed with formaldehyde, according to the procedure of Antón et al. (1999). Color plate, Fig. 7 (right panel) shows cells of *Hbt. salinarum* NRC-1, following staining with DAPI.

Legend to the color figures

Figure 5. Effect of low salt stress. Cells of *Halobacterium salinarum* NRC-1 were exposed to water for 5 min prior to staining with the LIVE/DEAD kit (right panel); cells shown in the left panel had not been exposed to water and were stained in the presence of 4 M NaCl. Bars, 2 µm.

Figure 6. Effect of low salt stress**.** Cells of *Halococcus dombrowskii* DSM 14522^T were exposed to water for 30 min (left panel) or 60 min (right panel) prior to staining with the LIVE/DEAD kit. Bar, 2 µm.

Figure 7. Staining of haloarchaea with DAPI. Coccoid haloarchaea were stained following fixing with ethanol; non-coccoid haloarchaea were stained following embedding in agarose and fixing with formaldehyde (Antón et al., 1999). Left: *Halococcus salifodinae* DSM 13070; middle: *Halococcus dombrowskii* DSM 14522^T; right: *Halobacterium salinarum* NRC-1. Bars, 2 µm.

Figure 8. Double staining with DAPI and LIVE/DEAD kit. Cells of *Halococcus dombrowskii* DSM 14522^T were fixed with ethanol, stained with DAPI as well as the LIVE/DEAD kit dyes and viewed with filters for DAPI (left), SYTO 9 (middle) and propidium iodide (right), respectively. Bar, 2 µm.

Figure 9. Fluorescent particles in environmental samples. Dissolved rock salt (a) from the Permian deposit at Altaussee, Austria, and Dead Sea water (b) were treated with the LIVE/DEAD kit without any prior preparation. Both types of samples contained colony forming units (see text). Hazy staining material in dissolved rock salt is shown in panels c and d. Bars, 2 µm.

Figure 10. Haloarchaea in fluid inclusions. Cells were stained with the LIVE/DEAD *Bac*Light kit prior to embedding in salt. Fluid inclusions were square or rectangular. Low (left panel) and high (right panel) magnification of *Halobacterium salinarum* NRC-1 cells, trapped in fluid inclusions for about 2 days. Epifluorescent microscopy was performed with a Zeiss Axioskop (for details, see Leuko et al., 2004).

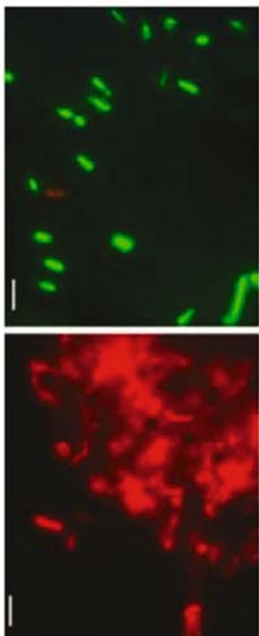

Figure 5

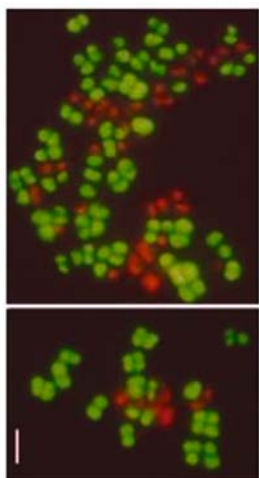

Figure 6

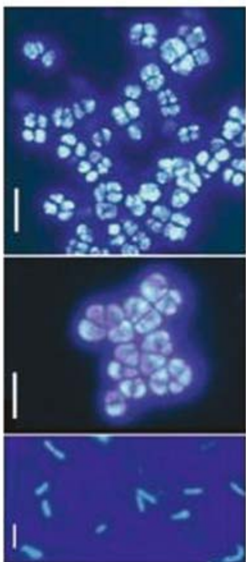

Figure 7

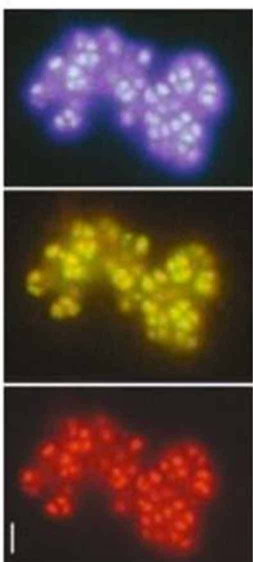

Figure 8

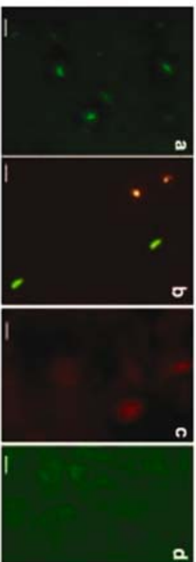

Figure 9

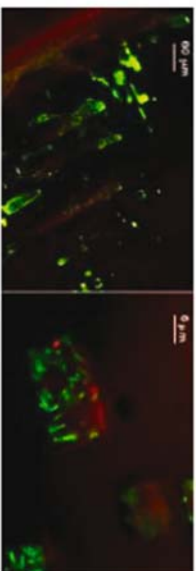

Figure 10

A combination of dyes was used for cells of *Hcc. dombrowskii* DSM 14522^T: ethanol-fixed cells were stained with the LIVE/DEAD kit and with DAPI (color plate, Fig. 8). All cells were of red fluorescence (color plate, Fig. 8, right panel), indicating loss of membrane integrity. The damaged cells took up DAPI, as has been observed before with dead bacterial cells (see Joux and Lebaron, 2000). Fluorescence of a yellowish colour was visible with the filter set for green fluorescence (color plate, Fig. 8, middle panel). Intermediate colours like yellow or orange have sometimes been observed for bacterial cells following staining with the LIVE/DEAD kit (Boulos et al., 1999). In our experiments, only coccoid haloarchaea, which often grow in clusters and aggregates, showed occasionally intermediate yellow-greenish colours; single cells of non-coccoid haloarchaea were of unambiguous (either red or green) fluorescence.

6. Environmental Hypersaline Samples

In this work we attempted to visualize directly the presence of halophilic microorganisms in dissolved rock salt from alpine deposits; in addition, samples from natural hypersaline brines such as the Dead Sea were examined. Figure 9a shows several particles of about 1-2 μm length with green fluorescence, which were detected in samples of dissolved rock salt from the salt mine in Altaussee, Austria, following staining with the LIVE/DEAD kit. According to their sizes and shapes, they could represent viable halophilic prokaryotes. Autofluorescent particles in dissolved rock salt were not observed with the microscopic settings used here; occasionally, larger irregular structures and hazy appearing materials were detected in dissolved rock salt, which apparently took up dyes to some degree and produced weak green or red fluorescence (color plate, Fig. 9c, d). The numbers of CFUs from dissolved rock salt varied - dependent on the origin of the salt and culturing conditions – between less than 10 CFU per ml in dissolved rock salt from the Salado formation to 130 ± 20 per ml in dissolved alpine salt (see Stan-Lotter et al., 2000).

Samples from Dead Sea water, which were taken in Jordan and had been stored at 4°C, contained rod-shaped particles of green fluorescence (color plate, Fig. 9b); some particles showed a transient yellow fluorescence following staining with the LIVE/DEAD kit. CFUs from the Dead Sea samples were between 180 and 240 per ml on agar plates containing M2S medium (Stan-Lotter et al., 2002).

7. Haloarchaea in Fluid Inclusions

Norton and Grant (1988) noted the preferential enclosure of halobacteria in fluid inclusions upon formation of crystals in the laboratory. Viability of cells under these conditions was retained for at least 6 months. The entrapment of haloarchaea in fluid inclusions could thus be a model system for the study of long term survival. To obtain micrographs of good contrast and clarity, we stained cells with the LIVE/DEAD kit prior to embedding in salt. Figure 10 shows examples of stained *Halobacterium* cells which were examined 2 days following their embedding in artificial halite. At low magnification, the bright green fluorescence of stained haloarchaea was outlining well

the morphology of the characteristic square or rectangular fluid inclusions of halite (Fig. 10, left panel). At higher magnifications, individual cells became visible (color plate, Fig. 10, right panel). The micrographs corroborated the entrapment and accumulation of haloarchaea in fluid inclusions rather than in the solid halite. The fluorescence of stained and salt-embedded haloarchaea remained stable for at least two weeks, when samples were stored in the dark at room temperature. Viability of unstained haloarchaeal cells in fluid inclusions, as judged from the determination of CFUs, was retained for at least 6 months, similar to the results of Norton and Grant (1988).

8. Summary

Viable haloarchaea were isolated repeatedly from Permo-Triassic rock salt; some strains were characterized in detail and found to represent novel species. PCR amplification of 16S rRNA genes of DNA extracts from the rock salt suggested the presence of a diverse halophilic community. Staining with the LIVE/DEAD kit was possible in the presence of high ionic strength, and the colours obtained with the LIVE/DEAD kit correctly predicted viable and thus culturable cells. The correlation between fluorescence and the presence of non-viable cells was unequivocal, as judged from haloarchaea damaged by water or ethanol, following staining with the LIVE/DEAD kit and DAPI. Particles of the size of microbial cells were detected by the LIVE/DEAD kit in hypersaline environmental samples. Staining with the LIVE/DEAD kit facilitated visualization of haloarchaea in fluid inclusions in salt.

The plans for the search for life in the solar system and beyond will constitute a challenge for microbiology and geomicrobiology. Fluorescent dyes which bind to nucleic acids – or similar molecules - may be suitable agents for life detection experiments. We suggest that terrestrial microorganisms ought to be tested, to find optimum stains and to develop methodology for future extraterrestrial materials - most likely return samples, or well-characterized meteorites. In addition, the methodology may be applicable to judging the forward contamination potential of spacecraft (see Rummel, 2001).

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HYDROCARBON DEGRADATION UNDER HYPERSALINE CONDITIONS

Some Facts, Some Experiments and Many Open Questions

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1. Introduction

The 1991 Gulf War oil spill – the largest in history – severely impacted not only Kuwait but also several hundred kilometers of the coast lines of Saudi Arabia, Bahrain and Qatar in an unprecedented manner (Fowler et al., 1993). It dramatically reminded the public and the scientific community of the still largely unsolved problem of hydrocarbon degradation under saline and/or arid conditions. The decrease of the total hydrocarbon concentration over the following years was mainly due to evaporation and weathering (Saeed et al., 1998; Sauer et al., 1998) and only slightly alleviated the severe environmental and socioeconomic consequences for the Arabian Gulf (Ahmed et al., 1998). As was shown recently, natural remediation appears to be extremely slow, especially in the coastal salt marshes. Reddy and coworkers found resilient hydrocarbons in the coastal sediments around West Falmouth, MA, USA, more than thirty years after a comparatively small spill of some 700,000 liters of fuel oil into the waters of Buzzards Bay (Reddy et al., 2002).

Since the first reviews on saline biodegradation (Oren et al., 1992) our understanding of the processes and organisms involved has considerably improved. It is, however, still far from comprehensive. Van Hamme et al. (2003) have provided an excellent summary of the latest developments in petroleum microbiology, Margesin and Schinner (2001a, 2001b) have discussed the biotechnological potential of halophilic microorganisms, and Berte-Corti and Fetzner (2002), as well as Ayala and Torres (2004) have concentrated on the mechanisms of hydrocarbon degradation. The status quo of anaerobic hydrocarbon metabolism was also presented recently (Boll et al., 2002; Widdel and Rabus, 2001).

This article now is focused on the aerobic degradation of aliphatic hydrocarbons under hypersaline conditions. Peyton and colleagues have provided a discussion of the degradation of aromatic hydrocarbons at high salt concentrations (Peyton et al., 2004). The present chapter first gives a general overview of the chemistry of aerobic microbial alkane degradations, followed by recent remediation studies under saline or hypersaline conditions, and finally an example of a wet hypersaline bioremediation is discussed in more detail: the clean-up of a former petroleum produced water pit in the Sultanate of Oman.

2. The Biological Chemistry of Aliphatic Hydrocarbon Degradation

Presumably the most common – but possibly only the most thoroughly studied – route for the microbial degradation of aliphatic hydrocarbons is the so-called α-hydroxylation pathway (= “terminal oxidation pathway”, Fig. 1). The alkane is hydroxylated at one terminal carbon by a monooxygenase. An alcohol dehydrogenase is thought to produce an aldehyde, which is oxidized to the corresponding long-chain carboxylic acid (= ”fatty acid”) by an aldehyde dehydrogenase. This acid is then converted into its coenzyme A-thioester and degraded into acetyl-CoA *via* the β-oxidation spiral before the carbon atoms are channeled into the primary metabolism through the tricarboxylic acid cycle.

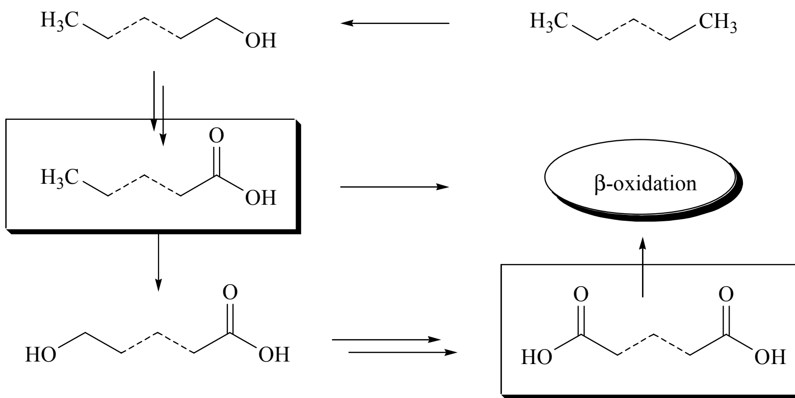

Figure 1. “Classical” pathways for the microbial degradation of aliphatic hydrocarbons: After an initial terminal hydroxylation a long-chain carboxylic acid is formed, which is either directly submitted to the β-oxidation spiral or undergoes prior bi-terminal oxygenation.

Alternatively, the fatty acid may be oxygenated at the other terminal carbon forming an ω-hydroxyalkanoic acid. This alcohol can be analogously oxidized through the corresponding ω-oxoalkanoic acid to an alkanedioic acid, which – after CoA ester formation – is submitted to degradation by β-oxidation. This modification is often referred to as “bi-terminal” or α,ω-oxidation pathway.

Since none of the non-polar substrates is freely water-soluble, all of the catabolic enzymes mentioned should be membrane proteins or at least membrane-associated proteins. For *Pseudomonas putida* Gpo1, this has recently been demonstrated (van Beilen et al., 2001). Two operons containing the genes for the oxidizing enzymes, as well as for rubredoxins, several regulatory proteins and a possible transport protein, were characterized and allowed to model the following scenario. The long-chain hydrocarbon diffuses through the outer membrane or is imported by a transport protein. The alkane hydroxylase is an integral membrane protein that scavenges the alkanes

from the inner membrane and hydroxylates them at the cytosolic side of the membrane. A soluble rubredoxin and rubredoxin reductase regenerate the monooxygenase. The fatty alcohols are then further oxidized by an alcohol and an aldehyde dehydrogenase, which are possibly also at least membrane associated (Van Hamme et al., 2003). The hydrophobic tail of the alkane would then only leave the membrane after its CoA thioester was formed (Fig. 2).

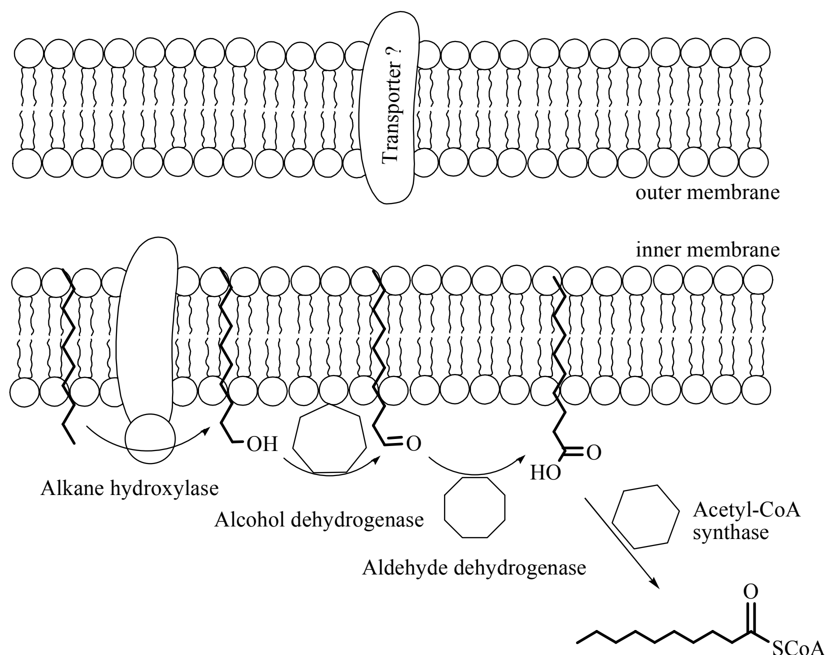

Figure 2. Possible scenario for the hydrocarbon activation in and at the inner membrane of *Pseudomonas pudida* Gpo1 (modified from Van Hamme et al., 2003).

Similar genes are being analyzed for a number of further organisms, such as an additional *P. putida* strain, *Burkholderia*, *Nocardioides* and *Acinetobacter*, and will soon shed more light on the mechanistic details of this pathway (e.g. Hamamura et al., 2001; Ishige et al., 2000; Marín et al., 2001; Tani et al., 2001). It must be emphasized again that the α-oxidation route is by no means the only pathway that has been described for the aerobic degradation of aliphatic hydrocarbons. Interestingly, different genes for multiple distinct alkane hydroxylases were even found in the same organisms (Van Hamme et al., 2003).

Relatively few mechanistic investigations have been reported for the subterminal (= ”internal”) oxidation pathway. Again, a monooxygenase is required to functionalize the alkane, but in this case the initial hydroxylation takes place at a methylene group - usually but not always - at carbon number two (Watkinson and Morgan, 1990) (Fig. 3).

The alcohol is oxidized to a ketone, which is transformed to an ester *via* an enzymatic Baeyer-Villiger reaction. Some of the enzymes involved in this pathway should be very promising for the biotechnological production of fine chemicals since chiral secondary alcohols are produced as intermediates (Bühler and Schmid, 2004; Bühler et al., 2003) and since the feasibility of scale-up and commercial exploitation of Baeyer-Villiger oxygenases has been recently demonstrated (Alphand et al., 2003).

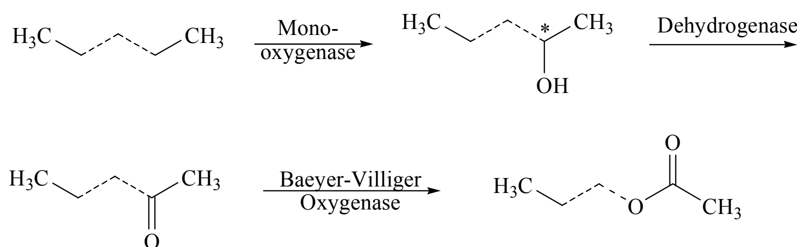

Figure 3. Internal (subterminal) oxidation of alkanes. Chiral secondary alcohols (*) are formed as intermediates during the process.

In 1988 Finnerty discovered that in certain *Acinetobacter* strains alkanes are converted to aldehydes *via* hydroperoxide instead of alcohol intermediates (Finnerty 1988). The dioxygenase, a homodimeric Cu-containing flavoprotein, utilizes molecular oxygen without the formation of radical intermediates (Maeng et al., 1996b). From the observation that peracids also occur in the cell extracts, it was suggested that the hydroperoxide produced is further oxidized, then reduced to an aldehyde and finally disproportionated to an alcohol and a carboxylic acid (Fig. 4) (Berthe-Corti and Fetzner, 2002; Finnerty, 1988).

Ample evidence for the existence of the discussed dioxygenases has accumulated (e.g. Maeng et al., 1996a). It does, however, appear questionable on the basis of the existing data that a further oxidation and reduction step is essential for the pathway. Whilst experimental data are missing, chemical logic suggests that the intermediate hydroperoxide – spontaneously or enzyme-catalyzed – may alternatively undergo an elimination to form the aldehyde directly without further intermediates (Fig. 5). This would also explain why stoichiometric investigations on the isolated dioxygenase have so far failed (Maeng et al., 1996b).

Finally, a conceptually different way of alkane functionalization was observed in a mutated *Rhodococcus* strain, designated KSM-B-3M – a desaturation instead of an oxygenation. Koike and coworkers isolated alkenes after administration of hexadecane, 1-chlorohexadecane and further 1-substituted alkanes (Koike et al., 1999). The double bond was always *cis* configurated and was found preferentially after the ninth carbon from the terminal methyl group, even if the substrate molecule contained further functionalities on the other end of the chain (Fig. 6). The authors hypothesize that the normal further degradation of the hydrocarbon would involve an oxidative cleavage of

the double bond, but that the alkenes accumulated because the genes for the oxygenating enzyme were destroyed by mutagenesis (Koike et al., 2000).

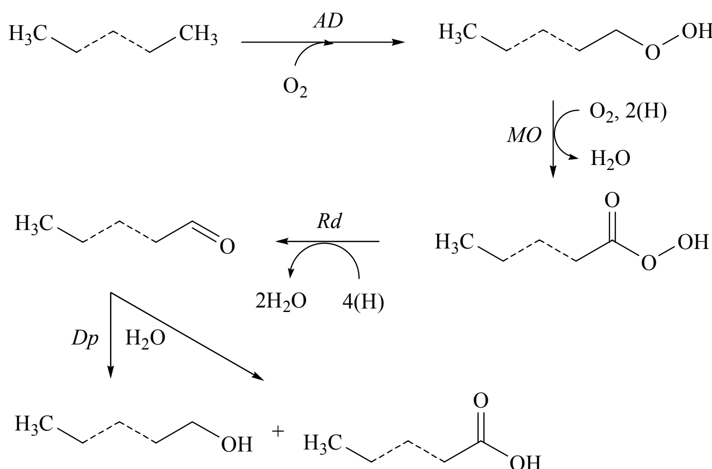

Figure 4. The so-called Finnerty Pathway for alkane degradation (modified from Berthe-Corti and Fetzner, 2002).

Apart from the α-oxygenation pathway all routes suggested leave many questions open. A number of genetic studies have recently shone light on gene clustering and regulation (Hara et al., 2004; Sei et al., 2003; van Beilen et al., 2004), but direct approaches to overproducing strains or isolated enzymes are still not available to a sufficient extent. Enzymatic hydrocarbon functionalization has an enormous industrial potential, both for remediation and for the conversion of petroleum compounds to higher value chemicals (Ayala and Torres, 2004). Thus further insight especially into non-conventional pathways could be invaluable.

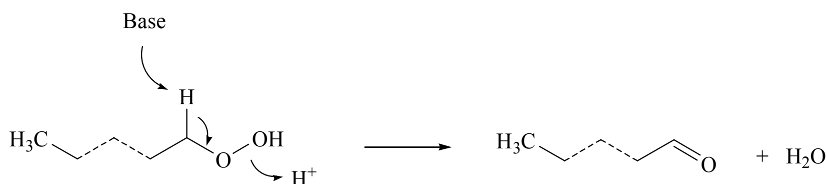

Figure 5. Suggested mechanistic short-cut for the so-called Finnerty pathway.

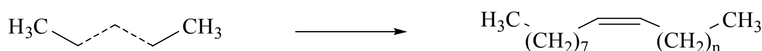

Figure 6. Central desaturation of alkanes according to Koike et al. (1999).

3. Hydrocarbon Metabolism in Hypersaline Environments

The first investigations on hydrocarbon degradation in sea water appeared in the 1970s (e.g. Soli and Bens, 1972), but until the beginning of the 1990s, only very few reports had described a possible hydrocarbon metabolism at hypersaline conditions. The general assumption that with rising salt concentrations – and thus reduced oxygen availability - the catabolic activity and the potential for hydrocarbon degradation decreased (Ward and Brock, 1978) led to a rather pessimistic assessment: "… attempts to demonstrate the degradation of otherwise relatively easily biodegradable pollutants in hypersaline lakes often yielded disappointing results (Oren et al., 1992)." Since then, however, a number of halophilic or at least halotolerant microorganisms have been characterized from hydrocarbon-contaminated saline ecosystems, and ecological or application studies have followed. However, most of this work is concentrated on marine or benthic ecosystems and was recently reviewed (Harayama et al., 1999, 2004).

The first two independent descriptions of a microbial alkane metabolism under truly hypersaline conditions appeared in 1990 (Bertrand et al., 1990) and 1991 (Kulichevskaya et al., 1991). Archaea isolated from a salt marsh in France and a saline oil field in Russia utilized petroleum hydrocarbons at salinities up to 32%. Two further extremely halophilic Archaea were found in association with petroleum hydrocarbons in former Soviet Republics: a *Haloferax* strain on a saline oil field in Kazakhstan (Zvyagintseva et al., 1995) and a *Halorubrum* on an oil field in Tatarstan (Zvyagintseva et al., 1998). Biodegradation experiments with these strains, however, were not reported.

Some further examples exist, where Bacteria were connected to alkane metabolism at high salt concentrations. Again from an oil field an extremely halotolerant streptomycete, *Streptomyces albiaxialis*, was isolated and shown to degrade petroleum hydrocarbons up to a salinity of 30% (Kuznetsov et al., 1992). A 15% salt concentration was tolerated by *Bacillus* and *Staphylococcus* strains that were employed in Japan in 1 m^3 batch fermentations to clean industrial waste waters contaminated with aliphatic hydrocarbons in a broad mixture of aromatics and phenols (Kubo et al., 2001). Presumably not aerobic but sulfur-reducing was the bacterial consortium that Bechtel et al. described from the Bahloul formation in Tunisia (Bechtel et al., 1996). Since it was isolated from hot hypersaline and sulfate-rich effluents of a salt dome it appears to degrade hydrocarbons close to salt saturation.

Finally, three recent publications describe the use of consortial halophiles for oil hydrocarbon degradation. The metabolism of diesel fuel by three bacterial mixed populations from contaminated saline Argentinean soils was monitored indirectly through the O_2 consumption. Although the cells remained viable up to 25% salt, hydrocarbon metabolism ceased at 17.5% and was maximal at below 10% (Riis et al., 2003). Catabolic activity up to 22% was described in Colombia for a bacterial consortium, which was immobilized on a solid support (Diaz et al., 2003). Four of the

strains from the consortium were reportedly able to grow in submersed cultures over a range of 0-32% salt. Finally, a rather unusual application for halophiles was suggested by Sakhno et al. (2003), who added halophilic Archaea to saline contaminated soil to increase the biological activity of the soil and stimulate the hydrocarbon degradation by *Pseudomonas* and *Rhodococcus* strains.

This short compilation may not be comprehensive. It is obvious, however, that detailed investigations on the mechanism of aerobic halophilic hydrocarbon degradation and on the biochemistry of the involved organisms are still not available. On the other hand, the increasing number of novel extremely halophilic or at least halotolerant oil-degrading microorganisms and the first applied investigations clearly show promise for further larger biotechnological applications. In the following section, one such application from the south of the Arabian Peninsula is described.

4. Remediation of a Former Produced Water Pit in Oman

The Sultanate of Oman is located on the south-eastern tip of the Arabian Peninsula, between 17-20°N and 52-60°E. The dominant landscapes of the country are arid gravel and sand deserts in the interior and salt marshes ("sabkhat") along the 2000 km long coastline to the Indian Ocean. The temperatures in summer may exceed 50 °C. Oil production is the major source of income in the country, and, although the modern oil-producing industry has gone a long way to prevent contaminations, small hydrocarbon spills can never be completely excluded. Bioremediation of contaminated soil and sand under the harsh climatic conditions of the Arabian dessert and the usually high salinity on oil fields can only be successfully attempted using indigenous extremophilic microorganisms.

On several visits to the Safah concession site of Occidental of Oman, Inc. (North-West Oman, 20 km from the border to the United Arab Emirates, Wilayat Ibri, 23.19° N, 55.45° E) between June 1999 and December 2000, soil, water and sand samples were collected from oil-contaminated sites in and around flare, drilling rig and produced water pits on the oil field, and from the "Modern Salt" solar salterns, which are associated with the oil field to produce drilling salt from the saline oil production water. Inoculation in halophilic standard media led to the isolation of 37 extremely halophilic microbial strains, both Bacteria and Archaea, from which 10 were virtually insensitive towards the presence of Oman Crude Oil.

On the Occidental concession site a former unlined produce water pit, the so-called "Halliburton Pit", had already been scheduled for clean-up and closure by the company prior to this study. The pit measures some 60 by 60 m, with a depth of some 10 m. The bottom consisted of a thick layer of salt and oil sludge, covered by fine dust and sand from the desert (Fig. 7). The hydrocarbon concentration of the first meter sediment varied between 10 and 40% (w/w). The infiltration was probably deeper, but no analytical data were available. Some of the isolated oil-tolerant halophilic microbes originated from the pit or its surroundings.

Figure 7. The "Halliburton Pit" in Safah before the start of the field experiment (top total, bottom details). The ground was covered with a thick layer of salt and oil sludge.

The pit was filled to an average depth of 1 m with slightly brackish water from a nearby well and fertilized with sludge from the camp's sewage plant. The ten selected "sturdy" microbial cultures were grown on a larger scale in the laboratory and were used to inoculate the water – creating a 3600 m^3 open-air bioreactor for the degradation of oil-contaminated sand and soil. Water loss due to evaporation was compensated by monthly refills (Fig. 8).

Figure 8. The water-filled "Halliburton Pit" in June 2001.

During regular visits to the Occidental site the physico-chemical and microbiological conditions in the Halliburton experimental pit were monitored. From all eight sides and corners of the pit, water was used to inoculate standard halophilic microbial growth plates on site, and sediment samples were collected for later analysis. Furthermore, temperature, pH and salt concentration were recorded weekly by Occidental's laboratory personnel.

4.1. GENERAL APPEARANCE OF THE PIT

The entire ground surface of the pit was covered with water with a depth of about 10-30 cm close to the edges and close to 2 m in the middle. After *ca* 3 months the water became greenish, indicating the presence of photosynthetically active green algae. Later, when the salinity rose, the greenish color disappeared, and the pit water appeared colorless and relatively clear.

During the weeks after the first filling, the salty sediments from the ground dissolved and, as expected, black viscous oil tar floated up to the surface, forming dense layers on the water surface along the banks of the pit (Fig. 9, left panel). After few months, however, no significant signs of hydrocarbon contamination could be observed any more (Fig. 9, right panel). About 20-30 cm below the water line, the sediments appeared slightly blackish. The color was not tightly absorbed but rather loosely mixed with the sediment since it was easily released into the water, when the sediments were poked or stirred with a rod. This gave rise to the concern that the tar was simply adsorbed by the sand. However, chemical analyses showed that these pigments did not contain significant amounts of hydrocarbons but consisted mainly of iron sulfide. Apparently, a consortium of sulfate-reducing microbes had formed in the microaerobic strata of the water body.

Figure 9. Details from the "Halliburton Pit": tar floating on the water surface some six weeks after filling and inoculation (left) and the clean banks after *ca* six months (right).

4.2. PHYSICO-CHEMICAL CHARACTERIZATION OF THE PIT WATER

Temperature, pH and chloride concentration of the water were recorded in weekly intervals over one year close to the surface at two opposing corners of the pit (Fig. 10). As anticipated, the temperature curve followed – with the expected short delay – the seasonal changes of the air temperature. The pH remained very stable and neutral throughout the observation period, indicating the presence of a “healthy”, stable microbial consortium.

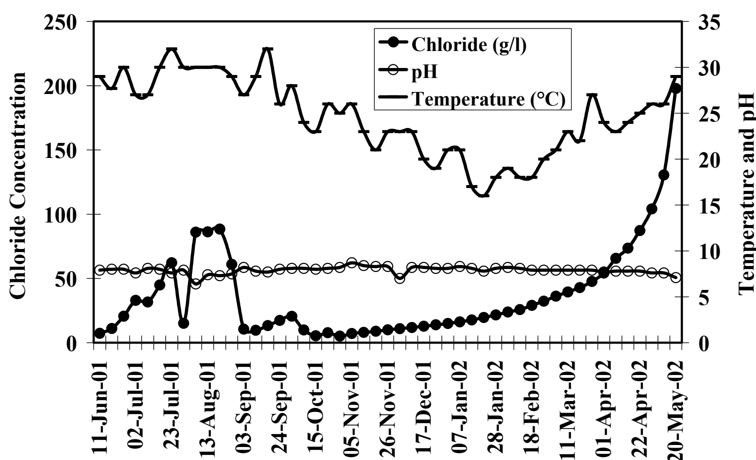

Figure 10. Physico-chemical monitoring of the pit from June 2001 to May 2002. Shown are the data for pH (open circles), water surface temperature (horizontal bars, both on the right ordinate), and chloride concentration (full circles, left ordinate).

The curve for the chloride concentration showed a remarkable behavior. Instead of the anticipated constant rise due to the dissolution of the salt from the ground sediments, a sudden drop of the salinity was observed following a refill some three months after the start of the experiment. It remained between 6-8 g l^{-1} chloride (corresponding to 10-14 g l^{-1} NaCl) for a period of several months. Obviously, stratification had occurred and, since for technical reasons the salinity had to be recorded close to the surface, relatively low values were obtained. In line with the principle of a solar pond, a layer of comparatively fresh or brackish water floated on a downward increasing concentration gradient of brine.

On November 15, 2001, temperature, pH, oxygen concentration and conductivity at different water depths in the north-western corner of the pit were recorded, using a tetrafunctional probe with a long cable. The probe was thrown into the water using varying cable lengths, and the reading was performed after the probe had reached the ground and the values had become stable (Table 1). The data disclosed the predictable extreme increase of the chloride concentration toward the bottom and toward the center of the pit. In addition, the temperature rose considerably with increasing depth. Both

observations strongly support the hypothesis of stratification and thus the formation of a solar salt pond.

TABLE 1. Temperature, pH, oxygen concentration and conductivity of the water in a transect starting in the north-western corner, facing towards the center of the Halliburton experimental pit on 15 November 2001. The readings for $c(O_2)$ at 1 and 2 m may be incorrect due to the extremely high conductivity.

Depth (m)	Temperature (°C)	pH	$c(O_2)$ (ppm)	Conductivity (μS)
"0"	24.6	8.1	9.2	30.9
ca. 0.25	28	8.36	13.8	30.6
ca. 0.5	31.9	7.2	6.7	70.3
ca. 1	41	6.4	>50	>999
ca. 2	42	6.4	>50	>999

Since a more homogeneous salt distribution was desired to facilitate the establishment of a stable microbial consortium, the construction of an aeration or agitation device was considered. However, after a period of strong winds, the gradient collapsed, and the salinity at the surface resumed its rise and leveled close to the saturation concentration at slightly above 200 g l^{-1}.

4.3. MICROBIOLOGICAL MONITORING

Viable cell counts (colony forming units CFU) in the pit waters were performed by direct aseptic spreading of the surface pit waters on plates (triplicates) at intervals of 2-3 months. Five different common halophilic media with salinities ranging from 10-25% were employed. The number of CFUs on the individual plates was not very high, but the microbial diversity in the pit must have been substantial since totally different organisms were isolated in any one analysis on the different media. Results such as those from 15 November 2001 (Table 2) were typical for the entire first year of the experiment. Interestingly, the number of CFUs from the water at various locations around the pit varied strongly between each other and for each location from visit to visit. It appeared to be much stronger influenced by the climatic conditions, such as the prevailing wind direction, than by the selection medium or the recorded salinity.

TABLE 2. Number of viable halophilic cells (colony-forming units) per ml in the surface water of the Halliburton experimental pit on 15 November 2001, plated on Yeast Seawater (YSW) and Halobacterial Standard Medium (HSM).

Location	Temperature (°C)	pH	CFU (YSW)	CFU (HSM)
East	23.3	8.4	5	0
Northeast	23.6	8.4	0	65
North	23.6	8.4	>100	>100
Northwest	23.8	8.4	>100	>100
West	24.2	8.4	18	3
Southwest	23.9	8.4	8	5
South	24.0	8.3	8	60
Southeast	23.9	8.0	0	5

The relatively low number of cells isolated from the water may well have been due to the limited number of selection media. A number of scanning electron micrographs, recorded of the surface of submersed pieces of metal or rock from the pit, always showed a dense coverage with cells and were optically free of hydrocarbon (Fig. 11). In agreement with earlier observations (Obuekwe and Alzarban, 1998) adherent microbes may have played an important role in the remediation.

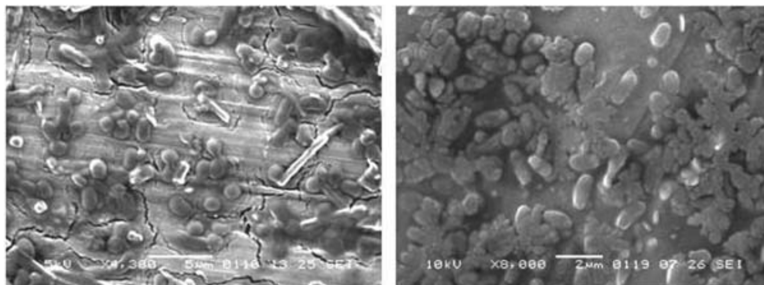

Figure 11. Scanning electron micrographs (gold-coated) of two small pieces of metal recovered from the pit.

Randomly chosen strains were submitted to biotransformation experiments in pure culture using pentadecane and eicosane. In most cases the mass spectrometric analyses of the metabolites suggested an α-degradation pathway (Fig. 1). Several strains produced small quantities of metabolites that could not yet be identified with the available instrumentation. In the medium extracts of one orange archaeon, however, aldehydes and carboxylic acids as well as long-chain esters were found. Since Archaea do not produce significant amounts of fatty acids (*cf. e.g.* Pugh and Kates, 1994, for examples), this can only be interpreted as an indication of a split pathway for hydrocarbon degradation: α-oxidation and subterminal routes proceed in parallel in the same organism at a salt concentration of 20%. Such split pathways are not frequently observed, but interestingly in another archaeon, *Haloarcula hispanica*, isoleucine biosynthesis appears to also proceed through parallel routes (Hochuli et al., 1999).

4.4. CLOSE-OUT ANALYSIS

In summer 2002 the hydrocarbon concentration close to the banks had fallen to below 1% (w/w), the legal limit for disposal in the Sultanate of Oman. Water refilling was halted, and the pit was allowed to dry out over the following months. Further analyses of the deeper sediments in the middle confirmed the drastic reduction of the hydrocarbon concentration in the pit. In January 2004, the entire sediments were excavated to a depth of about 5 m, where a layer of impermeable bedrock was reached (Fig. 12).

Figure 12. In January 2004 the sediments of the experimental pit were excavated, and the pit was closed starting from the northern side. The slope on the left used to be the southern bank.

Some very small pockets - totaling around 5 m^3 – were found, where the hydrocarbon concentration still ranged between 1 and 5%. The several thousand tons of the gravel, salt and sand mixture that remained contained only trace amounts of hydrocarbon. Due to the poor accuracy of the analytical data at the onset of the experiment a quantitative estimation of the total hydrocarbon degradation must remain tentative. From various but unsystematic analyses Occidental assumed a hydrocarbon contamination of the pit sediments between 10 and 40%, down to a depth of 1 m. No data existed from deeper layers. A very cautions calculation using the lowest values and equaling 1 m^3 to 1 t, gives for the 60x60 m surface area and 1 m depth a hydrocarbon content of 360 t. Presumably, however, the infiltration reached much deeper, and the average concentration was higher than 10%. After the end of the experiment the remaining contaminated material contained (5 m^3, now the higher concentration is used for the calculation) a maximum of 250 kg. Even if the trace amounts of the bulk material are taken into account, more than 300 t of weathered hydrocarbon were removed during the 18-months experimental period at extremely low costs.

5. Summary

In a so far unique field experiment, a former produce water pit in northern Oman was converted into an open-air bioreactor with the aim to device an economic and environmentally benign method for the bioremediation of oil contaminations in arid areas using a “wet” (= submerged culture) system. It clearly demonstrates the

feasibility of large-scale halophilic environmental biotechnology. A more detailed characterization of the involved microorganisms will follow.

This example, however, and the others presented above are still few in number. Much work remains to be done before halophilic microorganisms can be predictably employed in commercial environmental biotechnology or even in the production of fine chemicals. A number of further applications have been proposed (Margesin and Schinner, 2001b; Patzelt et al., 1998), but sometimes even the most basic biochemical knowledge, such as on the central metabolism of the organisms (Hochuli et al., 1999), is incomplete. Overall, only small additions have to be made to a conclusion that was drawn previously: “The above survey shows that our knowledge on the degradation of organic pollutants at high salt concentrations is still extremely limited (Oren et al., 1992)”.

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THE RELEVANCE OF HALOPHILES AND OTHER EXTREMOPHILES TO MARTIAN AND EXTRATERRESTRIAL ENVIRONMENTS

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1. Introduction

Life in various forms appears almost all over Earth, and most organisms live in environments considered "normal" from our anthropocentric point of view. There are other organisms, however, that are exceptional, living in very harsh conditions—they are termed extremophiles. The extremophiles live in extraordinary habitats; some of them are not able to grow in more moderate conditions or even to tolerate them for short periods. Such harsh environments are lethal for most other organisms. The majority of known extremophiles are unicellular—members of the domains Archaea and the Bacteria; there are also a few members of the Eukaryotes (protists and multi-cellular forms) among these exceptional organisms. The study of extremophiles and their habitats increases our understanding of the limits of life and the nature of the first organisms on early Earth, and in addition gives us an insight into the possibilities of extraterrestrial life (which is a part of the study of astrobiology).

Astrobiology, an interdisciplinary research field, is the new scientific branch of studies that includes the origin of life, various parameters of terrestrial environmental conditions and their inhabitants (extremophiles), astronomy, astrophysics and the possibilities for extraterrestrial life. In essence, astrobiology is all encompassing. From comparison of the physical and atmospheric conditions on some celestial bodies with the inhospitable niches and environments on Earth, we find that some extremophiles could serve as candidates or analogues for extraterrestrial life in the Solar System and beyond. Thus, the study and understanding of extremophiles is valuable for astrobiology. Among the current celestial places suitable as candidates for finding past and perhaps present life are Mars, the Jovian satellites such as Europa, and – for exploring initial stages of life as they may have occurred on young Earth – Saturn's moon Titan. Thus extremophiles, living at the edge of life, may serve as models for life forms in or beneath the surface of Mars, Jupiter's satellites (e.g. Europa), and other planetary bodies in the Solar System and beyond.

2. The Extremophiles

Most extremophiles are (micro)organisms that thrive in habitats which for other terrestrial life-forms are intolerably hostile or even lethal environments. They are

classified (see below) according to the conditions in which they grow as thermophiles, hyperthermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles, barophiles, etc. (Table 1). In addition to the various extremophilic factors one may add that microbial spores can withstand a variety of different hostile conditions in their dormant stage for long periods of time. There are some limits in long-term dormancy, namely, thermal decomposition and ionizing radiation (McKay, 2000). Extremophiles thrive in hot niches, ice, salt solutions, acid and alkaline media; some may grow in toxic waste, organic solvents, heavy metals while extremophiles are found as well in several other habitats that were previously considered inhospitable for life and toxic for organisms (see Table 1).

TABLE 1. The extremophiles on Earth and possibly on extraterrestrial bodies

Environmental parameter	**Type**	**Location and Conditions**	**Examples**
Salinity	Halophiles	In salt lakes (neutral, and alkaline pH). In salt solutions up to saturation. Synthesis of glycerol to balance the external osmotic pressure	Cyanobacteria, diatoms, flagellates, *Dunaliella*; *Halobacteriaceae.*
pH	Acidophiles Alkaliphiles	pH 0 - 4 some hot springs pH >9 in African soda lakes	*Picrophilus oshimae, Cyanidium caldarium*, *Dunaliella acidophila,* *Natronobacterium, Spirulina* sp., fungi
Temperature	Psychrophiles Thermophiles Hyperthermophiles	-20°C - <15°C 50°C - 80°C 80°C - >100°C	*Psychrobacter*, *Polaromonas*, cyanobacteria, diatoms and icy green algae, some insects. *Cyanidium*, prokaryotes as *Mastigocladus* *Pyrolobus fumarii* (113°C)
Hydrostatic pressure	Barophiles Piezophiles	Weight lovers Pressure lovers	Prokaryotic strain MT41 tolerates >100 MPa.
Radiation, Ultraviolet and ionizing radiation		High level of radiation	Cyanobacteria, desert plants *Deinococcus radiodurans*
Thriving in organic solvents and under various atmospheres	CO_2 gas Organic toxic solutions	Thriving at pure gas solutions of damaging agents	Cyanidiaceae (grown under pure CO_2)
Gravity	Hyper-gravity Hypo-gravity	>1 g < 1 g	Such microorganisms are not known on Earth (this factor may be related to extraterrestrial environments)
Desiccation	Xerophiles	Anhydrobiotic condition Low water activity	Prokaryotes, extreme halophiles or endoliths, fungi and lichens (a symbiotic system), nematodes

Based on Oren and Seckbach (2001), Seckbach and Oren (2004), and other sources.

Many of the extremophiles cope with more than one environmental parameter, and may be termed as multi- or poly-extremophiles (Rothschild and Mancinelli, 2001). For example, the unicellular rhodophytan *Cyanidium caldarium* (Seckbach, 1994, 1999; Seckbach and Walsh, 1999) and its cohorts are thermo-acidophiles growing in an acid media (pH 0.5 to 3.5) at elevated temperatures (maximal temperature 57°C); they tolerate salinity range from 3 to 10% (Pinto et al., 1994), 1 N H_2SO_4 (Allen, 1959) and thrive under pure CO_2 (Seckbach, 1994). Likewise the barophiles/piezophiles, the weight/pressure lovers are also either thermophiles, living near hydrothermal vents at the bottom of the oceans, or psychrophiles (cold lovers) in the depth of the seas and oceans.

Among the extremophiles are representatives of all domains of life [Prokarya (Archaea, Bacteria) and Eukarya]. Most extremophiles are prokaryotes (Archaea and Bacteria), but this group also includes eukaryotic cells (Roberts, 1999). Among the Eukaryotes are protists (e.g., algae, fungi, protozoa) and multicellular forms living in some of these harsh habitats. Some extremophiles are relegated to a particular habitat and are not able to survive milder conditions, while other are more flexible and can grow also in "normal" environments.

A number of these drastic environments may represent the primeval conditions that existed for the pioneering microbes evolving during the early periods in young Earth. For example, hyperthermophiles live in very warm or hot conditions and obtain their energy by heterotrophic and/or chemotrophic (with inorganic chemicals) metabolic pathways, while thermophiles of less extreme temperatures (i.e., below 74°C) may even be photosynthetic. The ability of thermophiles and hyperthermophiles to cope with such extreme environments may agree with the idea proposed by several scientists that the first organisms evolved in warm zones. The thermophiles are also placed at the roots of the archaeal branch in the tree of life based on molecular sequences. They may have evolved in the primordial soup of little ponds, in hot springs, inside rocks or even at the hydrothermal vents at the openings found around the black smokers at the bottom of the oceans. The hydrothermal vents are located at mid-ocean ridges where plates spread apart and spew out hot water containing dissolved minerals providing material and energy for a rich diversity of extremophilic life. Other extremophiles may have evolved at great depth of beneath the surface environments. Living in such places, these microorganisms (if they existed) may have been protected from the impact of the asteroid bombardment on the planet and shielded from high doses of UV irradiation during the early stages of Earth's evolution. Huge extraterrestrial asteroids and boulders from space hit young Earth between 4.5 Ga and 3.8 Ga (bya = billion years ago). During this period, some microbes may have found refuge by descending into underground areas where the temperature could reach ~93°C and around hydrothermal vents it could well over 100°C. There is some evidence that life appeared when this impact ceased at 3.5 to 3.8 Ga (Glausiusz, 2004). The conditions below Earth's surface were suitable for thermophiles deriving their energy via chemoautotrophic oxidation of inorganic chemicals, e.g., *Acidithiobacillus thiooxidans* oxidizes sulfur or sulfide to sulfate, *A. ferrooxidans* oxidizes ferrous to the ferric iron, while others oxidize hydrogen to water, nitrites to nitrates. Other microorganisms may have survived by blasting into space and returning to Earth later when the conditions became milder. The fact that there is almost no limit in the habitats on Earth brings the astrobiologists to

propose that extremophiles may serve as models for candidates for extraterrestrial life in planets and moons of the Solar System and beyond.

Some enzymes from extremophiles are tolerant of extreme parameters such as heat, salinity, acidity, alkalinity, and high or low pressures. They have been introduced recently into applied biotechnology systems and industry. We can find the extremophiles and their cellular productions in food and dairy products, health food, medical and pharmaceutical lines, antibiotics, detergents, in fermentation processes, and so on.

While this chapter covers most extremophiles, it does not deal with microorganisms thriving under anaerobic conditions. The anoxic atmospheres appeared as the original natural environment, while the oxygenated atmosphere is a secondary evolution. The oxidative atmosphere evolved after the appearance of the oxygenic photosynthesis process by cyanoprokaryota (Avers, 1989; Shapiro, 1986).

Additional reviews on extremophiles can be found in various chapters in the series *Cellular Origins, Life in Extreme Habitats and Astrobiology*; see: www.springeronline.com, and in Horikoshi and Grant (1998), Roberts (1999), Rothschild and Mancinelli (2001), and Seckbach (1999, 2000a, 2000b, 2003) and Seckbach and Walsh (1999).

2.1. HALOPHILES – THE SALT LOVERS

In the Solar System our Earth is considered the sole water-endowed planet, some 71% of its area are seas and oceans, mostly salt water. Similar water bodies have not yet been proven to exist outside the Solar System. The dawn of the appearance of the first organisms is unknown. They were most probably simple cells belonging to the Prokaryotes and ruled on Earth for 2–2.5 Ga (until the evolution of the eukaryotic cell). In this "Prokaryotic world" the Archaea and Bacteria were the sole organisms on Earth and they occupied almost every niche on the surface and subsurface of Earth. The prokaryotic microbes may have evolved 3.6 bya within saline solutions in the oceans; it seems, therefore, that at present too, all organisms "remember their roots" and use salt in their cells. Brines are the medium for halophilic microorganisms, and such environments occur ubiquitously on Earth. Halophiles represent a class of extremophilic organisms that are also adapted to high salt solution. Among the hypersaline halophiles are photosynthetic green algae (such as *Dunaliella* species), diatoms, and others. The enigmatic Cyanidiaceae, a group of thermo-acidophilic red algae, including *Cyanidioschyzon merolae*, *Cyanidium caldarium* and *Galdieria sulphuraria* tolerate NaCl solutions from 3 to 10% of NaCl (Pinto et al., 1994; Seckbach, 1994, and see above). Among the halophiles at different ranges of pH are also cyanoprokaryota, green and purple Bacteria, sulfur-reducing Bacteria, and methanogenic Archaea.

Most halophiles live in the marine environments (containing water of 3% salt), others thrive in inland lakes within bodies of salty water, while the hyper-halophiles grow in very high concentration salt solutions. The rod shaped halobacteria strains grow within a concentration of ~ 30% salt as salt concentration is present in the Dead Sea (Israel-Jordan) and in the Great Salt Lake (Utah, USA). Other may tolerate solutions of saturated salt solutions. As the water evaporates from salty aqueous bodies, high concentrations of salt solutions remain, then only halophiles will remain and obtain

these areas for themselves. Vreeland et al. (2000) revived bacteria that were enclosed in salt crystals for 250 million years. Assuming this report is authentic and fully reliable, we may have a new record of halophilic life in dry condition for vast periods.

The halophiles have to react against the high gradient gaps in osmotic pressure of their external solution vs. their internal cytosol medium. They have to avoid plasmolysis – the process where cell aqueous solution escapes due to the hypertonic external solution that causes the cytoplasm shrinkage. To avoid the escape (plasmolysis) of cell liquid, the halophiles balance these concentration differences of the solutes inside and outside the cell membrane by synthesizing intracellular organic compounds (e.g., glycerol, betaines, sucrose, K^+ ions, etc.). These compounds are synthesized and/or accumulated in response to osmotic stress at high salinity. Halophiles may regulate the movement of solutions by other means as well (Oren, 2002). The cell membrane may also regulate the osmotic pressure on both sides of the plasmalemma.

Some Antarctic lakes are salty at different concentration and some can reach saline concentration close to the Dead Sea level. Such saline lakes do not freeze during the Antarctic winter even when the temperature declines to -50°C. For further literature on the halophiles, see Oren (1999, 2002) and the chapters in this volume.

2.2. LIVING AT THE END RANGES OF TEMPERATURE AND pH

2.2.1. Thermophiles and Hyperthermophiles

The organisms living in warm or hot habitats are the thermophiles and hyperthermophiles, respectively. On the tree of life these microbes are placed close to the root of the prokaryotic position. It has been reported that among organisms there are some differences in heat tolerance (Table 1). For example, the upper temperature limit for eukaryotes is 62°C, while the thermo-cyanoprokaryotes can occur at < 70°C, thermophilic bacteria may thrive at 70°C to <100°C and hyperthermophilic Archaea can be detected at a maximum of 113°C (observed in the archaeon *Pyrolobus fumarii* – see also Seckbach and Walsh, 1999 and tables therein). This latter temperature point is considered the highest current temperature for life and might support the source where life has originated. Under special treatments and conditions the maximum temperature of life and the edge of stability can reach higher values, such as 121-125°C (Kashefi and Lovley, 2003; Seckbach and Oren, 2004). Such archaeal anaerobes gain their energy from reducing Fe^{+3} into Fe^{+2} via formation of magnetite.

The thermophiles thrive in geothermic sources, such as in hot springs, hot geysers, or at hydrothermal vents at the ocean floors. Black smokers on the sea floor expel hot water at 350°C, gases, minerals, and other materials from the inside of the Earth. One of the microbes associated with the walls of black smokers is *Methanopyrus* that produces methane (CH_4) gas.

2.2.2. The Psychrophiles Like Cold and Chilly Environments

Life cannot grow where liquid water does not exist. On the opposite scale from the spectrum of the thermophiles are the psychrophiles (cold-loving organisms) that grow at very cold ranges of temperature, even below freezing some of these may live in icy layers. In snow and ice layers one can find eukaryotic diatoms, green algae (e.g., *Chlamydomonas nivalis*) that "paint" snow with their pigmentation (green, red, orange).

Psychrophilic microorganisms are in permafrost of Siberia, in the Arctic zones, and in the icy depth close to the Vostok Lake (Antarctica). The Antarctic sea is populated with Bacteria, algae, and diatoms. In Antarctica the endolithic microbes—among them Bacteria, cyanobacteria, algae, and the symbiotic association-lichens live a few millimeters below the surface of rocks at very cold temperatures. In addition, at the ocean floor are worms dwelling in methane-enriched environments.

The psychrophiles can tolerate severe cold environments at the level of $-20^{o}C$ (Rivkina et al., 2000; Junge et al., 2004). Furthermore, lichens survive Antarctica winters at $-65^{o}C$. Many psychrophiles are also barophiles (poly-extremophiles) and such microorganisms possibly could survive on Mars when the air temperature reaches $-10^{o}C$ during daytime throughout the warmest part of the year.

2.2.3. Acidophiles

Acidophilic members are among all three domains of life. Some may be phototrophic others heterotrophic – all require acid media. Seckbach (2000a) presented a table of the lower pH limits for different groups of organisms. Most of the thermo-acidophiles are prokaryotes (Archaea and Bacteria) with the exception of some fungi and the algal members. Their habitats are in warm, hot (such as occur at volcanic fields, solfatara fields, hot springs), ambient and cold areas. Some acidic habitats are formed by microorganisms, such as after oxidation of elemental sulfur or ferrous ions and other compounds by bacteria (e.g., *Acidithiobacillus thiooxidans*, *A. ferrooxidans*), which leads to accumulation of sulfuric acid and ferric ions that precipitate $Fe(OH)_3$ with release of protons in various environments, according to the following equation:

$$2\ Fe^{2+} + 0.5\ O_2 + 5\ H_2O \rightarrow 2\ Fe(OH)_3 + 4\ H^+$$

Acid environments occur around active geothermal vents and springs, abandoned mines, or from sulfuric compounds' contaminations and other polluted places with acid wastes turning the area to acidic.

Several poly-extremophiles thrive in various ranges of pH, from milder solutions to very acidic media. Among the prokaryotes are thermo-acidic organisms (Oren and Seckbach, 2001; Seckbach and Walsh, 1999; Weiss Bizzoco, 1999; see tables therein). The archaeal hyperacidophilic *Picrophilus oshimae* and *Picrophilus torridus* grow down to pH –0.06, the lowest level of pH known to support life. *Thermoplasma acidophilum*, another archaeon, grows at pH 1.8-2 (min. at 0.4). The eukaryotic thermoacidophilic red alga *Cyanidium caldarium* thrives at pH 0-3.5 (Seckbach, 1994, 1999) and tolerates 1 N sulfuric acid (Seckbach, 1994, 2000b). Some green algae are acidophiles, such as *Dunaliella acidophila* at pH 0.5-3.0 (Pick, 1999), *Chlamydomonas acidophila* (pH 1-2) or *Euglena mutabilis* (pH 1-5). Among diatoms, *Pinnularia braunii* grows at pH 0-4. Cyanobacteria are known to thrive at neutral to alkaline pH. Yet there are some exceptions, such as the *Oscillatoria* and *Spirulina* discovered in Bavarian lakes at acidic condition measured a pH of 0.06 to 2.9 (Steinberg et al., 1998) but not shown to grow at these levels (Castenholz, personal communication). Hundreds of lakes in Sweden and Canada showed cyanobacteria present even in the most acidic lakes (Steinberg et al., 1998) which are very oligotrophic. There are three species of fungi growing at pH near 0 (Schleper et al., 1995): *Acontium cylatium*, *Cephalosporium*

spp., and *Trichosporon ceribriae*, which grow at pH –0.06 while their optimum growth is at pH ~ 0.5.

These acidic pH values are only external to the cells; the intracellular cyto-solution (cytosol) keeps its pH level around neutral pH (Beardall and Entwisle, 1984; Oren and Seckbach, 2001; Seckbach, 2000a). The cell membrane rejects and prevents the H^+ from entering the cell by using a variety of mechanisms for also pumping the protons out. Strong cyto-proton pump or low proton membrane permeability regulates the H^+ ions and stops them from entering the plasmalemma. The internal neutral pH has to protect some cyto-constituents like DNA, ATP, and chlorophyll from the acidification damage. Sasaki et al. (1999) reported about intracellular extract solutions from four species of Dictyotales to be highly acidic (at pH 0.5 to 0.9), similar values of pH were observed in some species of Desmarestiales (both groups are brown algae, Phaeophyta). Their intracellular pH measured to be 0.5-0.9, because their vacuoles contain sulfuric acid. Apparent these low values are due to the vacuolar content, but the true cytoplasmic pH is supposedly much higher.

Further data on acidophiles are reported in volumes 1, 2, and 6 of Cellular Origins, Life in Extreme Habitats and Astrobiology (COLE) and elsewhere. See website: www.spingeronline.com

2.2.4. Alkaliphiles

In some soils and soda lakes in east Africa and under dry climates (such as in Mojave desert), the soil is very alkaline. There are microorganisms (Archaea, Bacteria, cyanobacteria, and protists) that prefer to live on the high scale of the pH (alkaline levels). Such high pH levels were found together with high salt solutions. In highly alkaline-saline lakes, as in Mono Lake, CA, are diatoms, and green algae such as *Nanochloris, Chlamydomonas* and *Dunaliella.* The cyanobacterium *Spirulina platensis* is an obligate alkaliphile, and reaches a high population at pH values of 11 and above. *Plectonema nostocorum* has been reported to grow up to pH 13 which is considered the highest level at which life has been found. The diatoms are prominent components in the biota of many alkaline lakes.

These alkaliphiles keep the highly alkaline pH outside the cell and pump ions in and out the cells (similar to the acidophiles) to keep a lower and milder intracellular milieu which might reach up pH 9 (Horikoshi, 1998; see table 6.2 there) while the external pH level is much higher. For further data on alkaliphiles see Horikoshi (1998), Oren and Seckbach (2001), and Seckbach and Oren (2004). For additional data on alkaliphiles see Horikoshi and Grant (1998). The alkaline hypersaline lakes and their inhabitants may serve as analogues for ancient microbial habitats on Mars (see below).

2.3. XEROPHILES—THE DRY LIVING AND DESICCATED ORGANISMS

Some microorganisms have the ability to grow in low water activity and tolerate water stress. Low water availability can cause high concentration of salt and other solutes. Deserts and dry valleys of Antarctica or the Siberian permafrost support a variety of microorganisms in dry conditions. A survey (Davis, 1972) has shown that cyanobacteria (e.g., *Nostoc* sp.) survive desiccation for a record of 107 years. The diatom *Nitzschia palea* and the chlorophytes *Pleurococcus* sp. and *Cystococcus* sp. were shown to be viable after 98 years of dry storage in a herbarium.

Spores have a low if any rate of metabolism, and remain in long-term periods of dormancy before being revived. Well known are the cases claiming of viable bacterial spores resuscitated from the stomachs of insects imbedded in amber dated at 25–40 million years (Cano and Borucki, 1995). If the report (Vreeland et al., 2000) of the restoration to life of bacteria that had been encapsulated for 250 million years in a salt crystal is supported by unquestionable ground, then we have strong evidence on the long period during which life can be persevered.

2.4. ORGANISMS THRIVING AT HIGH PRESSURE

Barophiles (weight loving) and piezophiles (pressure loving) microorganisms both in the subsurface of Earth and in the depths of the ocean are obligated to live heterotrophically, chemoautotrophically or chemolithotrophically, in darkness and at high pressure. This elevated hydrostatic pressure can reach over a thousand atmospheres (at the bottom of the ocean). For every 10 m depth in the water column the hydrostatic pressure increases by about 1 atmosphere (0.1 MPa). The pressure at the deepest point in the ocean (at the bottom of Mariana Trench near the Philippines) is about 110 MPa. The barophilic bacteria are poly-extremophiles being psychrophilic in addition. Among the Archaea are the baro-thermophiles observed in the hydrothermal vents; *Themococcus barophilus* grows at temperatures up to 100^oC. Our knowledge about barophiles must be expanded for us to understand more about this fascinating group. For additional discussion on deep-sea Bacteria, see Yayanos (2000).

2.5. MORE EXTREME PARAMETERS FOR MICROORGANISMS

There are other factors of extreme environments that support life under various atmospheres. Some algae thrive under pure CO_2 (Seckbach, 1994), and other microorganisms grow under ammonia (Siegel, 1999) or on hydrogen gas (e.g., the methanogenic Archaea). Microorganisms can grow in environments with chemicals, heavy metals, organic solvents that are lethal for most other organisms. Another parameter is gravity, which might be relevant more to life in space or in celestial places, rather than to earthling organisms.

3. Astrobiology

In recent years the new multidisciplinary field of astrobiology has been established. Currently, in this area there are numerous books, publication of a plethora of articles in various journals and proceedings of conferences, meetings, workshops and a great deal of research being conducted. The wide variety of astrobiology-related disciplines include prebiotic chemistry, astronomy, astrophysics, origin of life, microfossil records, evolution, diversity of microorganisms, extremophiles, geobiology, molecular biology, extremophiles and extraterrestrial possibilities of life. It also includes life in the Universe, discovery of new stars, planets and satellites and the search for extraterrestrial intelligent life (SETI). Astrobiology has been referred also as bioastronomy, exobiology, extraterrestrial life and the Russian term cosmobiology.

The extremophiles that are ubiquitous may be considered as candidates for inhabiting celestial places. Furthermore, if there is life to based on silicon instead on carbon as well in solvents other than water (non-aqueous solvents), then life may perhaps exist on planets and satellites. The most popular extraterrestrial bodies within the Solar System that may carry living forms (as we know it), or their precursors are Mars and the Jovian moons such as Europa. Titan, the satellite of Saturn, may serve as a prebiotic laboratory resembling the Earth's prebiotic periods. Europa has a very cold environment (minus 162°C) and from its image one can distinguish huge cracked shells of ice floating and moving on water. These ice sheets may cover and float over an ocean of warm water originating from Europa's hydrothermal vents that may reach a couple of hundred degrees Celsius. The surface appearance of Europa resembles Alaska and Antarctica with their frozen seas. If Prokaryotes can survive such severe conditions on Earth, why not there in the heavenly bodies? One can ask the question: How unlikely is it that life does not exist elsewhere?

Similar to Jupiter, Saturn is a gas planet surrounded by hydrogen and helium (with traces of methane and ammonia). Its largest moon Titan is covered by a reddish fog over the icy continent and contains organic molecules including hydrocarbon oceans (while no oxygen is present in its atmosphere). Similar prebiotic organic chemistry may have led to life on Earth.

3.1. MARS

Our knowledge about Mars has increased but still not complete. Wherever there is free liquid water and other certain elements, there should be life. Water makes all cells "running" in their enzymatic reactions, natural processes and other functions in all cells. There are still debates among the astrobiologists whether Mars contains briny water on the upper layers of the surface (Möhlmann, 2004) or whether the Martian water is within layers present deeper in the subsurface. Such water might be accumulated in salty oceans warmed up by volcanic sources, while it could be also located as hydrated minerals. On Mars, similar to Earth, liquid water should goes together with life since water is vital for cell metabolism, enzymatic reactions, processes as photosynthesis, etc. The recently discovered methane in the Martian atmosphere provides encouragement for the scientists searching for life (Formisano et al., 2004). This detection of Martian methane may not originated from chemical reaction but rather it is believed that it may have evolved from biological sources

The images of Mars indicate that in the past it was warmer (during the caldera's age) and wetter, with plenty of running water in rivers, canyons, lakes and large oceans. The bulk of the Martian water might be tied up in the subsurface, either as permafrost in the craters in the polar latitudes, or in deep aquifers as reservoirs of water (McKay and Marinova, 2001). Possibly some forms of life may have flourished there in the past. Perhaps spores from the ancient "green Martian Planet" are still present as dormant spores and might be revived when environmental conditions will be improved by terraforming (Graham, 2004). If Martian surface conditions are at present non-inhabitable for living forms, the subsurface (lithologies in subterranean areas) may offer the only dwelling place for extant life forms. Therefore, finding of liquid water on Mars is vital for the presence of contemporary life on this planet.

Today the red planet is dry, cold (-50°C to +12°C); the Martian salty solutions are lowering the freezing point of its water so that microbes might grow in liquid water. Mars also has a very low atmospheric pressure (~ 6 to 10 mbars) and the oxygen in the atmosphere is very low (<0.15%). Its atmosphere contains 95% CO_2, 2.7% N_2, 1.6 % Ar and other minute ingredients. The soil of Mars contains mineral ions such as: Na, Mg, Ca, Cl and K. Organisms require sulfur, potassium, sodium, chloride, potassium, iron, and trace elements, all thought to be present on Mars.

Since it would be difficult for organisms to grow at the surface regions in the cryosphere and under UV radiation of Mars, if life does exist on Mars today, it could be located in the subsurface where warmer ground waters may be present. The thin Martian ozone layer does not significantly block UV rays, but the radiations of very short wavelengths penetrate all the way to the surface. If some species of Bacteria or Archaea may overcome Martian conditions (similar to those Bacteria existing in permafrost – see Möhlmann 2004), then this planet might perhaps harbor psychrophilic life. Also, microbial dormant spores may have life preserved there in 3.5 to 4 Ga old sediments that were left over from when the planet was warmer and had running water. Earthling Arctic and Antarctic life forms wait for the icy coverage to melt into liquid water (conditions that might be similar on Mars).

Mars (and other planets' moons) might carry life in hydrothermal lakes under the surface or in ice-rich regions similar to the way microbial life is harbored in the icy layer and in subsurface warm water which is assumed to exist in Vostok Lake (Antarctica).

As the early water evaporated from Mars, a high concentration of dissolved salt was left so that only halophiles remained and had the planet for themselves. By understanding osmophily (halophily) and extremophiles on Earth we may detect life that lives or has lived on other planets. Such osmophiles are best suited for life in brines and evaporates. Halophiles may possibly occur in the Martian icy soil, as they thrive in the hypersaline ponds of Antarctica. Therefore, a preparatory search for extant and extinct life of Mars should be a focused on osmophiles or on their fossilized remnants (Mancinelli, this volume). All the answers about Martian life can be found only after samples from Mars will be brought to Earth.

Recently there have been some proposals to create a greenhouse effect for warming Mars towards restoring habitable conditions on this planet. Such terraforming ideas for Mars have been published (Friedmann and Ocampo-Friedmann, 1995; McKay and Marinova, 2001). Only in time will we know if such proposals are realistic or just wishful dreams.

Is the future export or transfer of microbes from Earth to Mars practical? Some scientists believe that the red planet is currently too cold and dry to allow the survival of any terrestrial organisms. Terrestrial life forms could perhaps survive there only after warming up the planet to a minimal level. Such warming of Mars can take place by using artificial greenhouse gases (Marinova et al., 2004). Mars appears to have sufficient water, if warmed, to form large bodies, and to once again be capable of harboring life. There might be a possibility that genetic engineering may "create" and convert organisms and equip them with special features to resist extraterrestrial environments such as those existing on Mars. If introduced on Mars they may assist in the terraforming there.

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Biodata of **Rocco Mancinelli**, author of "***Halophiles: a Terrestrial Analog for Life in Brines on Mars - Halophiles on Mars***"

Dr. Rocco L. Mancinelli is a Senior Research Scientist with the SETI Institute. His research interests are broad, encompassing ecology, physiology, and biogeochemistry. Specifically he studies microbe-environment interactions with emphasis on the environmental limits in which organisms can live. He currently uses four systems in these studies: 1) Halophiles in evaporitic salt crusts that form along the marine intertidal; 2) Microbial mats inhabiting diverse environments (e.g., the intertidal area of the Baja coast, the alkaline and acid hot springs of Yellowstone National Park, hypersaline lakes and the perennially ice-covered lakes in the dry valleys of Antarctica); 3) Areas where rock (desert) varnish occurs; and 4) The space environment in Earth orbit. The results of such experiments are used to model microbe-microbe and microbe-environment interactions. The models are used in formulating hypotheses regarding mechanisms of survival of organisms in the space environment as well as the evolution of the nitrogen cycle and the role exogenous sources of fixed nitrogen play in the physiology of nitrogen metabolism, biogeochemistry and microbe community structure. Further, these models are extrapolated to what is known about the environment (geochemistry and climatology) of early Mars in an attempt to determine the potential for life to evolve on that planet.

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HALOPHILES: A TERRESTRIAL ANALOG FOR LIFE IN BRINES ON MARS

Halophiles on Mars

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1. Introduction

When water on a planet begins to evaporate the dissolved minerals become more concentrated, forming a brine, eventually precipitating out of solution and forming evaporite deposits. The brines and evaporites form a highly saline environment that is hostile to most life forms, but provides an ideal habitat for microorganisms that are osmophilic, or halophilic. Halophiles are salt "loving" microorganisms that inhabit environments with salt concentrations ranging from 15% to saturation. Osmophiles are organisms that “love” high osmotic environments, such as halophiles. On earth, representatives of halophiles occur in all three domains of life, the Archaea, Bacteria and Eukarya.

Data from recent Mars missions suggest that Mars almost certainly had abundant liquid water on its surface at some time in the past, which was eventually lost. The loss of water probably occurred when Mars lost its atmosphere causing the water to evaporate, or recede into the subsurface. As the water evaporated, the dissolved minerals became more concentrated forming brine pockets and evaporites within the permafrost. Because studies suggest that it may be relatively easy for halophilic, or osmophilic type organisms to evolve, if there were any life on Mars in water then there should have been the evolution of some type of osmophile, or halophile. Brine pockets containing high concentrations of dissolved salts would have selected for the survival of osmophiles, or halophiles. These brine pockets may either be "oases" for an extant Martian biota, or the last refuge of an extinct Martian biota.

Halophiles fall into two categories, extreme halophiles requiring 15% salt to saturation, or moderately halophilic organisms living in saline environments ranging from seawater to 15% salt. Organisms that are halotolerant prefer non-saline environments, but can grow from essentially no salt to nearly 10% salt. Halophiles form populations so dense that the red color associated with hypersaline lakes and ponds is due to the pigmentation of the halophilic Archaea and the eukaryote *Dunaliella*. Although these lakes and ponds contain populations of halophilic Bacteria and other members of the Eukarya they go unnoticed to the naked eye because they are not pigmented.

Halophiles are everywhere in the world there is salt. They represent a phylogenetically, physiologically, evolutionarily, and ecologically diverse group of organisms. Most halophiles are found interspersed among non-halophiles in the phylogenetic tree. They encompass heterotrophs, autotrophs, and some possess light harvesting pigments either for photosynthesis, or for energy production via rhodopsin. They live in cold or hot environments, wet environments (e.g. lakes and ponds), dry environments (e.g. soils and salt crusts), and alkaline as well as neutral environments. They can be aerobes, anaerobes, or facultative anaerobes. Some possess true cell walls (Bacteria and most Eukarya, except *Dunaliella*) and some do not, such as most of the Archaea. They even differ with respect to their modes of osmotic adaptation. The one characteristic they have in common is their ability to live in hypersaline environments. Adaptation to life at high salt concentrations can be achieved in different ways. The most commonly occurring strategy involves the accumulation of organic osmotic solutes without the need for specialized adaptation of intracellular proteins to high salt. This mechanism occurs in all three domains of life. The second option is the intracellular accumulation of high concentrations of K^+. This strategy, unlike the use of organic solutes, requires extensive adaptation of the intracellular enzymatic machinery to be functional in the presence of high ionic concentrations. The great diversity in strategies used by the halophiles to cope with the high salinity in their environment coupled with the fact that halophily occurs throughout the tree of life in all three domains suggests that adaptation to life at high salt concentrations is easy to evolve, and has probably arisen many times during the evolution of life.

Viable microbes have been found in Arctic permafrost that is 1 to 3 million years old (Gilichinsky and Wagener, 1995; Rivkina et al., 1998; Shi et al., 1997; Vorbyova et al., 1997), and in permafrost that is probably older in Antarctica (Wilson et al., 1998). These microbes are psychrotrophs and psychrotolerant mesophiles. Given what we know about Mars (see the following section) these organisms and their environments may be model systems for understanding if life could have originated and evolved on Mars.

2. Mars

Mars is a cold dry planet that is bombarded with UV radiation. Overall, it is currently considered inhospitable to life. Yet, Mars is considered the most likely planet, next to Earth, to either harbor life now in protected oases, or sometime during its past. It is thought that all of the constituents for life are present on the planet, and that the radiation regime would not have precluded the origin and evolution of life (Banin and Mancinelli, 1995; Mancinelli and Banin, 1995). The one factor that would preclude life as we know it on Mars at any stage is the lack of liquid water sometime during its past.

2.1. HISTORY OF WATER ON MARS

The surface of Mars today exhibits carved features such as out-flow channels, valleys

and lahars that date back to its early history (Baker and Milton, 1974; Carr, 1981; Christianson, 1989; Squyres, 1984; Squyres and Carr, 1986). These features have led many planetary geologists to hypothesize that liquid water once flowed across the martian surface (Anders and Owen, 1977; Baker and Milton, 1974; Carr, 1981, 1986; Christianson, 1989; Clark and Baird, 1979; Lewis, 1972; McElroy et al., 1977; Pieri, 1980; Pollack and Black, 1979; Squyers, 1984, Squyres and Carr, 1986). Results from NASA's current MER (Mars Exploration Rover) mission provide strong evidence that Mars once possessed standing bodies of liquid water (Squyres et al., 2004).

There are several possible sources of water for Mars. These include: 1. remnants of an original steam atmosphere condensed after a period of global melting had ended and the planet had cooled after accretion; 2. out-gassing of juvenile water from the planetary interior during volcanic activity; 3. water originating from the impact of asteroids with a composition similar to carbonaceous chondrites; and 4. water originating as part of a veneer of cometary material toward the end of the period of heavy bombardment. Arguments for all these possible sources have been proposed, based upon either observational evidence or geochemical evidence provided by studies of meteorites. Early hypotheses regarding the quantity of water present on Mars stemmed from evidence provided by images returned from spacecraft. Numerous studies of morphological features such as outflow channels, presumed to be cut by water, and relict basins, assumed to have been oceans, resulted in estimates (expressed as the depth of a hypothetical layer across the entire planet) ranging from a few tens of m up to 440 m (Baker et al., 1991; Carr, 1986).

2.2. EVAPORITES ON MARS

Evaporite deposits consisting primarily of halite (NaCl), gypsum ($CaSO_4.2H_2O$), or anhydrite ($CaSO_4$) and containing bacterial and algal assemblages are well known in the fossil record and are geographically widespread (e.g. Rothschild et al., 1994). It has been demonstrated that microorganisms entrapped in fluid inclusions of growing NaCl crystals may be motile for three weeks, and may remain viable for up to six months (Norton and Grant, 1988). Rothschild et al. (1994) demonstrated that microorganisms inhabiting gypsum-halite crusts perform carbon and nitrogen fixation while inside the dry crystals of the crust for at least a year. Although highly controversial, bacteria might survive for millions of years in the fluid inclusions of salt deposits including evaporites (Vreeland et al., 2000).

Several studies have speculated on the presence of evaporite deposits on Mars (e.g., Clark and van Hart, 1981; Rothschild, 1990). The potential for evaporites to occur on Mars and their relationship to liquid water and life has been reviewed by Rothschild (1990). More recently, the Opportunity rover of the MER mission found hematite concretions buried just beneath the surface in dirty, salt-laden deposits. These deposits were laid down as a shallow sea, or a series of puddles that had evaporated (Squyres et al., 2004). Analytical measurements performed in "Eagle Crater" near the Opportunity landing site revealed an abundance of sulfate salts. Small amounts of sulfate had been inferred from martian soil sulfur analyses since the Viking landers of the late 1970s, but remote sensing had not detected sulfates

from orbit. Opportunity's investigation of the large crater "Endurance" resulted in finding several meters of the same sulfate rich evaporite as at "Eagle Crater". Presumably it goes down the entire 300 meters of the light-toned, layered stratum seen from orbit underlying Meridiani Planum. The MER mission has detected sulfates, as well as other weathering products at the landing sites of both rovers (Spirit and Opportunity). Recently, the water-signature mineral goethite was detected in "Columbia Hills" at the Spirit landing site. Data from OMEGA, the visible and infrared mineralogical mapping spectrometer aboard ESA's Mars Express Mission, suggest that kieserite is ubiquitous in low-lying regions where water might have collected and evaporated, such as at the bottom of a canyon of the Valles Marineris. OMEGA also detected clays, produced by the water weathering of silicate rock, and serpentine, the weathering product of olivine. The distribution of both magnesium and calcium sulfates was mapped in the tiny fraction of Mars covered by OMEGA so far. The sulfates appear not only where ancient waters may have collected, but also in some, although not all, of the layered deposits beyond Meridiani Planum. Layered deposits have become the leading geological mystery on Mars. Some geologic force — water, wind, volcano, or impact — laid down light-toned material inside impact craters and in other low-lying regions. The MER and Mars Express discoveries suggest that at least some of the layered deposits were formed in standing water: ponds, lakes, or oceans (Squyres et al., 2004).

A sulfate-salty Mars tells a story of a young planet corroded by acid. It is possible that sulfuric acid derived from volcanic emissions would have mixed with any water that was about and chemically eroded rock to produce a variety of sulfates, in particular a potassium iron hydroxy sulfate called jarosite. The Opportunity rover's Mössbauer instrument did in fact identify jarosite in the evaporite at "Eagle Crater". Because the Meridiani Planum rocks are some of the oldest seen on the planet, the water on early Mars is interpreted to have been acidic. Signs that running water cut valleys during the first billion years or so of Martian history — when life was beginning on Earth — have convinced most researchers that early Mars was salty, warm, and wet or at least not so cold that all water was continually locked up as ice.

2.3. WATER AND LIFE ON MARS

Water in the liquid state serves as the solvent for many biogenic compounds and mediates important geochemical (abiotic), prebiotic and biological reactions. Liquid water is important to the reactions necessary for the origin of life and its continued evolution because it is the primary intracellular solvent. As a consequence, the temperature regime of at least some portion of a planet must be above the triple point of water for life to have evolved.

A key question for life is, was water present on the surface of Mars for sufficient time to allow life to originate and evolve? Current attempts to answer this question are based on the interpretation of photographs from recent missions to Mars. The valley networks in the southern highlands suggest that water flowed across the martian surface early in the history of the planet. Recently revealed gullies may be sites of present day near surface liquid water (Christensen, 2003; Malin and Edgett, 2000; Squyres et al., 2004). Photographs of Holden Crater show apparent layers that

may be remnants of former shorelines of a standing body of water on Mars (e.g. Parker et al., 2000). The change in albedo observed near Schiaparelli Crater may be due to evaporites (e.g. Cabrol and Grin, 2001). Data from MER provides further evidence for water activity in the past, as discussed previously.

Data collected during landed missions to Mars, as well as from analyses of the martian meteorites suggest that all of the necessary chemical constituents for life existed on Mars during its early history (e.g., Banin and Mancinelli, 1995). Further, it has been suggested that the environmental conditions on early Mars would not preclude the origin and evolution of life on that planet (Mancinelli and Banin, 1995). A primary habitat for any life that may have evolved on Mars would have been water, which would certainly have contained various dissolved minerals. Mars is thought to have lost most of its atmosphere as time progressed. As this process continued, the water on the planet's surface would have frozen and evaporated, and, high saline brine pockets and evaporite deposits would have formed. These brine pockets would most likely occur deep beneath the surface and may be an oasis for an extant biota, or the last refuge for an extinct biota. Because brine pockets would have a high osmolarity the most likely organisms to survive in a brine pocket or in an evaporite are osmophiles that could be halophiles if the salt is halide.

The presence of osmophiles and halophiles has been documented in terrestrial systems, such as in gypsum-halite crusts in intertidal areas of the ocean (Rothschild et al., 1994), and in NaCl crystals (Oren, 2002; Rothschild and Mancinelli, 2001). Additionally, halophiles have been shown to survive freeze-thaw cycles (Mancinelli et al., 2004). Therefore, since organisms that live in salt exist on earth and organisms have been shown to live and metabolize in permafrost (Rivkina et al., 1998, 2000) the next question to address is could these organisms survive and live on Mars.

During the past 40 years numerous microbiological investigations have been conducted on Antarctic Dry Valley soils, some in preparation for the Viking mission to Mars (Benoit and Hall, 1970; Cameron, 1972; Cameron et al., 1970; Hirsch et al., 1988; Horowitz et al., 1972). The data from these investigations indicated that the number of viable microorganisms in the soil of the Antarctic Dry Valleys declined as a function of soil moisture (more negative water potential). The data suggest that water is the factor limiting microbial activity in the soil rather than temperature (Horowitz et al., 1972). This observation is also true for the Atacama Desert (McKay et al., 2003). The same would be true for Mars if life existed on that planet.

Investigations were undertaken during the 1960s and 1970s to determine the effect of freeze-thaw cycles at one atmosphere and at reduced atmospheric pressures. For example, it was demonstrated that a variety of soil microorganisms survived being subjected to freeze-thaw cycles using temperature ranges from +25 to –60 °C (Foster et al., 1978; Green et al., 1971; Hawrylewicz et al., 1965, 1966; Kuzurina and Yakashina, 1970; Young et al., 1968), low atmospheric pressures down to 10 mbar (Foster et al., 1978; Hagen and Jones, 1963; Kuzurina and Yakashina, 1970; Roberts, 1963; Silverman et al., 1963), and levels of moisture in soils of ~0.5 % (Hawrylewicz et al., 1966). The results of these studies suggest that the most important factor for the survival and growth of life is the availability of liquid water. Much of this type of research stopped when the Viking mission search for life could not show that life existed on the planet's surface.

Recent interest in the survivability of microbes in permafrost as well as renewed speculation on the possibility of life beneath the surface of Mars, or on Europa has lead to new studies on microbial survival through a series of freeze-thaw cycles and life in permafrost. Permafrost, permanently frozen soil, contains unfrozen water (Ershov, 1998; Nersesova and Tsytovich, 1966) allowing mass transfer of ions (Ostroumov and Siegert, 1996). This transfer is greatest in microzones with low ice contents and least at sites where the ice content is high or in solid ice (Ostroumov and Siegert, 1996). Thus, the physical structure of permafrost makes metabolic activity possible. Using incorporation of ^{14}C-labeled acetate into lipids by samples of a natural population of bacteria from Siberian permafrost, Rivkina et al. (2000) showed that microbes could metabolize in permafrost down to $-20^{o}C$. Mancinelli et al. (2004 and unpublished data) have shown that halophiles can survive freezing in a liquid brine, dried in salt, as well as freeze-thaw cycles.

It is known that changes in water content of soils have profound effects on microbial activity (Griffin, 1981), which result in changes in soil microbial populations (Griffin, 1972). Water potential is the free energy of water in a system, relative to the free energy of a reference pool of pure, free water. Pure free water has, by definition, zero water potential, and for example, the potential energy of water in unsaturated soils is negative. The tendency for water is to flow from high to low potential, making the availability of water for microbes in the soil less as the potential is lowered. The water potential of a microbial cell in soil is likely to be near equilibrium with its microenvironment within the soil (see e.g. Elliott, 1981 for a review). The prospects for the origin of life on Mars would only have been favorable if the water potential was higher than it is today. Due to the necessity of liquid water, life undoubtedly arose in liquid water. Thus, life on Mars would be located in association with water-laden deposits, aquifers or soils that were once saturated with liquid water. Recent data from the MER mission indicates that the water was salty, an ideal habitat for a halophile.

3. Conclusions

Although the Viking mission found no evidence of life on the Martian surface (reviewed by Klein, 1979; Mancinelli, 1998), the search for extant and extinct life on Mars continues. There is mounting evidence from missions to Mars that there was liquid water on the surface of the planet early in its history (e.g., Malin and Edgett, 2000 and Squyres et al., 2004). Additionally, there may be liquid water on the surface today resulting from melting snow fields (Christensen, 2003). Further, data from recent missions suggest that evaporites should exist on Mars (Cabrol and Grin, 2001; Clark and van Hart, 1981; Edgett and Parker, 1998; Rothschild, 1990).

Halophily/osmophily is not rare and probably evolves easily, and arose many times. If life evolved on Mars, then osmophily probably evolved as well. Osmophiles are best suited for life in brines and evaporites. Halophiles/osmophiles are found in a variety of cold environments. Studies have shown that microbes can metabolize in permafrost (Rivkina et al., 1998). Halophiles/osmophiles are better adapted than other organisms to survive in brines, drying and freeze-thaw cycles, as may have

occurred on Mars. The last vestiges of an extinct Martian biota, or an extant biota could be present in a cold, desiccating environment, perhaps in brine pockets within permafrost. Therefore, it is possible that the last organisms to survive on the Martian surface were halophiles or osmophiles.

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Section II. Archaea

Comparative genomic survey of information transfer systems in two diverse extremely halophilic Archaea, *Halobacterium* sp. strain NRC-1 and *Haloarcula marismortui*
Brian R. Berquist, Jeetendra Soneja and Shiladitya DasSarma

Walsby's square archaeon; it's hip to be square but even more hip to be culturable
Henk Bolhuis

Gene regulation and the initiation of translation in halophilic Archaea
Felicitas Pfeifer, Peter Zimmermann, Sandra Scheuch and Simone Sartorius-Neef

Protein translation, targeting and translocation in *Haloferax volcanii*
Jerry Eichler, Gabriela Ring, Vered Irihimovitch, Tovit Lichi, Irit Tozik and Zvia Konrad

Enzymes of halophilic Archaea. Recent findings on ureases and nucleoside diphosphate kinases
Toru Mizuki, Ron Usami, Masayuki Kamo, Masaru Tanokura and Masahiro Kamekura

Osmoadaptation in methanogenic Archaea: recent insights from a genomic perspective
Katharina Pflüger, Heidi Wieland and Volker Müller

Biodata of **Brian R. Berquist**, **Jeetendra Soneja**, and **Shiladitya DasSarma**, authors of ***"Comparative Genomic Survey of Information Transfer Systems in Two Diverse Extremely Halophilic Archaea, Halobacterium sp. strain NRC-1 and Haloarcula marismortui"***

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COMPARATIVE GENOMIC SURVEY OF INFORMATION TRANSFER SYSTEMS IN TWO DIVERSE EXTREMELY HALOPHILIC ARCHAEA, *HALOBACTERIUM* SP. STRAIN NRC-1 AND *HALOARCULA MARISMORTUI*

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1. Introduction

Halophilic archaeal microorganisms (haloarchaea) thrive in extreme environments with salt concentrations approaching saturation, such as marine solar salterns or the Dead Sea (DasSarma and Arora, 2002). Conditions in these two environments are similar with respect to extreme high salinity, but differ in the composition and concentrations of salts present (thalassic *vs.* athalassic). While thalassic environments contain a proportion of salts similar to the sea, athalassic ones, e.g. the Dead Sea, deviate from that composition. Therefore, halophilic Archaea have adapted and evolved differently to reflect the differences in environmental conditions, since their divergence from a common ancestor (Oren et al., 1990). The availability of genome sequences from representative archaeal organisms from these two types of halophilic environments enables comparative genomic analyses between diverse haloarchaeal organisms.

The genome of *Halobacterium* sp. strain NRC-1, a thalassic organism isolated from sea salt, was the first to be completely sequenced and revealed a dynamic 2,571,010 bp distributed among three replicons: a 2 Mb large chromosome and two large extrachromosomal replicons pNRC200, 365 kb, and pNRC100, 191 kb (Ng et al., 2000). The second genome of a halophilic archaeon, *Haloarcula marismortui,* is that of an athalassic halophile isolated from the Dead Sea. The sequence is nearly complete and comprised of ~ 4,274,642 bp, almost 2 Mb greater than *Halobacterium* sp. strain NRC-1. The larger genome size for *H. marismortui* is somewhat reflected in its increased metabolic versatility, growing on sugars and in simple defined media (Oren et al., 1990). *H. marismortui* also contains three rRNA operons, two of which are diverged. In stark comparison, *Halobacterium* sp. strain NRC-1 requires 15 amino acids, with only five being synthesized *de novo*, as well as several supplemental vitamins. Strain NRC-1 also contains only a single rRNA operon.

As with other archaeal organisms, both *Halobacterium* sp. strain NRC-1 and *H. marismortui* exhibit significant similarities to eukaryotes in their macromolecular biosynthetic machinery, including DNA replication, repair, basal transcription, and translation (Olsen and Woese, 1997). Both organisms also display significant similarities to Bacteria in their two-component signal transduction systems and transcriptional regulators (Aravind and Koonin, 2001; Galperin, 2004). We utilized

genome sequence information from these two halophilic Archaea to identify and examine in detail the similarities and differences in information transfer proteins that are predicted to be involved in the cellular processes of DNA replication, repair, recombination, chromosome segregation, cell division, basal transcription, transcriptional regulation, and two-component signal transduction in these two diverse haloarchaea.

2. Methods

We performed computational analysis of two haloarchaeal genomic sequences to determine the number of conserved genes, orthologs and paralogs, contained within the two haloarchaeal genomes. BLASTable databases for both genomes were created using the NCBI BLAST server. All the predicted genes from one genome were used as querie sequences in BLAST (Altschul et al., 1997) analysis against the predicted genes from the other and vice-versa. The purpose was to find all the matches for each gene from one genome present in the other. The BLAST expect value cut-off of 10^{-6} was used to determine a match for a certain gene. Using Perl scripts, the BLAST results for each gene were parsed to find all matches with an e-value less than or equal to the cut-off. The pairs of genes that had each other as their reciprocal top hit were identified as the orthologs between the two genomes. All the matches in a genome for a particular gene that had an e-value of 10^{-6} or less were considered as candidate paralogous genes in that genome.

Analysis done to identify all proteins predicted to be involved in DNA replication, repair, cell division, transcription, translation and two component signal transduction was performed in the following manner: all *Halobacterium* sp. strain NRC-1 proteins predicted to be involved in these processes had their respective COG(s) identified (Tatusov et al., 1997, 2000, 2001) and were then BLASTed against a local database of *H. marismortui* predicted proteins (4,630). The top five BLAST hits were parsed using Perl scripts into an Excel table containing the *Halobacterium* sp. strain NRC-1 gene number, name, annotation, and COG. These results were examined, and any *H. marismortui* protein that had multiple hits to *Halobacterium* sp. strain NRC-1 proteins was eliminated, save for the top BLAST score. Any *H. marismortui* protein hits with an expected value greater than 10^{-6} to a *Halobacterium* sp. strain NRC-1 protein were not considered to be significant and were eliminated from the analysis. This identified orthologs and paralogs of specific proteins in the two haloarchaea. Predicted COGs for *H. marismortui* were then used to validate the BLAST analysis annotations and identify additional proteins that are present in *H. marismortui* but not *Halobacterium* sp. strain NRC-1. Any proteins whose COG prediction for *H. marismortui* did not correspond to the predicted function inferred by BLAST analysis were then subjected to a search of the nonredundant protein database at NCBI and a search of the conserved domain database (CDD) at NCBI (Marchler-Bauer et al., 2002, 2003) to identify protein domains, allowing a more detailed function prediction. The CDD was also used to analyze predicted transcriptional regulators in the two haloarchaea, while both CDD and Pfam (Bateman et al., 2004) protein domain databases were used in domain prediction of two component signal transduction proteins. Use of both the COG functional

prediction and the CDD/Pfam domain identification databases allowed for a more robust functional prediction.

For multiple sequence alignments, either CLUSTAL_X (Thompson et al., 1997) or TCoffee (Notredame et al., 2000) programs were used, depending on the number of sequences analyzed. If less than 30 sequences or 10,000 characters were present TCoffee was used for alignments; otherwise, alignments were made using CLUSTAL_X. All alignments were manually inspected and edited if necessary. Phylogenetic tree construction was performed using quartet-based maximum-likelihood phylogenetic analysis implemented in the program suite TREEPUZZLE (Schmidt et al., 2002) with the JTT amino acid substitution matrix.

3. Results

BLASTable databases were created using 2,626 *Halobacterium* sp. strain NRC-1 unique genes and 4,630 *Haloarcula marismortui* genes. All the *Halobacterium* sp. strain NRC-1 genes were blasted against the database of *H. marismortui* genes and the reciprocal BLAST using *H. marismortui* gene queries against a *Halobacterium* sp. strain NRC-1 gene database was performed. After parsing the BLAST output of all the genes from both genomes, we found 1,735 reciprocal hits between the two. Out of 1,735 reciprocal hits, 1,631 pairs of genes were such that both the genes had an e-value of 10^{-6} or less. The remaining 104 pairs of genes had at most one of the two genes with an e-value less than or equal to the cut-off.

The BLAST results for these 1,735 *Halobacterium* sp. strain NRC-1 genes were parsed to find the total number of matches present in the *H. marismortui* genome. These would be regarded as encoding paralogous genes in the much larger genome of *H. marismortui*. Initially, all the matches with e-value less than or equal to the cut-off were extracted from the BLAST output of the *Halobacterium* sp. strain NRC-1 genes. However, multiple *Halobacterium* sp. strain NRC-1 genes could have the same *H. marismortui* gene as a match. Therefore, while calculating the total number of matches in the *H. marismortui* genome, it was ensured that any *H. marismortui* gene is considered only once (even though it might have matched more than one *Halobacterium* sp. strain NRC-1 gene). For this, we used the hash table data structure in Perl, and stored the gene-ids as the keys of the hash table. We found a total of 2,529 *H. marismortui* genes matching the 1,735 *Halobacterium* sp. strain NRC-1 genes with an e-value less than or equal to the cut-off.

Specific protein sequences corresponding to genes predicted to be involved in the informational processes of DNA replication, repair, recombination, chromosome partitioning, cell division, basal transcription, transcriptional regulation, and two-component signal transduction were extracted from the genome sequence of *Halobacterium* sp. strain NRC-1. These were initial queries against a BLASTable database of all 4,630 predicted protein sequences of *H. marismortui*. In order to identify all of the homologous proteins in the two organisms the top five BLAST hits from the *Halobacterium* sp. strain NRC-1 queries were parsed and evaluated based on expect values and in some circumstances protein alignments. COG functional predictions were also correlated to both *Halobacterium* sp. strain NRC-1 and *H. marismortui* proteins identified through the initial BLAST analysis. All proteins were also subjected to

protein domain searches of CDD. In the case of two component signal transduction proteins, the Pfam database was also utilized. These analyses allowed for comprehensive identification and functional prediction of these informational proteins.

3.1. DNA REPLICATION AND CELL DIVISION

Genome sequencing efforts in Archaea have uncovered a chimeric nature of protein factors used in DNA replication and cell division (Bernander, 1998, 2000, 2003). Although Archaea are classified as prokaryotic organisms, many protein factors used in DNA replication are homologous to components of the eukaryotic machinery that likely perform similar functions. In addition, these archaeal factors are quite distinct from protein factors performing these functions in Bacteria. On the other hand, cell division factors in Archaea appear to be more like those factors which are used in Bacteria.

Archaeal organisms have been found to encode homologs of the eukaryotic replication initiation factor Orc1/Cdc6. Typically one or two genes for this factor are found encoded within a complete archaeal genome. However, it has been found that haloarchaea encode ten or more Orc1/Cdc6 homologs within their genomes (Berquist and DasSarma, 2003). Ten Orc1/Cdc6 eukaryotic type origin recognition proteins homologs are found in *Halobacterium* sp. strain NRC-1. Even more surprising was detection of a greater number of *orc1/cdc6* genes in the genome of *H. marismortui*, at least seventeen (Table 1, 2). Previous phylogenetic analysis of Orc1/Cdc6 proteins in sequenced archaeal genomes had indicated the presence of three major clades (Berquist and DasSarma, 2003). Two of these clades can be found in other sequenced Archaea, with one named for the Orc7 protein of *Halobacterium* sp. strain NRC-1, representing archaeal origin binding proteins and one named after the Orc6 protein of *Halobacterium,* whose function is not yet determined. The third clade identified is strictly specific to haloarchaea and named for the *Halobacterium* sp. strain NRC-1 Orc8 protein. Phyogenetic reconstruction of only haloarchaeal Orc1/Cdc6 proteins reveals five distinct clades of haloarchaeal Orc1/Cdc6 proteins (Fig. 1). Single homologs of Orc6 and Orc7 are present in both halophiles and each is distinct from other Orc1/Cdc6 proteins forming their own separate clades. Orc2, 3, 4, and 5 proteins from *Halobacterium* sp. strain NRC-1 form a larger clade together with four Orc1/Cdc6 homologs from *H. marismortui*. In *Halobacterium* sp. strain NRC-1 all of these *orc1/cdc6* genes are encoded on its large extrachromosomal replicon pNRC200, and some are predicted to be on extrachromosomal elements in *H. marismortui*. Whether they play a role in initiating DNA replication is currently unknown. Interestingly, one Orc5 clade protein coding gene in each organism (orc4 and 280160) is physically linked to a gene for a B family DNA polymerase homolog, implying that they may in fact play a role in the replication of those genetic elements. Another clade is formed by Orc1 and Orc9 *Halobacterium* sp. strain NRC-1 proteins, along with three related proteins from *H. marismortui*. These *Halobacterium* sp. strain NRC-1 genes are also both encoded on pNRC200, with *orc9* being in a region duplicated and also coded for on the pNRC100 extrachromosomal element (Ng et al., 1998, 2000). The last clade is the largest in *H. marismortui* with eight members and contains the chromosomally encoded *Halobacterium* sp. strain NRC-1 Orc8 and Orc10 (although this *orc10* gene has an IS element directly upstream and is therefore likely nonfunctional). It is clear that halophilic Archaea have undergone a lineage specific expansion of this chromosomally

encoded Orc1/Cdc6 clade (Fig. 1), although functions for representatives in this clade are yet unknown. The *orc8* gene of *Halobacterium* sp. strain NRC-1 is encoded proximal to a predicted a GC skew switch point (Kennedy et al., 2001; Zhang and Zhang, 2003), but that locus has been shown not to function as an autonomously replicating sequence (ARS) element (Berquist and DasSarma, 2003).

TABLE 1. Orc1/Cdc6 homologs in *Halobacterium* sp. strain NRC-1 and *H. marismortui.*

Halobacterium NRC-1 gene number*	*Halobacterium* NRC-1 gene name	Number(s) of *H. marismortui* equivalent(s)
45	*orc10*	7
1224	*orc8*	1
2271	*orc6*	1
2411	*orc7*	1
5091/6091*	*orc9*	1
6150*	*orc1*	2
6164*	*orc2*	0
6187*	*orc3*	1
6272*	*orc5*	1
6363*	*orc4*	2

* Denotes Orc1/Cdc6 homologs which are coded for on extrachromosomal elements in *Halobacterium* sp. strain NRC-1.

TABLE 2. Comprehensive listing of DNA replication, chromosome segregation, cell division, repair, recombination, putative cell cycle kinases, CRISPR associated, chromatin, restriction modification genes and pathways in *Halobacterium* sp. strain NRC-1 and *H. marismortui*. Numbers in italics indicate a greater number of homologs in that particular genome.

Gene Name	**COG(s)**	**COG Description(s)/ Putative Function**	**Number of Homologs in *Halobacterium* NRC-1**	**Number of Homologs in *H. marismortui***
Replication				
orc	COG1474	Cdc6-related protein, AAA superfamily ATPase	10	*17*
rep	No COG	Plasmid-type DNA replication initiator	3	*4*
mcm	COG1241	Predicted ATPase involved in replication control, Cdc46/Mcm family	1	*3*

rfa	COG1599/ COG3390	Single-stranded DNA-binding replication protein A (RPA), large (70 kD) subunit/small (32 kD) subunit	4, 2	*5, 3*
rfcA/rfcB/rfcC	COG0470	ATPase involved in DNA replication/ Processivity clamp loader subunit	1, 1, 1	1, 1, 1
pcn	COG0592	DNA polymerase sliding clamp subunit (PCNA homolog)	1	1
pri/pri2	COG1467/ COG2219	Eukaryotic-type DNA primase, catalytic (small) subunit/ large subunit	1,1	1,1
dnaG	COG0358	DNA primase (bacterial type)	1	1
polA1/polA2	COG1311/ COG1933	Archaeal DNA polymerase D family (small subunit)/ DNA polymerase II, large subunit/DNA polymerase δ, subunit B	1,1	1,1
polB	COG0417	DNA polymerase elongation subunit (family B)	2	2
rnh	COG0164	Ribonuclease HII	1	1
rnhA	COG0328	Ribonuclease HI	1	*2*
rad2(FEN1, XPG)	COG0258	5'-3' exonuclease (including N-terminal domain of PolI)/Flap endonuclease	1	1
lig	COG1793	ATP dependent DNA ligase	1	1
dnl3	COG1423	ATP-dependent DNA ligase, homolog of eukaryotic ligase III	0	*1*
ligA	COG0272	NAD-dependent DNA ligase (contains BRCT domain type II)	0	*1*
topA	COG0550/ COG0551	Type IA Topoisomerase/Zn-finger domain associated with topoisomerase type I	1	1
gyrA/gyrB	COG0188/ COG0187	Type IIA topoisomerase (DNA gyrase/topo II, topoisomerase IV), A subunit/B subunit	1, 1	1, 1
top6A/top6B	COG1697/ COG1389	DNA topoisomerase VI, subunit A/DNA topoisomerase VI, subunit B	1, 1	1, 1

Chromosome Segregation/ Cell Division				
smc1	COG1196	Chromosome segregation ATPases/SMC homolog	1	1
srl	COG1196/ COG0419	Chromosome segregation ATPases/ATPase involved in DNA repair	2	*4*
ucc	COG1340	Uncharacterized archaeal coiled-coil protein	1	1
mrp	COG0489	ATPases involved in chromosome partitioning/Mrp-like homolog	1	*2*
crcB	COG0239	Integral membrane protein possibly involved in chromosome condensation/CrcB-like homolog	2	2
ftsZ	COG0206	Cell division GTPase/Cell division septum	5	5
minD	COG2894/ COG0455	Septum formation inhibitor-activating ATPase/ATPases involved in chromosome partitioning	2	*6*
soj	COG1192	ATPases involved in chromosome partitioning/ParA-like homologs	6	*10*
Direct DNA Repair				
phr	COG0415	Deoxyribodipyrimidine photolyase	2	*3*
pll	COG3046	Uncharacterized protein related to deoxyribodipyrimidine photolyase	1	1
ogt	COG0350	Methylated DNA-protein cysteine methyltransferase/O-6-alkylguanine transferase	1	1
sod	COG0605	Superoxide dismutase	*2*	1
Mismatch Repair				
mutS1	COG0249	Mismatch repair ATPase (MutS family)	2	2
mutS2	COG1193	Mismatch repair ATPase (MutS family)	1	*2*
mutL	COG0323	DNA mismatch repair enzyme (predicted ATPase)	1	1

Nucleotide Excision Repair				
uvrA/uvrB/ uvrC/uvrD	COG0178/ COG0556/ COG0322/ COG0210	Excinuclease ATPase subunit/Helicase subunit of the DNA excision repair complex /Nuclease subunit of the excinuclease complex/Superfamily I DNA and RNA helicases	1, 1, 1, 1	1, 1, 1, 1
rad3a-c	COG1199	Rad3-related DNA helicases	3	3
rad25a-b (XPB)	COG1061/ COG1948	DNA or RNA helicases of superfamily II /ERCC4-type nuclease	2	*3*
uvdE	COG4294	UV damage repair endonuclease	0	*1*
Base Excision Repair				
ogg	COG0122	3-methyladenine DNA glycosylase/8-oxoguanine DNA glycosylase	1	1
mutT/mtl	COG0494	NTP pyrophosphohydrolases including oxidative damage repair enzymes/MutT/MutT-like	8	*11*
mutY	COG1194	A/G-specific DNA glycosylase	1	1
nthA,B	COG0177	Predicted EndoIII-related endonuclease	3	*4*
urg	COG1573	Uracil-DNA glycosylase	3	*4*
alkA	COG0122	3-methyladenine DNA glycosylase/8-oxoguanine DNA glycosylase	1	1
nfi	COG1515	Deoxyinosine 3' endonuclease (endonuclease V)	1	1
xthA	COG0648	Endonuclease IV	1	1
Homologous Recombination				
radA1-2	COG0468	RecA/Rad51 recombinase	2	2
hjr	COG1591	Holliday junction resolvase - archaeal type	1	1
rbl	COG1637	Predicted nuclease of the RecB family	3	3
recJ	COG1599	Single-stranded DNA-specific exonuclease	1	1
arj	COG1107	Archaea-specific RecJ-like exonuclease, contains DnaJ-type Zn finger domain	2	2

Non-Homologous End Joining				
mre11	COG0420	DNA repair exonuclease/Mre11/Rad 58 nuclease	1	1
rad50	COG0419	ATPase involved in DNA repair	1	1
eif4A	COG1111/ COG1948	ERCC4-like helicases MPH1/ERCC4-type nuclease	1	1
polIV	COG1796	DNA polymerase IV (family X)	0	*1*
Translesion Polymerase				
umuC	COG0389	Nucleotidyltransferase/ DNA polymerase involved in DNA repair	1	1
DNA/RNA helicases				
hel	COG1112	Superfamily I DNA and RNA helicases and helicase subunits	1	*3*
helA	COG1201	Lhr-like helicases	1	*4*
hepA	COG0553	Superfamily II DNA/RNA helicases, SNF2 family	1	1
brr2	COG1204	Superfamily II helicase	1	1
rad24a	COG1201	Lhr-like helicases	1	1
rad24b	COG1204	Superfamily II helicase	1	*3*
hcc	COG1205	Distinct helicase family with a unique C-terminal domain including a metal-binding cysteine cluster	0	*1*
Nucleases/ Phosphoesterases				
edn	COG2827	Predicted endonuclease containing a URI domain	1	1
exo	COG3359	Predicted exonuclease	1	1
mnl	COG1525	Micrococcal nuclease (thermonuclease) homologs	1	1
xseB	COG1722	Exonuclease VII small subunit	0	*2*
xseA	COG1570	Exonuclease VII, large subunit	0	*2*
rad24c	COG0622	Predicted phosphoesterase	1	*2*
ychR	COG1407	Predicted ICC-like phosphoesterases	2	2

Site Specific Recombinases/ Integrases				
ssr/xcd	COG0582/ COG4973/ COG4874	Integrase/Site-specific recombinase XerC/XerD	5	*16*
Putative Cell-Cycle Control Kinases				
stk	COG1718	Serine/threonine protein kinase involved in cell cycle control	1	1
stk2	COG0478	RIO-like serine/threonine protein kinase fused to N-terminal HTH domain	1	1
CRISPR associated proteins				
cas2	COG1343	Uncharacterized protein predicted to be involved in DNA repair	0	*1*
cas1	COG1518	Uncharacterized protein predicted to be involved in DNA repair	0	*1*
cas4	COG1468	RecB family exonuclease	0	*1*
cas3	COG1203	Predicted helicases	0	*1*
no homolog	COG1688	Uncharacterized protein predicted to be involved in DNA repair (RAMP superfamily)	0	*1*
no homolog	COG3649	Uncharacterized protein predicted to be involved in DNA repair	0	*1*
no homolog	COG1583	Uncharacterized protein predicted to be involved in DNA repair (RAMP superfamily)	0	*1*
Chromatin Structure				
hpyA	COG2036	Histone H3/H4 fusion	1	1
nhp	No COG	Nonhistone chromosomal protein MC1	1	1
Restriction Modification				
rmeM	COG0286	Type I restriction-modification system methyltransferase subunit	*1*	0
rmeR	COG0732	Restriction endonuclease S subunits	*1*	0

rmeS	COG0610	Type I site-specific restriction-modification system, R (restriction) subunit and related helicases	*1*	0
rme	COG1002	Type II restriction enzyme, methylase subunits	*1*	0
Other				
pelA	COG1537	PelA, Predicted RNA-binding proteins/Pelota	1	1

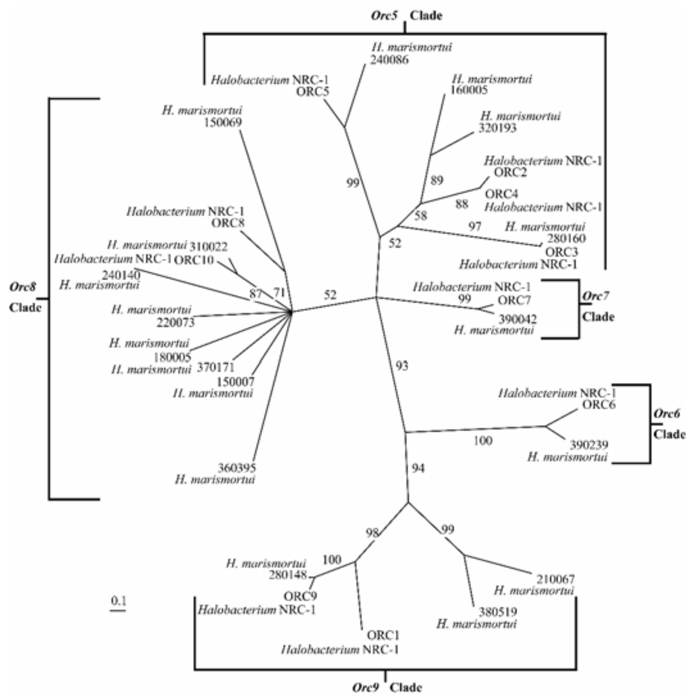

Figure 1. Quartet puzzling maximum likelihood consensus phylogenetic tree of Orc1/Cdc6 proteins from the genomes of *Halobacterium* sp. strain NRC-1 and *H. marismortui*. Clade designations (discussed in text) are outlined and named for the *Halobacterium* sp. strain NRC-1 Orc1/Cdc6 protein member(s). *Halobacterium* sp. strain NRC-1 gene names are used, while *H. marismortui* gene reference numbers are used.

In addition to eukaryotic type replication initiators, a single homolog of the heterohexameric eukaryotic-type replicative helicase proteins (MCM) is typically found in Archaea. This holds true for *Halobacterium* sp. strain NRC-1 which encodes a single chromosomal copy of *mcm* that has been found to contain an intein element (Ng et al.,

2000). For *H. marismortui,* three *mcm* genes were found, with two being located on the large chromosome, one with an intein and one without, and one homolog likely located on an extrachromosomal element (Table 2). It appears that the multiple Mcm homologs in *H. marismortui* are a result of lineage specific gene duplications, as phylogenetic analysis of these proteins depicts monophyly of the *Halobacterium* sp. strain NRC-1 Mcm and the three *H. marismortui* Mcm proteins (data not shown). The *H. marismortui* extrachromosomal homolog may in fact aid in the replication of this element by acting as its specific replicative helicase. Alternatively, *H. marismortui* Mcm proteins may come together to form a multimeric helicase complex, analogous to the eukaryotic MCM2-7 complex.

Single strand DNA (ssDNA) binding is accomplished in euryarchaea by homologs of replication factor A subunits Rfa32, Rfa41, and possibly Rfa14 (Kelly et al., 1998; Komori and Ishino, 2001). Four homologs of the Rfa41 subunit are found in *Halobacterium* sp. strain NRC-1, two with a zinc finger motif and two without. Two homologs of the Rfa32 subunit are also found and are arranged in tandem with the RFA41 homologs containing a zinc finger motif (Komori and Ishino, 2001), but no Rfa14 homolog(s) are readily detectable. *H. marismortui* contains five Rfa41 subunits, three of which contain a zinc finger motif while two do not. Associated with those three Rfa41 homologs are three Rfa32 homologs (Table 2). It is intriguing that both haloarchaea contain multiple copies of RFA complex genes. Perhaps these multiple copies serve specific functions within the cell, *i.e.* DNA replication, specific DNA repair pathways, and/or homologous recombination.

Both haloarchaea contain three subunits for the processivity clamp loader complex RFC, *rfcA, rfcB,* and *rfcC,* as well as a single gene (*pcn*) for the DNA polymerase processivity clamp, PCNA. For RNA priming, both organisms have single copies of the large and small subunits of the eukaryotic type primase, as well as a homolog of the bacterial type primase, DnaG. Whether these both act in the priming process or have a different cellular function is currently unknown. For RNA primer removal and Okazaki fragment maturation, homologs of a flap endonuclease, *rad2*, and two RNaseH homologs from both class I and class II types are present in these organisms (Ohtani et al., 2004). Although the replicative DNA polymerase for Euryarchaeota is yet to be determined, single homologs for the large and small subunit of the euryarchaeal specific D family polymerase are found in both *Halobacterium* sp. strain NRC-1 and *H. marismortui*. Both organisms also encode two homologs of B family polymerases. In both organisms, one gene is located on the large chromosome, while the other is found on large extrachromosomal replicons, (pNRC200 in *Halobacterium* sp. strain NRC-1) (Table 2). Both of these *polB2* genes are found adjacent to an *orc1/cdc6* homolog (*orc4*). It seems possible that the *polB2* homologs in these haloarchaea act in concert with *orc4* to initiate and replicate these particular extrachromosomal replicons.

For regulation of superhelical density and perhaps resolution of catenated chromosomes after DNA replication, *Halobacterium* sp. strain NRC-1 and *H. marismortui* encode both subunits of DNA gyrase, both subunits of the archaeal and plant type IIB topoisomerase, topoVI, and a type IA topoisomerase (Table 2). In *H. marismortui* TopA is interrupted by an intein, which must be excised out in order to form a functional type I topoisomerase. The only other example of an intein in a type I topoisomerase occurs in TopA of *Pyrococcus furiosus.* Both haloarchaea also possess multiple predicted integrases/site specific recombinases of the XerC/D family (Table 2).

All archaeal species sequenced to date contain an ATP-dependent DNA ligase. Some other Archaea, such as *Pyrococcus* sp., *Archaeoglobus,* methanogenic Archaea, and *H. marismortui* include a homolog of eukaryotic DNA ligase III, which appears to function in DNA repair and possibly recombination in eukarya (Martin and MacNeill, 2002). In addition to the two ATP dependent DNA ligases, *H. marismortui* also has been found to encode a homolog of a bacterial type NAD^+ dependent DNA ligase (Table 2). This is the only instance to date of a bacterial type NAD^+ dependent DNA ligase being found in an organism other than a bacterium, with the exception of a single eukaryotic virus, although the viral ligase lacks the zinc-binding tetracysteine domain and the BRCT domain (Sriskand et al., 2001). *H. marismortui* NAD^+ dependent DNA ligase shows 43% identity to LigA of *E. coli* and possesses all functional domains, including a C-terminal BRCT motif. No homologs of this protein can be identified in any other archaeon. It is quite likely that this gene has been laterally acquired by *H. marismortui*. The other possibility would be that all Archaea and eukaryotes have lost this NAD^+ dependent DNA ligase during evolution, whereas *H. marismortui* has retained it. Figure 2 displays phylogenetic analysis of DNA ligases from Bacteria and Archaea, which have previously been found to contain five conserved motifs and suggested to have evolved from a common ancestor (Aravind and Koonin, 1999). It clearly shows the phylogenetic place of this DNA ligase to be bacterial and quite distinct from the ATP dependent DNA ligases typically found in the archaeal domain of life. While the cellular function of this DNA ligase is not yet known, perhaps it has been acquired to act at the replication fork for ligation of Okazaki fragments, as in all Bacteria to date, or it may function in a specific DNA repair pathway.

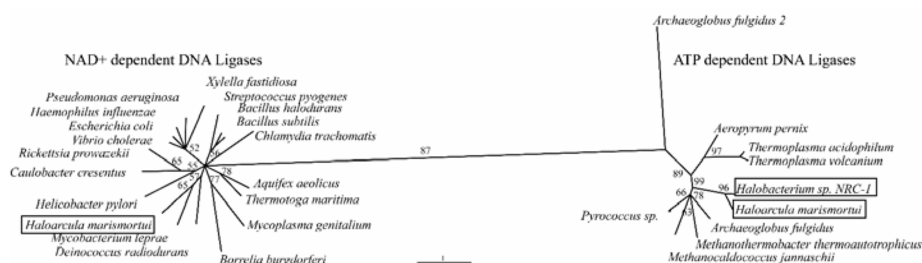

Figure 2. Quartet puzzling maximum likelihood consensus phylogenetic tree of NAD^+ (bacterial) and ATP (archaeal) dependent DNA ligase proteins. DNA ligases from *Halobacterium* sp. strain NRC-1 and *H. marismortui* are boxed.

Although much of the archaeal DNA replication apparatus is related to eukaryotic DNA replication apparatus, the archaeal cell division machinery appears to be more like the bacterial apparatus (Bernander, 1998, 2000, 2003). This may reflect the requirements for a prokaryotic cellular structure and division. Both *Halobacterium* sp. strain NRC-1 and *H. marismortui* contain five homologs of the bacterial cell division GTPase *ftsZ* as well as multiple homologs of the septum site determining protein MinD

(Table 2). *Halobacterium* sp. strain NRC-1 contains two of these latter homologs, whereas *H. marismortui* encodes six. Whether they all act in septum positioning and formation for cell division is currently unknown.

Another interesting finding from the genome sequence of *H. marismortui* was the presence of clustered regularly interspaced short palindromic repeats (CRISPR) along with CRISPR-associated (*cas*) genes. This particular class of repeats is found throughout the prokaryotic domain in both Bacteria and Archaea with 1 to 5 loci and anywhere from 1 to 128 repeats (Jansen et al., 2002). Previous analysis of halophilic Archaea had identified CRISPR loci in *Haloferax* sp. (Mojica et al., 1995). The DNA repeat sequence in *H. marismortui* is 30 bp in length with a DNA sequence of 5'-GTTTCAGACGGACCCTTGTGGGGTTGAAGC-3' and resembles those found in *Haloferax* sp. The actual function of these particular repeats is not yet known, although they have been speculated to play a role in partitioning (Mojica et al., 1995) or act as mobile genetic elements (Jansen et al., 2002). The CRISPR loci are typically physically associated with a core set of four genes (Table 2): a RecB family nuclease, a helicase, and two other genes predicted to be involved in DNA repair (Aravind et al., 1999). In *H. marismortui* three other genes are associated: two genes of the DNA repair RAMP superfamily and another gene predicted to be involved in DNA repair, which has previously only been detected in the genomes of three Bacteria (*Bacillus halodurans*, *Thermotoga maritima*, and *Streptococcus pyogenes*). *Halobacterium* sp. strain NRC-1 is lacking any CRISPR loci and accordingly it does not contain the set of genes typically found associated with these repeats.

For protein components used in DNA replicon partitioning, it seems likely that for both haloarchaea, their respective Smc homolog plays a large role in segregation of their large chromosome. Interestingly both organisms also possess additional genes which have homology to Smc and Rad50 (*srl)* (Table 2). In *Halobacterium* sp. strain NRC-1 both genes are found on the large extrachromosomal replicon, pNRC200 and possibly act as its segregation apparatus. Four such genes are encoded within the *H. marismortui* genome and perhaps play a similar role. It is also possible that these gene products are involved in some aspect of DNA repair, similar to Rad50. Also encoded in the haloarchaeal genomes are genes homologous to Soj (ParA) partitioning of *Bacillus* spp. These are all encoded for on the extrachromosomal replicons in *Halobacterium* sp. strain NRC-1 and could play a role in replicon partitioning.

3.2. DNA REPAIR AND RECOMBINATION

Both *Halobacterium* sp. strain NRC-1 and *H. marismortui* reside in environments which are subject to a number of DNA damaging agents, e.g. solar radiation, extreme aridity, and the presence of toxic heavy metals (Wang et al., 2004), and thus have evolved to deal with the presence of these damaging agents in their environments. Both organisms possess a suite of DNA repair enzymes to remedy the lesions caused by these damaging forces in order to maintain their genomic integrity.

In order to repair DNA damage due to exposure to UV radiation, both haloarchaea have light and dark repair processes. The complete genome sequence of *Halobacterium* sp. strain NRC-1 indicated the presence of two photolyase homologs. However, because photolyases cannot be distinguished from cryptochromes by their primary amino acid sequences, we cannot rule out the possibility that one of these homologs

functions in diurnal cycling. It has been shown that *Halobacterium* spp. have extremely efficient photoreactivation (Sharma et al., 1984) and one of the photolyase homologs (*phr2*) in strain NRC-1 encodes a functional cyclobutane dimer (CPD) photolyase (McCready and Marcello, 2003). The *phr2* gene is also physically linked to one of two homologs of superoxide dismutase (Takao et al., 1989). *H. marismortui* encodes three photolyase/chryptochrome homologs, with two most closely related to *phr2* of *Halobacterium* sp. strain NRC-1 and are likely CPD photolyases (Table 2). One of these *phr2* homologs is also physically linked to the only gene encoding a superoxide dismutase in *H. marismortui*, suggesting that this linkage has physiological importance relevant to the prevention and repair of DNA damage due to UV radiation and superoxide radicals in these two organisms. The function of *phr1* homologs in both organisms is currently unknown. Both haloarchaea also possess an ORF that is similar to photolyases (*pll*), but its role in the cell is yet unknown.

These organisms also possess the ability to repair UV damage under dark conditions. *Halobacterium* sp. strain NRC-1, *H. marismortui*, and some methanogenic Archaea possess bacterial type *uvrABCD* nucleotide excision repair complex. Like other archaeal organisms, the haloarchaea also encode homologs of the eukaryotic type nucleotide excision repair pathway, with three homologs each of *rad3* helicase and two homologs each of *rad25* helicase. As previously mentioned, both haloarchaea also possess a *rad2* flap endonuclease homolog. Whether these two systems act in concert to repair damaged DNA or act separately, is yet unknown. *H. marismortui* also is predicted to have a third alternative excision repair pathway for UV photoproduct repair (Table 2). This alternative excision repair pathway is dependent upon a UV endonuclease, Uvde, which forms an incision immediately 5' to the UV photoproduct and then a flap endonuclease (*rad2*) will excise the ssDNA region containing the photoproduct. This particular pathway of repair has been described in two eukaryotes, *Schizosaccharomyces pombe* and *Neurospora crassa*, as well as in *Bacillus subtilis* and *Deinococcus radiodurans* (Yasui and McCready, 1998). This is the first time it has been identified in a member of the archaeal domain of life. The presence of multiple UV damage repair pathways in these two haloarchaea underlies the importance of being able to repair photoproducts that arise due to solar UV radiation present in the environments which these organisms inhabit.

Classical mismatch repair systems are almost completely absent from archaeal organisms. No MutH homologs are readily identified, however MutS1 and MutL homologs can be identified in the genomes of *Methanosarcina acetivorans, Halobacterium* sp. strain NRC-1, and *H. marismortui*. It is tempting to speculate that these organisms acquired these genes through an ancient horizontal transfer, although it is possible that mismatch repair was lost in most other Archaea, as eukaryotes are known to possess *mutS* and *mutL* homologs. *Halobacterium* sp. strain NRC-1 and *H. marismortui* both contain two copies of *mutS1* along with a single copy of *mutL*. Two *mutS*2 homologs are also found in *H. marismortui*, with *Halobacterium* sp. strain NRC-1 containing a single homolog (Table 2). The actual function of MutS2 homologs is not known, but they are not likely to be involved in mismatch repair (Eisen and Hanawalt, 1999).

Both organisms possess a suite of genes predicted to be involved in base excision repair. Many MutT (NUDIX family pyrophosphohydrolase) homologs are coded for in both haloarchaea, with eight in *Halobacterium* sp. strain NRC-1 and eleven in *H.*

marismortui. It appears that elimination of oxidized bases in the dNTP pool may be a major mechanism for maintaining genetic fidelity, although the actual substrates for these homologs are currently unknown. A single homolog of an 8-oxo-guanine DNA glycosylase, Ogg (MutM) is present in both haloarchaea. MutY and Nth DNA glycosylases are present in both organisms, with both organisms possessing a single *mutY* gene, and three and four *nth* genes for *Halobacterium* sp. strain NRC-1 and *H. marismortui* respectively. Both organisms also possess multiple uracil DNA glycosylase genes, whose products act to remedy effects of spontaneous cytosine deamination and uracil misincorporation in DNA. A single alkylation damage glycosylase, AlkA, is present in both organisms as is a single gene, *xthA*, encoding an apurinic/apyrimidinic abasic site endonuclease (Table 2).

Halobacterium sp. strain NRC-1 and *H. marismortui* also possess a homolog of the UmuC translesion bypass DNA polymerase. *H. marismortui* also has been found to encode an X family DNA polymerase, homologous to DNA polymerase IV (Table 2). Members of this polymerase family are involved in non-homologous end joining in eukaryotes (Wilson and Lieber, 1999), so it is likely that the function is conserved in *H. marismortui*.

For homologous recombination and non-homologous end joining, both haloarchaea contain two *recA*/*rad51* homologs, RadA1 and RadA2, also called RadB. Both organisms also encode an archaeal type Holliday junction resolvase. RecB and RecJ homologs are also present, but their role in archaeal homologous recombination has yet to be determined (Table 2). Mre11 and Rad50 homologs are also present in single copies and are encoded by adjacent genes proximal to a B family DNA polymerase, PolB1, in both organisms. It is tempting to speculate that PolB1 homologs in these organisms interact with the MR complex and aid in the repair of DNA double strand breaks. Absent from the genomes of these organisms are the newly identified archaeal nuclease and helicase, NurA (Constantinesco et al., 2002) and MlaA (Manzan et al., 2004), whose genes are physically linked to *mre11* and *rad50* in the genomes of thermophilic Archaea. No homolologs of eukaryotic MR complex partners NBS1 and Xrs2 are present in archaeal genomes.

Overall, both *Halobacterium* sp. strain NRC-1 and *H. marismortui* possess both eukaryotic and bacterial features in DNA replication, repair, recombination, chromosome segregation, and cell division pathways. *H. marismortui* has been found to possess a larger number of paralogous genes in many categories, notably *orc1/cdc6*, *mcm*, *rpa*, *phr* (photolyase), and *mutT*. *H. marismortui* also was found to unexpectedly possess a bacterial NAD^+ dependent DNA ligase, the only microorganism that is not a bacterium which has been found to possess one to date. Additionally, *H. marismortui* is the first archaeon where an alternate UV repair endonuclease (Uvde) has been identified.

3.3. BASAL TRANSCRIPTION FACTORS

Basal transcription in Archaea is carried out by recognition of basal promoter elements, TATA box and Tfb recognition element (BRE), by TATA binding protein(s) (Tbp) and transcription factor IIB homolog(s) (Tfb). These basal transcription factors act to recruit the archaeal RNA polymerase (RNAP) complex, which is homologous to RNA polymerase II complex from eukaryotes. Archaeal RNA polymerase is made up of 11-

3 subunits that are homologs of their eukaryotic counterparts (Best and Olsen, 2001; Hickey et al., 2002). Both *Halobacterium* sp. strain NRC-1 and *H. marismortui* contain the A and A' homologs of RPB1 and also the β subunit of bacterial RNAP, B and B' homologs of RPB2 and β bacterial subunit, D homolog of eukaryotic RPB3 and bacterial α, E' and E'' homologs of RPB7, F homolog of RPB4, H homolog of RPB5, K homolog of RPB6, L homolog of RPB11 and bacterial α, N homolog of RPB10, P homolog of RPB12, and both haloarchaea possess two genes homologous to the M subunit and eukaryotic RPB9 and transcription elongation factor IIS (Tfs) (Table 3). It is possible that one homolog from each organism is a subunit of RNAP, while the other acts as Tfs, cleaving nascent RNA transcripts from RNAP at the 3' end (Hausner et al., 2000).

TABLE 3. Comprehensive listing of basal transcription factors in *Halobacterium* sp. strain NRC-1 and *H. marismortui*. Numbers in italics indicate a greater number of homologs in that particular genome.

Gene Name	COG	COG Description/Putative Function	Number of Homologs in *Halobacterium* NRC-1	Number of Homologs in *H. marismortui*
Basal Transcription Factors				
tbp	COG2101	TATA-box binding protein (TBP), component of TFIID and TFIIIB	*6*	1
tfb	COG1405	Transcription initiation factor TFIIIB, Brf1 subunit/Transcription initiation factor TFIIB	7	*9*
tfl	COG1405	Transcription initiation factor TFIIIB, Brf1 subunit/Transcription initiation factor TFIIB/Tfb like homolog	1	1
tfeA	COG1675	Transcription initiation factor IIE, alpha subunit	1	1
tfs	COG1594	DNA-directed RNA polymerase, subunit M/Transcription elongation factor TFIIS	1	1
RNA Polymerase Components				
rpoA	COG0086	DNA-directed RNA polymerase, β' subunit/160 kD subunit	1	1
rpoB'	COG0085	DNA-directed RNA polymerase, β subunit/140 kD subunit	1	1

rpoB''	COG0085	DNA-directed RNA polymerase, β subunit/140 kD subunit	1	1
rpoC	COG0086	DNA-directed RNA polymerase, β' subunit/160 kD subunit	1	1
rpb3	COG0202	DNA-directed RNA polymerase, α subunit/40 kD subunit	1	1
rpoE'	COG1095	DNA-directed RNA polymerase, subunit E'	1	1
rpoE''	COG2093	DNA-directed RNA polymerase, subunit E''	1	1
rpoF	No COG		1	1
rpoH	COG2012	DNA-directed RNA polymerase, subunit H, RpoH/RPB5	1	1
rpoK	COG1758	DNA-directed RNA polymerase, subunit K/omega	1	1
rpoL	COG1761	DNA-directed RNA polymerase, subunit L	1	1
rpoM	COG1594	DNA-directed RNA polymerase, subunit M/Transcription elongation factor TFIIS	1	1
rpoN	COG1644	DNA-directed RNA polymerase, subunit N (RpoN/RPB10)	1	1
rpoP	COG1996	DNA-directed RNA Polymerase, Subunit RPC10 (contains C4-type Zn finger)	1	1
Antitermination/ Termination factors				
nusA	COG0195	Transcription elongation factor	1	1
nusG	COG0250	Transcription antiterminator	1	1
Putative Chromatin Modifying Proteins				
sir2	COG0846	NAD-dependent protein deacetylases, SIR2 family	0	1
act	COG0454 / COG1243	Histone acetyltransferase HPA2 and related acetyltransferases/ELP3 Histone acetyltransferase	9	*18*

Archaeal organisms also possess a homolog of the N-terminal region of eukaryotic transcription factor IIEα (TfeA), including a single homolog in the genomes of *Halobacterium* sp. strain NRC-1 and *H. marismortui* (Table 3). *In vitro* studies of the *Sulfolobus solfataricus* TfeA homolog has indicated that this factor exhibits a slight stimulation on transcription, but that this stimulation is promoter specific (Bell et al., 2001). It seems likely that the haloarchaeal TfeA homologs function in a similar

manner, interacting with Tbp/Tfb complexes to stimulate transcriptional activity from certain promoters.

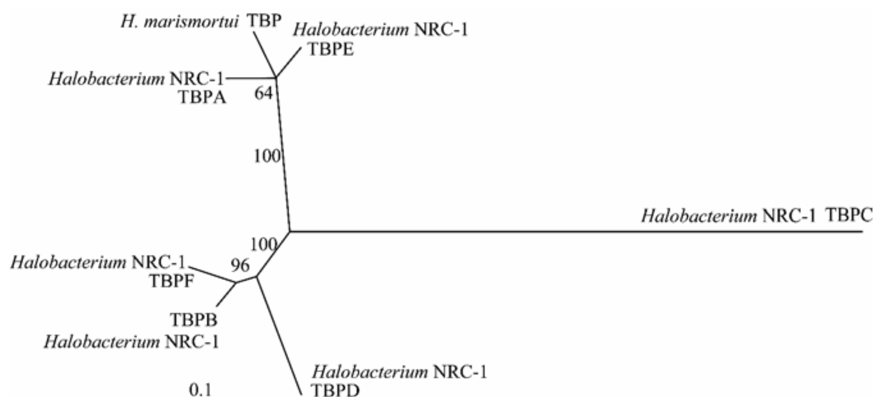

Figure 3. Quartet puzzling maximum likelihood consensus phylogenetic tree of Tbp proteins from the genomes of *Halobacterium* sp. strain NRC-1 and *H. marismortui*. *Halobacterium* sp. strain NRC-1 gene names are used, while the single *H. marismortui* Tbp is named.

One of the most surprising findings from sequencing the genome of *H. marismortui* was the identification of only a single *tbp* gene, similar to most other Archaea. This is in stark contrast to *Halobacterium* sp. strain NRC-1 which unusually encodes six different *tbp* genes (Table 3). The single Tbp protein from *H. marismortui* appears to be most closely related to the only chromosomally encoded Tbp protein in *Halobacterium* sp. strain NRC-1, TbpE (Fig. 3); all other *Halobacterium* sp. strain NRC-1 *tbp* genes are located on the extrachromosomal elements pNRC100 and/or pNRC200. Although TbpA from *Halobacterium* sp. strain NRC-1 groups with TbpE and *H. marismortui* Tbp, it is only half size, containing a single copy of the imperfect cyclin repeat which is duplicated in canonical Tbp proteins. This Tbp is likely to be functional through homodimerization, which would restore the two cyclin repeat "saddle" structure of a Tbp (Kosa et al., 1997). Although *H. marismortui* contains only a single Tbp, it contains more unique *tfb* genes than *Halobacterium* sp. strain NRC-1: 8 *vs.* 7, compared to 1 in most other Archaea (Fig. 4). The finding of a single *tbp* gene with a multitude of *tfb* genes raises questions regarding basal transcription and promoter selection in haloarchaea. *Halobacterium* sp. strain NRC-1, with six *tbp* genes and seven *tfb* genes, has been proposed to utilize different Tbp and Tfb combinations to select different gene promoter classes based upon environmental conditions and stimuli, analogous to sigma factors in Bacteria (Baliga et al., 2000; Ng et al., 1998, 2000). This hypothesis for Tbp/Tfb combinations for different promoter class recognition was borne not only out of the *Halobacterium* sp. strain NRC-1 complete genome sequence, but also through extensive mutagenesis of the *bop* gene promoter, whereby the TATA box contains a

noncanonical guanine and a consensus BRE could not be identified (Baliga and DasSarma, 1999, 2000). For *H. marismortui*, basal promoter selection would then seem to be regulated by the combination of a single Tbp with different Tfbs to recognize different promoter classes. Alternatively, for both organisms a single Tbp and a single Tfb could function to recognize all basal promoter elements, with other Tbps, for *Halobacterium* sp. strain NRC-1, and Tfbs for both acting to carry out other cellular functions.

Both *Halobacterium* sp. strain NRC-1 and *H. marismortui* also possess a single gene with some homology to Tfb, listed as Tbl in Table 3 and Fig. 4. This particular protein is placed into COG1405 with other Tfbs, but only possesses limited sequence homology to Tfb factors and is quite divergent from all other Tfb proteins found in *Halobacterium* sp. strain NRC-1 and *H. marismortui* (Fig. 4). This gene product may actually function as another Tfb in the haloarchaea, yielding another possible basal promoter recognition combination or it may serve a different cellular function.

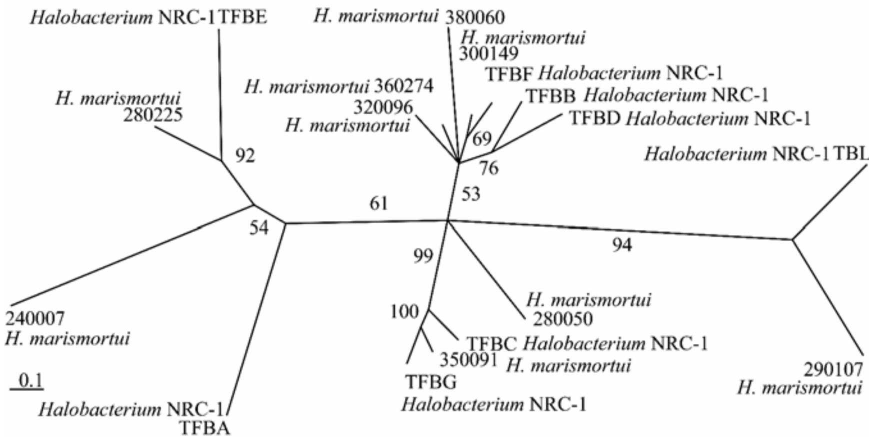

Figure 4. Quartet puzzling maximum likelihood consensus phylogenetic tree of Tfb proteins from the genomes of *Halobacterium* sp. strain NRC-1 and *H. marismortui*. *Halobacterium* sp. strain NRC-1 gene names are used, while *H. marismortui* gene reference numbers are used.

3.4. TRANSCRIPTIONAL REGULATION

Although most of the basal transcription machinery in these haloarchaea are homologous to eukaryotic factors, transcriptional regulators in these organisms appear to be more related to those used in Bacteria and the mechanisms of transcription regulation also appear to be similar in the prokaryotic domains of life, *i.e.* local regulation by repression or activation via a DNA binding protein. We used a combination of BLAST, COG, and CDD analyses to come up with a comprehensive listing of transcriptional regulators and placed them into families based upon helix turn

helix (HTH) homologies and COG groupings in the two haloarchaea, *Halobacterium* sp. strain NRC-1 and *H. marismortui* (Table 4).

TABLE 4. Comprehensive listing of predicted transcriptional regulators in *Halobacterium* sp. strain NRC-1 and *H. marismortui*. Numbers in italics indicate a greater number of homologs in that particular genome.

Gene Name	**COG(s)**	**COG Description(s)/Putative Function**	**Number of Homologs in *Halobacterium* NRC-1**	**Number of Homologs in *H. marismortui***
aamR	COG2150	Predicted regulator of amino acid metabolism, contains ACT domain	1	1
acaR	COG1545/ COG1478	Predicted nucleic-acid-binding protein containing a Zn-ribbon	2	*4*
acrR	COG1309	Transcriptional regulator/AcrR/TetR family	2	*8*
arcR	COG1414	Transcriptional regulator IclR	1	*11*
arsD	No COG	Arsenic resistence repressor	1	0
arsR/arl	COG0640/ COG1439/ COG1733/ COG1777/ COG1846/ COG1849/ COG1940/ COG2345/ COG3355	Predicted transcriptional regulators	24	*39*
asnC/aclR/ cinR/nirD/ nirH	COG1522	Transcriptional regulator/AsnC/Lrp family	12	*13*
balR	COG0271	Stress induced morphogen	1	1
bat/boa/ bolR	COG3413	Predicted DNA binding protein	10	*34*
birA	COG1654	Biotin operon repressor	1	1
calR	COG1497	Predicted transcriptional regulator	1	1
camR/hmr	COG3609/ COG0864	Predicted transcriptional regulators containing the CopG/Arc/MetJ DNA-binding domain/Predicted transcriptional regulators containing the CopG/Arc/MetJ DNA-binding domain and a metal-binding domain	4	*9*

cylR	COG4465	Pleiotropic transcriptional repressor,CodY, GTP-sensing transcriptional pleiotropic repressor CodY	0	*1*
dzr	COG1571/ COG3478	Predicted DNA-binding protein containing a Zn-ribbon domain	1	*2*
glcK	COG1940	NagC, Transcriptional regulator/sugar kinase	1	1
gul/imd	COG3620/ COG2524/ COG4109	Predicted transcriptional regulator, contains C-terminal CBS domains/Predicted transcriptional regulator containing CBS domains/	3	3
gvpD	COG0467	RecA-superfamily ATPases implicated in signal transduction/putative *gvp* operon repressor	*1*	0
idr/sirR/ troR	COG1321	Mn-dependent transcriptional regulator	4	4
marR/mrl	COG1510/ COG1846/ COG1733	MarR, Transcriptional regulators/Predicted transcriptional regulators	2	*3*
mbf	COG1813	Predicted transcription factor, homolog of eukaryotic MBF1	2	2
nacR	COG1308	Transcription factor homologous to NACalpha-BTF3	1	1
nbp	COG2402/ COG2405/ COG1848	Predicted nucleic acid-binding protein, contains PIN domain	*3*	2
pdb	COG2118	DNA-binding protein	1	1
pdm	COG1661	Predicted DNA-binding protein with PD1-like DNA-binding motif	1	1
phoU/prp	COG0704	PhoU, Phosphate uptake regulator	2	*8*
phrH	No COG	PhiHI Repressor Homolog	2	*4*
prlR/gvpE	COG1695	Predicted transcriptional regulators/padR-like	8	*12*
pspA	COG1842	Phage shock protein A (IM30), suppresses sigma54-dependent transcription	0	*2*
ptr1	COG4190	Predicted transcriptional regulator	1	1
ptr2	COG4742	Predicted transcriptional regulator	0	*1*
pzb	COG1439	Predicted nucleic acid-binding protein, consists of a PIN domain and a Zn-ribbon module	1	1
rflR	COG1339	Transcriptional regulator of a riboflavin/FAD biosynthetic operon	1	1
rspR	COG2002	Regulators of stationary/sporulation gene expression	1	*3*

smtR	COG1349	Transcriptional regulators of sugar metabolism	0	*5*
sstR	COG1378	Predicted transcriptional regulators	3	*10*
tenA	COG0819	Putative transcription activator	0	*2*
tzhR	COG3357	Predicted transcriptional regulator containing an HTH domain fused to a Zn-ribbon	1	1
vcl	COG1487	Predicted nucleic acid-binding protein, contains PIN domain	0	*2*
xrlR	COG1395/ COG1396/ COG1476/ COG1709/ COG2522	Predicted transcriptional regulator	5	*6*

The largest family of predicted transcriptional regulators in both *Halobacterium* sp. strain NRC-1 and *H. marismortui* is the bacterial-type ArsR (HTH) family. This particular HTH family of transcriptional regulators includes repressors that dissociate from DNA in the presence of metal ions to allow for transcription of the downstream genes (Wu and Rosen, 1993). In the metal ion rich environments where these two halophilic Archaea are found to reside, it is not terribly surprising that this family of transcriptional regulators is the major component of transcriptional regulation with thirty-nine genes in *H. marismortui* and twenty-four genes in *Halobacterium* sp. strain NRC-1. One of these regulators, ArsR in *Halobacterium* sp. strain NRC-1, has been shown to play a role in the arsenic resistance of this organism and whose transcription is induced by the presence of antimonite (Wang et al., 2004).

Another major family of transcriptional regulators found in haloarchaea is the AsnC/Lrp family. AsnC/Lrp family transcriptional regulators are typically small DNA binding proteins, whose target sequences lack inverted repeat elements and whose promoter binding capability is typically cooperative (Brinkman et al., 2003). Some biochemical and structural information on archaeal Lrp homologs is available for *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively, with a *S. solfataricus* homolog binding *in vitro* upstream of its own coding ORF and repressing transcription (Bell and Jackson, 2000; Napoli et al., 1999). A dozen genes encoding transcriptional regulators of this family are present in *Halobacterium* sp. strain NRC-1, with a baker's dozen (13) in *H. marismortui*, although the genes that they regulate are currently unknown.

A third large family of transcriptional regulators in haloarchaea belongs to a family named for *padR*, the repressor of *padA*, a phenolic acid decarboxylase of *Pediococcus pentosaceus* (Barthelmebs et al., 2000). PadR homologs have also been detected in both gram positive, e.g. *Bacillus subtilis,* and gram negative Bacteria, e.g. *Vibrio cholerae*. It appears that some members of this family act as repressors by binding inverted repeats directly upstream of the transcription start site of target genes (Barthelmebs et al., 2000). In *H. marismortui*, twelve genes encode *padR* family transcriptional regulators. In *Halobacterium* sp. strain NRC-1, eight genes encoding *padR* family transcriptional

regulators are present, including *gvpE*, a predicted regulator of gas vesicle synthesis (Jones et al., 1989; Kruger et al., 1998).

H. marismortui has fifteen transcriptional regulators predicted to be involved in sugar uptake (10) and utilization (5), while *Halobacterium* sp. strain NRC-1 encodes only three regulators for sugar specific uptake. This discrepancy is likely due to the metabolic versatility of *H. marismortui*, which is able to uptake and utilize a larger number of sugars than *Halobacterium* sp. strain NRC-1.

Homologs of the AcrR/TetR family are also present in both *Halobacterium* sp. strain NRC-1 and *H. marismortui*, with two and eight members being present respectively. ArcR/IclR family regulators are more prevalent in *H. marismortui*, with eleven members, than in *Halobacterium* sp. strain NRC-1, which contains just a single homolog.

Another family of regulators in these organisms falls under the xenobiotic response element (Xre) helix-turn-helix family. This type of transcriptional regulator was first identified in *B. subtilis* prophage PSBX as a repressor (Wood et al., 1990; McDonnell and McConnell, 1994) and has subsequently been identified in other Bacteria and a mold. *Halobacterium* sp. strain NRC-1 contains five members of this family of transcriptional regulators, while *H. marismortui* contains six.

A number of other transcriptional regulators/families are present in these two haloarchaea. *H. marismortui* contains nine regulators of the CopG/Arc/MetJ DNA binding domain, with four present in *Halobacterium* sp. strain NRC-1. Each halophile contains a single gene encoding regulators homologous to *bolA,* a regulator that induces morphogenic changes in *E. coli*; *birA*, a homolog of the biotin operon repressor and biotin-(acetyl-CoA carboxylase) ligase; a homolog with the CAP/CRP catabolite activator/repressor protein DNA binding domain; NagC homolog, *glcK*, which acts as both a sugar kinase as well as a transcriptional regulator; and a homolog which is predicted to regulate a FAD/riboflavin biosynthetic operon.

These aforementioned transcriptional regulators are predicted to mostly be repressors of transcription, based on their homology to bacterial repressors and the predominant positioning of the HTH motif in the N-terminus, which is also characteristic of transcriptional repressors (Perez-Ruenda and Collado-Vides, 2000, 2001). The transcriptional regulator families were identified by comparison of HTH sequences. Identification of these transcriptional regulators and families provides a solid foundation to identify *cis* acting regulatory DNA motifs, to identify sets of coordinately regulated genes, and their transcriptional profiles in response to environmental changes.

Interestingly, both *Halobacterium* sp. strain NRC-1 and *H. marismortui* contain some regulators that are homologous to factors found in eukaryotes (Table 2). Examples are their two homologs each of eukaryotic MBF1, multiprotein bridging factor. In eukaryotes, this protein factor acts to bridge transcriptional activators to TBP, thus serving as a co-activator (Takemaru et al., 1997). With a least one Tbp homolog in each genome, it is possible that these proteins function in a similar manner.

Although transcriptional repression seems to be a large mechanism of transcriptional regulation in these haloarchaea, both organisms encode a family of transcriptional regulators that all contain their HTH in the C-terminus and appear to act as transcriptional activators based upon genetic studies. This family of transcriptional regulators is related to *bat*, the bacterio-opsin activator of transcription, from

Halobacterium sp. strain NRC-1. Ten homologs are present in *Halobacterium* sp. strain NRC-1 and an astounding thirty-four homologs are present in *H. marismortui.* The Bat protein of *Halobacterium* sp. strain NRC-1 is the founding member of this family of transcriptional activators. It contains both sensor domains (PAS/PAC and GAF motifs), as well as the specific pfam HTH10 family DNA binding domain. The Bat protein is responsible for coordinate regulation of bacteriorhodopsin (purple membrane, bacterio-opsin protein + retinal cofactor) production in this organism. It acts by binding to a 15 bp consensus upstream activator sequence (UAS) to promote transcription of not only the *bop* (bacterio-opsin) gene, but also *blp* (converts β-carotene to retinal) and *brp* (converts β-carotene to retinal), the last step in retinal synthesis; and *crtB1* (phytoene synthase), the first committed step in retinal synthesis (Baliga et al., 2001; Peck et al., 2001). Another family member (*dmsR*) in *Halobacterium* sp. strain NRC-1 appears to act locally on an operon responsible for DMS utilization. DmsR appears to act by stimulating transcription of the *dmsEABCD* operon (Mueller and DasSarma, 2004). Of the ten proteins in this transcriptional activator family, Bat and two other members possesses known sensory domains in addition to the HTH DNA binding region. Bat contains a redox/oxygen sensing PAS/PAC motif and light sensing GAF domain. Boa1 (bacterio-opsin activator-like) contains 2 PAS domains as well as a GAF domain and Boa4 contains a GAF domain. Boa4 and its ortholog in *H. marismortui* are also predicted to have a phage integrase domain. Whether this domain is functional in these organisms and the role it would play in a transcriptional activator, or a remnant of a phage integration event is not known. In *H. marismortui,* the *bat* family regulators (bfr) comprise a large portion of their predicted transcriptional regulators. Seven of the thirty-four regulators also contain sensor domains in addition to the HTH DNA binding domain. One protein contains an N-terminal response regulator domain, 2 PAS/PAC (redox sensing) motifs, 2 GAF (light responsive) domains, and the C-terminal HTH. Another member contains an N-terminal response regulator domain, 2 PAS/PAC (redox sensing) motifs, a GAF (light responsive) domain, and the C-terminal HTH. A third member containing an N-terminal response regulator receiver domain also contains 3 GAF (light responsive) domains and the C-terminal HTH. A member contains a GAF domain followed by a PAS/PAC motif followed by another GAF domain and the C-terminal HTH. One member contains a single PAS/PAC motif, a single GAF domain, and the C-terminal HTH. The last member containing sensing domains has 2 PAS/PAC (redox sensing) domains along with the C-terminal HTH. It is likely that these proteins are a large part of light, oxygen, and redox sensing for the cell and act to activate transcription of genes necessary for adaptation to the environmental conditions. They may act as Bat does in *Halobacterium* sp. strain NRC-1 by coordinately regulating a number of genes through their own sequence specific upstream activator sequences. The additional twenty-nine bfr homologs most likely also play a role in activating transcription of a number of genes, but whether they act globally or locally is not currently known.

3.5. TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS

Two-component signal transduction systems are ubiquitous throughout the three domains of life. They serve as mechanisms for cells to recognize changes in environmental conditions and to elicit a proper response. A typical two-component

signal transduction system is comprised of a histidine protein kinase and a response regulator receiver protein. The histidine kinase protein senses environmental stimuli, whereby a phosphoryl group is transferred to the response regulator receiver thereby activating an effector domain which can then activate transcription of genes to provide a specific cellular response (Stock et al., 2000). Halophilic Archaea inhabit dynamic environments with gradients of salinity, nutrients, oxygen, and light among other variable conditions, and are able to sense and respond to these variables. In addition to integral membrane halotransducer proteins (*htr*), 17 in *Halobacterium* sp. strain NRC-1 and at least 14 in *H. marismortui*, both organisms possess a number of sensory histidine kinase homologs and response regulator receiver homologs.

Halobacterium sp. strain NRC-1 encodes twelve sensory histidine kinase homologs, containing a histidine kinase A phosphoacceptor domain (HisKA) and a histidine kinase ATPase domain (HATPase), in its genome and has six predicted response regulator receivers, including the CheY response regulator of chemotaxis. Three sensory histidine kinases are physically linked in the genome to a response regulator, and these presumably act in concert to sense stimuli and provide the impetus for a cellular response. One sensory histidine kinase contains the cGMP binding, light responsive GAF domain, while the other two contain light, redox, and oxygen sensing PAS/PAC domains. Two gene products from *Halobacterium* sp. strain NRC-1 are both sensory histidine kinases as well as the response regulator receivers. Both proteins are made up of multiple sensory motifs; with one containing an N-terminal response regulator receiver domain, two PAS motifs, a GAF domain followed by 3 PAS/PAC motifs and the other, encoded on both extrachromosomal elements, contains an N-terminal response regulator receiver domain, 3 PAS motifs and a PAS/PAC motif; along with the HisKA and HATPase domains. Two additional sensory histidine kinase homologs contain a GAF domain, another contains a HAMP motif, while two have no recognizable sensory motifs and another contains only a HisKA domain. Also within the genome is a gene product which has a stand alone GAF domain and another which contains a stand alone PAS/PAC motif. The occurrence of these domains in sensory histidine kinases, as well as the aforementioned *bat* family transcriptional regulator family (bfr), and as stand alone modules underlies the tremendous importance of sensing light, oxygen, and redox potential for *Halobacterium* sp. strain NRC-1.

Similarly, sensing light, oxygen and redox potential is extremely important for *H. marismortui*. This organism has devoted fifty-eight ORFs to signal transduction sensory histidine kinases and twenty ORFs to response regulator receivers, which do not contain HisKA or HATPase domains. The percentage of genes devoted to signal transduction (excluding *htr* genes) is approximately double that of which is devoted in *Halobacterium* sp. strain NRC-1 (~1.70% to ~ 0.75% respectively). Twelve sensory histidine kinases are physically linked in the *H. marismortui* genome to a response regulator, and these pairs presumably act in concert to sense stimuli and provide the impetus for a cellular response. Twenty-two signal transduction histidine kinases, which do not contain a response regulator receiver domain, contain either PAS/PAC and/or GAF motifs. This is ~ 7 times more than what is found in *Halobacterium* sp. strain NRC-1. Three of these sensory histidine kinase proteins contain both PAS/PAC redox sensing motifs and GAF light sensing domains, with one member having nine PAS motifs and two GAF domains. Twenty of the predicted sensory histidine kinase homologs also contain response regulator receiver domains in their N-terminus. Of

those twenty, four have no known additional sensory motifs, two contain only a single GAF domain, nine contain one to three PAS/PAC motifs, and five contain both PAS/PAC motif(s) and a GAF domain(s). *H. marismortui* encodes twenty response regulator receiver homologs that do not contain HisKA or HATPase domains. Seven of these contain PAS/PAC and/or GAF motifs as well, while the remainder appears to be simple response regulator receiver proteins with no additional protein domains. Two other proteins are coded for which do not contain sensory histidine kinase motifs, nor do they contain response regulator receiver domains. One contains a stand alone PAS/PAC motif, while the other contains two GAF domains and four PAS/PAC motifs. As stated for *Halobacterium* sp. strain NRC-1, it seems that a large portion of environmental responses for *H. marismortui* involves light, oxygen, and redox. Over half of the sensory histidine kinases contain motifs which are involved in sensing changes in these environmental conditions. Approximately a third of the response regulator receivers also contain these domains, six bfr transcriptional regulators contain these motifs, two of those also containing N-terminal response regulator receiver domains, and two additional proteins contain these light, oxygen, and redox sensing domains.

H. marismortui again, has devoted additional genes to both transcriptional regulators and two-component signal transduction systems, although it has limited basal promoter selection to a single Tbp protein. From the comparative genomic analyses it appears as though *H. marismortui* contains more complex regulatory networks, due to the larger number of transcriptional regulators and two component signal transduction systems, than *Halobacterium* sp. strain NRC-1. An overriding theme in the two organisms is an importance on sensing and responding to light, oxygen, and redox conditions.

4. Conclusions

The availability of genome sequences from two diverse extremely halophilic Archaea, *Halobacterium* sp. strain NRC-1 and *H. marismortui*, has allowed for a detailed examination into information transfer processes and proteins involved in DNA replication, repair, recombination, chromosome segregation, cell division, basal transcription, transcriptional regulators and two component signal transduction systems. These two diverse haloarchaea inhabit harsh and extreme environments in which they must be able to utilize nutrients for growth, respond to a number of DNA damaging agents, and respond to dynamic changes in light, oxygen, redox, nutrients, and salinity, among other environmental conditions. A general finding is that *H. marismortui* encodes a larger number of genes in every category examined. *H. marismortui* also possesses added capabilities, with the exception of TATA box recognition. By analyzing these two organisms from a comparative genomic perspective allows for common conserved elements to be recognized. The availability of facile genetic and genomic manipulation of *Halobacterium* sp. strain NRC-1 provides for a strong experimental platform to interrogate these conserved haloarchaeal characteristics. Utilizing *ura3* based gene knockout/gene replacement methodologies (Peck et al., 2000; Wang et al., 2004), microarray technologies, and traditional molecular biological techniques coupled to comparative genomic and bioinformatic approaches it is possible for extremely fruitful post-genomic studies on the model halophile *Halobacterium* sp. strain NRC-1. These results can be extended to other halophilic Archaea, *i.e. H.*

marismortui, providing useful data for a better understanding of the archaeal domain of life.

5. Summary

Halophilic Archaea inhabit environments containing high solar (UV) radiation and nearly saturated salts. Some flourish in thalassic environments containing 4-5 M NaCl, e.g. *Halobacterium* sp. strain NRC-1, while others, like *Haloarcula marismortui*, prosper in athalassic environments containing less than M NaCl and nearly 2 M $MgCl_2$. The availability of genome sequences for these two diverse haloarchaeal organisms enabled a "halocentric" comparative genomic survey to be conducted. We examined a number of predicted gene products involved in informational cellular processes; DNA replication, repair, recombination, chromosome segregation, cell division, basal transcription factors, transcriptional regulators, and signal transduction; in these two organisms for similarities and differences using reciprocal best hit, clusters of orthologous genes (COG), and protein domain predictions via the conserved domain database (CDD) and Pfam database. We found common themes in these two haloarchaea, such as expansion of *orc1/cdc6* origin recognition complex homologs, redundant pathways for nucleotide excision repair, and many signal transduction components containing light, oxygen and/or redox sensing domains. We also found significant differences including the presence of a bacterial-type NAD^+ dependent DNA ligase and only a single gene encoding a TATA binding protein (Tbp) in *H. marismortui*, while *Halobacterium* sp. strain NRC-1 contains a restriction modification system which is absent in *H. marismortui.* The larger genome size of *H. marismortui* is not only devoted to additional metabolic capabilities, but also to DNA replication, repair, transcriptional regulation, and signal transduction proteins and pathways.

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WALSBY'S SQUARE ARCHAEON

It's Hip To Be Square, But Even More Hip To Be Culturable

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"The mask that you wore - My fingers would explore
The costume of control - Excitement soon unfolds"
Jim Morrison, Easy Ride

1. Historical Background

Twenty-five years ago Anthony Walsby and his wife Fausta paid a short visit to Eilat in Israel, principally to look at the Solar Lake on the Sinai Peninsula. Wolfgang Krumbein, who at that moment was working in Eilat, invited them for a two-day trip to Ophira and the Sabkha Gavish in the south of Sinai. Walsby collected samples from salty puddles that were used by Bedouin to collect solar salt. Upon return to the Marine Science Department in Menai Bridge, North Wales, he analysed the samples by light microscope in search for gas-vacuolate cyanobacteria. It was then that he saw the square 'bacteria' for the first time (Walsby, 1980). The original report of this discovery, which he published in Nature, was received with some scepticism since at that time cells with that unique shape were unknown. It is now well established that these square structures are living micro-organisms although they are still the only known examples with such a perfectly square morphology. Walsby told me that in his manuscript he originally proposed to name the organism "*Quadra fausta*", for his wife, but with the pretence that "fausta" (Latin "fortunate") reflected the serendipity of the discovery. However, one of the referees suggested not to name it until it was in axenic culture and so this name was left out of the final 1980 publication. Walsby said he spent several months trying to isolate the squares, but without success. Thereafter, many others tried to isolate these intriguing squares for further characterisation. Until now nobody succeeded, despite the fact that these square cells were quite large, easily recognised by microscopy, and highly abundant in crystalliser ponds, which were easily accessible. This enigmatic organism stubbornly remained in the long list of unculturables.

2. The Unculturables

Hampered by the fact that many micro-organisms are not easily cultivated, and due to the introduction of novel molecular biological tools, microbial ecological research has

shifted from the more traditional cultivation based analysis of microbial communities to a state of the art molecular discipline. The molecular approach has focused largely on the conserved 16S rRNA gene encoding the prokaryotic ribosomal small subunit RNA molecule, as a molecular identifier for micro-organisms (Woese and Fox, 1977). One of the major conclusions to be drawn from the culture independent analysis of various ecosystems is that the extant microbial diversity is far greater than previously thought. Analysis based on 16S rRNA gene sequence comparisons led to the discovery of many novel microbial taxa and species. Nevertheless, these studies also have important limitations. Often they do not get beyond assigning putative relationships with other organisms and speculations on their eco-physiological role are based on similarities with related cultivated species, which is very unreliable since microbes can be very diverse in their physiology and genetic makeup even within a narrow range of variation of the 16S rRNA gene sequence (Rodríguez-Valera, 2002). Therefore cultivation is still the method of choice to fully understand the physiology and complex ecological interactions in which micro-organisms engage.

However a common problem in microbiology is the fact that a large number of micro-organisms that are present in an ecosystem resist cultivation with the conventional tools. Many of these "unculturables" are well known to microbiologist either by molecular detection, visual observation or by their biochemical signatures of reactions they catalyse. Unfortunately, the species that seem less important for the ecosystem functioning are relatively easily cultivated, whereas the ecologically relevant and dominant organisms often resist cultivation. Nevertheless, improvement of cultivation techniques and insight in their specific requirements from physiological studies led to a number of recent successes in which micro-organisms previously thought to be unculturable were isolated. Well known examples are species of "Pelagibacter" belonging to the abundant marine SAR11 cluster of bacteria (Morris et al., 2002; Rappé et al., 2002) and the anaerobic ammonium oxidising planktomycetes (anammox) (Strous et al., 1999).

In this overview I will summarise our current knowledge about one of the most intriguing micro-organism that for long could not be cultivated, the enigmatic halophilic square archaeon, better known as "Walsby's Square Bacterium". The square archaeon has recently been isolated in both my lab in Groningen, The Netherlands (Bolhuis et al., 2004) and in the lab of Mike Dyall-Smith, Melbourne, Australia (Burns et al., 2004b).

Although the subject of this review has become widely know as Walsby's Square Bacterium, I will refer hereafter only to Walsby's Square Archaeon since phylogenetically this organism belongs to the domain of Archaea and further confusion about its phylogenetic position should be prevented.

3. Culture Independent Characterisation of Walsby's Square Archaeon

As described above, the square Archaea are found in hypersaline ecosystems. These include naturally formed or man-made ecosystems in which seawater evaporates, leading to a concentration and sequential precipitation of calcium carbonate and calcium sulphate, and leaving a hypersaline sodium chloride rich brine. Further evaporation and concentration of the brine result in the precipitation of sodium chloride (halite), where

after a dense magnesium chloride brine develops (bittern) that eventually becomes hostile to life (Oren, 1994). It is especially at the last stage of halite formation and before the brines get sterile, where the squares dominate (Benlloch et al., 1995). The original finding place of Walsby's Square Archaeon in the Sinai Peninsula was visited by Walther Stoeckenius in August 1980 who described it as a pool of which most of the 500 m diameter depression was evaporated. At that time of the year, it solely consisted of a 10 to 15 meter wide and 150 m long pool that was not more than 20-50 cm deep (Stoeckenius, 1981). However as Walsby remarked he suspected that Stoeckenius would not have had to travel this far and that similar organisms are likely to be found in other hypersaline ecosystems as well (A. Walsby, personal communication). This was indeed true since Stoeckenius later found square Archaea close to his own lab in the San Francisco Bay salterns (Stoeckenius, 1981). It is currently known that these organisms are widely distributed and can be found in a variety of hypersaline environments that are close or above sodium chloride saturation such as the well studied crystalliser ponds in Spain (Benlloch et al., 1995, 1996, 2001, 2002; Bolhuis et al., 2004), near San Francisco Bay and in Baja California (Stoeckenius, 1981), Australia (Burns et al., 2004a, 2004b), Israel (Oren, 1993) and in Lake Saxkoye, Russia (Oren, 1994).

In the absence of molecular tools and isolation procedures, the square archaeon has initially mainly been studied by (electron-) microscopy or on basis of physiological tests on samples that were naturally enriched in the abundantly present square Archaea. However, since none of these samples were pure, caution had to be made in drawing conclusions from the experiments. Below I will summarise a number of the initial observations and physiological test.

3.1. STRUCTURAL FEATURES

3.1.1. The Cell Envelope

In order to provide evidence to criticasters that the square cells were indeed living micro-organisms rather than some crystalline artefacts, Walsby conducted an electron microscopic analysis of the cells in collaboration with Ken Parkes. They showed that the surface of the squares was covered with ~20 nm particles that were arranged in a regular hexagonal lattice later to be identified as a surface layer (S-layer) and a characteristic feature for haloarchaea (Parkes and Walsby, 1981). The presence of this S-layer in the square cells led Walsby to his, as was shown later correct, assumption that the squares were related to haloarchaea (Walsby, 1980).

In general, S-layers are formed from identical protein subunits arranged into a monolayer of simple and repetitive patterns (Eichler, 2003). Archaeal S-layers are dominated by glycosylated proteins that, like other haloarchaeal proteins, are enriched in acidic residues relative to S-layer glycoproteins originating from non-halophilic Archaea. This enhanced number of acidic residues is thought to promote proper protein folding in high-salt conditions (Eichler, 2003). Archaeal S-layer glycoproteins consist of a C-terminal transmembrane domain that anchors the glycoprotein to the plasma membrane. The extracellular part of the protein can undergo both *N*- and *O*-glycosylation, a process that apparently takes place after the protein has been transported through the cytoplasmic membrane (Zhu et al., 1995).

The S-layer of the square archaeon was studied in more detail (Kessel and Cohen, 1982; Stoeckenius, 1981), confirming the regular array of 20 nm particles typical of an S-layer. Kessel and Cohen (1982) presented optical diffraction patterns of S-layers from two adjacent square Archaea that revealed both a hexagonal and tetragonal array of subunits and different lattice constants. They suggested that these lattices might have been derived from different square Archaea but not necessarily belonging to different species.

A single function for S-layers has not yet been identified. Instead S-layers may serve various roles, such as protective coats, structures involved in cell adhesion and surface recognition, molecular sieves, scaffolding for enzymes or to determine cell shape (Sleytr and Beveridge, 1999). Nevertheless, in Archaea the S-layer most likely serves as the cell-envelope. Especially in Archaea such as *Methanococcus*, *Sulfolobus*, *Halobacterium* and *Thermoproteus* the S-layer seems to be the exclusive cell-wall component outside the plasma membrane and therefore determine the shape of the cell. It is likely that in square Archaea the S-layer, in combination with other cell wall components, plays an important role in maintaining their unique shape. One of these alternative cell wall components that may contribute to the square shape is a fibrillar sheath that has been observed (Kessel and Cohen, 1982; Parkes and Walsby, 1981). However, a fibrillar sheath is not always observed and was not reported to be present in cells from the pure cultures. This discrepancy might be indicative for the presence of more than one species of square Archaea, or fibrillar sheaths may only be expressed under yet unknown physiological conditions.

3.1.2. Gas Vesicles

One of the most eye-catching features of the square Archaea besides their perfect square shape is the abundant presence of intracellular white refractile bodies (Fig. 1). As mentioned above, Walsby showed that these refractile bodies were so called gas-vesicles, which at that time and currently still Walsby's major scientific topic of interest (Walsby, 1994). Gas vesicles are hollow proteinaceous structures that usually have cylindrical tubes and are closed by conical end caps. Gas vesicles can be found in several planktonic species of Bacteria and Archaea, in which they provide buoyancy. They are impermeable to liquid water, but highly permeable to gases and are normally filled with air. Al though there are currently up to 14 genes identified that have been implicated in gas vesicle production, only two proteins play a dominant role in the actual gas vesicle structure. GvpA is the dominant protein, making up ~ 95% of the gas vesicle and forming a rigid rib structure. GvpC binds to the outside of the ribs and stabilizes the structure against collapse (Beard et al., 2002; Walsby, 1994). Gas vesicles from different organisms vary in shape from cylindrical with conical ends to spindle shaped forms and in size from 45 up to 1400 nm (Walsby, 1994). Through regulation of their relative gas vesicle content aquatic microbes are able to perform vertical migrations. Apparently, in slow growing organisms, which the square archaeon most certainly is, the vertical movement by means of gas vesicles is more efficient than by swimming with flagella, which in fact are absent in the square archaeon.

The gas vesicles observed in the square Archaea appear morphologically similar to those described for other haloarchaeal and bacterial species (Bolhuis et al., 2004; Parkes and Walsby, 1981). They vary in length from 80 to almost 1000 nm and are frequently

concentrated around edges of the squares. In other occasions the square cells appear to be completely filled with gas vesicles which are distributed evenly over the cells. Similar to their role in other organisms the gas vesicle aid the cell to optimally position itself within the water column, i.e., close to the surface so that they can optimally scavenge the scarce oxygen in these hypersaline brines and benefit from the light as an alternative source of energy (see below). Although gas vesicle negative mutants might occur in their natural environment, the absence of gas vesicles have been often used as a selective marker to distinguish "square like micro-organisms" from Walsby's square archaeon (Javor et al., 1982; Oren et al., 1999).

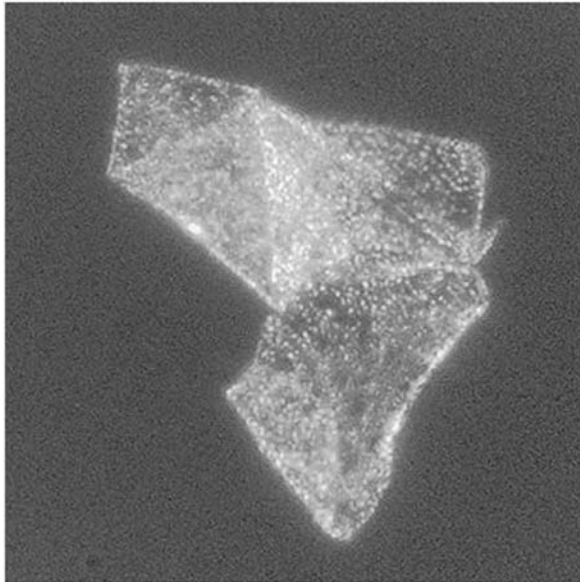

Figure 1. Darkfield microscopic image of a large folded sheet of 'Haloquadratum walsbyi', revealing its sharp edges and straight corners. Size is ~40x40 micrometer. White spots are gas vesicles.

3.1.3. Poly-(β-Hydroxy-Butyric Acid) Granules

Besides gas vesicles, the square archaeon also contain large granular intracellular structures that give the flat cells sometimes a lumpy appearance and that are in contrast to gas vesicles not refractile but rather appear dark when studied by phase contrast microscopy (Kessel and Cohen, 1982; Stoeckenius, 1981). It was speculated that these granules consisted of poly-β-hydroxybutyric acid (PHB). This was recently confirmed by analysis of pure cultures in which the PHB granules were stained with the PHB specific stain Nile Bleu A (Burns et al., 2004a). The formation of PHB granules were

initially considered to be a unique feature of the squares since at that time these had not been observed in any other halophilic Archaea. However, PHB production has later been well characterised in *Haloferax mediterranei* (Fernandez-Castillo et al., 1986) and species of *Haloarcula* (Hezayen et al., 2000; Kirk and Ginzburg, 1972; Nicolaus et al., 1999).

PHB and related compounds function as storage compounds of carbon and energy that are accumulated during unbalanced growth by prokaryotes, i.e. in the presence of an excess of a carbon source in combination with limitation of another nutrient such as nitrogen, phosphor, sulphur or oxygen (Kolibachuk et al., 1999). In the hypersaline ecosystems, especially oxygen may be one of the limiting factors because of the low dissolution of oxygen in the brines. PHB may be reutilised by the organism when the carbon source gets exhausted. Some PHB polyesters have physical properties similar to those of polypropylene, making them a relatively cheap source of biodegradable plastic from renewable resources (García Lillo and Rodriguez-Valera, 1990; Kolibachuk et al., 1999).

4. It's Hip To Be Square! But Is It Also Useful?

There have been several speculations on how these cells are able to generate and maintain their square shape and their, as Walther Stoeckenius stated, "amazing acute edges and corners". The consensus idea at the moment is that the shape is most likely determined by the rigidity of the S-layer in combination with the absence of turgor pressure (Walsby, 1980). Kessel and Cohen (1982) noted from their electron microscopic images that the edges are curved or rounded rather than straight, as would be expected for a perfect square or rectangular box with a very narrow width. They envisioned the organism as a flattened cylinder, which, to my opinion, is the most likely explanation for the square shape.

Being flat and square is hip as the saying goes and the media attention about the initial discovery and recent successful cultivation shows, but it also provides the most optimal surface to volume ration for a single celled micro-organism. Many essential processes take place at the cell membrane, e.g. uptake of nutrients and oxygen, disposal of waste products and catching light for photosynthetic processes. Unlike Eukaryotes, micro-organisms have no means of intracellular transport of waste products and nutrients to the appropriate part in the cell but instead rely on the process of diffusion. However, diffusion is a rather slow process and rapidly becomes the rate-limiting step when cells increase in volume. This is one of the major reasons why micro-organisms stay as small as they are (Koch, 1996). For micro-organisms it is therefore essential to maintain an optimal surface to volume ratio. As can be seen in Fig. 2A, the surface to volume ratio for spherical cells rapidly decreases with increasing cell size, a very unfavourable situation. Instead, flat organisms such as the square Archaea maintain an optimal surface to volume ratio no matter how large they would become as long as they stay flat. The same would be true for flat circular, disk-like micro-organisms (e.g. *Haloferax volcanii* (Mullakhanbhai and Larsen, 1975). Therefore, with respect to the optimal surface to volume ratio it's rather hip to be flat. Being square instead of

circular may have an additional benefit in most efficiently occupying the available space at the surface of a water column (Fig. 2B).

This optimal surface to volume ratio may also explain why square Archaea can become as large as they do. Regularly cell sizes are measured in natural samples ranging from 5 x 5 to 40 x 40 micrometers. These larger cell structures may be in the process of cell division, as was clearly demonstrated in a unique image by Kessel and Cohen (1982), showing a group of square Archaea that have divided but not yet separated (similar to the top sketch in Fig. 2B). However, I identified sheets in standing cultures of the pure isolate that were extraordinarily large and that undoubtedly were the square Archaea (Fig. 1). They measured from 100 x 100 up to 1000 x 1000 micrometers, the latter being clearly visible with the unaided eye. Independent of their size, these sheets are always equally thin, measuring between 0.1 and 0.5 micrometer.

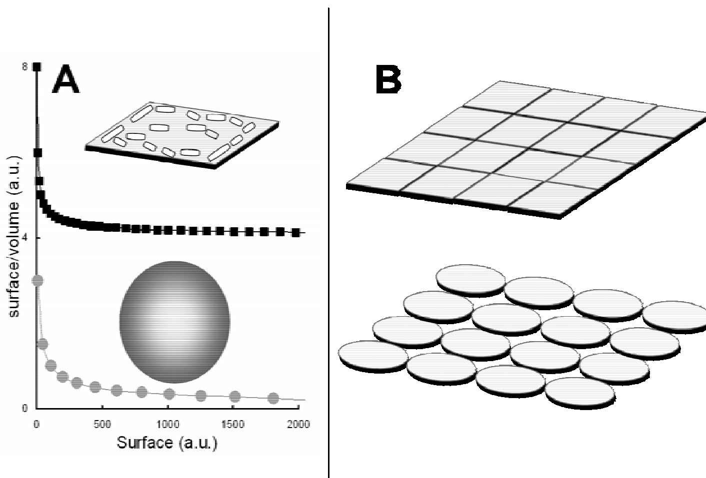

Figure 2. A) Plot comparing the 'surface to volume' ratio of a spherical (circles) and a flat square organism (square). The surface/volume is plotted against the surface. B) Schematic occupation of space comparing a square with a disk-shaped organism pointing out the efficient occupation of the available space by square organisms.

Surprisingly no clear division walls were visible not even when using higher resolution microscopic techniques (i.e. confocal laser scanning microscopy). In addition I did not observe the characteristic alignment of gas vesicles along a putative division wall as can be clearly seen in the micrographs published in Walsby's initial description. It might well be possible that these large sheets already consist of small compartments in which the formation of division walls is not yet triggered. This will be further investigated in the near future. Similar to the small cells, the large sheets are characterised by straight edges and perfectly square corners. These sheets are quite flexible and often folded when studied by microscopy (Fig. 1) (Bolhuis et al., 2004; Kessel and Cohen, 1982). In standing cultures of the pure isolates, these sheets appear as folded rosettes rather than flat sheets.

5. SQUAREBOP; The Square's Way to Life from Light

Halophilic Archaea and especially *Halobacterium salinarum* became widely known largely due to one of the best studied integral membrane proteins, bacteriorhodopsin (BR) (for a review see Oesterhelt, 1998). Together with the related halorhodopsin (HR), these seven transmembrane domain containing proteins function as light driven ion pumps translocating either protons out of the cell (BR) or chloride ions into the cell (HR). The outward directed proton translocation over the membrane mediated by BR results in the generation of an electrochemical gradient that can be used to drive several energy dependent processes, essentially the generation of ATP via the proton dependent ATPase. Ion translocation in BR and HR is initiated by a conformational change upon photon absorption by the covalently linked chromophore retinal. Bacterio-opsin, the protein lacking the retinal chromophore, which gets covalently linked to the protein at a later stage, is encoded by the *bop* gene (Dunn et al., 1981). Although initially thought to be specific for halophilic Archaea (but not necessarily present in all haloarchaea), homologs of BR have been found in other prokaryotes as well. A bacterial counterpart called proteorhodopsin was discovered through genomic analyses of naturally occurring marine bacterioplankton (Béjà et al., 2000).

The first indication that Walsby's square archaeon also contained bacteriorhodopsin-like pigments came from the work of Walther Stoeckenius (1981). He applied flash spectroscopy on a natural sample enriched in squares and observed a transient absorbance change indicative for the presence of bacteriorhodopsin-like pigments. However, he did not observe ordered arrays of intra-membrane particles with the typical appearance of purple membrane patches such as described for *Halobacterium salinarum* (Oesterhelt and Stoeckenius, 1974). Molecular proof for the presence of a bacterio-opsin homologue came from an environmental study in which PCR techniques were applied to study the distribution of prokaryotic rhodopsin genes in hypersaline environments (Papke et al., 2003). Using *bop*-specific primers, a unique cluster of bacterio-opsin genes were amplified from DNA retrieved from the crystalliser ponds and clustered with the proton translocation family of bacterio-opsins. These abundant *bop*-like genes co-occurred with the dominantly present square cells, leading the authors to the suggestion that these genes are most likely derived from the squares. Their assumption was correct as was shown later when the same set of primers was used to amplify the *bop* gene from one of the pure square strains (Bolhuis et al., 2004). Comparison of the "*squarebop*" gene with the *bop* gene of *Halobacterium salinarum* supports the assumption that the protein it encodes functions as a light driven proton pump, since all amino acid residues essential to accommodate the retinal chromophore and translocate protons are conserved (data not shown).

6. Molecular Detection of the Square Community

Until about 10 years ago, characterisation of square haloarchaea depended largely on microscopic observations and physiological tests with a mixed halophilic microbial community. These studies suggested that the squares are the dominant population in crystalliser ponds and natural salt lakes at various geographic locations (Benlloch et al.,

1995, 2002; Bolhuis et al., 2004; Burns et al., 2004a, 2004b; Oren, 1993; Stoeckenius, 1981; Walsby, 1980). However, their distribution and abundance could be studied to their full extent only after the introduction of molecular tools and after identification of their 16S rRNA gene sequences. PCR amplification, cloning and sequencing of 16S-rRNA genes from DNA obtained from a crystalliser pond located south of Alicante, Spain, revealed a dominant clone, the X84084 or SPhT sequence, that was linked to the square organism (Benlloch et al., 1995).

The X84084 sequence was used by Antón et al. (1999) to construct several probes for fluorescence in situ hybridisation (FISH), of which one probe (CS1337) specifically labelled the gas vesicle containing square Archaea described by Walsby. Thus the phylogenetic position of the square archaeon could now be clarified. The squares were shown to be unrelated to any of the know haloarchaea within the Halobacteriaceae. They probably make up a new genus with as closest cultivated relative haloarchaeon strain T1.3 isolated from an ancient salt deposit (McGenity et al., 2000) with which the squares have only 93% similarity at the 16S rRNA gene sequence level (Bolhuis et al., 2004).

Knowledge about the 16S rRNA gene sequence and the application of FISH proved to be powerful tools to study the presence of square organisms in hypersaline ecosystems in various geographical locations. Using FISH, it was confirmed that the square Archaea, that at that time could not be cultivated, are the dominant prokaryotes in the crystalliser ponds, making up over 60% of the total prokaryotic community. On the other hand, species that are readily and frequently isolated from these ponds, e.g. *Haloarcula* sp., account for less that 0.1% of the total prokaryotic population in crystalliser ponds (Antón et al., 1999). This furthermore showed that even in relatively low diversity ecosystems, cultivation is biased towards minority species. The estimated 60% of squares in the crystalliser ponds of the multi-pond solar saltern 'Braç del Port', Santa Pola near Alicante, Spain, is in good agreement with the estimated 40-60% derived from microscopic and molecular analysis of crystalliser ponds from other salterns (Antón et al., 1999; Benlloch et al., 2002; Burns et al., 2004a; Casamayor et al., 2002; Oren et al., 1996; Øvreås et al., 2003; Rosselló-Mora et al., 2003). Total cell numbers in the brine pool of Sinai investigated by Walsby (1980) were in the order 10^7 per ml. Similar numbers are commonly encountered in saltern crystallizer ponds. In some cases, however, the number of square Archaea seems to be very low. Ochsenreiter et al. (2002) only occasionally observed square-shaped prokaryotes during microscopic inspection, and the SPhT phylotype was not amplified in their study. The reason for this remains unclear and it would be worthwhile to investigate which factors (geochemistry, nutrients or rainfall) were responsible for the absence of the normally worldwide distributed squares in these particular crystalliser ponds in La Palma, Spain.

Rosselló-Mora et al. (2003) applied the CS1337 FISH probe in a novel technique called FISH-MAR in which cells can be identified with species specific FISH probes but at the same time metabolic processes can be measured by following the incorporation of specific radio-labelled substrates (Lee et al., 1999). They showed that in their sample also the squares were the dominant species in the archaeal population and that these squares metabolised both acetate and amino acids. Autotrophy or uptake of glycerol was not observed, which is in contrast to the observed (but slow) growth on glycerol in pure cultures (Bolhuis et al., 2004).

Another intriguing observation that resulted from molecular analysis of crystalliser ponds was the insight into the apparent co-existence of square Archaea with a group of closely related clones of Bacteria, the so called *Salinibacter ruber* cluster. Together the square archaeon and *Salinibacter* account for most of the microbial biomass in crystalliser ponds with salinities ranging from 22 to 32% (Antón et al., 1999, 2002; Benlloch et al., 2002; Casamayor et al., 2002; Rosselló-Mora et al., 2003). The relative abundance of the square archaeon and *Salinibacter* as well as their co-existence was confirmed by the analysis of Øvreås et al. (2003), which applied a broad set of techniques (terminal restriction length polymorphism, transmission electron microscopy and denaturing gradient gel electrophoresis) with similar results. More remarkable, however, was their analysis of community diversity by determining thermal melting and re-association of community DNA, from which they could calculate the community DNA base composition. The crystalliser pond with a salinity of 37% in which the square archaeon and *Salinibacter* dominate, a major population with a peak around 45-50 mol% G+C and a smaller population with a peak around 65 mol% G+C were found. Despite the fact that other haloarchaea have DNA with a generally high G+C content (~ 60-65%), they concluded that this low GC population might correspond to the dominance of square bacteria, whereas the smaller peak at 65 mol % GC correspond with *Salinibacter* and other less abundant haloarchaea. Surprisingly, initial analysis of DNA obtained from the Spanish pure isolate suggests that the GC content of this organism is indeed in the order of 47%, whereas the GC content of *Salinibacter* is close to 68% (Antón et al., 2002), thus showing that Øvreås and co-workers were right in their assumption. Now it is of course of great interest to understand what this low GC content means for the square organism in terms of amino acid codon-usage and overall acidity of the proteins.

7. Isolation and Cultivation of Walsby's Square Archaeon; An End to The Quest

As mentioned above, many researchers have tried to cultivate this intriguing square prokaryote and not only because of its unique shape but also because of its ecological relevance in crystalliser ponds. This organism is the most abundant prokaryote especially at the end of the evaporation process where NaCl precipitates and close to the onset where the $MgCl_2$ concentration in the remaining "bitterns" becomes too high to support live. A first announcement of isolating Walsby's Square Archaeon came in 1986 (Torrella, 1986). The organism was isolated from a Spanish saltern and apparently only grew under low nutrient conditions. Unfortunately this culture was not deposited in a culture collection and was lost. Walter Stoeckenius observed growth in enrichment cultures by adding peptone medium to natural brine, but he was never able to find colonies on agar plates, the commonly applied method to check for purity of an isolate (Stoeckenius, 1981). Other square Archaea that have been described and isolated did not contain the characteristic gas vesicles and where never as perfectly square as the organisms first observed by Walsby. These pleiomorphic haloarchaea belonged to the genera *Haloferax* and *Haloarcula* (Alam et al., 1984; Javor et al., 1982; Kessel et al., 1985; Oren et al., 1999) and were unrelated to the SPhT phylotype.

Fortunately I was able to obtain a pure culture after enriching and isolating a square archaeon from a Spanish solar saltern. The square cells were initially enriched in cultures containing low amounts of nutrients (0.1% C-source and 0.01% yeast extract). In addition, colonies were only visible on plates when agarose instead of agar was used as solidifying agent (Bolhuis et al., 2004). Microscopic and molecular characterization confirmed that this gas vesicle containing and perfect square organism was indeed Walsby's square archaeon. The organism was named 'Haloquadratum walsbyi' (salt square of Walsby).

One month after the manuscript describing the Spanish isolate was accepted for publication, a second manuscript was submitted, accepted and published, describing the isolation of a square archaeon from an Australian saltern (Burns et al., 2004b). Whereas cultivation attempts were futile in the previous 24 years, now two strains have been isolated independently and within a short time period of each other. A common theme in the isolation procedures of both strains was the use of low concentrations of yeast extract and the use of Na-pyruvate as carbon and energy source.

Being able to cultivate Walsby's square archaeon now allows us to test several of previously made assumptions based on observations in natural samples (see above). We will be able to determine its ecological function, biochemical properties, and the specific adaptations to its extreme environment, and we can now obtain insight in the molecular basis of its unique morphology. Another intriguing question to be addressed in the near future is the apparent syntrophic relationship of 'Haloquadratum walsbyi' with the bacterium *Salinibacter ruber*. Since there are now two strains available we can compare these strains by standard physiological tests, molecular analysis such as genetic fingerprinting and ultimately by comparing their genome sequences. Comparing the two strains might also give insight in their evolution and dispersal since this enigmatic organism can be found in several continents, ranging from Europe, North America, the Middle East and Australia. Traveling such large distances through normal seawater would be rather impossible since 'Haloquadratum walsbyi' cannot survive at salinities below 15% of total salt (Bolhuis et al., 2004). Instead, dispersal more likely occurs via cells in salt crystals hitchhiking with migrating birds that frequent these salterns, via salt crystals that are carried by the wind or alternatively through the ancient human manufacturing and use of sea-salt.

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GENE REGULATION AND THE INITIATION OF TRANSLATION IN HALOPHILIC ARCHAEA

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1. Introduction

Halophilic Archaea offer a variety of possibilities to investigate the regulation of gene expression *in vivo*, since vector plasmids and also an expression vector are available, and the *bgaH* gene derived from *Haloferax lucentense* encoding an enzyme with β-galactosidase activity is useful as reporter gene (Holmes and Dyall-Smith, 2000).

We use the genes involved in the gas vesicle formation of *Halobacterium salinarum* und *Hfx. mediterranei* as a model system to investigate haloarchaeal gene expression. Fourteen *gvp* genes are involved in gas vesicle formation, arranged as two clusters *gvpACNO* and *gvpDEFGHIJKLM* (DasSarma et al., 1994; Englert et al., 1992a). These genes constitute the 10 kb vac region. The P_A promoter located in front of *gvpACNO* drives the synthesis of the major (GvpA) and minor (GvpC) gas vesicle structural proteins (and two other proteins of unkown functions), whereas the upstream and oppositely oriented P_D promoter is responsible for the expression of the two regulatory proteins GvpD and GvpE, and additional proteins involved in the formation of the gas vesicle structure (Pfeifer et al., 1997; Shukla and DasDarma, 2004). *Hfx. mediterranei* contains the single mc-vac region, whereas the distinct p-vac and c-vac regions are found in *Hbt. salinarum*. The latter two vac regions are related but not identical to the *gvp1* (p-vac) and *gvp2* (c-vac) gene clusters of *Halobacterium* sp. NRC-1 (Ng et al., 2000).

The gas vesicle genes offer a suitable model system to investigate archaeal gene expression, since the formation of these proteinaceous particles depends on the salt concentration in the medium and also the growth phase of the producer. The moderately halophilic *Hfx. mediterranei* synthesizes cylinder-shaped gas vesicles exclusively in the stationary growth phase in media containing more than 17% salt (Englert et al., 1990; Rodriguez-Valera et al., 1983), and the extremely halophilic *Hbt. salinarum* PHH1 produces spindle-shaped gas vesicles constitutively by expression of the p-vac region. The c-vac region is only expressed in the p-vac deletion mutant *Hbt. salinarum* PHH4, leading to cylinder-shaped gas vesicles in the stationary growth phase (Krüger and Pfeifer, 1996). The expression of these vac-regions is regulated by at least two endogenous regulatory proteins, namely the transcriptional activator GvpE, and GvpD which is involved in repression. For functional analyses we use the related *Hfx. volcanii* (Mullakhanbhai and Larsen, 1975) that offers a clean genetic background. The

following review summarizes various aspects of our investigations on *gvp* expression at the transcriptional and the translational level.

2. Regulation of *gvp* Gene Expression at the Level of Transcription

2.1. FACTORS INVOLVED IN TRANSCRIPTION INITIATION IN ARCHAEA

Archaeal promoters consist of a TATA-box centred around position -28 upstream of the transcription start site. The initiation of transcription requires the multicomponent RNA polymerase (RNAP), the TATA-box binding protein TBP and the transcription factor TFB that binds at the BRE sequence found upstream and adjacent to the TATA-box in many archaeal promoters (Bell et al., 1999b; Hausner et al., 1996; Qureshi et al., 1995; Soppa and Link, 1997; Thomm, 1996). Hyperthermophilic and methanogenic Archaea usually contain a single TBP and one or a few TFB proteins, whereas multiple and divergent genes encoding TFB and TBP proteins are present in *Halobacterium* sp. NRC-1 (Ng et al., 2000) and *Hfx. volcanii* (Thompson et al., 1999; and TIGR sequence of the *Hfx. volcanii* genome). Very likely these transcription factors also have some influence on the gene regulation (Baliga et al., 2000). Most studies on promoter regulation in haloarchaea have been done *in vivo* (Danner and Soppa, 1996; Gregor and Pfeifer, 2001; Hofacker et al., 2004; Patenge et al., 2000), in contrast to hyperthermophilic and methanogenic Archaea where *in vitro* transcription systems are available to study the basal transcription and the action of regulatory proteins (Bell et al., 1999a; Brinkmann et al., 2000; Enoru-Eta et al., 2000; Hochheimer et al., 1999; Lee et al., 2003; Leonard et al., 2001; Ouhammouch et al., 2003; Vierke et al., 2003). The high salt requirement (up to 4 M KCl) of halophilic proteins and also the possession of multiple transcription factors presumably complicate the establishment of such an *in vitro* system for haloarchaea.

2.2. ANALYSIS OF THE *gvp* GENES AND THEIR PROMOTER REGIONS

The 14 *gvp* genes of the p-vac region are transcribed from four promoters (P_{pA}, P_{pD}, P_{pF}, and P_{pO}) located upstream of p-*gvpA*, p-*gvpD*, p-*gvpF* and p-*gvpO* (Fig. 1). Only P_{pA} and P_{pD} are activated by GvpE, and a negative effect is imposed by GvpD on the GvpE-mediated activation. In contrast, P_{pF} and P_{pO} are not influenced by either regulatory protein (Hofacker et al., 2004). Only two promoters drive the expression of the c-vac and the mc-vac regions (P_{cA} + P_{cD}, and P_{mcA} + P_{mcD}), and except for P_{cD} all of these are activated by GvpE (Gregor and Pfeifer, 2001; Röder and Pfeifer, 1996; Zimmermann and Pfeifer, 2003).

Northern analysis and also the use of *bgaH* as reporter gene to analyse the strength of the p-vac promoters determine P_{pA} as strongest promoter of the p-vac region, followed by P_{pO}, P_{pF} and P_{pD} (Fig. 2; Hofacker et al., 2004; Offner et al., 1996). A scanning mutagenesis encompassing 50 basepairs upstream of the start site of transcription in P_{pA} and determination of the BgaH activities in *Hfx. volcanii* transformants support that the promoter strength is affected by the sequences of the TATA-box and the putative BRE element (Hofacker et al., 2004). Mutations in these elements lead to a lack of basal transcription, whereas the alteration of the P_{pA}-BRE

element to the consensus sequence results in an enhanced transcription. A high GC content in the sequence around position -10 yields a reduced P_{pA} activity (Hofacker et al., 2004), presumably because RNAP-mediated melting of this region during transcription initation is affected.

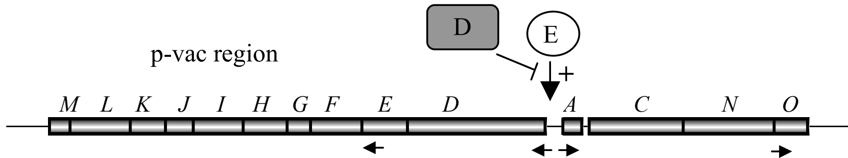


Figure 1. Genetic map of the p-vac region and regulatory proteins. The fourteen *gvp* genes are depicted as boxes labelled A and C through O. Arrows mark the start and direction of transcription. GvpE is a transcriptional activator, whereas GvpD is involved in repression.

BRE **TATA**

*cRnaAnt***TTTAWA** **+1**

pA CAGGAGACATAACGACTGGTGA**AACCA**T*ACACATC***CTTATG**TGATGCCCGAGTATAGTTAGAGAT*

pD CACCAGTCGTTATGTCTCCTGTAATGAG*TCGTCAT***TCTAAG**TACGGAGAGTGTAAAGCTTCTTAG*

pF GGGGCAGACCTGAGTCCGGGTACAGTAT*ACCCGCA***TTTAAA**TGACCTTGCAGTCGAAGGTGTACT*

pO TAGAATCCGCGATCGACGACATGGAAGT*CGCCCTT***TCTTAA**GATCCGGGGTCTCTACATAGAAGC*

promoter activities: pA > pO > pF > pD

Figure 2. Sequences of the four promoters of the p-vac region and relative BgaH activities determined in *Hfx. volcanii* transformants. The TATA-box (bold) and BRE elements (*italics* and underlined) are marked, and the archaeal consensus sequences of both elements are given on top (R = A, G; W = A, T; n = any base). The start site of transcription is marked by +1 and a star. The sequence affecting the GvpE-mediated activation in pA (upstream of BRE) is indicated in bold (after Hofacker et al., 2004).

The GvpE-mediated regulation of P_{pA} has been investigated in P_{pA}-*bgaH*/pE^{ex} transformants using the 50-bp scanning mutant series (Hofacker et al., 2004). Construct pE^{ex} contains the p-*gvpE* reading frame expressed under the ferredoxin promoter control in the expression vector pJAS35. These experiments suggest that the sequence AACCA located upstream of the P_{pA}-BRE element is crucial for GvpE-mediated activation (Fig. 2). However, this mutant analysis encompasses only 13 bp upstream of BRE. As demonstrated later, the sequence AACCA is part of a sequence element that is similar to the sequences involved in the GvpE-mediated activation of P_{mcA} (see below).

With respect to the promoter activity, the strongest transcript formation of any *gvp* promoter is achieved with P_{mcA} in the presence of the cGvpE protein, which is the strongest of the three activator proteins (Gregor and Pfeifer, 2001). Such P_{mcA}-

bgaH/cE^{ex} transformants yield similar amounts of BgaH activity as determined for P_{fdx}-*bgaH* transformants, where the expression of *bgaH* is driven by the ferredoxin promoter (Gregor and Pfeifer, 2005). A strong expression of the *gvp* genes is only achieved in the presence of GvpE. The weakest promoter is P_{cA} of the c-vac region that completely lacks a basal promoter activity and is only active in the presence of the homologous cGvpE (Gregor and Pfeifer, 2001). To determine whether the undetectable basal P_{cA} promoter activity is due to the relatively minor conservation of the TATA-box and the BRE-element in P_{cA}, these elements were substituted with the respective sequences of the stronger P_{pA} promoter. The resulting P_{cApA}-*bgaH* chimera still lack BgaH activity, but gain an enhanced cGvpE-mediated activation and also an induction by the heterologous pGvpE and mcGvpE proteins (Gregor and Pfeifer, 2005).

A 4-bp scanning mutagenesis throughout the 50-bp region separating the TATA-elements of P_{mcD} and P_{mcA} defined the interaction site of GvpE. These analyses determined the sequence TGAAACGG-n4-TGAACCAA as being important for the GvpE-mediated activation in P_{mcA} (Fig. 3A). Portions of this sequence are conserved in all three P_A promoter regions, and the AACCA sequence determined earlier in P_{pA} is part of the respective element (Fig. 3B). All GvpE-inducible promoters exhibit related sequence elements at this position, whereas P_{cD}, P_{pF} and P_{pO} (i.e. promoters not affected by GvpE) are different.

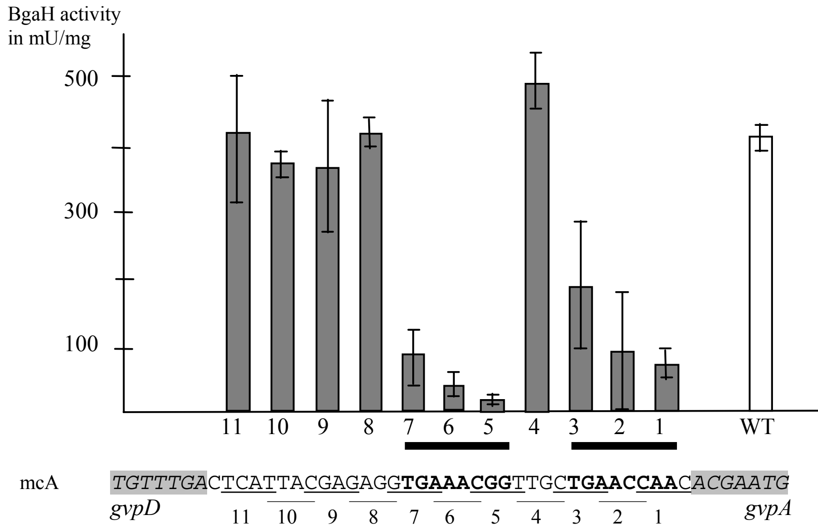
2.3. THE REGULATORY PROTEINS GvpD AND GvpE

The transcriptional activator GvpE (21 kDa) resembles a basic leucine zipper protein as suggested by molecular modelling of the C-terminal AH6 helix and mutation analyses (Krüger et al., 1998; Plößer and Pfeifer, 2002). Mutants of cGvpE with alterations of the leucine zipper helix AH6, or of the two putative DNA binding sites DNAB and AH4 are unable to stimulate the expression of P_{cA}-*bgaH* in *Hfx. volcanii* transformants. GvpE appears in late exponential growth in *Hbt. salinarum* PHH4 and *Hfx. mediterranei* as demonstrated by Western analyses (Krüger and Pfeifer, 1996; Zimmermann and Pfeifer, 2003).

The larger GvpD protein (54-60 kDa) is involved in the repression of gas vesicle formation, since ΔD transformants containing an mc-vac region with a 918-nt deletion internal to mc-*gvpD* are gas vesicle overproducers, and the overproduction is reduced to the level of wild type in ΔD/D transformants (Englert et al., 1992b; Pfeifer et al., 1994). A p-loop motif found near the N-terminus of GvpD is essential for this repression (Pfeifer et al., 2001). cGvpD encoded by c-vac is present during the early growth phases of *Hbt. salinarum* PHH4 (Krüger and Pfeifer, 1996), whereas in *Hfx. mediterranei* mcGvpD appears shortly after mcGvpE in late exponential growth (Zimmermann and Pfeifer, 2003).

The GvpE protein can be isolated by affinity chromatography from the lysate of *Hfx. mediterranei* using an Ni-NTA matrix tagged with mcGvpD$_{his}$, and v*ice versa* mcGvpD is purified using an mcGvpE$_{his}$-tagged matrix (Zimmermann and Pfeifer, 2003). Similar results have been obtained with the respective Gvp proteins of c-vac, and also with the heterologous pairs (mcGvpD/cGvpE and cGvpD/mcGvpE) (Scheuch, 2003). Thus, GvpE and GvpD are able to interact, and this interaction might also occur *in vivo* as part of the negative P_A promoter control. Currently we are trying to identify the contact sites in both proteins, and to answer the question whether the ATP binding

(and hydrolysis?) plays a role in this process. ATP hydrolysis could yield the energy for structural alteration of GvpE resulting in a protein unable to bind to the promoter region or to contact the basal transcription apparatus for activation. It is possible that this alteration is due to a phosphorylation of GvpE, since p-loop mutants of GvpD are unable to repress gas vesicle formation (Pfeifer et al., 2001).



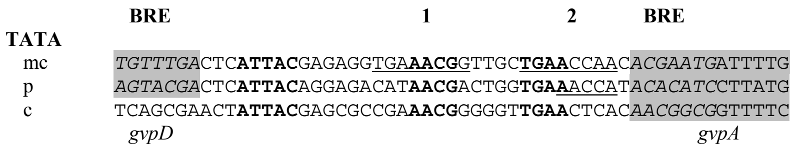


Figure 3. (A) Summary of the results of a 4-bp scanning mutagenesis with the P_D - P_A region in mc-vac. The BgaH activities are drawn relative to the mutations in the promoter region (underlined and numbered in the sequence given at the bottom). The BRE-elements of P_{mcD} (left) and P_{mcA} (right) are shaded in grey. The sequences affecting the GvpE-mediated activation are written in bold (after Gregor and Pfeifer, 2005). (B) Alignment of the P_D - P_A region of the mc- vac (mc), p-vac (p) and c-vac (c) regions. The TATA-box and BRE elements of P_A (right) and the BRE element of P_D (left) are shaded in grey. Conserved sequences (≥4 nt) are marked in bold. The regions determined as GvpE interaction site in P_{mcA} and P_{pA} are underlined.

An unusual observation led to the assumption that the interaction of GvpD and GvpE induces an enhanced proteolytic degradation: both Gvp proteins (and especially GvpE) are absent in DE^{ex} transformants, whereas D^{ex}, E^{ex}, or ΔDE^{ex} transformants

(carrying the 918 bp deletion internal to mc-*gvpD*) contain large amounts of GvpD or GvpE protein (Zimmermann and Pfeifer, 2003). This apparent instability of GvpE in the presence of GvpD could also be the mechanism underlying the GvpD-mediated repression of gas vesicle formation.

The results obtained so far lead to the following model on the regulation (Fig. 4):

(1) GvpE activates the P_A promoter without the help of any other Gvp protein (Gregor and Pfeifer, 2001). The additional presence of GvpD results in a reduced GvpE-mediated activation, indicating that GvpD is involved in the repression of P_A (Hofacker et al., 2004; Zimmermann and Pfeifer, 2003).

(2) GvpD and GvpE are able to interact, and the "repression" of the *gvp* gene expression could be the result of an interaction, leading to a modified GvpE which has lost the activator function. Another possibility could be that a GvpD-induced proteolytic degradation of GvpE is the reason for the reduced P_A promoter activity.

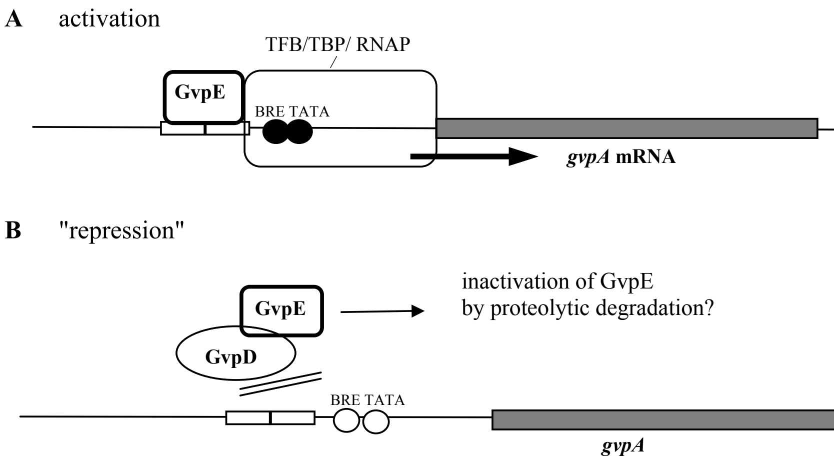


Figure 4. Model proposed for the regulation of the P_A promoter by GvpE and GvpD. See text for further explanations.

3. Translation Initiation in Halophilic Archaea

3.1. COMPARISON OF THE INITIATION OF TRANSLATION IN BACTERIA, EUKARYA AND ARCHAEA

The machinery involved in archaeal translation has both bacterial and eukaryotic features (Bell and Jackson, 1998; Dennis, 1997). More than 10 genes encoding proteins homologous to eukaryotic translation initiation factors are present in Archaea, whereas only three initiation factors (IF1, IF2, IF3) are required in Bacteria (Gualerzi and Pon, 1990). Similar to bacterial mRNAs, archaeal transcripts are polycistronic and lack a CAP structure at the 5'-end. The CAP structures of the eukaryotic transcripts are

recognized by the 80S ribosome that finds the translation start codon by a scanning mechanism (Kozak, 1999, 2002). In Bacteria, the mRNA is recognized by the 30S ribosomal subunit involving an mRNA-rRNA interaction that depends on an 8 nt sequence element found near the 3′-end of the 16S rRNA, complementary to a sequence in the 5' untranslated region (5'-UTR) of the mRNA (Gualerzi and Pon, 1990; Shine and Dalgarno, 1974). The spacing between this Shine-Dalgarno (SD) sequence and the start codon in *E. coli* is optimal at 8-10 nt, but ranges from 5 to 13 nt (Chen et al., 1994). However, there are also conflicting data with regard to the concept that the SD element is strictly required for translation initiation in Bacteria. Ribosomes reconstituted with 16S rRNAs lacking an anti-SD element are still able to initiate translation at the correct site *in vitro*, and another study reports that mRNAs with and without the SD element form the 30S initiation complex (Calogero et al., 1988; Melancon et al., 1990).

Also archaeal transcripts exhibit putative SD elements upstream of the translation initiation codon, especially with reading frames that are co-transcribed (Bell and Jackson, 1998; Dennis, 1997; Watanabe et al., 1997). However, the 5'-UTR of the first reading frame in such a co-transcript often does not indicate an SD element (Tolstrup et al., 2000). An example is the p-*gvpA* transcript that occurs as monocistronic, but also as part of the p-*gvpACNO* mRNA (Englert et al., 1992a; Offner et al., 1996). The 20-nt 5'-UTR lacks an SD sequence element, but the p-*gvpA* transcript is efficiently translated.

A significant percentage of archaeal transcripts completely lack the 5′-UTR and are nevertheless efficiently translated. Leaderless mRNAs are found in all three domains of life, but their amount is the highest in Archaea (up to 30% in hyperthermophilic Archaea; Sensen et al., 1996; Slupska et al., 2001). The translation of leaderless mRNAs cannot depend on an mRNA-rRNA interaction, and the initiation of translation must follow another mechanism (La Teana et al., 2000; Moll et al., 2001, 2002; O'Connor et al., 1999). Recently, it has been shown that in *E. coli* leaderless mRNAs are recognized by the 70S ribosome including IF2-fMet-tRNA, and their efficient translation depends on the presence of the start codon AUG (Moll et al., 2002, 2004; O'Donnel and Janssen, 2002; Udagawa et al., 2004). As leaderless mRNAs regardless of their origin can be translated by archaeal, bacterial and eukaryotic ribosomes, the recognition of such transcripts must be an evolutionary conserved function of the translation apparatus (Grill et al., 2000; Moll et al., 2002). Examples of haloarchaeal leaderless mRNAs are the transcripts of the *brp* and the *bop* gene encoding bacterio-opsin and a regulatory protein (Betlach, et al., 1984; DasSarma et al., 1984), the *fdx* mRNA (Pfeifer et al., 1993), and the monocistronic p-*gvpO* mRNA of the gas vesicle gene cluster (Offner et al., 1996). All these transcripts start only one nucleotide upstream of the AUG start codon and are efficiently translated in *Hbt. salinarum*.

The first report dealing with the translation initiation in Archaea and the role of the putative Shine-Dalgarno sequences has been published by Condò et al. (1999). A cell-free system derived from the hyperthermophilic crenarchaeote *Sulfolobus solfataricus* was used to analyse translation. Two mutations in the putative SD motif of an mRNA completely abolish the translation *in vitro*. However, the complete deletion of this altered 5'-UTR resulting in a leaderless mRNA relieves the block of translation (Condò et al., 1999). An additional report of the same group concludes that two different mechanisms exist for the ribosome-mRNA interaction in *S. solfataricus* (Benelli et al., 2003). These authors show that leadered mRNA (+ SD element) forms a stable binding complex with the 30S subunit without the aid of initiator tRNA (tRNAi) or any other

factor *in vitro*, and that the SD motif is sufficient to direct the 30S subunit to the translation initiation region. In contrast, leaderless mRNAs are unable to interact with the 30S subunits and require the presence of tRNAi, suggesting that codon-anticodon pairing is important for positioning the ribosome at the AUG start codon (Benelli et al., 2003). We have started to investigate the signals required for translation initiation in haloarchaea *in vivo*, using the *gvp* genes of the p-vac region involved in gas vesicle formation of *Hbt. salinarum* PHH1 as a model system.

3.2. PUTATIVE SHINE-DALGARNO ELEMENTS IN *gvp*-mRNA

Three of the four major transcripts of the p-vac region contain relatively long untranslated regions at the 5'-terminus (p-*gvpA*: 20 nt, p-*gvpD*: 72 nt, and p-*gvpF*: 169 nt), but the fourth transcript, p-*gvpO*, starts only 1 nt upstream of the AUG start codon (Englert et al., 1992a; Offner et al., 1996). Except for p-*gvpA* and p-*gvpO* all mRNA leaders contain sequences complementary to the 3′-end of the small ribosomal subunit RNA (Fig. 5; Sartorius-Neef and Pfeifer, 2004).

SD element:	GGAGGUGA
gvpO	CGGGGUCUCUACAUAGAAGC***AUG****GCU*
gvpN	CUUCGGCCGGAUGAUAAAAC***AUG****ACG*
gvpC	UCACAGACAAACGCGACGAG***AUG****AGU*
gvpA	GGGUUAAUCCCAGAUCACCA***AUG****GCG*
gvpD	CCAUCGACUGGAGAGAAGUA***AUG****AGU*
gvpE	CUCACGGAGAUGGUCUAACA***AUG****GAC*
gvpF	AUCCCAAGGAAGAGAGACCA***AUG****ACU*
gvpG	AACAAGGAGGCCGAUAAUGC***AUG****UUC*
gvpH	CGCAUGGAGGUCAAGAAAUA***AUG****GUA*
gvpI	ACCGAUGGAGGUGAAUCCAC***AUG****AGC*
gvpJ	CACUGAGGACGAGGUGAACG***AUG****AGU*
gvpK	AGCGGAGGAGACCAAATGAG***AUG****GAA*
gvpL	GAAUCCCAGCGUGAUGACGU***AUG****ACU*
gvpM	GCGCCAGAUAUUGGUAAAUA***AUG****GAG*

Figure 5. Shine-Dalagarno motifs found upstream of the *gvp* reading frames in the p-vac region. The SD element on top derives from the anti-SD sequence in the 16S rRNA. Nucleotides of the putative SD element are marked in grey, with identical nucleotides underlined. The AUG start codon of the respective *gvp* gene is given in bold. The leader region of *gvpH* has been investigated in further detail.

The leader region of p-*gvpH* has been investigated in further detail. A 4-nt scanning mutagenesis done with the 5'-UTR underlines the importance of the putative SD element for the efficiency of translation (Sartorius-Neef and Pfeifer, 2004). The various mutant mRNA leaders were fused to the *bgaH* reading frame and tested in *Hfx. volcanii* transfomants with respect to the resulting BgaH activity. The results clearly demonstrate a contribution of the SD element to the efficiency of translation (Fig. 6). Mutations up- or downstream of this sequence have no influence on translation, whereas only 5-50% of the BgaH activity obained with the wild type 5'-UTR are seen with the

mutated SD elements (Sartorius-Neef and Pfeifer, 2004; examples are shown in Fig. 6). The most significant reductions (5-20% of the BgaH activity found with the wild type 5'-UTR) are observed when the GGAGG-portion of the putative SD element is altered. However, the complete alteration of the SD element in mutant SD7 still yields 20% of the BgaH activity found in wild type, indicating that such leadered mRNAs lacking a sequence complementary to the 16S rRNA are still translated. These results suggest that the SD element has a significant impact on the efficiency of translation, but does not strictly control translation as described for the *Sulfolobus* cell-free system (Condò et al., 1999).

SD sequence	GGAGGUGA	
p-*gvpH*	CGCAUGGAGGUCAAGAAAUA**AUG**	100%
SD0-6	.CACC..................	94%
SD1-6	...GGAC................	10
SD2-4	.....ACCA..............	15
SD3-2	.......CCUA............	10
SD4-4	.........CGUC..........	50
SD5-1	.............UAUG......	96
SD7	.....CCUAAA.C..........	20

Figure 6. Scanning mutagenesis of the p-*gvpH* mRNA leader region. Dots mark nucleotides identical to the 5'-UTR of p-*gvpH*. The AUG start codon is marked in bold. The numbers on the left indicate BgaH activities in the respective transformants in percent (after Sartorius-Neef and Pfeifer, 2004).

All these scanning mutants contain only 4-nt alterations in the 22-nt leader region, whereas the remainder of the leader is kept unchanged. Interestingly, an inversion of the 22-nt 5'-UTR of p-*gvpH* results in the complete loss of translation, suggesting that the leader sequence harbours additional information for the recognition of the AUG initiation codon (unpublished). Alterations of the spacing of the SD element relative to the translation initiation codon of p-*gvpH* yields an optimal distance between 4 and 10 nt, while a distance of 1 nt appears to be too close for translation (Sartorius-Neef and Pfeifer, 2004), which is similar to the results obtained with *E. coli* (Chen et al., 1994).

3.3. LEADERLESS TRANSCRIPTS

A complete deletion of the 5'-UTR resulting in a leaderless *gvpH* mRNA (Δlead, i.e. expressing the leaderless H-*bgaH*) yields a 15-fold enhanced BgaH activity compared to the H-*bgaH* mRNA carrying the 20-nt leader discussed above, demonstrating that the initiation of translation in leaderless transcripts is very efficient (Sartorius-Neef and Pfeifer, 2004). Similarly, a leader-containing and a leaderless p-*gvpA* transcript was investigated in *Hfx. volcanii* transformants, and for more quantitative studies also as translational fusion with *bgaH.* The latter construct pA_A-*bgaH* encodes the fusion protein 6A-BgaH carrying the first 6 amino acids of GvpA fused to BgaH. The BgaH activity derived from pA_A-*bgaH* transformants was with 104 ± 40 mU mg^{-1} more than

100-fold enhanced compared to the transformants containing the respective leadered mRNA (1.0 ± 0.3 mU mg^{-1}) (unpublished). From these results it appears that leaderless mRNAs are most efficiently translated, whereas an mRNA leader reduces the translation efficiency and thus confers a regulatory effect on translation.

The efficient translation of leaderless transcripts induces speculations on the evolution of mRNA leaders and the mechanism of the mRNA recognition by the ribosome. The translation of leaderless transcripts is most likely an ancient trait since ribosomes of all three domains are able to translate such mRNAs (Grill et al., 2000; Moll et al., 2002). Recent analyses in bacteria demonstrate that leaderless mRNAs are recognised by the complete 70S ribosome and tRNAi rather than by the 30S subunits in *E. coli* (Moll et al., 2004; Udagawa et al., 2004). Since intact 70S ribosomes rather than the dissociated subunits are important here, one could speculate that Archaea contain a larger portion of intact 70S ribosomes as opposed to the dissociated subunits. Further analyses will shed more light on this subject.

4. Summary

The genes involved in the formation of gas vesicles are a useful model system to study the regulation of gene expression at the level of transcription and translation in halophilic Archaea. Two endogenous regulator proteins, the transcription activator GvpE, and GvpD involved in repression, have been characterized. Scanning mutagenesis determined a sequence element located upstream of the BRE element of the P_A promoter that is important for the GvpE-mediated promoter activity. The GvpD and GvpE proteins interact, and this interaction might lead to an inactive GvpE. It is possible that the repression of P_A is caused by a GvpD-induced proteolytic degradation of GvpE. Most *gvp* mRNAs contain a putative Shine-Dalgarno (SD) sequence upstream of the translation start codon that determines the efficiency of translation. However, mRNAs containing a complete mutation of the SD sequence still exhibit 20% of the translation, demonstrating that the SD element is not essential. A very efficient translation is found with leaderless transcripts, and the translation of these must follow a mechanism other than a specific mRNA-rRNA contact.

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Biodata of **Jerry Eichler,** author (with **Gabriela Ring**, **Vered Irihimovitch**, **Tovit Lichi**, **Irit Tozik** and **Zvia Konrad**) of ***"Protein Translation, Targeting and Translocation in Haloferax volcanii"***

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PROTEIN TRANSLATION, TARGETING AND TRANSLOCATION IN *HALOFERAX VOLCANII*

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1. Introduction

In Archaea, a variety of proteins must either insert into or traverse the plasma membrane. As in Bacteria and Eukarya, translocation of such proteins requires that they first be identified as destined to reside outside the cytosol and that they then be delivered to membranous translocation sites where they ultimately cross the membrane. Although still limited, relative to what is known in the other two domains of life (Johnson and van Waes, 1999; Manting and Driessen, 2000), major advances in our understanding of archaeal protein translocation have been made of late, largely thanks to studies involving halophilic Archaea (Pohlschröder et al., 2004; Ring and Eichler, 2004a).

2. Archaeal Protein Translocation: A Co- or Post-Translational Event?

At the onset of translocation, the kinetic relation to protein translation must be chosen. In a co-translational translocation system, translating ribosomes are targeted to the translocon, the site of protein translocation in the membrane, such that translation and translocation become coupled processes. In post-translational translocation, proteins cross the membrane only after much, if not all of the protein has been first synthesized in the cytoplasm. In Archaea, the relation of protein translation to protein translocation remains unclear. Indeed, it seems that in halophilic Archaea both systems exist. The biosynthesis of *Halobacterium salinarum* bacterioopsin, the apoprotein form of the multi-membrane-spanning light-driven proton pump bacteriorhodopsin, involves co-translational translocation of the N-terminal region of the protein (Dale and Krebs, 1999; Dale et al., 2000). These findings are in agreement with earlier reports of co-sedimentation of 7S RNA (a component of the signal recognition particle; see below), and bacterioopsin mRNA with membrane-bound ribosomes, as well as of the puromycin-induced release of 7S RNA from such ribosomes (Gropp et al., 1992). The C-terminal region of bacterioopsin is, however, inserted in a post-translational manner (Dale and Krebs, 1999). In contrast, the need for the seventh and final transmembrane domain for membrane insertion of the entire bacterioopsin molecule when found as part of a larger fusion protein heterologously expressed in *Haloferax volcanii* suggested that

bacterioopsin insertion takes place post-translationally (Ortenberg and Mevarech, 2000). It should be considered though, that as the sole component of the purple membrane in *Hbt. salinarum* and the major protein expressed in later growth stages (Gropp et al., 1992), bacterioopsin may rely on a dedicated insertion machinery and as such may not reflect the general translocation process. Indeed, bacterioopsin is synthesized with an unusually short, glutamate-containing signal peptide that lacks the hydrophobic core and positively-charged amino acids contained in the signal peptides of proteins recognized and translocated by the general Sec secretory pathway (Gropp et al., 1992). Furthermore, the relation between translation and translocation of a membrane protein may not hold true for protein secretion.

Accordingly, in studies aimed at defining the temporal relation between archaeal protein translation and secretion, *Hfx. volcanii* cells were transformed to express chimeras containing the signal peptide of the major exported protein in this species, the surface layer glycoprotein, fused to different reporter proteins. By following the secretion of these chimeras in either the absence or presence of anisomycin, an antibiotic shown to inhibit protein synthesis in halophilic Archaea, it could be demonstrated that protein translation and secretion occur independently. The ability of Archaea to secrete proteins in a post-translational manner is unexpected, given that SecA, the ATPase that drives post-translational translocation in Bacteria, is apparently not found in Archaea (Pohlschröder et al., 2004; Ring and Eichler, 2004a).

The twin-arginine translocation (Tat) system, predicted on the basis of genomic sequence analyses to serve as the major vehicle for export of halophilic archaeal secretory proteins (Bolhuis, 2002; Rose et al., 2002), also operates in a post-translational manner. The use of the Tat system, capable of translocating folded substrates (Robinson and Bolhuis, 2001), has been proposed as offering a strategy whereby secretory proteins could overcome the potential dangers stemming from remaining in a 'loosely-folded' conformation in a highly saline cytoplasm prior to translocation, as would be required for transport by the Sec protein translocation system. In non-halophilic microorganisms, the Sec pathway serves as the main route for proteins crossing the plasma membrane (Dilks et al., 2003).

3. The Signal Recognition Particle (SRP) Pathway in Archaea

In Eukarya and Bacteria, SRP and the SRP receptor serve to link protein translation to protein translocation (Keenan et al., 2001). Based on a combination of genetic, structural and biochemical approaches, it appears that the archaeal SRP system corresponds to a mosaic, adopting facets of the parallel eukaryal and bacterial systems, while also incorporating traits unique to Archaea (Eichler and Moll, 2001; Moll, 2004; Zwieb and Eichler, 2002). Like all SRPs, the archaeal particle includes SRP RNA and the SRP54 protein. Despite a low extent of sequence conservation overall, archaeal SRP RNA assumes a secondary structure essentially identical to that of higher eukaryal SRP RNA. The archaeal molecule can, however, be discriminated from its eukaryal counterpart by the presence of helix 1, formed upon pairing of the 5' and 3' ends of the molecule, and the absence of helix 7. In addition to SRP54, shown to be an essential protein in *Hfx. volcanii* (Rose and Pohlschröder, 2002), the archaeal SRP also includes SRP19, a protein component that can be found in the eukaryal but not the bacterial

particle. The archaeal SRP19 can nonetheless be distinguished by the absence of certain regions present in the eukayal protein (Bhuiyan et al., 2001).

Using purified components, recent reconstitutions of archaeal SRP and SRP subcomplexes from several different strains, including *Hfx. volcanii* (Tozik et al., 2002), have allowed for detailed study of the assembly and structure of the archaeal particle (Bhuiyan et al., 2000; Hainzl et al., 2002; Maeshima et al., 2001; Oubridge et al., 2002). Such studies have revealed that during SRP assembly in *Hfx. volcanii* and other Archaea, SRP19 proteins interact with SRP RNA in order to facilitate SRP54 binding to SRP RNA, as is the case in Eukarya. However, unlike what occurs during eukaryal SRP assembly, the interaction between SRP RNA and SRP54 in Archaea is not entirely SRP19-dependent, as a significant degree of archaeal SRP RNA-SRP54 binding takes place without SRP19. The ability of archaeal SRP RNA and SRP54 to interact in the absence of SRP19 could reflect a need for a more stable SRP in Archaea, possibly related to the extreme environments inhabited by these microorganisms.

Whereas archaeal SRP recalls its eukaryal counterpart, the archaeal SRP receptor is more similar to the bacterial SRP receptor, FtsY. As in Bacteria, archaeal FtsY exists in both a soluble and membrane-associated form (Lichi et al., 2004; Moll et al., 1997), yet given the apparent absence of a FtsY receptor in prokaryotes, it remains unclear how FtsY interacts with the membrane. In *Escherichia coli*, membrane binding of FtsY has been suggested to be mediated via clusters of lysine and arginine residues situated close to the N-terminus of the protein (de Leeuw et al., 1997; Powers and Walter, 1997; Zelazny et al., 1997). Examination of FtsY sequences from a variety of archaeal species has also revealed the presence of clusters of positively-charged residues at the beginning of the protein (Lichi et al., 2004). In halophilic Archaea, however, far fewer of such residues are detected, suggesting that archaeal FtsY may rely on additional portions of the protein for membrane association. Indeed, it has been shown that a truncated version of *Hfx. volcanii* FtsY, comprising the C-terminal NG domain, i.e. the portion of the protein responsible for the SRP54-binding and GTPase activity, is capable of membrane binding (Lichi et al., 2004).

While SRP must interact with its receptor at some point during protein targeting, the order of events that take place during the archaeal SRP cycle remains to be defined. In the hyperthermoacidophilic archaeon *Acidianus ambivalens*, the formation of a soluble SRP54-FtsY complex was reported, as was the membrane binding ability of both SRP54 and FtsY (Moll, 2003). In contrast, the membrane binding of *Hfx. volcanii* SRP54 was shown to be FtsY-dependent (Lichi et al., 2004).

4. The Archaeal Ribosome Receptor

During eukaryal co-translational protein translocation, SRP delivers selected translating ribosomes to the ER membrane through the affinities of SRP for its receptor (Connolly and Gilmore, 1993; Miller et al., 1993) and of the ribosome for the translocon (Gorlich et al., 1994; Kalies et al., 1994). Similarly, bacterial ribosomes have been shown to specifically bind to SecYEG, the membrane protein complex comprising the core of the bacterial translocon (Prinz et al., 2000; Zito and Oliver, 2003). Recent *in vitro* studies with *Hfx. volcanii* have revealed the ability of haloarchaeal ribosomes to bind to SecYE-based sites, thus confirming the binding of ribosomes to the translocon in all three

domains of Life (Ring and Eichler, 2004b). In these studies, the ability of translation-competent ribosomes to bind to inverted membrane vesicles, both prepared from *Hfx. volcanii*, was demonstrated using a floatation approach. Quantitative analysis of the binding revealed an affinity comparable to that found in Eukarya and Bacteria, pointing to the existence a proteinaceous receptor for the archaeal ribosome. Accordingly, proteolytic pretreatment of the vesicles essentially eliminated ribosomal binding. When the inverted membrane vesicles were, however, prepared from cells expressing chimeras of translocon components SecE or SecY fused to a cytoplasmically-oriented cellulose binding domain, reduced ribosome binding, likely due to steric hinderance by the cellulose binding domain, was observed. Moreover, pretreatment with cellulose drastically reduced ribosome binding to chimera-containing, but not to wild type vesicles. What is more, and in striking contrast to what has been reported in non-halophilic organisms (Borgese et al., 1974; Connolly and Gilmore, 1993; Prinz et al., 2000), the *in vitro* ribosome binding to membranes prepared from *Hfx. volcanii* required molar salt concentrations, with no binding occurring at lower salt levels. While the basis for this salt-dependent binding is not known, the availability of a crystal structure of the 50S large subunit of the *Haloarcula marismortui* ribosome (Ban et al., 2000) could provide structural insight into ribosome-translocon interactions in halophilic Archaea.

5. The Archaeal Translocon

As in the other domains of Life, passage of archaeal secretory proteins across the membrane, like insertion of membrane proteins, occurs at a dedicated protein complex, the translocon. In Bacteria, the translocon is based on the SecYEG complex (Brundage et al., 1990), while in Eukarya, a translocon based on the core Sec61αβγ complex, embedded in the membrane of the endoplasmic reticulum (ER), serves as the entrance to the secretory pathway (Gorlich and Rapoport, 1993). Analysis of completely sequenced genomes, as well as cloning of individual genes from other strains, has revealed the presence of homologues of these proteins in Archaea (Pohlschröder et al., 2004; Ring and Eichler, 2004a).

Archaeal SecY proteins, predicted to span the membrane ten times (Auer et al., 1991; Cao and Saier Jr., 2003; Irihimovitch et al., 2003; Kath and Schafer, 1995), likely form the pore through which translocating proteins cross the membrane, as is accepted for bacterial SecY and eukaryal Sec61α (Joly and Wickner, 1993; Mothes et al., 1994). Sequence comparison reveals that archaeal SecY proteins are more reminiscent of eukaryal Sec61α proteins than of their bacterial counterparts (Rensing and Maier, 1994; Cao and Saier Jr., 2003), despite being named according to the prokaryal nomenclature. Similarly, archaeal SecE is closer to the eukaryal version of the protein, i.e. Sec61γ (Cao and Saier Jr., 2003). Indeed, the similarities of the archaeal and eukaryal proteins, together with the similar relative positions of archaeal and bacterial *secE* genes, were used together to reveal the homology between bacterial SecE and eukaryal Sec61γ (Hartmann et al., 1994). Together with SecYE/Sec61αγ, the bacterial and eukaryal translocons also include SecG (Brundage et al., 1992) or Sec61β (Gorlich and Rapoport, 1993), respectively. However, unlike SecY/Sec61α and SecE/Sec61γ, SecG and

Sec61β are not very similar (Cao and Saier Jr., 2003). In Archaea, advanced bioinformatic techniques identified an archaeal version of Sec61β (Kinch et al., 2002).

At the protein level, only a limited number of studies on the archaeal translocon or its components have been reported. Complementation of a temperature-sensitive bacterial *secY* mutant with the *Methanococcus vannielii* SecY-encoding gene allowed growth of the mutant strain at the non-permissive temperature (Auer et al., 1991), suggesting not only that archaeal SecY can functionally replace the bacterial protein, but also that archaeal SecY is active despite the absence of the ether-based phospholipids that comprise the archaeal membrane. The salt-insensitive interaction between a cellulose binding-domain and cellulose was used for purification of chimeras containing *Hfx. volcanii* SecY or SecE fused to the said binding domain (Irihimovitch et al., 2003). These studies revealed both the SecY- and SecE-containing proteins to be stably expressed and exclusively localized to the membrane. Most recently, the three-dimensional structure of the methanoarchaea *Methanocaldococcus jannaschii* SecYEβ complex has been solved, providing the first high-resolution view of a translocon (van den Berg et al., 2004).

In Bacteria, SecYEG can be found as part of a larger complex that includes SecDF, although not all strains express the latter proteins (Tseng et al., 1999). Genome analysis has revealed, similarly, that SecDF is present in some, but not all Archaea (Eichler, 2003; Tseng et al., 1999). Sequence comparison of bacterial and archaeal SecDF, including those of *Halobacterium* sp. NRC-1, reveals that the two versions of the proteins present similar membrane topologies and positioning of conserved sequence elements. The composition of these conserved motifs can, however, be clearly divided along Archaea-Bacteria lines (Eichler, 2003). While the role(s) of SecDF in protein translocation have yet to be clearly defined, these proteins have been reported to modulate the *in vitro* membrane-associating behavior of SecA, the ATPase component of the bacterial protein translocation apparatus (Duong and Wickner, 1997; Economou et al., 1995). Considering the apparent absence of archaeal SecA (Pohlschröder et al., 2004; Ring and Eichler, 2004a), the domain-specific character of conserved SecDF sequence elements could reflect differences in the functions of these proteins in Bacteria and Archaea.

6. What's Next?

As has been shown to be the case with many other aspects of archaeal biology, work with halophiles has also provided insight in protein translocation in this domain of life. Now, with many of the tools needed for *in vitro* reconstitution of protein translocation, such as inverted membrane vesicles (Ring and Eichler, 2001), SRP (Tozik et al., 2002), FtsY (Lichi et al., 2004), functional ribosomes (Ring and Eichler, 2004b) and reporter proteins (Irihimovitch and Eichler, 2003) available from *Hfx. volcanii*, it should be soon possible to recreate and study the translocation process in a controlled manner. However, before such efforts can proceed, additional tools and techniques require attention. The most pressing of these is the development of an *in vitro* protein translation system for halophilic Archaea. While such systems designed for use with haloarchaeal components have been reported (Bayley and Griffith, 1968; Sanz et al., 1988; Saruyama and Nierhaus, 1985), they lack the versatility and efficiency called for

in an *in vitro* translation/translocation setup. At the same time, improved molecular tools for genetic manipulation of halophilic Archaea, such as an increased number of selectable markers, enhanced gene knockout techniques and plasmids for protein over-expression, will help address the process of archaeal protein translocation *in vivo*. Such studies could, for example, provide answers to questions related to the relative use of the Sec and Tat translocation pathways in haloarchaea. Thus, while much remains to learned before we fully comprehend how Archaea transport proteins into and across their membranes, continued investigation using halophiles as a model system will likely bring us closer to this goal.

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ENZYMES OF HALOPHILIC ARCHAEA

Recent findings on Ureases and Nucleoside Diphosphate Kinases

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1. Introduction

The majority of strains of halophilic Archaea (family Halobacteriaceae) grow best at NaCl concentrations of 3.5-4.5 M. To compensate for the high salt in the environment, halobacteria accumulate salts, mainly KCl, up to 5 M. Most of the halobacterial proteins are thus adapted to function in the presence of high salt concentrations, and lose their activities at low salt concentrations (Madern et al., 2000).

We here report our recent studies on two proteins from halophilic Archaea: urease and nucleoside diphosphate kinase. Urease, an enzyme composed of heterogeneous subunits (Mobley et al., 1995), is an interesting target protein to investigate the relationship between salt concentration and subunit interaction. Nucleoside diphosphate kinase of halophilic Archaea was first studied as a major cytosolic protein of *Natrialba magadii* that bound to ATP-agarose and eluted with 5 mM ATP in the presence of 3.5 M NaCl (Polosina et al., 1998). Further detailed experiments have demonstrated that nucleoside diphosphate kinase is one of the few halobacterial proteins that do not require high salt concentrations to maintain their stability (Ishibashi et al., 2002; Polosina et al., 2002). A comparison of their salt response patterns and stabilities may provide insights into the way these enzymes have adapted to function at different salt concentrations.

2. Ureases

The presence or absence of urease activity has been one of the criteria used in the taxonomic studies of prokaryotes including halobacteria. A survey of the literature on the taxa of halophilic Archaea showed that some strains have been reported to possess the urease activity. For example, 33% of the strains of the genus *Haloarcula*, and 14% of the strains of the genus *Halobacterium* were claimed to be urease-positive (Torreblanca et al., 1986). In our survey, ammonia released from urea was determined after incubation with cell free extract obtained by sonication. Only a few strains of the genus *Haloarcula* were shown to be urease producers, while more than 60 strains of the genera *Halobacterium*,

Halococcus, *Haloferax*, *Halogeometricum*, *Halorubrum*, *Haloterrigena*, *Natrialba*, *Natrinema* and some species of *Haloarcula* were urease negative. Alkaliphilic halobacteria of the genera *Natronobacterium*, *Natronococcus*, *Natronorubrum* and *Natronomonas*, and some fastidious strains of five other genera were not tested. *Har. hispanica*, which showed highest activity, was selected for further characterization of halobacterial urease.

2.1. PURIFICATION AND CHARACTERIZATION OF *HALOARCULA HISPANICA* UREASE

The urease of *Har. hispanica* was purified using hydrophobic chromatography, gel permeation chromatography, and finally preparative polyacrylamide gel electrophoresis. No activity was detected in the purified preparation, probably owing to inactivation in the PAGE step. A partially purified urease was shown to be a typical halophilic enzyme in that it irreversibly lost its activity in the absence of salt. The optimal temperature of the urease activity was 70°C in the presence of 23.3% NaCl, while with 8.3% NaCl, 60°C was the optimum. The maximum activity was obtained at 18-23 % NaCl when measured at 50°C. The activity in the presence 2.84 M (21.2%) KCl + 0.43 M (2.5%) NaCl (3.27 M salt in total) was 88.5% of that in the presence of 3.26 M (19.1%) NaCl. We are now further trying to purify the *Har. hispanica* urease.

2.2. PCR AMPLIFICATION OF GENES OF *HALOARCULA* UREASES AND THE UREASE GENE CLUSTER OF *HALOARCULA MARISMORTUI*

We partially succeeded in the PCR amplification of urease genes from nine strains of the genus *Haloarcula*, including urease-negative ones. However, urease-negative strains of several other genera gave no PCR products. We may conclude that only strains of the genus *Haloarcula* possess the urease genes.

The genome sequence of *Har. marismortui* suggest that the urease genes that encode the three subunits are arranged in the order of β, α, and γ. The order of the subunits is unique, to the authors' best knowledge, in that the order in the genes of other microorganisms is γ, β, and α. Although the entire gene structure of the urease of *Har. hispanica* is still to be elucidated, our present data suggest that the three subunits of the eight strains of *Haloarcula* are also encoded in the same order as in *Har. marismortui*. We also detected an accessory protein gene cluster of *Har. marismortui* in the order of UreG, UreD, UreE, and UreF immediately downstream of the γ subunit. There were no accessory protein genes upstream of the β subunit. The order of the accessory protein genes is also unique, since the order in other microorganisms is mostly UreE, UreF, UreG, and UreD. Altogether, the cluster of urease genes of *Har. marismortui* is β - α - γ -UreG-UreD-UreE-UreF, while the typical microbial urease gene cluster is γ - β - α - UreE-UreF-UreG-UreD, suggesting that a gene shuffling had occurred somewhere during the evolution of the halophilic Archaea (Mizuki et al., 2004a).

3. Nucleoside Diphosphate Kinases

Nucleoside diphosphate kinase has been shown to be almost ubiquitous in living

organisms with just a few exceptions. The main function of nucleoside diphosphate kinase is to exchange γ-phosphates between nucleoside triphosphates and diphosphates, thus playing a key role in maintaining cellular pools of all nucleoside triphosphates (Bernard et al., 2000). Polosina et al. (1998) and Ishibashi et al. (2001) independently purified nucleoside diphosphate kinase from *Nab. magadii* and a strain of *Halobacterium salinarum*, respectively, as a protein that bound to ATP-agarose in the absence of salt and eluted with ATP. An alignment of more than 100 sequences revealed a fairly well conserved primary structure throughout the three domains, Eukarya, Bacteria, and Archaea. We also noticed some signature amino acids residues specific to Eukarya (Kamekura, unpublished data).

At the initial stage of our work before the contig sequences of *Har. marismortui* and *Haloferax volcanii* became available, we purified the nucleoside diphosphate kinases from strains representing three genera of halobacteria: *Halobacterium* sp. NRC-1, *Haloarcula hispanica*, and *Hfx. volcanii*. Preliminary data showed they differed significantly in the salt concentration-activity profiles and in the behavior on native-PAGE. *Halobacterium* sp. NRC-1 and *Hfx. volcanii* always gave sharp bands with a relative motility of 0.54 and 0.19, respectively. The nucleoside diphosphate kinase of *Har. hispanica*, on the other hand, gave either a single, diffuse band with a relative mobility of 0.2 to 0.3, or a mixture of the diffuse band with an additional sharp band having the same mobility as that of *Hfx. volcanii* nucleoside diphosphate kinase. These data prompted us to expand the scope of investigation to strains of other genera, as well as to several strains of a single genus. For the latter aspect, members of the genus *Haloarcula* were chosen, as this genus contains as many as nine species (including species whose names have not been validly published). We expected that the variation in the amino acid sequences within the same genus might be lower than that among genera, allowing the identification of residue(s) responsible for the differences in the behavior in PAGE and in salt response pattern of the activities, if any.

3.1. PURIFICATION AND N-TERMINAL AMINO ACID SEQUENCING

Ishibashi et al. (2001) have shown that *Hbt. salinarum* nucleoside diphosphate kinase was separated from molecular chaperone DnaK, another major ATP binding protein by affinity column chromatography using ATP-agarose column chromatography in the absence of NaCl. We demonstrated that nucleoside diphosphate kinase proteins were purified to homogeneity from halophilic archaeal strains belonging to six genera only with ATP-agarose chromatography in the presence of 3 M NaCl. Although we do not know the reason halophilic archaeal nucleoside diphosphate kinases can so easily be purified by a single ATP-agarose chromatography, Polosina et al. (2002) suggested that "although the nucleoside diphosphate kinase is a minor protein, its strong affinity for ATP makes it a major component among the proteins that bind to ATP-agarose."

Twelve halobacterial strains belonging to six genera were cultivated in 200 ml of appropriate media, cells were disrupted by sonication, and nucleoside diphosphate kinase was purified by applying to a small column of ATP-agarose, yielding sufficient amounts of purified nucleoside diphosphate kinase for amino acid sequencing and polyacrylamide gel electrophoresis. The N-terminal amino acid sequences of the nucleoside diphosphate kinase preparations suggested that the initiation methionine residues were deleted post-translationally in all strains, and a completely conserved sequence, ERTFVMVKPD

was found at amino acid #4 to 13. It was concluded that the nucleoside diphosphate kinases of *Halobacterium* sp. NRC-1, *Har. marismortui*, and *Hfx. volcanii* are composed of 160, 153 and 153 amino acid residues, respectively.

3.2. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed using 15-25% precast gels under various conditions (Fig. 1).

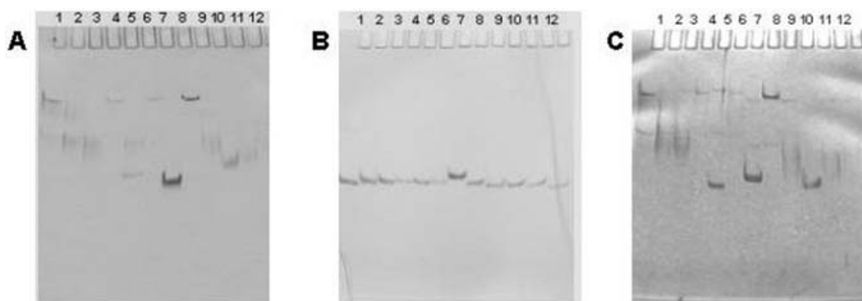

Figure 1. Polyacrylamide gel electrophoresis of nucleoside diphosphate kinase preparations from 12 halobacterial strains. A: Native-PAGE, B: SDS-PAGE (boiled for 3 min), C: SDS-PAGE (not heated). Lane 1: *Haloarcula vallismortis*; Lane 2: *Haloarcula argentinensis*; Lane 3: *Haloarcula quadrata*; Lane 4: *Haloarcula aidinensis*; Lane 5: "Haloarcula sinaiiensis"; Lane 6: *Haloarcula hispanica*; Lane 7: *Halobacterium* sp. NRC-1; Lane 8: *Haloferax volcanii*; Lane 9: *Natrialba asiatica*; Lane 10: *Natrialba magadii*; Lane 11: *Natrinema pellirubrum*; Lane 12: *Haloterrigena* sp. GSL-11.

Native-PAGE of these preparations again revealed a variety of patterns (Fig. 1A). Nucleoside diphosphate kinases from *Halobacterium* sp. NRC-1 and *Natrialba magadii* each gave relatively sharp single bands with relative mobilities of about 0.5. SDS-PAGE of the preparations heat treated at 80°C or higher for 3 minutes with SDS showed that all were almost completely homogeneous and they had almost the same molecular mass except for that of *Halobacterium* sp. NRC-1 (Fig. 1B). Figure 1C is an SDS-PAGE in which the samples were not heat-treated before loading to the gel. A separate run with a molecular marker indicated that the relative mobility of *Hfx. volcanii* nucleoside diphosphate kinase corresponded to a molecular mass of 56 kDa, which seemed to be a trimer of 17.4 kDa (calculated) monomers. Heating of *Hfx. volcanii* nucleoside diphosphate kinase with SDS at 50°C for up to 3 min gave a trimer, 70°C for 3 min gave a mixture of trimer and monomer bands, and 10 min heating resulted in a complete shifting to the monomer. On the other hand, heating of *Haloterrigena* sp. GSL-11 nucleoside diphosphate kinase with SDS at 70°C for 2 seconds gave a diffuse band (smear), 30 seconds heating gave a mixture of smear and monomer, and 3 minutes heating resulted in a complete shift to the monomer. These data demonstrate the considerable diversity in the nucleoside diphosphate kinases from different genera, and among different species of

the genus *Haloarcula*. It must be pointed out that the behavior on the native-PAGE and SDS-PAGE without heat-treatment differed slightly upon repeated experiments.

Nucleoside diphosphate kinase has been shown to be a multi-subunit enzyme composed of identical monomers (Giartosio et al., 1996). Eukaryotic nucleoside diphosphate kinases (human, bovine retina, *Drosophila melanogaster*, *Dictyostelium discoideum*) are hexamers, whereas bacterial enzymes (*Escherichia coli*, *Myxococcus xanthus*) are reported as tetramers. The nucleoside diphosphate kinase from the hyperthermophilic archaeon *Methanocaldococcus jannaschii* has been overexpressed in *E. coli* and crystallized. Preliminary crystallographic data suggests it has a homohexameric structure (Min et al., 2000), but its detailed enzymatic properties have not been reported. The nucleoside diphosphate kinase from *Nab. magadii* has been suggested to be homooligomeric hexamer in the range of NaCl concentration from 0.45 to 3.5 M, based on analytical ultracentrifugation (Polosina et al., 2002). On the other hand, *Hbt. salinarum* nucleoside diphosphate kinase, an equivalent of that of *Halobacterium* sp. NRC-1, has been shown to change its subunit assembly depending on the concentration of NaCl, from dimeric at 0.2 M to hexameric at 3.8 M, as determined by the sedimentation equilibrium method (Ishibashi et al., 2002). In this context, the results of the native- and SDS-PAGE of *Halobacterium* sp. NRC-1 and *Nab. magadii* nucleoside diphosphate kinase are of interest but also puzzling. Relative mobilities of *Halobacterium* sp. NRC-1 nucleoside diphosphate kinase in native-PAGE, SDS-PAGE (not heated), and SDS-PAGE (boiled) were exactly the same (Fig.1 A, B, C). Preliminary data suggest that the nucleoside diphosphate kinase stays dimeric even in the absence of NaCl (Ishibashi, personal communication). These data may suggest that the nucleoside diphosphate kinase migrated as a dimer in the native-PAGE. Behaviors of other nucleoside diphosphate kinases are also puzzling, including the diffuse band (smear) to monomer and trimer to monomer conversion upon heat treatment with SDS, but we need more information on the three dimensional structures of these proteins in the high and low NaCl concentrations.

3.3. DETERMINATION OF AMINO ACID SEQUENCES OF NUCLEOSIDE DIPHOSPHATE KINASES FROM *HALOARCULA* SPP.

Genes encoding nucleoside diphosphate kinases of *Haloarcula* species were PCR amplified with a primer set; sense primer was designed from the conserved sequence mentioned above and the antisense primer was designed from the downstream region of *Har. marismortui* nucleoside diphosphate kinase gene. Figure 2 is the alignment of the complete amino acid sequences, which are combinations of the N-terminal sequences and the deduced sequences from the amplified genes. The nine nucleoside diphosphate kinases differed only at one to four residues each other, except that *Har. quadrata* and "Har. californiae" have the same sequence.

3.4. EFFECT OF NaCl CONCENTRATION ON NUCLEOSIDE DIPHOSPHATE KINASE ACTIVITY

The effects of NaCl concentration on the activity (the salt response patterns) of nucleoside diphosphate kinases were determined by measuring ATP generated from ADP with GTP as a phosphate donor. Nucleoside diphosphate kinases from *Halobacterium* sp.

NRC-1, *Hfx. volcanii* WFD11, and *Haloterrigena* sp. GSL-11 were dialyzed thoroughly against a buffer containing 0.05 M NaCl. Protein concentrations of dialysates were 16-26 μg ml^{-1}. Figure 3A depicts the salt response patterns of the enzymes. *Halobacterium* sp. NRC-1 and *Haloterrigena* sp. GSL-11 nucleoside diphosphate kinases exhibited essentially the same salt-response pattern, with optimal activities at 1 M NaCl. The specific activity of the *Halobacterium* sp. NRC-1 nucleoside diphosphate kinase at 1 M NaCl was 60 U mg $protein^{-1}$. On the other hand, *Hfx. volcanii* nucleoside diphosphate kinase showed a distinctly different pattern in that higher NaCl concentrations inhibited enzyme activity. The amino acid sequences of *Halobacterium* sp. NRC-1 and *Hfx. volcanii* nucleoside diphosphate kinases were 78.4 % similar, i.e., there was a 33 amino acids difference.

```
 Har. vallismortis  SDHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
"Har. sinaiiensis"  SEHERTFVMVKPDGVQRGLIGDIVSRFEDCGLKMVGGKFMQIDQELAEEH
 Har. quadrata      SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
 Har. marismortui   SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
 Har. japonica      SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
 Har. hispanica     SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
"Har. californiae"  SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
 Har. argentinensis SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
 Har. aidinensis    SEHERTFVMVKPDGVQRGLIGDIVSRFEDRGLKMVGGKFMQIDQELAEEH
                    *.*************************** ********************

 Har. vallismortis  YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
"Har. sinaiiensis"  YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
 Har. quadrata      YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
 Har. marismortui   YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
 Har.japonica       YGEHADKPFFDGLVDFITSGPVFAMVWEGQDATRQVRNMMGETDPAESAP
 Har. hispanica     YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
"Har. californiae"  YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
 Har. argentinensis YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRNMMGETDPAESAP
 Har. aidinensis    YGEHEDKPFFDGLVDFITSGPVFAMVWEGQDATRQVRTMMGETDPAESAP
                    **** ********************************.************

 Har. vallismortis  GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDAPW
"Har. sinaiiensis"  GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDSSW
 Har. quadrata      GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDSSW
 Har. marismortui   GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEAELVDWDQIDSSW
 Har.japonica       GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDSSW
 Har. hispanica     GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDAPW
"Har. californiae"  GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDSSW
 Har. argentinensis GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDSSW
 Har. aidinensis    GTIRGDYGLDLGRNVIHGSDHEDEGANEREIELFFDEDELVDWDQIDAPW
                    ************************************ **********. *

 Har. vallismortis  LYE
"Har. sinaiiensis"  LYE
 Har. quadrata      LYE
 Har. marismortui   LYE
 Har. japonica      LYE
 Har. hispanica     LYE
"Har. californiae"  LYE
 Har. argentinensis LYE
 Har. aidinensis    LYE
                    ***
```

Figure 2. Alignment of amino acid sequences of nucleoside diphosphate kinases from nine strains of the genus *Haloarcula*. The sequence of *Har. marismortui* was deduced from contig 131 and the N-terminal amino acid was determined experimentally.

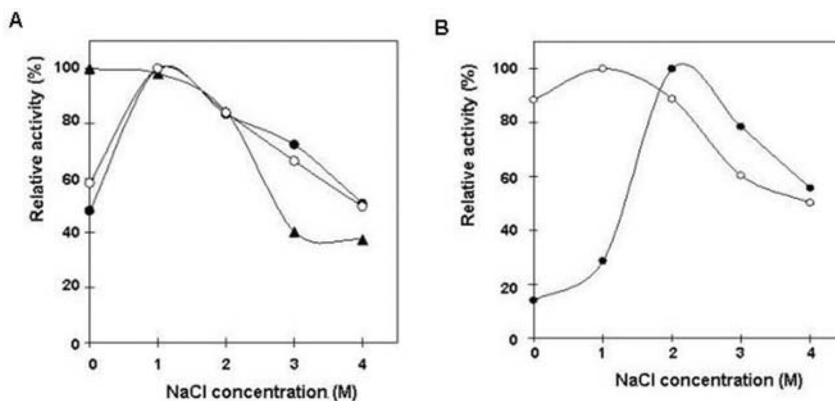

Figure 3. Salt response patterns of halophilic archaeal nucleoside diphosphate kinases. A: *Halobacterium* sp. NRC-1 (●), *Haloferax volcanii* (▲), and *Haloterrigena* sp. GSL-11 (○). 100% relative activities corresponding to 550,886 RLU, 597,917 RLU, and 539,810 RLU, respectively, as measured in a Lumitester K-210. B: "Haloarcula sinaiiensis" (●) and *Haloarcula quadrata* (○). 100% relative activities correspond to 580,972 RLU and 372,173 RLU, respectively.

An interesting difference in salt response pattern was found between *Har. quadrata* and "Har. sinaiiensis" nucleoside diphosphate kinases. The two nucleoside diphosphate kinases differed only at the 30th amino acid; arginine in the former and cysteine in the latter, as depicted in Fig. 2. The former showed an optimal activity at 1 M NaCl, whereas the activity of the latter was highest at 2 M NaCl (Fig. 3B). (Mizuki et al., 2004b).

3.5. CRYSTALLIZATION

"Har. sinaiiensis" was cultivated in 72-liter cultures, nucleoside diphosphate kinase was purified, and a 10 mg preparation of the protein was subjected to crystallization. Two crystals appeared in the following conditions. Crystal 1, 20°C, [20 % (w/v) PEG 8,000, 0.1 M Tris-HCl pH 8.5 and 0.2 M $MgCl_2$] (Fig. 4), Crystal 2, 4°C, [30 % (v/v) isopropanol, 0.1 M HEPES-NaOH pH 7.5 and 0.2 M $MgCl_2$].

4. Discussion

Though various halophilic enzymes are supposed to exhibit halophilic properties by different mechanisms, comparative analysis of amino acid sequence of enzymes combined with mutational analyses is a powerful approach for analyzing the relative importance of certain residues in protein stabilities and halophilicity. For example, malate dehydrogenase from *Har. marismortui* is one of the most thoroughly investigated halobacterial enzymes (Irimia et al., 2003). Some time ago, Madern et al. (1995) showed that a mutant malate dehydrogenase protein with single amino acid change (Glu243Arg)

was more halophilic than the wild type enzyme, and required significantly higher concentrations of NaCl or KCl for equivalent stability. In another example, the halophilic serine proteinase halolysin R4, Kamekura et al. (1996) showed that replacement of two cysteine residues by two serine residues in the C-terminal extension decreased the stability of the enzyme, and the mutant Halolysin R4 also required significantly higher concentrations of NaCl for optimum activity.

Figure 4. Crystals of nucleoside diphosphate kinase from "Haloarcula sinaiiensis".

However, these two examples are perhaps not the most useful representatives for studying the relative importance of specific residues on the activity and stability of halophilic enzymes as they lose activity below 1 M NaCl. As shown above, we have found that ureases from *Haloarcula* spp. also lose activity at low salt concentration. The ideal protein for the study of halophilicity should be an enzyme that is stable in the absence of salt and exhibits highest activity in the presence of salt. For example, genes of such an ideal protein could be expressed in *E. coli* to readily access large quantities of pure and active halophilic enzymes (Ishibashi et al., 2001). In this sense, the nucleoside diphosphate kinase proteins are quite promising in the future investigation of halophilicity at the single-amino acid-substitution level.

The difference in the salt response patterns of the *Har. quadrata* and "Har. sinaiiensis" nucleoside diphosphate kinases is quite interesting. The 30th amino acid was arginine in *Har. quadrata* and cysteine in "Har. sinaiiensis". The arginine at this position is conserved in all halobacterial nucleoside diphosphate kinases except the one of "Har. sinaiiensis" and that of *Halobacterium* sp. NRC-1, which is substituted by another basic amino acid, lysine. In almost all of the archaeal and eukaryal nucleoside diphosphate kinases, this position is occupied by arginine or lysine, whereas in bacterial nucleoside diphosphate kinases it is lysine or alanine. In this sense the cysteine in this position is quite extraordinary.

Although the survey of the relationships between primary structure and salt-response pattern of halobacterial nucleoside diphosphate kinases has just begun, our present data suggest that a single-amino acid substitution may cause a drastic shift in the optimal salt concentration of halobacterial enzymes by as much as one molar.

So far, several halobacterial enzymes have been crystallized and three-dimensional

structures determined; malate dehydrogenase (Irimia et al., 2003), dihydrofolate reductase (Pieper et al., 1998), catalase-peroxidase (Yamada et al., 2001), 2Fe-2S ferredoxin (Frolow et al., 1996), bacteriorhodopsin (Pebay-Peyroula et al., 2000), and ribosomal particles and some r-proteins (Yonath, 2002). The nucleoside diphosphate kinase protein of "Har. sinaiiensis" has just been crystallized in two conditions, and we are presently attempting to improve the quality of the crystals based on suggestions by Costenaro et al. (2002). When it will be possible to obtain good-quality crystals, a more thorough study of the structure and the salt relationship of halophilic archaeal nucleoside diphosphate kinase will be possible.

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OSMOADAPTATION IN METHANOGENIC ARCHAEA: RECENT INSIGHTS FROM A GENOMIC PERSPECTIVE

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1. Principles of Osmoadaptation

In their natural environments microorganisms have to cope with changing conditions of various kinds. Apart from the availability of nutrients, varying temperatures and pH, another frequently changing factor is the osmolarity. This is not only the case in saline environments, but also common in soil, in which evaporation and rainfall causes drastic changes in the environmental osmolarity.

Two general strategies are known that living cells use to reestablish turgor pressure and to circumvent the detrimental consequences of water loss when exposed to increasing osmolarity. One is the so-called "salt-in-cytoplasm" type, where inorganic ions, mainly K^+ and Cl^-, are accumulated in the cytoplasm to a level which resembles the external salt concentration. This strategy is found in halophilic *Halobacteriales* (Archaea), the anaerobic halophilic *Halanaerobiales* (Bacteria), and the recently described *Salinibacter ruber* (Oren, 1999; Oren et al., 2002). However this strategy requires far reaching adaptations of intracellular machineries to high salt concentrations and, therefore, limits growth to certain osmolarities. The second strategy, which is used by the majority of the living cells, is the accumulation of compatible solutes, which are defined as small, soluble, organic molecules that do not interfere with the central metabolism of the cell, even if they are accumulated to high concentrations (Brown, 1976). This strategy is wide spread and found in all lines of descent of life (Bohnert, 1995; Kempf and Bremer, 1998; Roberts, 2000; Martin et al., 1999). Halophilic cells accumulating compatible solutes can basically preserve the same enzymatic machinery as non-halophiles and are, therefore, more flexible than cells using the "salt-in-cytoplasm" strategy. They cannot only cope with high but also tolerate lower osmolarities.

2. Distribution of Compatible Solutes in Methanogens

Most methanogens react to changes in the extracellular NaCl concentration with the accumulation of compatible solutes. Studies with *Methanosarcina* species adapted to elevated salt concentrations showed that at low osmolarities (0.3 osmol kg^{-1}) potassium

and α-glutamate are accumulated as predominant compatible solutes (Sowers and Gunsalus, 1995). A further increase in the salt concentration to 1.0 osmol kg^{-1} also led to a further increase in the potassium and α-glutamate concentration of the cytoplasm. On the one hand, this contributes to an increase of the osmotic potential of the cytoplasm, and on the other hand, by the co-accumulation of potassium and glutamate the net charges are counterbalanced. A further increase of the extracellular NaCl concentration to 2.0 osmol kg^{-1} had no remarkable effect on the potassium and glutamate concentration. Instead, the accumulation of a novel osmolyte, N^{ε}-acetyl-β-lysine was observed. However, when glycine betaine was present in the environment, this compound was taken up by the cells and the synthesis of N^{ε}-acetyl-β-lysine was reduced (Sowers and Gunsalus, 1995).

NMR analysis in *Methanosarcina mazei* Gö1 showed that this organism accumulates at moderate salt concentrations (400 mM NaCl) mainly glutamate and only very little N^{ε}-acetyl-β-lysine. At high salt concentrations (800 mM NaCl), however, the glutamate concentration increased only slightly (1.4-fold), but N^{ε}-acetyl-β-lysine was accumulated 5.8-fold compared to moderate salt concentrations and became the predominant compatible solute (Pflüger et al., 2003). The influence of externally provided glycine betaine on the solute concentrations has not been analyzed up to now. However, as *M. mazei* Gö1 possesses at least one glycine betaine transporter, this solute is most likely taken up and contributing to the solute pool, when present in the medium.

Methanohalophilus portucalensis uses a broader range of compatible solutes. This methanogen accumulated at salt concentrations of 1.7 M NaCl α-glutamate, glycine betaine, β-glutamine, and N^{ε}-acetyl-β-lysine, as it was shown by analyses of the internal solute pool (Lai et al., 1991). At higher NaCl concentrations the amount of glycine betaine, β-glutamine, and N^{ε}-acetyl-β-lysine accumulated increased. The α-glutamate concentration, however, stayed on a similar level. Furthermore, it was shown that the internal potassium concentration was about 1.8-fold higher in cells adapted to 2.7 M NaCl compared to cells grown at 1.7 M NaCl.

The thermophilic methanogen *Methanococcus thermolihtotrophicus* synthesized and accumulated α- and β-glutamate when the external salt concentration was below 1 M NaCl (Robertson et al., 1992). Concomitantly, the internal potassium concentration is adjusted to the level of the negatively charged amino acids to counterbalance the negative charges. A further increase of the external NaCl concentration led to an increase in the N^{ε}-acetyl-β-lysine level, and the internal concentrations of α- and β-glutamate were slightly reduced.

Taken together, this shows that methanogenic Archaea, like Bacteria, are not restricted to only one compatible solute to maintain turgor pressure, but use a set of these compounds. This allows them to react much more flexible to changes in the extracellular salt concentration by adjusting the solute pool to the given condition.

3. How Methanogens Cope with Hypersalinity

In bacteria, the response to a hyperosmotic shock can be divided into two general phases (Martin et al., 1999). In a first phase a rapid influx of potassium could be observed to maintain turgor pressure and prevent plasmolysis. In contrast to cation

uptake, there is very little information about the anion accumulated along with potassium. For *Escherichia coli*, a role of glutamate was discussed, but recently it was suggested that not glutamate but phosphate is accumulated (Kornberg et al., 1999). Studies revealed large accumulations of inorganic phosphate (poly P) in cellular responses to high salt. Poly P is a linear polymer of many orthophosphate residues linked by high energy phosphoanhydride bonds and is formed by dehydration from P_i.

In *E. coli* also putrescine excretion was observed following a hyperosmotic shock (Schiller et al., 2000). Following hyperosmotic shock, *E. coli* strain AN387 started immediately to excrete putrescine, and internal putrescine decreased with a rate of approximately 1 nmol per mg (dry weight) per min, while external putrescine concentrations increased. It was calculated that the increase in external putrescine exactly corresponded to its decrease in the cell.

For other bacteria, the nature of the anion is unknown. In the second phase, cellular potassium levels are reduced and the accumulation of compatible solutes begins. The first phase is mainly independent of transcriptional activity and based on already existing systems.

Up to date, *Methanococcus thermolithotrophicus* is the only methanogenic archaeon in which the process of salt adaptation has been studied after a hyperosmotic shock in a time dependent manner (Martin et al., 2001; Roberts, 2004). Cells of *Methanococcus thermolithotrophicus* react to a hyperosmotic shock with a lag phase of cell growth. The duration of the lag phase is depending on the magnitude of the shock. It is assumed that the first response of the cells to an osmotic upshock is a very rapid efflux of water. At the same time potassium is internalized and the amount of potassium internalized overshoots what would be needed to balance the external sodium ions if an appropriate counterion were also internalized. In the following 10-20 minutes the potassium concentration slightly decreased and a new steady state was achieved, which was about 1.5-fold higher than in non-shocked cells. The speed of this response is pointing to the internalization of potassium via already built transporters and not to higher expression and production of transport proteins. In the second phase of the response to a hyperosmotic shock the accumulation of compatible solutes was observed. Thus, in *Methanococcus thermolithotrophicus* the accumulation of organic osmolytes begins once the intracellular potassium is stabilized. At about 20 min after the shock the accumulation of α-glutamate started. Furthermore, β-glutamate and aspartate also seemed to be accumulated, but on a much lower level. N^{ε}-acetyl-β-lysine was found in very low amounts, but did not contribute significantly to the pool of compatible solutes right after the shock. Longer periods, i.e. several cell cycles, are required to have maximal accumulation of N^{ε}-acetyl-β-lysine. That this zwitterion is accumulated only in osmoadapted cells is consistent with the hypothesis that activity of one of its biosynthetic enzymes is limiting in cells grown and adapted to lower NaCl concentrations. In salt adapted cells N^{ε}-acetyl-β-lysine is the predominant compatible solute and the amount of α-glutamate is reduced (Robertson et al., 1992). This is pointing to α-glutamate and potassium playing a role as transient compatible solutes at the beginning of the second phase of the answer to a hyperosmotic shock in *Methanococcus thermolithotrophicus*.

3.1. ACCUMULATION OF POTASSIUM IN METHANOGENS

In bacteria, the first response to an osmotic upshock is the accumulation of potassium via a primary, ATP-driven potassium uptake system (Kdp) (Bakker, 1992). The Kdp-ATPase is activated by osmolarity. In addition, expression of the genes encoding the Kdp ATPase is induced by an osmotic upshock. Signal transduction is mediated via a two component system. The membrane bound sensor KdpD senses changes in osmolarity as well as changes in intracellular potassium (Sugiura et al., 1994). This signal is then transmitted to the response regulator KdpE that undergoes phosphorylation. When KdpE is phosphorylated, it takes on an active form that exhibits high affinity for the *kdpABC* promoter, which in turn results in activation of the *kdpABC* operon. It was also suggested that the central hydrophobic domain of KdpD, which is conceivably responsible for membrane anchoring of this protein, plays a role in the signalling mechanism underlying KdpE phosphorylation in response to hyperosmotic stress. So far, Kdp-like transport activities have not been detected in methanogens and, in addition, the genomes of *Methanosarcina mazei* Gö1, *Methanosarcina barkeri* or *Methanosarcina acetivorans* do not encode Kdp-like ATPases. Therefore, the question is how methanogens accumulate potassium. The genome of *M. mazei* Gö1 (Deppenmeier et al., 2002) encodes two homologues of the *trkA* and *trkG* genes of *E. coli*. The gene products could form the key complex of the Trk transport system that is catalyzing the low affinity, high rate potassium accumulation in *E. coli*. Furthermore there are four genes in the *Methanosarcina mazei* genome that code for putative K^+ channels. Mutational inactivation is required to identify the role of these proteins in potassium uptake and osmoadaptation. The nature of the anion accumulated along with potassium is unknown, but glutamate or phosphate might be involved.

3.2. EXPORT OF SODIUM

Methanogenic Archaea are Na^+-dependent (Perski et al., 1982), and non-halophiles such as *Methanosarcina mazei* or *Methanosarcina barkeri* require Na^+ for growth and methane formation in concentrations of about 0.1 mM. At low salt, Na^+ is expelled from the cells by action of the primary Na^+ pump methyltetrahydromethanopterin: coenzyme M methyltransferase (Becher et al., 1992). This enzyme creates a sodium ion gradient across the membrane ($Na^+_i < Na^+_o$) that is essential for methanogenesis. At elevated Na^+ concentrations additional mechanisms have to apply. Inspection of the genome sequence of *Methanosarcina mazei* revealed three genes coding for potential Na^+/H^+ antiporters and a primary, ATP-driven Na^+ pump (MM1056). The expression of only one of the Na^+/H^+ antiporter genes and MM0294 was induced at high salt which suggests their involvement in Na^+ homeostasis (unpublished).

3.3. EXPORT OF POTASSIUM

If there is a first phase of osmoadaptation by a fast accumulation of potassium in *Methanosarcina*, potassium would be exported in the second phase and compatible solutes accumulated instead. The genome of *Methanosarcina mazei* encodes a couple of different potential K^+ export systems. There are three glutathione-regulated

potassium-efflux system proteins (MM0933, MM1909, MM0929) that have similarities to KefC and are located upstream of the *abl* operon (Fig. 1). The KefC protein is a member of the CPA2 family of monovalent cation/proton antiporters and plays a role in responding to changes in osmotic pressure and in protecting the cell from electrophile toxicity in *E. coli*. It is interesting to note that the lysine-2,3-aminomutase and the β-acetyltransferase genes (*abl* operon, see below) that are involved in the biosynthesis of the compatible solute N^{ε}-acetyl-β-lysine are preceded by genes encoding potential potassium efflux systems along with regulators. The potential potassium transporters are divergently transcribed from the *abl* operon which would be consistent with a co-regulation of both clusters.

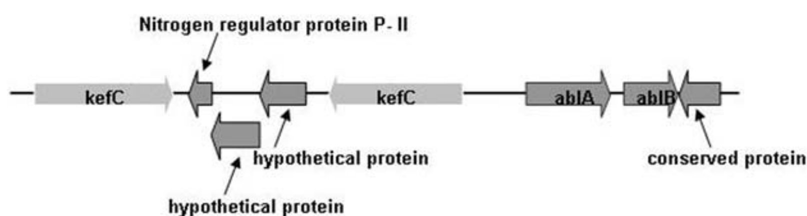

Figure 1. Genetic organization of the *kefC* and *abl* operon in *Methanosarcina. mazei* Gö1. *ablA* is coding for the lysine-2,3-aminomutase and *ablB* for the acetyltransferase. *kefC* is coding for the glutathione-regulated potassium-efflux system protein.

4. Accumulation of Compatible Solutes in Methanogens

As a long time adaptation to hypersalinity, methanogenic Archaea accumulate compatible solutes to maintain turgor. This can either be achieved by uptake or by biosynthesis, but uptake is preferred over biosynthesis due to energetic reasons (Oren, 1999).

4.1. TRANSPORT OF COMPATIBLE SOLUTES IN METHANOGENS

Methanosarcina thermophila TM-1 was the first methanogenic archaeon in which transport of glycine betaine in response to an osmotic upshock was shown (Proctor et al., 1997). This methanogen can adapt and grow at salinities ranging from 0.05 up to 1.2 M NaCl. When glycine betaine is provided externally it can be taken up into the cell and, at the same time, the synthesis of compatible solutes like N^{ε}-acetyl-β-lysine or α-glutamate is downregulated. Furthermore, the presence of glycine betaine in the medium led to increased growth rates at high salinities, proving its role as compatible solute. Kinetic analyses suggest a single, high affinity transporter, which seems to be highly specific for glycine betaine, as several compounds like choline, dimethyl glycine, sarcosine, or proline did not affect transport of glycine betaine (Proctor et al., 1997). It seems to be a secondary transporter linked to a proton and/or sodium ion

gradient. Unfortunately, up to date the genes encoding this transporter have not been identified.

Glycine betaine transport was also found in *Methanohalophilus portucalensis* strain FDF1, an obligate halophilic methanogen, which grows optimally within the salt range of 1.2 to 2.9 M NaCl (Boone et al., 1993). *Methanohalophilus portucalensis* was the first organism of the class of obligate halophilic methanogenic Archaea in which solute uptake was examined. This organism is able to synthesize glycine betaine *de novo*, but if this solute is present in the culture medium uptake is preferred over *de novo* synthesis (Lai et al., 2000). As with cultures of *Methanosarcina thermophila* TM-1 the addition of glycine betaine to the growth medium of *Methanohalophilus portucalensis* led to increased growth rates at elevated salinities, confirming its role as a compatible solute. The uptake of glycine betaine followed the kinetics characteristic for the presence of a single transporter (Lai et al., 2000). However, in this case molecular data are also missing up to now.

The first transporter for compatible solutes, which was identified on a molecular level, is the glycine betaine transporter Ota of *Methanosarcina mazei* Gö1 (Roeßler et al., 2002) (Fig. 2). This methanogenic archaeon is able to adapt and to grow up to 1 M NaCl, and the addition of glycine betaine to the growth medium facilitated adaptation to higher salt concentrations, showing that it has a function as compatible solute. Glycine betaine uptake increased with increasing salt concentrations, indicating a salt-dependent regulation of the transporter and/or its genes (Pflüger and Müller, 2004; Roeßler and Müller, 2001). Transport studies argued for an ATP dependent primary transport system. This transporter, Ota, is encoded by an operon consisting of three genes arranged in the order 5'-*otaA*-*otaB*-*otaC*-3' (Fig. 2). A promoter structure consisting of a TATA box and the factor B recognition element (BRE) was identified *in silico* upstream of the start codon of the first gene, *otaA*. *otaA* is coding for the ATP hydrolyzing subunit of this ABC transporter. The second gene of this operon, *otaB*, encodes the membrane spanning domain, and overlaps 1 bp with *otaA*. A short intergenic region of 228 bp separates *otaB* from the gene encoding the substrate binding protein, *otaC*. Immediately downstream of *otaC* is a potential rho-independent transcriptional terminator. *otaA*, *otaB*, and *otaC* are 1353, 834, and 921 bp, respectively. Transcriptional analyses revealed a salt dependent transcription of *otaC*. Expression of *otaC* at the standard NaCl concentration of 38.5 mM was very low, but it drastically increased with increasing NaCl concentrations, clearly demonstrating a salt dependent regulation of the glycine betaine transporter Ota on a transcriptional level (Roeßler et al., 2002).

Regulation was also examined on protein level (Hoffmann et al., submitted). In the absence of salt, cellular Ota concentrations were low, but increased with increasing salt concentrations. A maximum was reached at 300-500 mM NaCl (3-3.5-fold increase compared to 38.5 mM). Thereafter, it declined somewhat but still remained 2 to 2.5-fold higher at 700 and 800 mM NaCl. These data clearly demonstrate a salt-dependent increase in the cellular level of OtaC. After a hyperosmotic shock the first significant increase in OtaC levels were detected 3 h after the shock and a maximum was obtained 4 h after the shock. This level remained unchanged up to stationary growth phase. It should be noted that in non-shocked cells OtaC levels began to increase during late exponential growth phase until a maximum was reached in early stationary phase, indicating a second, growth phase-dependent regulation of the cellular OtaC content.

The induction of OtaC is triggered by an increase in osmolarity and independent of Na^+ and/or Cl^-, as other osmolytes than NaCl also led to increased OtaC levels. Production of the transporter is not regulated by the solute transported, as externally supplied solutes such as glycine betaine or glutamate had no effect on cellular Ota levels. However, at present time it is too early to speculate about salt-dependent signal transduction chains in methanogenic Archaea. Experiments aimed to identify salt (osmo) sensors and signal transduction chains in methanogens have just begun.

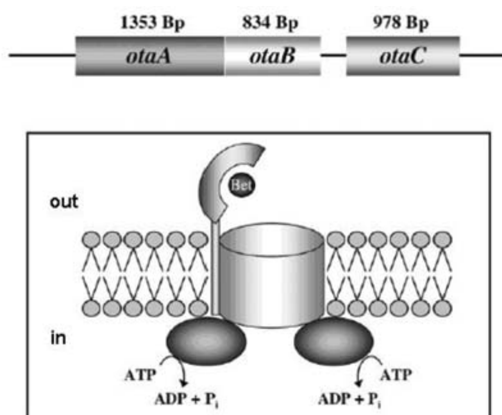

Figure 2. Genetic organization of the *ota* operon in *M. mazei* Gö1 and hypothetical structure of Ota. *otaA* is coding for the ATP binding protein, *otaB* for the membrane spanning protein, and *otaC* for the extracellular glycine betaine binding protein.

4.2. BIOSYNTHESIS OF COMPATIBLE SOLUTES IN METHANOGENS

By means of NMR analyses hypothetical pathways for the biosynthesis of N^{ε}-acetyl-β-lysine, β-glutamine, and glycine betaine have been proposed in *Methanohalophilus portucalensis* (Roberts et al., 1992). According to this, N^{ε}-acetyl-β-lysine is synthesized from α-lysine, which is converted to β-lysine and subsequently acetylated. This requires the activity of two enzymes, a lysine-2,3-aminomutase and a β-acetyltransferase. The formation of glycine betaine in *Methanohalophilus portucalensis* is postulated to happen via the methylation of glycine. *Methanothermobacter marburgensis* also has the ability of glycine betaine synthesis (Ciulla et al., 1994).

4.2.1. Biosynthesis of N^{ε}-Acetyl-β-Lysine

The compatible solute N^{ε}-acetyl-β-lysine is wide spread among methanogenic Archaea. Amongst others it was found in *Methanogenium cariaci*, *Methanosarcina* spp., *Methanohalophilus* ssp., and *Methanococcus* spp. (Lai et al., 1991; Roberts et al., 1992;

Robertson et al., 1992; Sowers et al., 1990; Sowers and Gunsalus, 1995). The biosynthetic pathway of N^{ε}-acetyl-β-lysine has recently been studied in *Methanosarcina mazei* Gö1 (Pflüger et al., 2003). In this methanogen, as in other methanogenic Archaea, α-lysine seems to be built via the diaminopimelate pathway, as four genes that are similar to α-lysine biosynthesis genes from the diaminopimelate pathway were found in the genome of *Methanosarcina mazei* Gö1. α-Lysine is converted to β-lysine by the activity of a lysine-2,3-aminomutase, which is then acetylated to N^{ε}-acetyl-β-lysine by a β-acetyltransferase (Fig. 3). These enzymes are encoded by the *abl* operon. Two genes, *ablA* and *ablB,* are organized in this operon in the order 5'-*ablA*-*ablB*-3'. They are separated by a short intergenic sequence of 204 bp. A promoter structure consisting of a TATA box and the factor B recognition element (BRE) was identified *in silico* upstream of the start codon of the first gene, *ablA*. Downstream of *ablB* is a potential rho-independent transcriptional terminator. This *abl* operon is also found in other methanogens, like *Methanosarcina acetivorans*, *Methanococcus maripaludis*, and *Methanosarcina barkeri*. This leads to the assumption that all these organisms are capable of N^{ε}-acetyl-β-lysine synthesis. The function of the *abl* operon was studied with deletion mutants in *Methanococcus maripaludis*. A deletion of the *abl* operon resulted in mutants that were no longer able to adapt to elevated osmolarites. Furthermore, they no longer accumulated N^{ε}-acetyl-β-lysine, showing that this operon is essential for the production of N^{ε}-acetyl-β-lysine and that N^{ε}-acetyl-β-lysine is essential for the adaptation to high osmolarity (Pflüger et al., 2003).

Transcriptional studies of the *abl* operon in *Methanosarcina mazei* Gö1 showed high expression of the *abl* operon at 400 and 800 mM NaCl, while expression was apparently impaired at 38.5 mM NaCl (Pflüger et al., 2003). These data clearly show that the regulation of N^{ε}-acetyl-β-lysine is via the expression of the *abl* operon. In addition, it was found that the formation of N^{ε}-acetyl-β-lysine is also regulated via the activity of the aminomutase and/or the acetyltransferase (Martin et al., 2001; Roberts et al., 1992), the lysine-2,3-aminomutase activity in cells of *Methanococcus thermolithotrophicus* shifted from 0.68 to 1.4 M NaCl was induced about 8-fold (Martin et al., 2001).

4.2.2. Biosynthesis of Glutamate and Glutamine

The amino acids α-glutamate and β-glutamine are accumulated by *Methanosarcina thermophila* (Sowers and Gunsalus 1995). When *M. mazei* Gö1 is grown at 400 mM NaCl, the intracellular glutamate concentration is 0.41 μmol mg protein^{-1}. However, an increase in the external salt concentration up to the maximum tolerated (about 1 M NaCl) led to an increase in N^{ε}-acetyl-β-lysine, but the internal glutamate/glutamine pool stayed constant. The genome of *M. mazei* Gö1 revealed two genes coding for potential glutamine synthetases, one gene coding for glutamate synthase and three genes encoding the large subunit of the glutamate synthase. Therefore, it is well possible that the two isogenes are regulated differently by osmolarity/nitrogen and one isoenzyme is involved in the synthesis of the compatible solutes glutamate/glutamine and regulated by osmolarity, whereas the other is regulated by the nitrogen regulon. In addition, the genome encodes three genes coding for glutamate dehydrogenases. Therefore, accumulation of glutamate/glutamine might be *via* glutamate dehydrogenase

or the glutamine synthetase/GOGAT pathways. Conversion of α-glutamine to β-glutamine might be via the lysine-2,3-aminomutase or a yet to be identified glutamine aminomutase. The elucidation of the pathways involved and their regulation is under way.

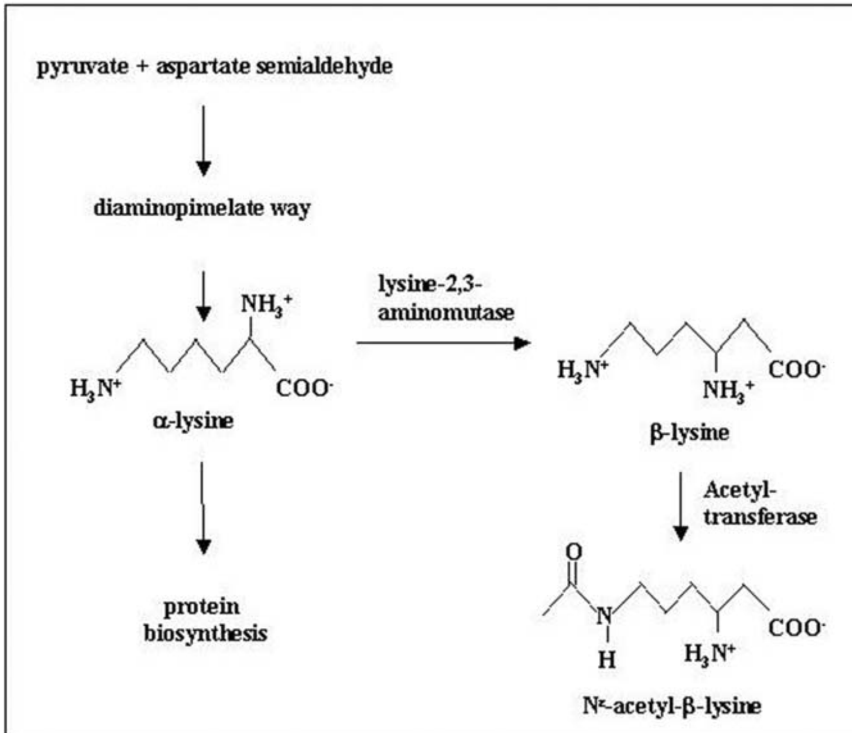

Figure 3. Hypothetical pathway of N^ε-acetyl-β-lysine synthesis in methanogenic Archaea. α-Lysine is converted by the activity of a lysine-2,3-aminomutase to β-lysine, which is then acetylated to N^ε-acetyl-β-lysine.

5. How Methanogens Cope with Hyposalinity

When bacteria are shifted to a medium of lower osmolarity, they release ions (mainly potassium) and small molecules (e.g. osmoprotectants) to reduce the membrane turgor. Channels, activated by an increase in membrane turgor, may provide the pathway for these effluxes; so called mechanosensitive (MS) channels appear in all three domains of life. MscS (small mechanosensitive channel) and MscL (large mechanosensitive channel) are found in bacterial cytoplasmic membranes. These channels function to alleviate excess cell turgor invoked by rapid osmotic downshock (Levina et al., 1999). The two channels give a specific response after a hypoosmotic shock. MscS selectively

opens small pores to release small osmolytes, whereas MscL opens great pores when the membrane tension increases and releases osmolytes and small proteins non-selectively (Berrier et al., 1992).

Until recently nothing was known about MS channels in Archaea. Changes in environmental osmotic pressures can also be expected to occur in the deep sea near hydrothermal chimneys, the natural habiat of *Methanocaldococcus jannaschii*. Two sequenced MscS homologues from *Methanocaldococcus jannaschii* (MscMJ) have recently been functionally characterized (Kloda and Martinac 2001), and they exhibit properties expected for MscS channels. The partial rescue of bacterial cells in media of higher osmolarity suggests that the level of cellular turgor needed to activate MscMJ, relative to the extracellular environment, may indeed be higher in *E. coli* than in the marine *Methanocaldococcus jannaschii*. MscMJ is activated by mechanical force transmitted via the lipid bilayer. Altough it is functionally behaving similar to MscS rather than MscL, this channel contains stretches of amino acids with a high proportion of identical residues not only to MscS but also to the TM1 transmembrane domain – the most highly conserved region among MscL proteins – of MscL.

In the genome of *Methanosarcina mazei* there are two genes coding for putative mechanosensitive ion channels (MM0236, MM3139).

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Section III. Bacteria

Salinibacter ruber: genomics and biogeography
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What we can deduce about metabolism in the moderate halophile *Chromohalobacter salexigens* from its genomic sequence
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The chloride regulon of *Halobacillus halophilus*: a novel regulatory network for salt perception and signal transduction in bacteria
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Jorge Quillaguamán, Bo Mattiasson and Rajni Hatti-Kaul

Biodata of **Josefa Antón**, author of **"*Salinibacter ruber: Genomics and Biogeography*"**

Josefa Antón is associate professor of microbiology at the University of Alicante, Spain. In the summer of 1998 she joined for three months the group of Prof. Rudolf Amann at the Max-Planck Institute for Marine Microbiology in Bremen, to try to develop a FISH protocol to study microorganisms in hypersaline environments, in collaboration with Dr. Ramon Rosselló-Mora (at that time, postodoctoral researcher at MPI and currently at IMEDEA in Mallorca, Spain). The development of these techniques allowed the unexpected discovery of *Salinibacter* in the salterns. Lately, this bacterium was isolated and described in collaboration with Profs. Oren and Rodríguez-Valera. Since the discovery of *Salinibacter*, Josefa Antón's team works together with Drs. Amann and Rosselló-Mora on the ecology, biogeography and genomics of *Salinibacter ruber*.

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SALINIBACTER RUBER: GENOMICS AND BIOGEOGRAPHY

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1. Introduction

Salinibacter ruber is an extremely halophilic bacterium that was first isolated from crystallizer ponds of solar salterns located in Alicante and Mallorca, Spain. These environments have a very high salt concentration (from 25% to saturation) and are populated by two types of microorganisms: the numerically dominant square Archaea and different phylotypes of *Salinibacter* (Antón et al., 2000; Øvreås et al., 2003). Until recently, no representatives of these two majority populations had been brought into pure culture, while a wide collection of haloarchaea had been isolated from the very same environments, showing that even in a very low diversity environment, culture techniques can be biased towards minority microorganisms. *S. ruber* was first described as "*Candidatus* Salinibacter" based on molecular ecology techniques and was finally isolated in 2002, enabling validation of the species name (Antón et al., 2002). More extensive documentation has recently become possible by the study of a wider collection of strains isolated around the world (Peña et al., in press). On the other hand, Walsby's square archaeon has been reluctant to cultivation, although many researchers had attempted different isolation approaches for years. However, many data about the squares are available from structural and molecular techniques (Antón et al., 1999; Kessel and Cohen, 1982; Oren et al., 1996), that identify them as Archaea belonging to the family *Halobacteriaceae*. More specifically they are related to SPhT (or Susana's phylotype, named after its discoverer, Susana Benlloch), a 16S rDNA sequence that had been recovered from different salterns around the world (Rodríguez-Valera et al., 1999). Very recently, Mike Dyal Smith and coworkers have finally isolated the square archaeon (Burns et al., 2004). Now, the two main players in crystallizer ponds are available for complete characterization.

S. ruber was thus the first aerobic extremely halophilic member of the domain Bacteria whith ecological relevance in crystallizer ponds. This was unexpected, since hypersaline environments had been described as almost monospecific cultures of

halophilic Archaea (Guixa-Boixareu et al., 1996). Surprisingly, *S. ruber* was very easy to isolate: only a lower nutrient concentration had to be used in the very same medium used for isolating haloarchaea and, after screening of the red colonies (very much haloarchaeal like), a high percent of them turned out to be members of this new genus. Once isolated in pure culture, it was evident that the similarity of *S. ruber* and haloarchaea could have hampered the isolation of the bacterium since both types of prokaryotes are extremely similar at the phenotypic level: both are extreme halophiles, aerobes, heterotrophs and pigmented by carotenoids (Lutnaes et al., 2002; Oren and Rodríguez-Valera, 2001). More detailed studies showed that *S. ruber*, like haloarchaea, accumulates high concentrations of K^+ to counterbalance the osmotic pressure of the medium (Antón et al., 2002; Oren et al., 2002), has a high proportion of acidic amino acids in its proteins, as well as enzymes functional at high salt concentrations (Oren and Mana, 2002), and even has a high proportion of G+C in its genome (65-70% for *S. ruber*, in the range of 59-70 for haloarchaea; Oren, 2002). All these similarities, together with the fact that *S. ruber* and haloarchaea share the same habitat in which prokaryotic density is very high, poses interesting questions about the evolutionary processes leading to a high degree of similarity between these two groups of organisms. As pointed out by Oren and Mana (2002), a comparative analysis of *Salinibacter* and haloarchaea would be of great interest to understand the mechanisms of this apparent convergent evolution and to ascertain the extent of lateral gene transfer between these members of the two prokaryotic domains.

2. Genomic and Proteomic Analysis of *Salinibacter ruber*

A few years ago we undertook a low coverage shotgun sequencing of *S. ruber* M31^T genome for data mining purposes. Besides, we have been isolating *S. ruber* strains form salterns around the world (from the Peruvian Andes to Eilat in Israel) to ascertain the degree of intraspecific diversity using different genetic and phenotypic tools, including the distribution of "interesting" genes newly found when sequencing the M31 genome.

S. ruber M31 has a genome of 3.4 ± 0.2 Mbp of which we have sequenced approximately 60% and assembled into contigs ranging from 100 to 16,000 bp. *S. ruber* has a typically prokaryotic ribosomal operon 16S-tRNA(Ile-GAU)-tRNA(Ala-UGC)-23S-5S that, according to our hybridization experiments, is present in a single copy. Among the genes identified in the sequence we have found many *che* and *fla* genes (EM studies had previously revealed the presence of flagella in *S. ruber*) as well as a high quantity of membrane transporters, including haloarchaeal-like K^+ transporters and, most interestingly, a homologue to the precusor of light-driven chloride pump halorhodopsin (Antón et al., in preparation). Besides, as found in the genome of the haloarchaeon *Halobacterium* NRC-1 (Ng et al., 2000), the genome of *S. ruber* shared a large number of homologues with the radiation-resistant bacterium *Deinococcus radiodurans*.

Comparing the complete proteomes available at the time of analysis (16 from Archaea and 43 from Bacteria), we found that 6% of the proteins coded in the genes in our library had a best match with archaeal proteins (considering only BLAST hits with E-values lower than e^{-20}). However, when the more restrictive conditions of Gophna et al. (2004) were used, only 1% of the proteins we had annotated could be of archaeal origin. Obviously, since we only have partial data, these results are not conclusive.

One very interesting question was whether *Salinibacter* proteins shared indeed the characteristics of haloarchaeal proteins, something that had already been studied for some *Salinibacter* enzymes and for bulk proteins (Oren and Mana, 2002). In order to address this point we have randomly chosen several proteins (more precisely, predicted gene products) from *Salinibacter* and compared their isoelectric points and their contents of acidic and basic amino acids and serine with their homologues of haloarchaeal origin, as well as from some non extremely halophilic Bacteria and Archaea. Results are shown in Fig. 1 and Table 1. All the proteins were of "bacterial origin", except the Na^+/Pro symporter and PyrE, that were of "archaeal origin" according to Gophna et al. (2004). Except for RecA, in every case analyzed, *S. ruber* proteins had isoelectric points closest to their haloarchaeal homologues and always lower than those for the rest of archaeal and bacterial proteins analyzed. *Halobacterium* NRC-1 proteome analysis had shown an average pI of approximately 5. Acidic proteomes were also predicted from partial genome sequences of *Haloarcula marismortui* and *Haloferax volcanii* (DasSarma, 2004). Most of the selected *Salinibacter* proteins resemble proteins from haloarchaea since they contain more acidic amino acids (and less basic amino acids) than their homologues from non halophilic Archaea and Bacteria. The evolutionary modifications required to re-engineer a protein so that it becomes halophilic appear to involve the introduction of additional acidic residues (Dennis and Shimmin, 1997). This seems also to be the case in the analyzed *S. ruber* proteins, although the genealogies of these proteins do not relate them with halophilic Archaea. However, we could not see a clear trend regarding the serine content. The amount of serine in bulk proteins from *Salinibacter* has been shown to be higher than that of proteins from the non-halophilic *Escherichia coli* and the moderately halophilic *Halomonas elongata* (Oren and Mana, 2002). However, the amount of serine in bulk protein is obviously influenced by the relative amounts of the individual cellular proteins, that are not considered in our analysis.

3. Halorhodopsin of *Salinibacter ruber*

As mentioned above, one of the more interesting findings in the *S. ruber* genome was the presence of a homologue to the haloarchaeal halo-opsin (called SalHO, for *Salinibacter* halo-opsin), the precusor of the retinal derivative light-driven chloride pump halorhodopsin. Together with the light-driven proton pump bacteriorhodopsin and two sensory rhodopsins involved in light sensing for phototaxis (Oren, 2002), halorhodopsin is one of the four type-1 retinal pigments that until recently were believed to be unique for the halophilic Archaea. However, homologues for bacteriorhodopsin and sensory rhodopsin have been found among members of the Bacteria, and even in the eukaryotic domain (Bieszke et al., 1999; Gärtner and Losi, 2003). In fact, the presence of a wide variety of proteorhodopsins, the bacterial homologue of bacteriorhodopsin, as a way of using solar energy seems to be a very common feature in prevalent components of marine microbiota (Béjà et al., 2001; De la Torre et al., 2003; Venter et al., 2004). So far, no halorhodopsin homologues had been found in bacterial genomes.

The halo-opsin homologue was found in a contig containing other genes of putative archaeal origin, as shown in Fig. 2A. Most likely, these different archaeal-like genes had a different evolutive history, since they are not simultaneously present in all the *S. ruber* strains from our collection (Peña et al., in preparation). A phylogenetic tree reconstructed

by comparing the amino acid sequence of the translated SalHO gene with microbial rhodopsins (Fig. 2B) shows that SalHO is clearly affiliated with the archaeal halorhodopsins. As previously described (Ihara et al., 1999), four clusters were observed in the tree corresponding to four different functional groups (i.e. H^+ pumps, Cl^- pumps, sensory rhodopsin I and sensory rhodopsin II).

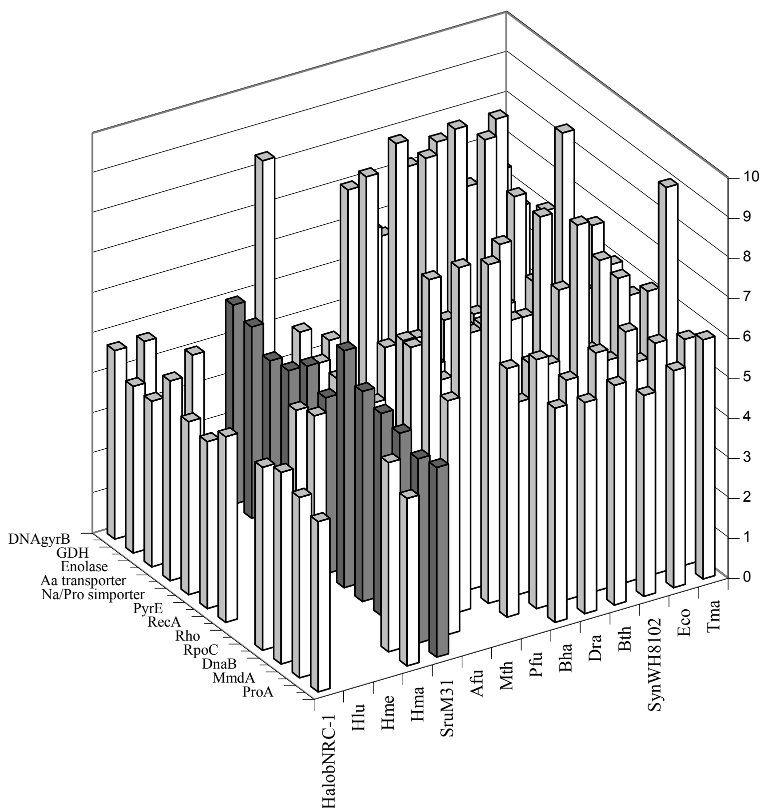

Figure 1. Calculated pI of proteins predicted from gene sequences: *Halobacterium* sp. NRC-1 ATCC 700922 (HalobNRC-1), *Haloferax lucentense* (Hlu), *Haloferax mediterranei* (Hme), *Haloarcula marismortui* ATCC 43049 (Hma), *Haloferax volcanii* (Hvo), *Salinibacter ruber* DSM 13855 (SruM31), *Archaeoglobus fulgidus* DSM 4304 (Afu), *Methanothermobacter thermautotrophicus* ΔH (Mth), *Pyrococcus furiosus* DSM 3638 (Pfu), *Bacillus halodurans* C-125 (Bha), *Deinococcus radiodurans* R1 (Dra), *Bacteroides thetaiotaomicron* VPI-5482 (Bth), *Synechococcus* sp. WH8102 (SynWH8102), *Escherichia coli* K12-MG1655 (Eco), *Thermotoga maritima* MSB8 (Tma). The proteins investigated were DNA gyrase B (DNAgyrB), glutamate dehydrogenase (GDH), enolase, the Na^+/proline symporter (Na^+/Pro symporter), orotate phosphoribosyltransferase (PyrE), recombination protein A (RecA), transcription termination factor (Rho), DNA-directed RNA polymerase beta' subunit (RpoC), replicative DNA helicase (DnaB), methylmalonyl-CoA decarboxylase alfa subunit (MmdA), and gamma-glutamyl phosphate reductase (ProA).

TABLE 1. Calculated percent of acidic amino acids (%ac), basic amino acids (%ba) and serine (%Ser) of predicted proteins shown in Fig. 1.

	DNAgyrB	GDH	Enolase	Aa transporter	Na^+/Pro symporter	PyrE
	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser
SruM31	16.41 / 10.65 / 6.8	14.28 / 9.44 / 3.2	17.21 / 6.55 / 5.2	4.38 / 9.92 / 4.55	7.01 / 5.17 / 6.6	19.05/8.57/5.7
HalobNRC1	17.83 / 10.47 / 4.7	17.78 / 7.45 / 4.8	17.58 / 8.29 / 3.0	4.99 / 10.30 / 7.42	10.45 / 4.51 / 5.2	19.34/7.55/6.1
Hlu	18.46 / 10.64 / 4.7					
Hme		17.00 / 8.61 / 5.4				
Hma						19.52/8.57/6.7
Afu	18.14 / 12.82 / 4.9		16.29 / 10.09 / 5.0	4.35 / 12.26 / 6.13	6.38 / 3.19 / 6.4	
Mth			16.82 / 7.93 / 5.5	8.46 / 8.83 / 9.24	4.71 / 6.22 / 4.1	16.41/14.36/5.1
Pfu			16.12 / 11.33 / 4.0		3.99 / 6.84 / 5.7	16.11/13.89/6.1
Bha	15.07 / 12.40 / 5.0	10.91 / 10.04 / 6.3		8.61 / 5.85 / 6.40	7.40 / 9.13 / 5.7	18.13/15.39/3.3
Dra	14.03 / 12.67 / 3.9	12.26 / 10.14 / 5.4	15.16 / 10.18 / 6.4	9.01 / 5.00 / 6.09	4.73 / 7.10 / 7.1	13.33/10.00/8.1
Bth	15.00 / 11.48 / 5.5	12.61 / 11.94 / 4.7	15.15 / 12.12 / 6.3	7.68 / 5.91 / 6.14	5.59 / 6.88 / 4.5	11.34/9.28/5.7
SynWH8102	15.04 / 12.64 / 5.3		13.48 / 7.90 / 7.0	9.15 / 4.98 / 6.71		12.50/11.50/4.0
Eco	15.04 / 12.81 / 5.2	11.18 / 9.84 / 4.9	13.65 / 11.11 / 5.1		5.96 / 5.96 / 5.5	10.03/12.60/6.9
Tma	16.67 / 15.88 / 5.0	13.46 / 12.26 / 3.6				13.90/13.90/5.9

	Rec A	Rho	RpoC	DnaB	MmdA	ProA
	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser	%ac / %ba / %Ser
SruM31	14.81 / 14 / 7.1	16.5 / 13.25 / 6.5	15.61 / 11.74 / 5.8	17.82 / 12.52 / 6.4	17.42 / 10.41 / 4.2	16.43 / 9.8 / 4.9
HalobNRC1	17.84 / 9.63 / 4.8		18.85 / 10.09 / 3.8	17.42 / 11.69 / 5.6	18.44 / 10.15 / 5.1	17.18 / 5.93 / 5.5
Hlu						
Hme						
Hma	18.36 / 9.32 / 6.4				16.70 / 10.82 / 5.3	18.09 / 6.86 / 6.0
Afu	18.5 / 7.6 / 6.6		14.99 / 14.42 / 4.4	16.82 / 17.44 / 6.6	13.73 / 12.02 / 3.1	
Mth	13.76 / 11.96 / 5.4		16.20 / 13.10 / 4.9	14.49 / 15.11 / 7.0		
Pfu	16 / 11 / 6.7		15.22 / 14.55 / 3.2	15.42 / 16.34 / 6.0	12.64 / 11.88 / 3.3	
Bha	14.34 / 13.12 / 6.4	16.35 / 14.74 / 6.4	13.93 / 14.43 / 4.5	16.52 / 11.89 / 5.3	12.60 / 11.63 / 4.8	13.94 / 10.82 / 6.2
Dra		14.3 / 13.14 / 4.2	15.72 / 12.48 / 5.4	13.84 / 10.94 / 6.2	13.27 / 11.73 / 5.4	12.27 / 10.19 / 4.4
Bth	12.94 / 11.84 / 5.5	15 / 15.4 / 4.6	13.81 / 13.6 / 5.0	12.58 / 13.65 / 6.1	12.26 / 11.28 / 5.1	12.95 / 10.79 / 6.2
SynWH8102	14.24 / 12.79 / 5.8		15.03 / 11.44 / 6.4	13.59 / 11.04 / 7.4	12.63 / 11.60 / 5.8	11.83 / 8.12 / 4.4
Eco	12.23 / 11.34 / 4.8	13.76 / 13.54 / 5.4	13.51 / 13.22 / 5.0	15.71 / 11.68 / 7.2	14.42 / 13.17 / 5	12.71 / 10.55 / 5.3
Tma	13.76 / 13.2 / 6.2	14.75 / 13.81 / 5.4	15.09 / 14.32 / 4.7	15.39 / 16.77 / 6.0	13.59 / 11.65 / 4.1	16.39 / 15.18 / 4.8

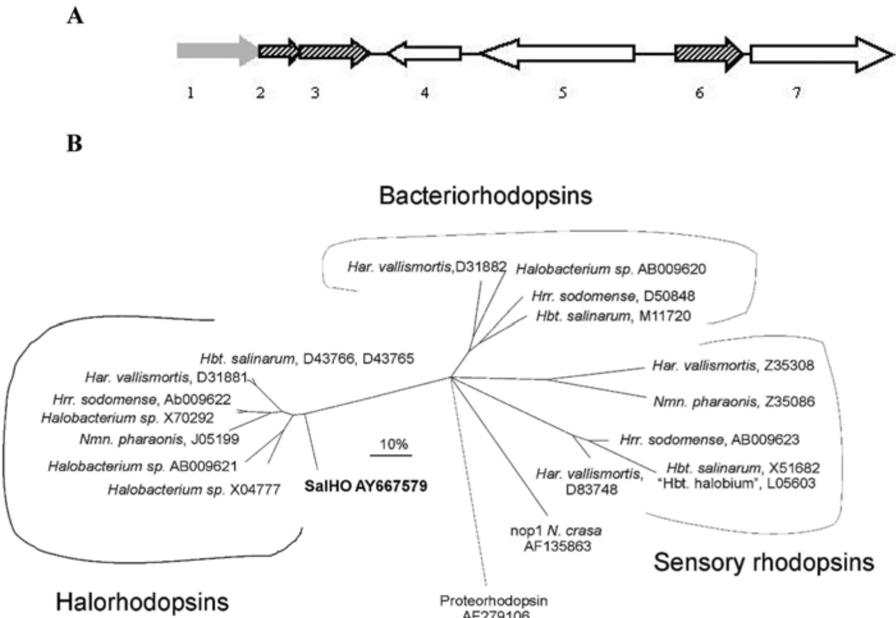

Figure 2. A. Description of the contig where the halo-opsin homologue was found: hypothetical (1), hypothetical (2), short chain alcohol dehydrogenase of unknown specificity (3), halo-opsin (4), sodium:proline symporter (proline permease) (5) oxidorreductase, short chain dehydrogenase/family (6), hypothetical (7). Putative origin of the ORFs: unknown (grey arows), archaeal (white), bacterial (black bars). B. Phylogenetic tree based on amino acid sequences of all homologous archaeal rhodopsins, *Neurospora crassa nop*-1 gene product, and bacterial proteorhodopsins available in public databases. The tree shown is based on neighbour joining approach by using the Kimura algorithm. Results were simultaneously evaluated by the maximum parsimony and maximum likelihood treeing approaches using the same data and subsets thereof. Multifurcations show such positions where the branching order could not be resolved. The SalHO sequence appeared consistently in the position shown in the tree independently of the algorithm or the dataset used. However, the position of the proteorhodopsin branch remained unstable and its position was dependent on the approach used. Fig. 2B was derived from Antón et al. (in preparation). *Har.* = *Haloarcula*; *Hbt.* = *Halobacterium*; *Hrr.* = *Halorubrum*; *Nmn.* = *Natronomonas*.

However, there must be another system for chloride transport since, as explained below, not all the *Salinibacter* strains harbour SalHO genes in their genomes. In fact, the extremely halophilic archaeon *Halobacterium salinarum* possesses both halorhodopsin and a light-independent chloride transport system which probably acts by coupling the inward transport of chloride with the influx of Na^+ (Duschl and Wagner, 1986).

4. Biogeography of *Salinibacter*

Since the description of *S. ruber* species, we have been isolating different strains (now, up to 50) from crystallizer ponds around the world. A collection of 17 strains (including the five used for species description: M1, M8 and M31 from Mallorca, Pola13/P13 and Pola 18/P18 from Alicante) was used to take a look at the intraspecific diversity (Peña et al., in press). Several techniques were used for this study, including sequencing of 16S rDNA and the 16S-23S rRNA gene spacer regions, as well as genomic fingerprinting with randomly amplified polymorphic DNA (RAPD; Sikorski et al., 1999) and pulsed field gel electrophoresis analysis (PFGE; Grothues and Tummler, 1991).

The 16S rRNA gene sequences of all new isolates were found to be identical to those of the already sequenced *S. ruber* strains. No representative of the second *Salinibacter* spp. phylotype EHB-2 (Antón et al., 2000) was obtained. The 16S-23S rRNA gene spacer regions were amplified for all strains and enzymatically digested with *Taq*I. In all cases, the digestion patterns were identical for all tested strains. Accordingly, their sequences were also very similar with values always higher than 97% sequence identity. With these similarities, no phylogenetic reconstruction could be performed to infer reliable genealogies within *S. ruber*. When the phylogeny of the gene spacer region of M31 was reconstructed with respect to a selection of the available homologous regions, we found that the closest relative sequence was that of *Rhodothermus marinus* in accordance with the 16S rRNA gene sequence reconstructions (Peña et al., in press).

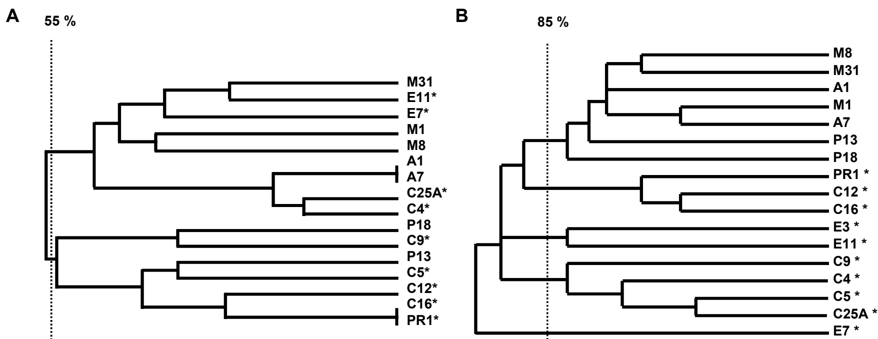

Figure 3. Dendrograms generated after UPGMA analyses of PFGE patterns (A) and RAPD profiles (B) from 17 *S. ruber* isolates. Source of isolation: S'Avall salterns, Mallorca, Spain (A1 and A7); Salinas de Levante salterns, Mallorca, Spain (M1, M8 and M31); Bras del Port salterns, Alicante, Spain (P13 and P18); Canary Islands salterns, Spain (C4, C5, C9, C12, C16, and C25); San Carles de la Rápita salterns, Ebro Delta, Spain (E7 and E11); Maras salterns, Peruvian Andes (Pr1). Black boxes indicate strains harbouring SalHO genes. From Peña et al., in press.

Genomic fingerprinting could however give a more detailed picture of intraspecific diversity. According to PFGE separation of genomic macrorestriction products, all the strains grouped in two clusters with more than 55% banding pattern similarity (Fig. 3).

Cluster 1 harboured all the isolates from the Mallorca and Ebro Delta salterns, whereas cluster 2 harboured all strains isolated from Alicante and the Peruvian Andes. Strains isolated from the Canary Islands appeared to be spread across both clusters. On the other hand, a dendrogram was constructed based on the analysis of 260 independent positions resulting from 8 different RADP analyses. One single independent cluster of 7 strains sharing at least 85% pattern similarity was found. All strains isolated from the Alicante and Mallorca salterns were grouped within the same cluster, whereas the rest of the strains were spread in 4 additional clusters. Interestingly, this unique cluster harboured all strains for which a copy of a SalHO gene was found. This result could indicate that the presence of a SalHO is related to the origin of isolation, although we have recently isolated some *S. ruber* strains from Canary Island that harbor this gene in their genomes.

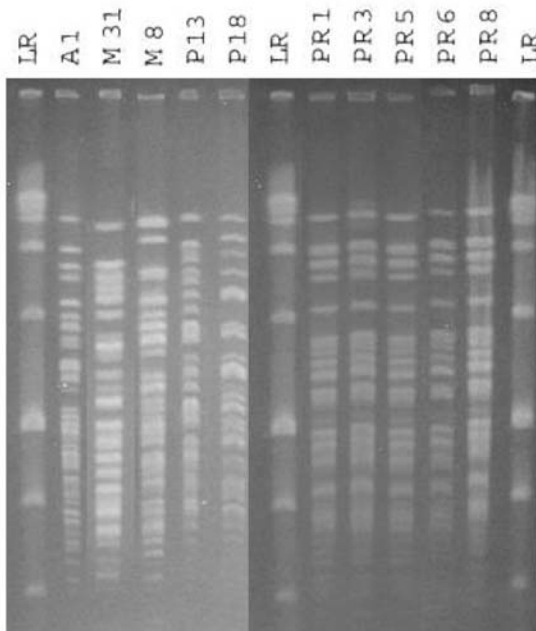

Figure 4. *Xba*I digestion of agarose embeded genomic DNA from different *S. ruber* strains. Source of isolation: S'Avall salterns, Mallorca, Spain (A1); "Salinas de Levante" salterns, Mallorca, Spain (M31 and M8); Bras del Port salterns, Alicante, Spain (P13 and P18); Maras salterns, Peruvian Andes (Pr1, Pr3, Pr4, Pr6, Pr8). LR: Biorad low-range marker for PFGE.

From the PFGE analysis of the different strains, it seems that *S. ruber* populations present different degrees of homogeneity depending on the location of isolation. This is particularly noticeable for strains isolated from a Peruvian saltern located at 3000 m of altitude in the Andes. In this environment, *S. ruber* can not be detected by FISH and

therefore must be present in very low numbers, although it can be readily isolated (Maturrano et al., in preparation). When genomic macrorestriction products of five Peruvian strains (isolated by different members of our group in different years) were compared by PFGE (Fig. 4), their patterns were found to be identical. This finding was in contrast with the diversity of PFGE patterns displayed by the otherwise very similar strains isolated from other salterns. There are thus many unanswered questions about how this species diversify in the environment and why very closely related, but not identical, strains thrive sharing the same habitat. Again, comparative genomics could help to understand how and why intraspecific diversity arises. For this purpose, we continue now to explore genomic differences of representatives of *S. ruber*, both uncultured and cultured. Hopefully, these studies will tell us more about the meaning of microdiversity in hypersaline environments.

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WHAT WE CAN DEDUCE ABOUT METABOLISM IN THE MODERATE HALOPHILE *CHROMOHALOBACTER SALEXIGENS* FROM ITS GENOMIC SEQUENCE

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1. Introduction

A draft sequence of the genome of the moderate halophile *Chromohalobacter salexigens* (formerly *Halomonas elongata*) DSM3043 (Fig. 1) has been determined to an 8X coverage by the Joint Genome Institute (JGI) of the US Department of Energy. From this sequence, 3370 predicted protein-coding genes were identified and provisionally annotated by computer analysis at the JGI. The draft sequence and annotation are available at http://genome.jgi-psf.org/microbial/index.html. In this chapter, we present a brief overview of insights we gained into metabolism in *C. salexigens* from the sequence. In view of the close phylogenetic relationship of *C. salexigens* to the Pseudomonaceae and other γ-Proteobacteria (see below), we used known enzyme sequences from Pseudomonaceae, *Acinetobacter*, *Escherichia coli*, other Enterobacteriaceae, and other bacteria (in this order of priority) as query sequences for orthologs in *C. salexigens*. Any match with a probability ("expect value" in tblastn; http://www.ncbi.nlm.nih.gov/BLAST) ≤ e-20 was considered to be highly significant, values between e-5 and e-20 to be marginal, and ≥ e-5 to be not significant. The usual disclaimers about inferences from genomic sequence are in order: that all of the annotations that we are assigning are provisional and await confirmation by biochemical or functional genetic analyses, and that the predictions of the functions of the *C. salexigens* gene products are only as valid as the accuracy of the assignment of the function of matching proteins in other organisms.

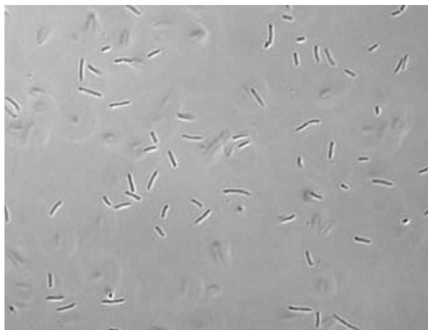

Figure 1. *Chromohalobacter salexigens* DSM3043, grown in LB medium supplemented with 1 M NaCl. Photograph: Kathleen O'Connor and Christopher Staiger.

2. Overview of the Sequence of the *C. salexigens* Genome

Pulsed-field gel electrophoretic analysis of total DNA cut with the rare-cutting enzymes *Pac*I and *Pme*I suggested that *C. salexigens* possesses a main chromosome of ~3.9 Mbp and a low copy plasmid of < 100 kbp (data not shown). The JGI generated a total of 3.7 Mbp unique sequences, and therefore, depending on the accuracy of our estimate of the genome size, we may be very near the complete coverage of the genome. The *C. salexigens* genome is 63.9% G + C. This value is close to the 66.6% G + C content found in *Pseudomonas aeruginosa* (Stover et al., 2000), a related non-halophilic γ-proteobacterium, but differs substantially from the 40.3% G + C composition in a second closely related γ-proteobacterium, *Acinetobacter* ADP1 (Barbe et al., 2004). However, despite the difference in nucleotide composition, the genome of *C. salexigens* is more similar in its size to the 3.6 Mbp (3325 ORF) genome of *Acinetobacter* than to the 6.3 Mbp genome of *P. aeruginosa*. The classification of *C. salexigens* among the γ-Proteobacteria is confirmed by the high similarity that the ORFs of this organism show to orthologs from other organisms in this subdivision (Table 1).

As can be seen in this Table, > 60% of the ORFS of *C. salexigens* show the highest similarity to orthologs from γ-Proteobacteria. In spite of the halophilic characteristics of *C. salexigens*, 37% of its ORFs are most similar to orthologs from Pseudomonaceae (which include *Acinetobacter*), in accord with the close phylogenic relationship between these organisms.

3. Carbon Source Metabolic Enzymes

We used the sequence data to make predictions about the carbon source metabolic repertoire of *C. salexigens*. The constituent enzymes of various pathways that we searched in *C. salexigens* were derived from the following references: central metabolism

was based on the chapter by Fraenkel (1996), the tricarboxylic acid (TCA) cycle by Cronan and LaPorte (1996), peripheral carbon source metabolism by Lin (1996) and Gottschalk (1985), and aromatic compound metabolism by Horn et al. (1991) and Nakazawa and Yokota (1973). For the tracing of the aromatic degradative pathways we also made extensive use of the University of Minnesota Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu). Most of the amino acid sequences of enzymes that were used as to search the *C. salexigens* genome were obtained from the Expert Protein Analysis System (ExPASy; http://www.expasy.org).

TABLE 1. The similarity of the predicted ORFs of *C. salexigens* to ORFs from other organisms.

Organism	% of ORFs of *C. salexigens* that show highest tblastn similarity to an ORF in the indicated organism
γ-Proteobacteria	61
Pseudomonaceae	37
Enterobacteriaceae	9
Vibrionaceae	6
Alteromonaceae	4
Xanthomonaceae	2
Other γ-Proteobacteria	3
α-Proteobacteria	13
β-Proteobacteria	11
Bacilli	3
Eukaryotes	3
δ-Proteobacteria	2
Actinobacteria	2
Cyanobacteria	1
Other Eubacteria	4
Archaea	0.5

3.1. ENZYMES OF CENTRAL METABOLISM

We found good matches in *C. salexigens* for all of the enzymes of the Embden-Meyerhof-Parnas pathway from phosphoglucose isomerase to pyruvate kinase. *C. salexigens* has the "standard" suite of glycolytic enzymes as found in *E. coli*, and it differs from *P. aeruginosa*, which lacks phosphofructokinase, and from *Acinetobacter*, which is devoid of both phosphofructokinase and pyruvate kinase (Barbe et al., 2004). We detected all of the enzymes of the oxidative and non-oxidative branches of the hexose monophosphate shunt as well as the Entner-Doudoroff pathways.

C. salexigens has good matches to the three subunits (E1, E2, and Lpd) of the NAD-dependent pyruvate dehydrogenase. There is no indication that it has an ortholog for pyruvate formate lyase, which is used in other organisms under anaerobic conditions. We found good matches to all of the usual TCA cycle enzymes. The one curiosity in this regard is the presence of both an NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) and an NAD-linked isoenzyme (EC 1.1.141). The latter enzyme is not present in *E. coli*

and *Salmonella typhimurium*, and the difference in the function of the two is not known. *C. salexigens* has the capacity to form oxalacetate to fixing CO_2 via phosphoenolpyruvate (PEP) carboxylase.

3.2. GLUCONEOGENESIS

Based on our understanding of metabolism in other organisms, growth on gluconeogenic carbon sources requires enzymes that enable the cell to circumvent "irreversible" reactions to generate pyruvate and PEP from TCA cycle intermediates and other 3-carbon compounds and to form fructose-6-P from fructose-1,6-P_2.

3.2.1. TCA Cycle to PEP

We found good matches for phosphoenolpyruvate synthase, which gives PEP from pyruvate, and PEP caboxykinase, which decarboxylates oxalacetate to yield PEP. The organism has three potential malic enzymes, which carry out the oxidative decarboxylation of malate to pyruvate, two of which appear to NADP-linked and one NAD-linked. Finally there is a membrane-bound oxalacetate decarboxylase, which produces pyruvate from oxalacetate. The latter reaction is catalyzed by a membrane bound-enzyme whose activity is linked to Na^+ extrusion, and therefore it may play a role in the halophilism or halotolerance of the organism.

3.2.2. Fructose-1,6-bisphosphatase

In contrast to the pyruvate or PEP-forming enzymes, we have not been able to find a good match to fructose-1,6-bisphosphatase (Fbpase) that can generate fructose-6-phosphate during gluconeogenic growth. There are at least six classes of proteins which have been recognized as having Fbpase activity. Class I Fbpase is the standard AMP-sensitive enzyme (Fraenkel, 1996) that is present in plants, animals, a variety of Eubacteria, and the extremely halophilic Archaea *Halobacterium salinarum* NRC-1 and *Haloarcula marismortui* ATCC 43049. This protein has been demonstrated by extensive genetic and biochemical analysis to be the physiologically important activity in *E. coli* used during gluconeogenic growth. Class II is represented by the GlpX protein, which was discovered as part of the *glp* (glycerol-3-phosphate) regulon in *E. coli* (Donahue et al., 2000) and shown to be the important Fbpase in *Mycobacterium tuberculosis* and a number of other Actinomycetes (Movahedzadeh et al., 2004). Although the GlpX protein of *E. coli* has Fbpase activity, its function is a mystery, because it is not adequate to support growth on gluconeogenic carbon sources in *fbp* mutants (which lack the Class I Fbpase), even when the *glp* regulon is induced by glycerol. Class III Fbpase contains the *yydE* gene product of *Bacillus subtilis* (Fujita et al., 1998). This enzyme has been shown to have Fbpase activity by in vitro assays and can complement a *Δfbp* mutation in *E. coli*, but its function is also unclear, because *B. subtilis* strains carrying a deletion of the *yydE* gene are not defective in growth on gluconeogenic carbon sources. Class IV of Fbpase has been identified in Archaea as a protein that has both Fbpase and inositol 1-phosphatase (I-1-Pase) activities (Stec et al., 2000). In addition to this enzyme, there is a Class V Fbpase in some but not all Archaea, which has nearly exclusive affinity for fructose-1,6-bisphosphate (Sato et al., 2004). *Thermococcus kodakaraensis* is one of the

Archaea that has orthologs of both the Class IV and Class V enzymes. Deletions were targeted to the genes encoding these proteins, with the result that the loss of the Class V enzyme abolished gluconeogenic growth, whereas removal of the Class IV enzyme did not result in an obvious phenotype (Sato et al., 2004). The implication is that the latter is the important Fbpase, at least in the Archaea that have both the Class IV and Class V enzymes. Finally, Class VI comprises sedoheptulose-1,7-bisphosphatase, a member of the Calvin-Benson-Bassham cycle, which has been found to be able hydrolyze fructose-1,6-bisphosphate also (http://us.expasy.org).

To make a long story short, we did not find significant matches (Expect values < e-5) in the *C. salexigens* genome to representatives of any of the classes of Fbpases, except for Class IV, the archaeal type Fbpase/I-1-Pase. However, even here, the matches were mostly borderline: when used as query in a tblastn search against the 23 sequenced Archaea in the data base, the *C. salexigens* ortholog of the Fbpase/I-1-Pase gave a match with an expect value of e-29 against a gene in the extreme halophile *Har. marismortui* ATCC 43049, but the other 22 had matches with expect values in the range of e-19 to e-0.8. The *C. salexigens* Fbpase/I-1-Pase gene has good matches to a gene found in most of not all Bacteria (e-20 to e-40). It was demonstrated in vitro that the product of the *E. coli* orthologous gene, called *suhB* (expect value of match to *C. salexigens* orthologs = 6e-34), has I-1-Pase activity, but the function of this enzyme in *E. coli* is yet to be discovered (Chen and Roberts, 2000).

In view of the facts that this putative *C. salexigens* Fbpase/I-1-Pase gene shows only low similarity to orthologs in Archaea and that the counterpart gene in *T. kodakaraensis* has been shown to be unnecessary for gluconeogenesis, we feel that that probably this gene product is not the authentic Fbpase in *C. salexigens*, and that the real Fbpase has not been revealed by the genomic analysis. In this regard, *C. salexigens* is similar to *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) in which it has not been possible to infer an Fbpase gene from genomic analysis (B. Goodner, unpublished). Thus, there may be yet another form of Fbpase (Class VII?) in *C. salexigens* (and *A. tumefaciens*) that needs to be identified. However, another more interesting, albeit speculative possibility could be that there might be a novel pathway from 3- or 2-carbon compounds to fructose-6-phosphate or a pentose phosphate that bypasses fructose-1,6-bisphosphate.

3.3. SPECIFIC CARBON SOURCES

The enzymes in the likely metabolic pathways of various carbon and energy sources that we found in the *C. salexigens* are summarized in Table 2. This analysis is mainly concentrated on the catabolic pathways of simple sugars and other common carbon sources, and is not intended to be comprehensive. *C salexigens* can degrade a number of amino acids and nucleotides (Arahal et al., 2001), which are not considered. Because of difficulties in assigning the specificities of transport systems, we were not able to identify the transport genes for most carbon sources with certainty. In the subsequent analysis, we made the assumption that if all of the components of a pathway subsequent to transport can be identified, the organism would be able to metabolize the respective carbon sources. NAD-linked dehydrogenases were also problematic, because the queries for these enzymes often showed significant high similarity to more than one ORF in *C.*

salexigens, complicating the unambiguous assignment of enzymes catalyzing dehydrogenase reactions.

TABLE 2. Our predictions about metabolic pathways in *C. salexigens* based on the genomic sequence.

Compound	Enzymes in that could be predicted from the sequence	Predicted enzymes in catabolic pathway that could not be found in the sequence	Can we account for all the necessary metabolic enzymes?
1. Compounds observed to support growth			
D-Glucose[1]	several potential ABC and Δψ-coupled permeases; two glucokinases; PQQ-dependent glucose oxidase; gluconolactonase		Yes
D-Gluconate[2]	H^+-coupled transporter; gluconate kinase		Yes
D-Fructose[1,2]	PTS enzyme I; PTS Hpr; PTS Enzyme $IIBC^{Fru}$; fructose-1-phosphate kinase; fructose-6-phosphate kinase		Yes
D-Mannose[1]	hexokinase?; phosphomannose isomerase		Yes
D-Galactose[1,2]	hexokinase?; UDP-glucose pyrophosphorylase; UDP-glucose 4-epimerase	galactose-1-P uridyl transferase	No
myo- (*meso*)-Inositol[1]	inositol dehydrogenase, poor match; 2-keto-*myo*-inositol dehydrase, poor match; possible 2,3-diketo-4-d-inositol hydratase; possible 2-deoxy-5-ketogluconate-P aldolase; malonyl (methylmalonyl?) semialdehyde dehyrogenase; *myo*-inositol 1-P phosphatase (I-1-Pase), function unknown	2-d-5-ketogluconate kinase	No
Lactose[3]		β-galactosidase 6-P-β-galactosidase	No
Maltose[1,2]	α-glucosidase (maltase)		Yes
Trehalose[1]	α,α-phosphotrehalase (α-glucosidase?)	periplasmic trehalase	Maybe
Mannitol[1]	mannitol → fructose dehydrogenase		Yes
Sorbitol[1,2]	sorbitol → fructose dehydrogenase		Yes
Galactitol (dulcitol)[1]	tagatose-6-P kinase; possible tagatose (fructose?)-1,6-P_2 aldolase	transport or phosphorylation system; galactitol-P dehydrogenase	No
D-Glucarate (saccharate)[1]	glucarate dehydratase; 5-keto-4-d-D-glucarate aldolase		Yes
Sucrose[1,2]	α-glucosidase (maltase)		Yes

L-Arabinose[1,2]	ribulokinase?	arabinose ↔ ribulose isomerase; ribulose-5-P epimerase	No
D-Ribose[1,2]	ribokinase		Yes
D-Xylose[1]	xylulose kinase	xylose ↔ xylulose isomerase	No
D-Erythritol[1]	kinase?	erythritol-1-P dehydrogenase; erythrulose-1-P dehydrogenase	No
TCA cycle intermediates: citrate[1,2], α-ketoglutarate[1], succinate[1,2], fumarate[1], malate[1]	several potential Na^+ and H^+ linked tri- and dicarboxylic acid transporters, specificity can't be inferred; subsequent metabolism via TCA cycle		Yes
Acetate[1,2]	acetyl CoA synthetase; isocitrate lyase; malate synthase		Yes
Ethanol[1,2]	alcohol dehydrogenases (uncertain specificity); aldehyde dehydrogenases (uncertain specificity)		Maybe
Glycerol[1,2]	glycerol kinase; glycerol-3-phosphate dehydrogenase		Yes
D-Tartrate[1]	tartrate dehydratase α, β subunits		Yes
D,L-Glycerate[1]	D-glycerate kinase		Yes on D-glycerate
Propionate[1]	propionyl CoA synthetase; 2-methylcitrate synthase; 2-methylcitrate dehydratase; 2-methylisocitrate lyase		Yes
Malonate[1]	malonyl-CoA: ACP-SH transferase malonate decarboxylase β, γ subunits, poor match	malonate decarboxylase α, γ subunits 2-(5''-triphosphoribosyl)-3'-dephospho-CoA synthase; phosphoribosyl-dephospho-CoA transferase	No
Benzoate[2]	See Figure 1		Maybe
Protocatechuate (3,4-dihydroxy-benzoate)[2]	protocatechuate 3,4-dioxygenase α, β subunits; 3-carboxy-*cis*,*cis*-muconate cycloisomerase; 4-carboxy- muconolactone decarboxylase		Yes, if 3-oxoadipate enol-lactone can be metabolized; See Fig. 2
4-Hydroxy-benzoate[2]	4-hydroxybenzoate 3-monooxygenase		Yes; see protocatechuate
Toluene[2]	toluene 2,3-dioxygenase α subunit toluene *cis*-dihydrodiol dehydrogenase catechol 2,3-dioxygenase I, II 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; poor match to 4-hydroxy-2-oxovalerate aldolase	toluene 2,3-dioxygenase β subunit; 2-oxopent-4-enoate hydratase; toluene 2-monooxygenase; toluene 3-monooxygenase; toluene 4-monooxygenase; toluene / o-xylene monooxygenase; 4-cresol dehydrogenase; p-hydroxybenzaldehyde dehydrogenase	No

2. Compounds observed as not being able to support growth

D-Glucono-lactone[1]	periplasmic gluconolactonase		Yes
Cellobiose[1]		cellobiose phosphorylase; cellobiose-6-P hydrolase (6-β-glucosidase)	No
Melibiose[1]		α-galactosidase	No
L-Fucose[1]	fuculose kinase; fuculose-1-P aldolase	fucose ↔ fuculose isomerase	No
L-Rhamnose[1]		rhamnulokinase; rhamnulose-1-P aldolase	No
Raffinose[1,2]		raffinose invertase (β-fructosidase); α-galactosidase	No
Inulin[1]		endo- or exoinulinase; inulin fructotransferase	No
Ribitol (adonitol)[1]	ribitol kinase ribitol dehydrogenase (specificity questionable)	ribitol-5-P dehydrogenase	Yes
Lactate (D-, L-form not specified)[1]	NAD-linked D-lactate dehydrogenase, probably fermentative; cytochrome-linked L-lactate dehydrogenase	FAD-dependent D-lactate dehydrogenase	No growth on D-lactate; possibly growth on L-lactate
Oxalate[1]	possible formyl-CoA, oxalate CoA-transferase; possible oxalyl-CoA decarboxylase	oxalyl CoA reductase	No

3. Not tested

N-Acetyl-D-glucosamine		N-acetylglucosamine-6-P deacetylase; glucosamine-6-P deaminase	No
D-Glucosamine	glucosamine-fructose-6-P aminotransferase, but this is probably biosynthetic	glucosamine-6-P deaminase	No
N-Acetyl-D-galactosamine		N-acetylgalactosamine-6-P deacetylase	No
D-Galactosamine		galactosamine-6-P isomerase / deaminase	No
Sorbose	possible sorbitol-6-P dehydrogenase	sorbose-1-reductase	No
Tagatose	possible tagatose-6-P kinase; possible tagatose (fructose?)-1,6-P_2 aldolase		Maybe
D-Altronate	altronate (mannonate?) dehydrates; 2-keto-3-d-gluconokinase		Yes
D-Tagaturonate	tagaturonate ↔ altronate oxidoreductase		Yes
D-Glucuronate		glucuronate ↔ fructuronate isomerase	No
D-Galacturonate		galacturonate ↔ tagaturonate isomerase	No
D-Mannonate	mannonate (altronate?) dehydrates; 2-keto-3-d-gluconokinase		Yes

D-Fructuronate	fructuronate → mannuronate reductase		Yes
D-Arabinose	D-arabinose-P-isomerase	specific kinase	Maybe
D-Xylulose	xylulose kinase		Maybe
Arabitol	arabitol dehydrogenase		Maybe
Hydroxypyruvate	hydroxypyruvate isomerase; tartronate semiladehyde reductase		Yes
Methanol	alcohol dehydrogenases (uncertain specificity)	PQQ-dependent methanol dehydrogenase	Maybe
Xylene		xylene monooxygenase, hydroxylase and electron transfer components; aryl alcohol dehydrogenase; aryl aldehyde dehydrogenase	No
Salicylate	salicylate 1-mono-oxygenase; poor match		Maybe
Benzene		benzene 1,2-dioxygenase α, β subunits; *cis*-1,2-dihydrobenzene-1,2-diol dehydrogenase	No
Vanillin	vanillin dehydrogenase; vanillate O-demethylase oxidoreductase		Yes, if we can explain the protocatechuate pathway
Naphthalene		1,2-dihydronaphthalene demethylase / oxidoreductase	No
Phenol	possible phenol 2-monooxygenase subunit P5	phenol 2-monooxygenase subunits P0 to P4	No

[1]Reported by Arahal et al. (2001).
[2]K. O'Connor and L.N. Csonka, unpublished observations.
[3]We found that lactose supported growth (K. O'Connor and L.N. Csonka, unpublished observation), whereas Arahal et al. (2001) reported that lactose supported acid production but not growth.

3.3.1. Glucose

Like pseudomonads and *Acinetobacter*, *C. salexigens* does not appear to have a PEP-dependent phosphotransferase system (PTS) for the uptake and phosphorylation of any sugars, except fructose (see below). It has several genes specifying ABC-type and ion co-transport driven transport systems for sugars, but the substrate specificities of these gene products are not evident from their sequences. There are two predicted genes for glucokinase that could generate glucose-6-phosphate, but these may have broader specificities for other sugars. As discussed above, the internal glucose-6-phosphate could be catabolized via glycolysis, the hexose monophosphate shunt, or the Entner-Doudoroff pathway.

C. salexigens has predicted open reading frames for the pyrroquinoline quinone (PQQ)-dependent glucose oxidase, gluconolactonase, and glucokinase, as well as the entire pathway for the synthesis of PQQ. Thus, like its pseudomonad and *Klebsiella* relatives, *C. salexigens* might be able to catabolize glucose by the non-phosphorylated route to gluconate and then by the Entner-Doudoroff pathway.

3.3.2. Fructose

We found genes for only one PTS system, which in *C. salexigens* appears to be specified by two genes. The first of these genes, which we designate as *fruA*, specifies a multi-functional protein that contains an N-terminal domain that is orthologous to the Enzyme IIA^{Fru} of *E. coli*, a central domain that is similar to the Hpr and Fpr components of PTS systems in other organisms, followed by a domain that is orthologous to Enzyme I. The second gene, *fruB*, specifies a protein that is similar to Enzyme $IIBC^{Fru}$ of *E. coli* and *P. aeruginosa*. In between *fruA* and *fruB*, there is another gene, *fruK*, which encodes a fructose-1-phosphate kinase. The similarity of these genes to loci in other organisms that have known function in fructose metabolism suggests that the PTS system specified by these genes recognizes fructose as its substrate, but in view of the generous substrate specificities of PTS systems in other organisms, this system might accept other sugars. On the basis of the sequence similarities to *P. aeruginosa* and *E. coli*, we predict that the fructose metabolic pathway in *C. salexigens* consists of uptake and phosphorylation to fructose-1-phosphate by the PTS and a second phosphorylation to fructose-1,6-bisphophosphate by the *fruK* gene product.

3.3.3. Problem Areas

Table 2 was based on the list of compounds that were tested by Arahal et al. (2001) and our laboratory (K. O'Connor and L.N. Csonka, unpublished data) for their ability to serve as carbon/energy sources for *C. salexigens*. From the sequence information, we were able to confirm the observed phenotypes of growth, or lack thereof, on various compounds, and in addition, we were able to make predictions on the ability of the organisms to metabolize several compounds that have not yet been tested. However, in a number of cases, we cannot account satisfactorily for the observed growth characteristics of the organism. In these problematic cases, *C. salexigens* could have the enzymes with the expected biochemical function, but these may not have sufficient similarity to the counterparts in organisms that were used as queries in the searches, or *C. salexigens* may use novel biochemical reactions for some of the missing steps that have not been recognized in other organisms. In the section below, we discuss the compounds for which there is a discrepancy between the observed growth phenotype and our inferences about the metabolic pathways from the genome sequence.

D-Gluconolactone. It is not obvious why *C. salexigens* cannot grow on gluconolactone: there is a predicted periplasmic gluconolactonase, and the organism is able to metabolize gluconate, the expected product of this enzyme.

D-Galactose. This compound can clearly support growth, and we were able to find three of the enzymes of the Leloir pathway of galactose metabolism (two of which also have anabolic functions in the synthesis of galactose residues in the lipopolysaccharide). However, we were not able to see a good match for galactose-1-P uridyl transferase (*galT* gene product in *E. coli*), which converts galactose-1-P + UDP-glucose to glucose-1-P + UDP-galactose.

myo-Inositol. The *myo*-inositol catabolism has been best characterized in *B. subtilis*, and we looked for orthologs of the enzymes from that organism (Yoshida et al., 2004). We found only marginal matches for five of the enzymes of the *B. subtilis* pathway and no ortholog for a sixth enzyme (2-deoxy-5-ketogluconate kinase). It is not clear whether the poor match between to the *B. subtilis* enzymes is due to sequence divergence between the Gram-negative and Gram-positive organisms, or whether *C. salexigens* uses a different pathway. As discussed in connection with Fbpase, there is a good match to I-1-Pase, and it is not clear that this enzyme is required for the metabolism of inositol.

Lactose. There is a discrepancy in the data on the metabolism of lactose. Arahal et al. (2001) reported that *C. salexigens* cannot use lactose as sole carbon and energy source but can produce acid from it, and we found that it can grow on it as sole carbon source (K. O'Connor and L.N. Csonka, unpublished data). However, we could not detect a candidate gene for either β-galactosidase or 6-P-β-galactosidase and cannot account for the metabolism of this disaccharide.

Trehalose. There is no predicted periplasmic or cytoplasmic trehalase, but we found an α,α-phosphotrehalase, which could split trehalose-6-P to glucose and glucose-6-P. However, there is no obvious trehalose kinase, and so our ability to rationalize a trehalose metabolic pathway depends on identifying the transport and phosphorylation enzymes.

Galactitol. We did not observe a good match to a galactitol kinase or PTS, but it is possible that these reactions could be handled by enzymes that have a broader substrate specificity (e.g. the fructose PTS). In *E. coli*, galactitol-1-P is oxidized to tagatose-6-P, phosphorylated to tagatose-1,6-P_2, and cleaved to dihydroxyacetone-P + glyceraldehyde-3-P. There is no obvious galactitol-1-P dehydrogenase in *C. salexigens*, so we cannot rationalize how the organism metabolizes this hexitol.

L-Arabinose. *C. salexigens* is able to grow on L-arabinose, but we were not able to confirm the presence of arabinose isomerase and ribulose-5-P epimerase, which are part of the degradative pathway in *E. coli*. Two different pathways of L-arabinose catabolism that proceed through the non-phosphorylated intermediates L-arabinolactone, L-arabonate, and 2-keto-3-deoxy-L-arabonate, have been described in *Sinorhizobium meliloti* (Duncan and Fraenkel, 1979) and *Bradyrhizobium japonicum* (Pedrosa and Zancan, 1974). However, sequences of the enzymes of these pathways are not available in the ExPASy database, and we cannot tell whether L-arabinose is metabolized via the pathways found in the latter organisms.

D-xylose. In *E. coli*, D-xylose is metabolized via xylulose. There does not appear to be an appropriate isomerase in *C. salexigens*, so we cannot account for the metabolic pathway of this pentose.

D-Erythritol. A pathway for D-erythritol metabolism, consisting of a kinase, erythrose-1-P dehydrogenase, and erythrulose-1-P dehydrogenase, has been worked out in *Brucella*

(Sangari et al., 2000). Genes for the latter two enzymes were not detected in *C. salexigens*, and therefore the erythritol metabolic pathway needs to be established.

Malonate. An aerobic pathway of malonate degradation has been determined in *Klebsiella pneumoniae*, which entails a complicated set of reactions for the decarboxylation of malonate to acetate (Dimroth and Hilbi, 1997). Of the seven enzymes of this pathway, four could not be identified in *C. salexigens* with query proteins from *K. pneumoniae* or *P. putida*. *Malonomonas rubra*, which can grow on malonate anaerobically, has a Na^+ pumping membrane-bound malonate decarboxylase that is needed to generate a membrane potential (Dimroth and Hilbi, 1997). The malonate metabolic enzymes of *M. rubra*, including the Na^+ pumping decarboxylase, also did not show adequate similarities to predicted *C. salexigens* proteins.

Benzoate. This compound is oxidized to catechol (1,2-dihydroxybenzene), which can be metabolized by the *meta*- or *ortho*-cleavage pathways (Fig. 2; Nakazawa and Yokota, 1973). For the *meta*-pathway, the genome was queried with ORFs present on the *Pseudomonas putida* TOL plasmid pWWO (Horn et al., 1991), and for the *ortho*-pathway, the genome was searched against the enzymes from *Acinetobacter* sp. (Barbe et al., 2004).

Although *C. salexigens* can grow on benzoate as a single carbon source (K. O'Connor and L.N. Csonka, unpublished data), we cannot account satisfactorily for the metabolism of this important compound. In common pathway to from benzoate to catechol, we could not find a good ortholog for the β-subunit of benzoate 1,2-dioxygenase. Furthermore, 2-oxopent-4-enoate hydratase seems to be missing from the catechol *meta*-cleavage pathway, and muconolactone isomerase and the A subunit of 3-oxodaipate CoA transferase are missing from the *ortho*-pathway. In addition, some of the matches are very borderline, at least to the *meta* pathway enzymes specified by the *P. putida* TOL plasmid and the *ortho* pathway enzymes found in *Acinetobacter*. However, overall the matches are better for the *ortho*-pathway enzymes from *Acinetobacter* than for the *meta*-pathway enzymes from the TOL plasmid. Therefore, we feel that it is more likely that *C. salexigens* uses the former route than the latter one. However, it is possible that we could find better quality matches if we searched the *C. salexigens* genome with benzoate degradative enzymes from a broader spectrum of organisms. In *Acinetobacter*, the benzoate degradative enzymes are arranged as a tight cluster (Barbe et al., 2004), but this does not seem to be the case in *C. salexigens*.

Protocathecuate (3,4-dihydroxybenzoic acid). This compound is metabolized to 3-oxoadipate enol-lactone, an intermediate in the catechol *ortho*-cleavage pathway (Fig. 2). We can find excellent orthologs for the three enzymes that bring protocatechuate into the *ortho*-pathway. So, provided that the latter pathway exists in *C. salexigens*, we could account for the necessary enzymes of protocatechuate metabolism. Again, unlike in *Acinetobacter* (Barbe et al., 2004), the three unique enzymes of protocatechuate metabolism are not adjacent to each other in our organism.

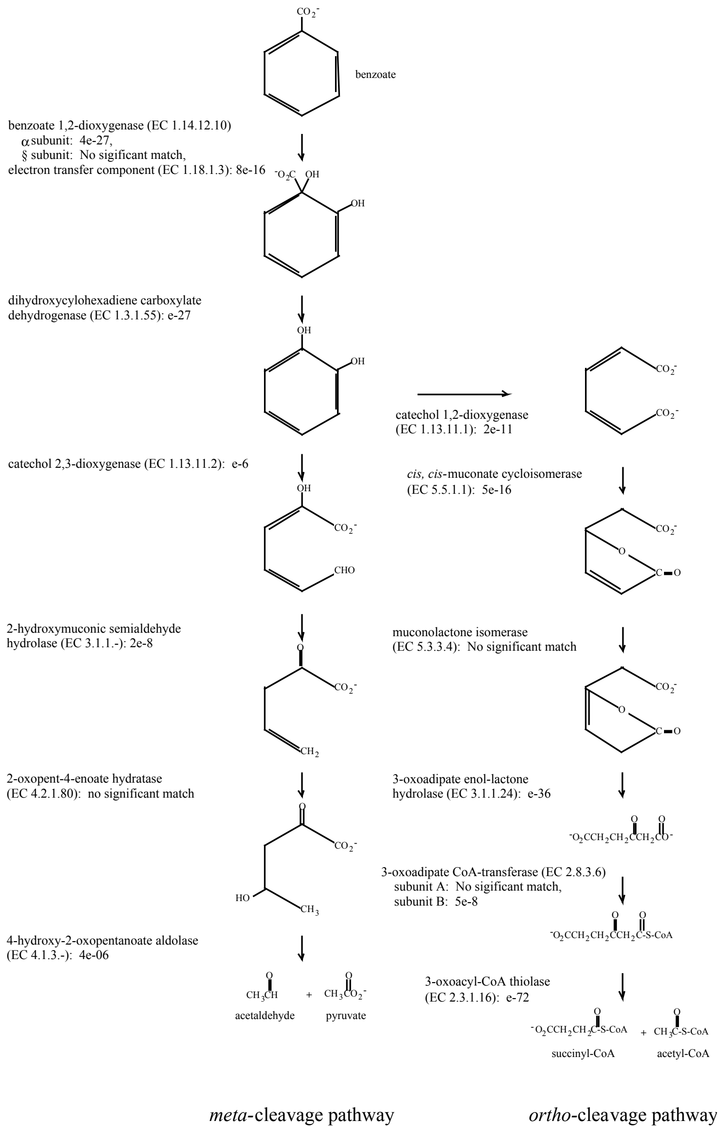

Figure 2. Benzoate metabolic pathways. The expect values indicate the tblastn similarity scores of *C. salexigens* ORFS against queries from genes from the *P. putida* TOL plasmid (for the reactions from benzoate to catechol and for the *meta* pathway) and from *Acenitobacter* (for the *ortho* pathway).

Toluene. There are five pathways of toluene degradation, beginning with the five enzymes xylene monooxygenase, toluene 2-monooxygenase, toluene 3-monooxygenase, toluene 4-monooxygenase, and toluene 1,2-dioxygenase (the University of Minnesota Biocatalysis / Biodegradation Database; http://umbbd.ahc.umn.edu). We did not detect acceptable similarities to enzymes of the first four pathways, but were able to recognize some of the enzymes of the fifth pathway, which goes through toluene-*cis*-1,2-dihydrodiol, 3-methylcatechol, *cis,cis*-2-hydroxy-6-oxohept-2,4-dienoate, *cis*-2-hydroxypenta-2,4-dienoate, and 4-hydroxy-2-oxovalerate to acetate, acetaldehyde plus pyruvate. We found good matches to all of the enzymes of this pathway, except for the β subunit of the first enzyme (toluene 2,3-dioxygenase) and the last enzyme (2-oxopent-4-enoate hydratase), and therefore we cannot properly account for the toluene metabolic pathway.

Xenobiotic compounds. We found suggestions for the presence of ORFs specifying various enzymes that are involved in the metabolism of the following xenobiotic compounds: acrylamide, benzamide, cyclohexanol, 1,2-dichloroethane, ethylbenzene, nitrophenyl phosphate, and phthalate. However, the complete metabolic pathways of these compounds could not be identified. Therefore, *C. salexigens* may be able to partially degrade these compounds, but it probably would not be able to grow on them as single carbon sources.

4. 1C metabolism

4.1. RIBULOSE BISPHOSPHATE CARBOXYLASE, LARGE SUBUNIT

The *C. salexigens* chromosome contains an ORF that shows high similarity to the large subunit of the Calvin-Benson-Bassham cycle ribulose bisphosphate carboxylase / oxygenase (RubisCO). Orthologous proteins exist in a variety of organisms, and among these, the *C. salexigens* sequence has highest similarity (E values in from e-119 to 5e-96) to ORFs in *Bordetella bronchiseptica* RB50, ND *Mesorhizobium loti* MAFF30309, and *Sinorhizobium meliloti* 1021. *B. subtilis* has a related enzyme, encoded by the *mtnW* (*ykrW*) gene that has been shown to carry out an enolase reaction in the S-adenosylmethionine to methionine salvage pathway (Ashida et al., 2003; Sekowska et al., 2004). However, we have not been able to find good matches for the other enzymes of this pathway, as it exists in *B. subtilis*. A RubisCO-like protein is also found in the *Chlorobaculum tepdium* and related green sulfur bacteria (Hanson and Tabita, 2001). Although these organisms are photoautotrophic, they use the reductive TCA cycle for CO_2 fixation instead of RubisCO. Rather, this enzyme has been suggested to have an as of yet undetermined role in sulfur metabolism or oxidative stress response (Hanson and Tabita, 2001). These RubisCO-like proteins in *B. subtilis* and *Chlorobaculum* do not have several amino acid residues that are required for carboxylase / oxygenase activity and they have been shown to lack these enzymatic activities. The RubisCO-like protein in *C. salexigens* contains non-conservative substitutions at 6 of 19 amino acid residues that are required for the binding of the carboxylase substrates and it also has a deletion of

11 residues in “loop 6”, which is essential for carboxylase activity (Ashida et al., 2003). Thus, it is unlikely that this enzyme operates as a carboxylase in *C. salexigens*, but we have no insights as to its true function. We have not observed an ortholog for the small subunit of RubisCO. Interestingly, *C. salexigens* appears to have gene for phosphoribulokinase, which is a member of the Calvin-Benson-Bassham cycle in CO_2 fixing organisms. However, there is no evidence for sedoheptulose-1,7-bisphosphatase, providing a second reason why it is unlikely that *C. salexigens* can fix CO_2 with the RubisCO-like protein.

4.2. CARBON MONOXIDE DEHYDROGENASE

There were good matches to the three subunits of the aerobic type carbon monoxide dehydrogenase found in *P. aeruginosa*; as is the case with many of the our predictions, the function of this enzyme needs to be addressed experimentally.

4.3. FORMALDEHYDE DEHYDROGENASE

The genome contains information for a glutathione-dependent formaldehyde dehydrogenase and two glutathione-independent formaldehyde dehydrogenases. It is not known whether *C. salexigens* can metabolize formaldehyde, but a closely related *Halomonas elongata* strain has been observed to be able to do so (Azachi et al., 1995). Thus, we can account for the metabolism of formaldehyde in *C. salexigens*.

4.4. FORMATE DEHYDROGENASE

We found evidence for two selenocysteine-containing formate dehydrogenases, one of which may contain molybdenum and the second may contain tungsten. As *C. salexigens* cannot grow fermentatively, these formate dehydrogenases are presumably coupled to an electron acceptor (O_2, NO_3^-), but we cannot deduce the nature of the electron acceptor from the amino acid sequence. We observed eight genes that could specify the components of formate hydrogen lyase (FdhF, HycA through HycG), but because these showed marginal similarity to orthologs from *E. coli* (e-13 to e-19), we are not certain of this identification. There was no evidence of a hydrogenase, which could either form or consume H_2.

5. Summary

From the draft genomic sequence of *C. salexigens*, we were able to find good indication that the organism has all of the enzymes of glycolysis, hexose monohophoshate shunt, Entner-Doudoroff pathway, and the TCA cycle. We were able to rationalize the pathways of metabolism of many of the common sugars and mono- and dicarboxylic acids. We could account only partially for the metabolic pathway of benzoate, 4-hydroxybenzoate, and 3,4-hydroxybenzoate. The sequence information gave hints that *C. salexigens* might be able to metabolize a number of other aromatic and xenobiotic

compounds at least partially. The latter suggestion, which needs to be followed up experimentally, presents the exciting possibility that *C. salexigens* might be exploited for the biological cleanup of polluted environments that are also high in salinity.

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K^+ TRANSPORT AND ITS ROLE FOR OSMOREGULATION IN A HALOPHILIC MEMBER OF THE *BACTERIA* DOMAIN: CHARACTERIZATION OF THE K^+ UPTAKE SYSTEMS FROM *HALOMONAS ELONGATA*

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1. Introduction

Halophiles have developed two different basic strategies of osmoregulatory solute accumulation to cope with water stress caused by high salt-concentrations in their environment: the ”salt-in-cytoplasm mechanism” and the ”organic-osmolyte mechanism”. The ”salt-in-cytoplasm mechanism”, first discovered in halobacteria, is considered the typical archaeal strategy of osmoadaptation. Halobacteria and related microorganisms accumulate K^+ in molar concentrations to cope with high external osmolality (Eisenberg and Wachtel, 1987; Larsen, 1973). However, some anaerobic halophilic Bacteria are known to employ this strategy as well and accumulate either K^+ or Na^+ depending on the growth phase (Oren et al., 1997; Rengpipat et al., 1988). The recently discovered genus *Salinibacter* as a member of the *Cytophaga-Flavobacterium-Bacteroides* phylum also accumulates K^+ within the cytoplasm and displays a salt tolerance comparable to that of extremely halophilic Archaea (Antón et al., 2002). In contrast to these organisms, methanogenic Archaea (e.g., *Methanohalophilus*) as well as phototrophic and aerobic chemoheterotrophic Bacteria employ the organic osmolyte mechanism and accumulate polar or zwitterionic, highly water-soluble, organic molecules called compatible solutes (Galinski, 1995; Lai et al., 1991; Severin et al., 1992).

1.1. THE ROLE OF K^+ IN NON-HALOPHILIC *BACTERIA* ACCUMULATING ORGANIC OSMOLYTES

Despite the abundance of inorganic ions like Na^+ and K^+, respectively, in high-salt growth media, these ions are largely excluded from the cytoplasm of microorganisms of the osmolyte strategy (Sadler et al., 1980; Ventosa et al., 1998). For a variety of non-halotolerant and halotolerant Bacteria it was shown that this state is altered only after a sudden increase in salinity, resulting in a transient accumulation of K^+. This inorganic cation accumulates on a short-term basis after a sudden increase in salinity.

It transiently acts as an osmolyte to restore cell volume and turgor pressure until it is replaced by compatible solutes (Dinnbier et al., 1988; Reed et al., 1985; Whatmore et al., 1990). Additionally, K^+ is thought to serve as a cellular messenger in the regulation of enzyme activity and gene expression (Sutherland et al., 1986; Booth and Higgins, 1990). It is also known that the accumulation of K^+ supports the respiration of cells exposed to an osmotic upshock (Meury, 1994). K^+ influences the generation of a proton gradient (ΔpH) as a component of the proton electrochemical gradient across cytoplasmic membranes (Padan et al., 1976; Tokuda et al., 1981). It is also required for the generation of a Na^+ electrochemical gradient in the uptake of organic acids (Tokuda et al., 1982).

1.2. K^+ UPTAKE SYSTEMS IN NON-HALOPHILIC BACTERIA

Although K^+ accumulation is essential for halophilic Bacteria which thrive over a wide range of salinity, the K^+ transport systems in these organisms have been analyzed so far only in one member of the γ-Proteobacteria, namely *Halomonas elongata* (Kraegeloh et al., 2005). Instead, the majority of investigations in this filed were carried out with non-halophilic and halotolerant Bacteria such as *Escherichia coli* (Dosch et al., 1991), *Vibrio alginolyticus* (Nakamura et al., 1998a, 1998b), and recently *Bacillus subtilis* (Holtmann et al., 2003), where K^+ transport systems have been studied intensively at the genetic and physiological level.

The major transport systems for K^+ accumulation in these organisms are the transporter Kdp (*E. coli*), the Ktr system (*V. alginolyticus*, *B. subtilis*), and the Trk transporter (*E. coli*, *V. alginolyticus*). Kdp is an inducible, high affinity K^+ translocating P-type ATPase (K_m = 2 μM) encoded by the *kdpABC* operon. Homologues of Kdp were found in many other Bacteria than *E. coli* (Walderhaug et al., 1989). Furthermore, it is now known that P-type ATPases different from Kdp are involved in K^+ uptake in Bacteria as well (Sebestian et al., 2001).

The Ktr system consists of two components: a transmembrane spanning subunit named KtrB forming the actual pore and a cytoplasmic membrane-associated KtrA-protein containing an NAD^+-binding domain. Ktr transporters, identified in many Bacteria and in at least one member of the archaeal domain (Roosild et al., 2002), allow for medium to low affinity K^+ uptake, which is Na^+ dependent (Tholema et al., 1999).

The Trk system has an evolutionary relationship to Ktr, is widespread in both Bacteria and Archaea (Durell et al., 1999), and has a medium to low affinity for its substrate K^+. Trk systems are secondary transporters and the uptake of K^+ is thought to be linked to H^+ symport. Trk consists of a transmembrane protein named TrkH or TrkG, which is the actual K^+ translocating subunit; and the cytoplasmic membrane surface protein TrkA, which is a NAD^+ binding protein. In *E. coli* the TrkH system requires an ATP-binding protein named TrkE (SapD), which is thought to activate transport. TrkE, in *E.* coli, is expressed by *sapD* located in the *sapABCDF* operon coding for an ABC transporter of unknown function (Harms et al., 2001). Not all Trk systems need *sapD* for activity. It is assumed that TrkG and Trk systems in other bacteria can use ATP-binding proteins stemming from ABC transporters different than SapABCDF.

2. The Role of K^+ for Osmoregulation in Halophilic Bacteria

2.1. K^+ CONTENT IN ADAPTED AND OSMOTICALLY STRESSED CELLS

Halomonas strains synthesize ectoine as their main compatible solute and require 3% NaCl (0.51 M) or more for optimal growth. Although ectoine is synthesized as the major compatible solute over a wide range of salinities to cope with constant osmotic stress, at a low salt concentration of 3% NaCl adapted cells of *H. elongata* contain more K^+ glutamate than ectoine. Only at salinities above 3% NaCl does ectoine become the predominant cytoplasmic solute in salt-adapted cells (Kraegeloh and Kunte, 2002). It was proven that the K^+ level in adapted, exponentially growing halophilic cells is independent of the NaCl concentration of the environment and remains unchanged over a wide range of salinity as estimated for *H. halodenitrificans* growing in complex medium (Sadler et al., 1980) and *H. elongata* grown in mineral salt medium (Kraegeloh and Kunte, 2002). In case of an osmotic upshock, however, the cytoplasmic K^+ content will rapidly increase (Fig. 1). The increase in K^+ is equivalent to the increase in glutamate and at any time the K^+ content is 2.4 times higher than the glutamate-level. This strongly indicates that glutamate is the counter-ion to K^+ to achieve electroneutrality. The K^+ glutamate content reaches its maximum at about 15 min after the hyperosmotic upshock and stays at this elevated level for at least 2 hours. Concurrently with the increase of K^+ glutamate, the compatible solute ectoine is synthesized and accumulated in the cytoplasm. Thirty minutes after the upshock 50% of the additional ectoine is already synthesized and after 100 min the final content of about 2.3 μmol ectoine mg^{-1} protein is reached. It has to be stressed that the increase in the cytoplasmic *concentration* (mol l^{-1}) of K^+ and also ectoine might be significantly faster than the increase in solute *content* (μmol ectoine per weight of protein or cell mass) due to the massive loss of water caused by osmotic upshock.

2.2. THE ROLE OF K^+ FOR OSMOREGULATION

It is debatable what role K^+ plays during the osmoregulatory response to osmotic upshock in moderately halophilic Bacteria. Initially, it seems that K^+ is accumulated as an osmolyte (like in enteric bacteria), bridging the time until sufficient compatible solutes such as ectoine are synthesized after osmotic shock. However, the K^+ content (μmol K^+ mg^{-1} protein) does not change but remains at an elevated level even when ectoine is accumulated to the maximum concentration. K^+-limitation experiments carried out with *H. elongata* showed that the organism failed to synthesize ectoine after osmotic upshock. However, the fact that osmoregulatory responses like compatible solute synthesis depend on the presence of K^+ is inconclusive as to the specific role of K^+ in osmoadaptation. Upshock experiments under K^+ limitation in the presence of the compatible solute glycine betaine revealed that *H. elongata* was still able to transport glycine betaine from the medium. Although betaine was accumulated in the cytoplasm, *H. elongata* was not able to resume growth after osmotic upshock, indicating that K^+ is needed not only as an osmolyte. Indeed, in the absence of sufficient K^+, there is a significant decrease in respiration of *H. elongata* (Kraegeloh and Kunte, 2002). This is an interesting observation, since, in response to osmotic upshock, the respiration rate of *H. elongata* declines whether K^+ is available or not. Furthermore, it is in the presence

of K^+ that the organism is able to establish respiration to a level existing prior to upshock. Similar observations were made with the marine bacterium *Vibrio alginolyticus*, where the addition of K^+ to K^+-depleted cells also increases the respiration rate (Tokuda et al., 1981). It was shown that the generation of a ΔpH across the cytoplasmic membrane is dependent on K^+ as a counterion for H^+. It was hypothesized that respiration is stimulated by a potassium-dependent, increased proton ejection, which is necessary to establish a ΔpH.

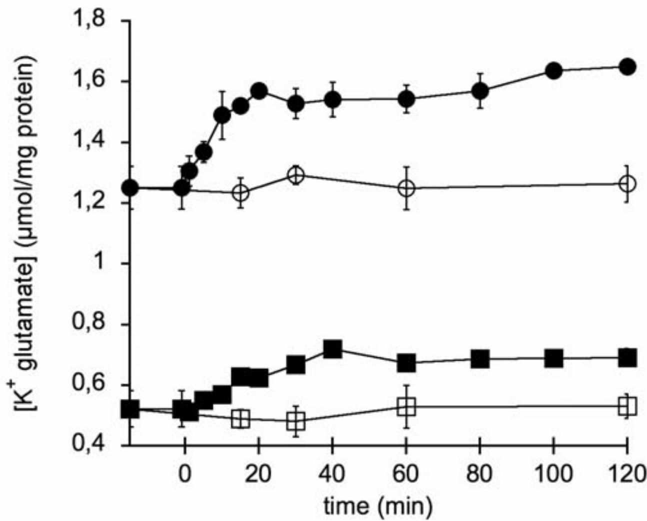

Figure 1. Effect of osmotic upshock on the cytoplasmic K^+ glutamate content. Cells were grown in Na-MM63-medium at 0.51 M NaCl containing 5 mM K^+. At time zero exponentially growing cells were exposed to osmotic upshock by increasing the salinity to 1.03 M NaCl. Solutes were determined by atomic absorption spectroscopy (K^+) and HPLC (glutamate). Presented values of K^+ (●, ○), and glutamate (■, □) in upshocked cells (closed symbols) and in control cells (open symbols) are the means of at least 3 independent experiments.

3. K^+ Transporter from Halophilic Bacteria: The Trk-Systems TrkI and TrkH Identified in *Halomonas elongata*

3.1. IDENTIFYING THE Trk-TRANSPORT SYSTEMS OF *H. ELONGATA*

Growth experiments carried with *H. elongata* under K^+ limitation determined the affinity of whole cells for K^+ and led to the conclusion that *H. elongata* must be equipped with K^+ transporter(s) of medium to low affinity (Kraegeloh and Kunte, 2002). Tn*1732* mutagenesis was successfully employed in characterizing the osmoregulated ectoine transporter TeaABC of *H. elongata* (Grammann et al. 2002). However, we did not succeed using this method in order to identify genes encoding K^+

transporters in *H. elongata*. Therefore, a plasmid-encoded gene bank of *H. elongata* was constructed and transferred into mutant strains of *E. coli*, defective in K^+ transport. By functional complementation as described by Kraegeloh et al. (2005), three genes coding for K^+ transporter proteins from *H. elongata* were cloned. Two adjacent genes named *trkA* (1374 bp) and *trkH* (1449 bp) were identified on an 8.5 kb DNA fragment, while a third gene called *trkI* (1479 bp) located at a different site of the *H. elongata* chromosome was found on a second 8.5 kb fragment (Fig. 2).

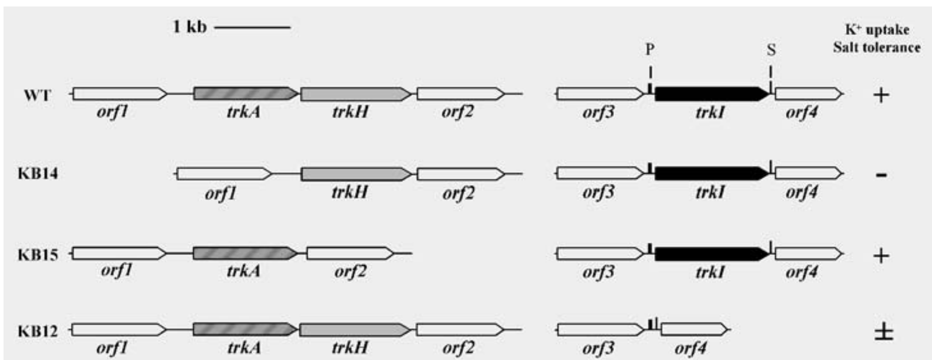

Figure 2. Gene organization at the *trkAH* and *trkI* loci of *H. elongata*. The relevance of the *trk* genes for K^+ uptake was determined by K^+ transport experiments (3.3, Fig. 4) and growth experiments on minimal medium under K^+ limitation (3.3, Fig. 3). K^+ transport and growth like wild-type, (+); reduced K^+ uptake and growth, (±); no K^+ uptake via TrkH and TrkI, no growth under K^+ limitation in mineral salt medium, (–). Open reading frame *orf1* upstream of *trkAH* encodes a potential RNA-methyltransferase, *orf2* downstream of *trkH* as well as *orf3* and *orf4* adjacent to *trkI* are of unknown function. P, putative promoter sequence; S, putative stem-loop sequence. Sequence analysis did not reveal promoter or stem-loop sequences for *trkAH* (Kraegeloh et al., 2005)

3.2. CHARACTERIZATION OF THE PUTATIVE Trk-PROTEINS AND THE GENETIC ORGANIZATION OF THE *trk* LOCI

The deduced amino acid sequence of *trkA* shares 65% identical amino acids with the TrkA protein of *Vibrio alginolyticus* and 63% identical amino acids with TrkA of *E. coli*. The *trkA* gene of *H. elongata* encodes a putative NAD/NADH binding protein and has a calculated molecular mass of 50 kDa (457 amino acids). The 1449 bp ORF named *trkH*, which begins 43 bp downstream of *trkA*, encodes a protein of 52 kDa (482 amino acids) and has the closest similarity to transmembrane proteins of the Trk transporter family. TrkH of *H. elongata* shows the highest comparison score with the transmembrane protein TrkH of *E. coli*, having 55% identical amino acids and still shares 39% identical amino acids with TrkG, the second Trk transporter of *E. coli*. The 1479 bp ORF named *trkI* is preceded by a potential σ^{70}-dependent promoter sequence and a likely ribosome binding site, and is followed by a potential stem-loop structure.

Like *trkH*, *trkI* codes for a putative transmembrane protein of the TrkH-type. Comparison of the potential TrkI protein, which has a calculated molecular mass of 53 kDa (492 amino acids), revealed a high degree of identity to the TrkH protein of *V. alginolyticus* (48% identical amino acids), but only 32% and 29% identity, respectively, to the *E. coli* transporters TrkH and TrkG. TrkI and its counterpart, the TrkH transmembrane protein of *H. elongata*, share 36% identical amino acids.

The genetic organization of the *trkAH* site of *H. elongata* resembles the *trkAH* locus of *V. alginolyticus*. Both organisms belong to the few prokaryotes described so far where the *trkA* and *trkH* genes are clustered together (Fig. 2). Not only is the *trkAH* cluster similar in structure and sequence, but also the genes adjacent to *trkAH* are similar in *H. elongata* and *V. alginolyticus*. The *orf1* upstream of *trkAH* in *H. elongata* codes for a potential protein containing a tRNA/rRNA-m^{5}C-methyltransferase domain. Enzymes that function as RNA-methyltransferases are encoded by open reading frames named *fmu* and *fmv*, respectively (Tscherne et al., 1999). Upstream of the *trkAH* cluster in *V. alginolyticus* three open reading frames are located named *fmt*, *fmu*, and *fmv* (Nakamura et al., 1998a). Interestingly, similarities in this respect were also found in *E. coli*, where *fmt* and *fmu* precede the *trkA* gene as well (Schlosser et al., 1993). In all three organisms the arrangement of the genes *fmu*/*fmv* and *trkA* is similar. However, in contrast to *H. elongata* and *V. alginolyticus*, the *trkA* gene in *E. coli* is located separately from *trkH* and the second system of this type *trkG*. For *trkAH* of *V. alginolyticus*, it is assumed that the gene cluster is organized as an operon and, since *V. alginolyticus* encodes only one Trk-system, a coordinated transcription of *trkAH* would be advantageous (Nakamura et al., 1998a). Transcriptional analysis of the *trkAH* locus in *H. elongata* by Northern hybridization using *trkA* and *trkH* RNA-probes, and RT-PCR revealed that *trkAH* is organized as an operon as well. However, a single *trkA* transcript was also detectable, which can arise either by partial termination of the transcription after *trkA* or from processing of the RNA to separate *trkA* and *trkH* (Kraegeloh et al., 2005).

3.3. THE ROLE OF TrkA, TrkI, AND TrkH FOR GROWTH OF *H. ELONGATA* AND K^+ UPTAKE

3.3.1. Effect of trk-mutations on growth of H. elongata

To test the role of the proteins encoded by *trkAH* and *trkI* for K^+ uptake in *H. elongata*, a set of different *trk*-deletion mutants were constructed (Fig. 2). Strains KB12 (Δ*trkI*), KB14 (Δ*trkA*), KB15 (Δ*trkH*), KB12.2 (Δ*trkH*, Δ*trkI*), and KB16 (Δ*trkA,* Δ*trkH*, Δ*trkI*) were used for growth experiments on agar medium (Fig. 3) and in liquid medium with different K^+ concentrations and varying osmolarity. At a K^+ concentration of 5 mM, deletion strain KB12 (Δ*trkI*) showed similar growth behavior as the wild-type even at elevated salinity of 2.05 M NaCl. However, growth of Δ*trkI* mutant KB12 was greatly diminished compared to the wild-type at a K^+ concentration of 0.1 mM. With increasing salinity the growth inhibition was even more pronounced and strain KB12 failed to grow in 0.1 mM K^+ medium at a salinity of 2.05 M NaCl (Fig. 3a). Additional deletion of *trkH* created double mutant KB12.2 (Δ*trkI*, Δ*trkH)*, which could only grow in high potassium medium of 100 mM or higher (Fig. 3b) and showed a similar phenotype as the triple mutant KB16 (Δ*trkA,* Δ*trkH*, Δ*trkI*). This indicates that the

trkH-encoded transporter is involved in K^+ uptake and allows for the reduced growth of strain KB12 (Δ*trkI*) in low potassium medium. In contrast, Δ*trkH* mutant KB15 displayed the same phenotype as the wild-type strain at all salt- and K^+-concentrations. Strain KB14, missing the potential NAD^+/NADH binding protein TrkA, also failed to grow in medium with low K^+ concentration (Fig. 3b). The growth experiments revealed that TrkI and TrkH are involved in K^+ uptake in *H. elongata*. As judged by the growth behavior of the *trk* mutants, TrkI appears to have a higher affinity for its substrate K^+ than does TrkH, and can compensate the knock out of *trkH* at least under the conditions tested. Furthermore, the knock out of *trkA* appears to affect both transporters TrkI and TrkH.

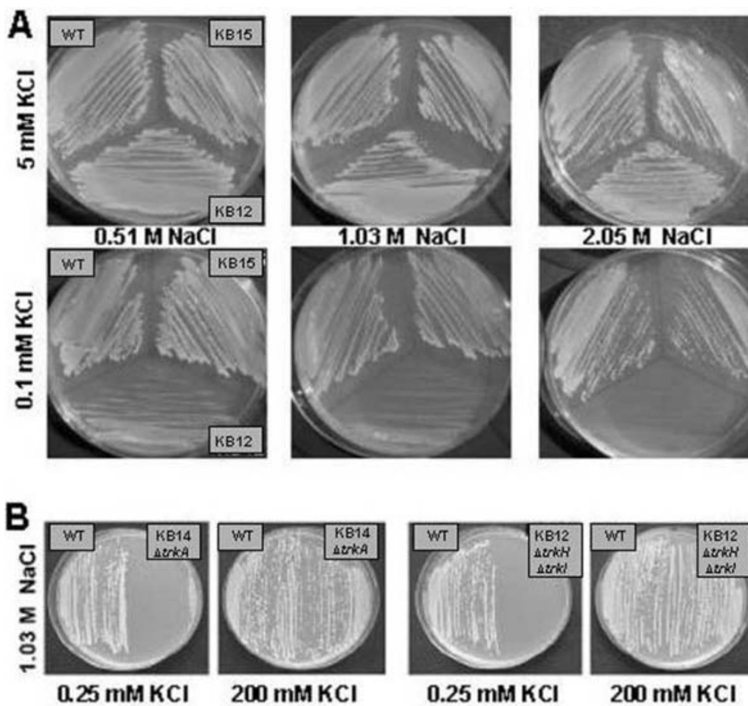

Figure 3. (A) Growth of *H. elongata* wild-type (WT), *trk*-deletion mutants KB12 (Δ*trkI*), and KB15 (Δ*trkH*) on minimal medium at different salinities (0.51 M, 1.03 M, 2.05 M NaCl) and low (0.1 mM) and high (5 mM) K^+ concentration. Growth was scored after 3 days (0.51 M and 1.03 M NaCl) and 4 days (2.05 M NaCl) incubation at 30 ˚C. Mutant KB15 grew similar to the wild-type in low and high K^+ at all salinities, while growth of KB12 accumulating K^+ only via TrkH was hampered in low K^+ medium especially at high salinity. (B) Growth of *H. elongata* wild-type (WT), *trkA* deletion mutant KB14, and KB12.2 (Δ*trkH* Δ*trkI*) on minimal medium at 1.03 M NaCl and different K^+ concentrations (0.25 mM and 200 mM). For (B) growth was scored after 3 days incubation at 30 ˚C (Kraegeloh et al., 2005).

3.3.2. TrkAH and TrkAI are K^+ transport systems with different substrate affinity and transport kinetics

To investigate the role of both TrkI and TrkH transporters in more detail, transport experiments were carried out and the kinetic parameters of K^+ uptake for the two Trk-systems were determined. To carry out K^+ uptake experiments, *H. elongata* wild-type, strain KB12 (Δ*trkI*), KB12.2 (Δ*trkH*, Δ*trkI*), and KB15 (Δ*trkH*) were grown in mineral salt medium containing 0.51 M NaCl and 5 mM K^+. The cells were depleted of more than 70% of their cytoplasmic K^+ content by washing twice in DEA buffer and tricine buffer according to the method described by Tokuda (1986). The washing procedure had no deleterious effect on K^+ transport and all cells were able to accumulate about 1.2 µmol K^+ per mg cell protein, which corresponds to the natural K^+ content found in exponentially growing cells of *H. elongata* (Kraegeloh and Kunte, 2002). After washing, the K^+ uptake in K^+ depleted cells was initiated by adding K^+ to the cells and the initial rates for K^+ uptake were determined.

K^+ uptake rates of TrkI determined with strain KB15 (Δ*trkH*) were fitted by non-linear regression and the uptake kinetics were best fit to the Michaelis-Menten model (Fig. 4a). Analysis of the transport kinetics of TrkI revealed a K_m value of 1.12 mM and a V_{max} of 176 nmol of K^+ taken up min^{-1} mg^{-1} protein. In contrast, the kinetic parameters for TrkH determined by transport experiments with strain KB12 (Δ*trkI*) were different from TrkI (Fig. 4a), showing a lower affinity for the substrate (half saturation constant of 3.36 mM K^+) and a lower transport velocity (V_{max} of 137 nmol K^+ min^{-1} mg^{-1} protein). Furthermore, the K^+ uptake did not follow the Michaelis-Menten kinetics, but was best described by:

$$V(S) = \frac{aS^2}{b + S^2}$$

in which V is the transport rate, S is the substrate concentration, a is the limiting rate (maximum velocity), and the square root of b is the substrate concentration at which $V = 0.5\ a$ (rate is half-limiting). The different kinetics of TrkH for K^+ uptake compared to TrkI was confirmed by the Hill plot (Fig. 4c), where the Hill coefficient was estimated to be 0.98 for TrkI and 2.1 for TrkH. The transport data of the wild-type strain were similar to strain KB15 (Δ*trkH*) having a K_m value of 1.18 mM and a V_{max} of 170 nmol K^+ min^{-1} mg^{-1} protein. Although the wild-type is equipped with both Trk systems, TrkH and TrkI, the V_{max} was not additive and K^+ uptake followed the Michaelis-Menten kinetics as described for strain KB15, where K^+ was accumulated only via the single transporter TrkI (Fig. 4).

To clarify the role of the *trkA* gene product for the transport of K^+ through TrkH and TrkI, *trkA* was deleted in the wild-type of *H. elongata*. Transport experiments carried out with the resulting mutant KB14 revealed that the loss of the *trkA* abolished any K^+ uptake activity via TrkH and TrkI (Fig. 4a). These results show that there is only one type of TrkA protein in *H. elongata* expressed from the *trkAH* locus, on which both TrkH and TrkI rely for transport of K^+.

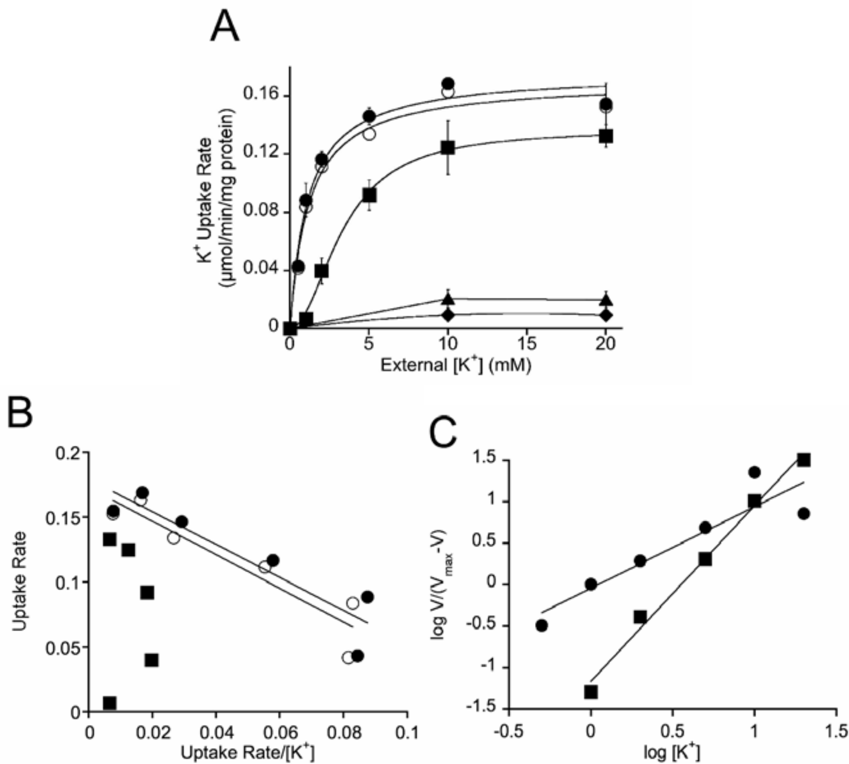

Figure 4. Kinetic analysis of K^+ transport via TrkH and TrkI in *H. elongata* wild-type (wt), strain KB12 (Δ*trkI*), KB15 (Δ*trkH*), KB12.2 (Δ*trkH,* Δ*trkI*) and KB14 (Δ*trkA*). Osmolarity of growth medium and transport buffer was adjusted by 0.51 M NaCl. Transport was started by adding potassium to the assay. (A) Lowering the K^+ concentration from 20 mM to 500 µM led to a decreasing transport rate. The K^+ uptake data were fitted by non-linear regression. The curve for K^+ transport via TrkI into KB15 (●) and transport by the wt (O) were fitted best by the Michaelis-Menten model and showed a K_m value of 1.12 mM and 1.18 mM K^+, respectively. The transport data for TrkH in strain KB12 (■) showed a sigmoidal dependence of transport rate versus K^+ concentration. The half saturation constant was determined to be 3.36 mM. Deletion of *trkI* and *trkH* abolished transport activity in strain KB12.2 (▲) proving that transporters TrkH and TrkI are responsible for the observed uptake of K^+. No transport activity was measured for the Δ*trkA* deletion mutant KB14 (◆) proving that *trkA* encodes the potential NAD binding protein for both TrkH and TrkI. (B) The plot of K^+ uptake rate versus K^+ uptake rate/K^+ concentration (Eadie-Hofstee plot), which is very sensitive to any deviation of Michaelis-Menten kinetics, confirmed the results of the non-linear regression and proved that TrkH uptake kinetics differ from the kinetics of TrkI. (C) Hill plot based on transport data from TrkI (●, KB15) and TrkH (■, KB12) to evaluate the Hill coefficient h, which was estimated to be 0.98 for TrkI and 2.1 for TrkH. Values of h greater than 1 indicate a deviation of the Michaelis-Menten kinetics and can be used as an index of enzyme cooperativity. Symbols: O, wt; ■, KB12; ▲, KB12.2; ◆, KB14; ●, KB15. Error bars indicate standard deviation (Kraegeloh et al., 2005).

Summarizing, these findings suggest that TrkI is the dominant K^+ uptake system in *H. elongata* wild-type, while TrkH does not contribute to K^+ uptake at least under the conditions tested, where salt adapted cells were used. Based on transport kinetics observed with different *trk*-mutant strains of *H. elongata* and the unknown transport mechanism(s) for K^+ uptake through Trk transporters, the following hypotheses to explain the different transport behavior of TrkAH and TrkAI are offered: i) TrkAH but not TrkAI could be a cooperative transport enzyme, which typically show sigmoidal rate kinetics as a function of substrate concentration and a Hill coefficient greater than 1; ii) the differences in kinetics could also be explained by random-order binding of the substrates K^+ and H^+ to TrkH, which would also lead to a sigmoidal curve and a different Hill plot, respectively, in contrast to the compulsory-order mechanism (Segel, 1975). The different transport kinetics of TrkI and TrkH also help to explain the reduced growth of strain KB12 (Δ*trkI*) in low potassium medium compared to strain KB15 (Δ*trkH*). The transport velocity of TrkI and TrkH differed only by the factor of 1.3 when the transport systems were saturated. However, at low K^+ concentrations of 100 μM to 250 μM the transport rates of TrkH were estimated to be at least 40-times lower than the rates of TrkI. The large differences in growth of strain KB12 and KB15 were observed only at these low concentrations, while at K^+ levels of 5 mM or higher both mutants were phenotypically identical.

Still to be resolved is why *H. elongata* has two Trk-uptake systems, of which one, TrkH, shows lower transport rates, less affinity for the substrate, and does not contribute significantly to K^+ accumulation in adapted cells of *H. elongata*. In *E. coli* K12 strains, often two Trk-systems are found, of which the gene *trkG* encoding the second system was most likely acquired through phage insertion. However, not all *E. coli* strains are equipped with two Trk transporters. The combination of a constitutively expressed Trk uptake system (Rhoads et al., 1976) transporting K^+ at a high rate and an inducible high affinity Kdp system (K_m = 2 μM), allows *E. coli* osmoregulated K^+ uptake even in low K^+ medium. Such high affinity transporters, like Kdp from *E. coli* are absent in organisms from soil and marine environments. This can be explained by the abundance of K^+, at least in the marine and saline environment where K^+ is found in concentrations of 10 mM or higher. Similar to *H. elongata*, a couple of low to medium affinity transport systems are found in *V. alginolyticus* (TrkAH, KtrAB, K_m = 50 μM) and *B. subtilis* (KtrAB, KtrCD). In the case of *H. elongata*, the different transport kinetics of the two K^+ transporters might help to explain the requirement for a second Trk system. TrkI seems to be the major K^+ transporter in exponentially growing cells adapted to a low salt concentration of 3% NaCl (w/v). However, one has to keep in mind that enzymes following the Michaelis-Menten kinetics, like TrkI, can only be regulated by comparatively large environmental changes (e.g. substrate concentration). In contrast, enzymes with sigmoidal kinetics can react to small changes in their environment and are often known as well regulated enzymes. To describe the two K^+ uptake systems TrkI and TrkH in even more detail, their behavior during environmental changes like osmotic shifts has to be examined and such studies might reveal additional information about the role and importance of these two transporters for adaptation and osmoregulation of *H. elongata*.

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THE CHLORIDE REGULON OF *HALOBACILLUS HALOPHILUS*: A NOVEL REGULATORY NETWORK FOR SALT PERCEPTION AND SIGNAL TRANSDUCTION IN BACTERIA

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1. Introduction

The moderately halophilic bacteria are a specialized group of organisms that require NaCl for growth. They grow with nearly the same rate over a rather wide range of external salt concentrations (0.5-2.0 M) which is evidence for effective mechanisms to cope with changing external salinities. Generally, growth of marine bacteria and moderate halophiles can be considered as being Na^+-dependent, and Na^+ is used for various bioenergetic reactions. However, of importance is not the presence of the ion *per se*, but the cells must maintain a sodium ion gradient ($Na^+_i < Na^+_o$) across their cytoplasmic membranes. Export of Na^+ can be achieved by primary and secondary transport systems, but the latter, like Na^+/H^+ antiporters, for bioenergetic reasons are better suited as effective Na^+ export systems operating at high external salt concentrations.

In contrast to the well established role of the cation Na^+ in the physiology of prokaryotes, little is known about the specific requirement for anions for growth and for the operation of specific cellular functions. The use of chloride salts in physiological studies with halophilic microorganisms is generally taken for granted and little thought is then devoted to the possibility that chloride may have more specific functions in the cell beyond serving as the counterion for the high concentrations of cations used in the growth media. In this review we will summarize the current status on the role of chloride in prokaryotes with a focus on the recently described novel function as a signal compound in the moderately halophilic bacterium *Halobacillus halophilus*.

2. Do Non-Halophilic Prokaryotes Have a Specific Requirement for Chloride? - A Survey of the Literature

The first indications for a specific chloride requirement in prokaryotes came from studies by MacLeod and his coworkers in the 1950s. They isolated a number of marine bacteria that proved to be highly stimulated or even strictly dependent on the presence of chloride for growth (MacLeod and Onofrey, 1956, 1957). Unfortunately no further

physiological studies were performed with these isolates, and the strains have since been lost.

There are only a few reports on a specific requirement for chloride in physiological processes in prokaryotes. Chloride seems to be essential for photosynthesis in some cyanobacteria, and an active transport of chloride was demonstrated (Ritchie, 1992a, 1992b; Zdrou and Tromballa, 1981). Acidophilic bacteria require chloride for the maintenance of a membrane potential (McLaggan et al., 1990), and the bo_3-type chinol oxidase of *Escherichia coli* contains chloride, but the physiological function is unknown (Ralle et al., 1999). The lactic acid bacterium *Lactococcus lactis* was shown to have chloride-induced genes, and one of the loci was identified to encode a glutamate antiporter and a glutamate decarboxylase. These findings led to the speculation that chloride is involved in acid response/resistance mechanisms in *L. lactis* (Sanders et al., 1998). This hypothesis is corroborated by the fact that a mutant of *E. coli* with a defect in a ClC-type chloride channel was impaired in surviving acid stress (Iyer et al., 2002). In summary, there are only few examples on specific functions of chloride in non-halophiles. In contrast, some halophiles have specific requirement for chloride.

2.1. CHLORIDE REQUIREMENT IN HALOPHILIC PROKARYOTES

Living cells are adapted to a certain salinity or salinity range for optimal cellular function. Despite their different salt requirements, living cells have developed only two mechanisms to cope with water stress. The majority of prokaryotes accumulate compatible solutes in response to increasing external salt concentrations (Kempf and Bremer, 1998; Roberts, 2000; Roeßler and Müller, 2001). Compatible solutes are defined as small, neutral, highly soluble organic molecules that do not interfere with cellular metabolism, and they are accumulated by either salt-induced *de novo* synthesis or salt-induced uptake from the medium (Brown, 1976). In contrast, some anaerobic Bacteria and some Archaea (the halobacteria) accumulate salt (KCl) in the cytosol to counterbalance the external salt concentration (Galinski and Trüper, 1994; Oren, 1999). Potassium is taken up by primary or secondary transporters (Epstein, 2003). In every case reported so far, intracellular KCl concentrations reported were in the molar range, comparable to the medium chloride concentration. Two systems for active uptake of Cl^- have been identified in the *Halobacteriaceae*. One is a light-independent transport system in which inward transport of Cl^- is coupled with the influx of Na^+ ions (Duschl and Wagner, 1986). Relatively little is known about this chloride pump. The second, much better characterized transport system is the light-driven chloride pump halorhodopsin (Kolbe et al., 2000; Oesterhelt and Tittor, 1989).

Some halophilic representatives of the bacterial domain such as the extremely halophilic red bacterium *Salinibacter ruber* (*Cytophaga*/*Flavobacterium*/*Bacteroides* group) and the anaerobic fermentative *Halanaerobium praevalens* also accumulate KCl in the cytoplasm at high external salinities (Rengpipat et al., 1988). *Salinibacter ruber* was recently isolated from a saltern in Spain (Antón et al., 2002). It is a rather interesting organism for it is a member of the Bacteria but has some properties in common with the red halophilic Archaea. *S. ruber* has a high salt requirement (optimum growth being achieved between 2.5-3.9 M NaCl, and a minimum of 1.7 M being needed for growth) and accumulates Cl^-, in addition to high concentrations of K^+ and Na^+.

Furthermore, no organic osmotic solutes could be demonstrated in significant concentration within the cells (Oren et al., 2002).

3. Chloride Requirement in the Bacterium *Halobacillus halophilus*

The aerobic, endospore-forming, Gram-positive bacterium *Halobacillus halophilus* was isolated from salt marsh sediments at the North Sea coast of Germany and originally described as *Sporosarcina halophila* (Claus et al., 1983). Based on 16S rRNA homologies, it was later reclassified as *Halobacillus halophilus* (Spring et al., 1996). Growth of *H. halophilus* is strictly salt dependent and optimal at a concentration of 0.5 to 2.0 M NaCl. Growth of *H. halophilus* is strictly dependent on Na^+. Although this has not been addressed experimentally, Na^+ is likely to be involved in bioenergetic reactions such as pH regulation or others. However, the most interesting point is that growth is also strictly dependent on Cl^-. No growth was observed at Cl^- concentrations of 0.2 M, but addition of chloride (to a medium with constant osmolarity) restored growth in a Cl^--dependent manner. Optimal growth occurs at 0.8 to 1.0 M Cl^-. Moreover, not only growth rates but also cell yields (final optical densities) were strictly chloride dependent. *H. halophilus* was the first bacterium for which a specific chloride dependence was demonstrated (Roeßler and Müller, 1998). In addition to growth, germination of endospores as well as flagella production and motility were identified to be chloride dependent (Dohrmann and Müller, 1999; Roeßler et al., 2000).

It should be noted that growth and germination of endospores was also observed in chloride-free but bromide containing media, indicating that bromide can substitute chloride. Moreover, after prolonged incubation in chloride-free but nitrate containing media, cells started to grow but the final optical densities are lower in nitrate-containing media. In contrast, nitrate could not substitute for chloride in germination or motility. It should be noted, however, that the substitution by bromide or nitrate is of no physiological significance since nitrate and bromide concentrations in the ecosystem are far below those values required for optimal activity.

4. Chloride-Induced Gene Expression and Protein Production in *H. halophilus*

In principle, the role of chloride in *H. halophilus* could be that of an osmolyte accumulated to counterbalance external salinity. However, this seems to be unlikely since *H. halophilus* uses various organic molecules in different concentrations as compatible solutes to counterbalance the external salt concentration (see below; Severin, 1993). What could then be the function of Cl^-? The very different functions of Cl^- (motility, flagellation, spore germination, growth) point to a more global, regulatory role such as in gene activation. To test this, the effect of Cl^- on the production of flagella was determined on a molecular level. Western blot analyses revealed that the production of the structural component of the flagellum, flagellin, was impaired in NO_3^- containing media (Roeßler and Müller, 2002). However, upon addition of Cl^- the cellular flagellin pool increased in a concentration dependent manner. Optimal flagellin production was achieved at 0.8 to 1.0 M Cl^-.

To test whether the transcription of the encoding gene, *fliC*, was also chloride dependent, it was cloned and sequenced. Subsequent Northern blot and RT-PCR analyses with RNA from cells grown at different chloride concentrations unequivocally demonstrated a chloride-stimulated expression of *fliC* (Roeßler and Müller, 2002). These experiments demonstrated that Cl^- influences the cellular flagellin pool by acting on both the transcriptional and translational level, but the effect on translation is much more pronounced. In addition, this was the first time that a Cl^--dependence of gene expression and protein production was shown in a moderate halophile.

5. A Chloride Regulon in *H. halophilus*

The above mentioned experiments clearly demonstrated a specific chloride-dependence of flagellin production in *H. halophilus*. However, flagellation is not essential for growth and thus the question remains why chloride is essential for growth of *H. halophilus*. One explanation would be that chloride is part of a global regulatory network in *H. halophilus*. To test this, cells were grown at 1 M NaCl or 1 M $NaNO_3$, and two-dimensional gel analyses was employed to identify Cl^--induced proteins. Indeed, five more proteins were specifically produced in Cl^- grown cells of *H. halophilus* (Roeßler and Müller, 2002). These were tentatively identified by BLAST searches as YvyD, a modulator of σ^S sigma factor, and SodA, a superoxide dismutase. Both proteins belong to the σ^B regulon in *B. subtilis*. σ^B is the sigma factor that is responsible for the general stress response in *B. subtilis* (Drzewiecki et al., 1998; Petersohn et al., 2001). Furthermore, a protein (LuxS) was identified that plays a role in the biosynthesis of autoinducers of the furanone family. This family of autoinducers is found in Gram-negative as well as in Gram-positive bacteria. The fourth protein was found to be the ATP-binding subunit of an ABC transporter of unknown substrate specificity. These experiments gave clear evidence that Cl^- affects the synthesis of several proteins in *H. halophilus* and it is speculated that Cl^- is a novel environmental signal of a global regulatory network in *H. halophilus*. A hypothetical model of Cl^- dependent functions in *H. halophilus* is presented in Fig. 1.

6. Possible Functions of the Chloride Regulon in *H. halophilus*

What could be the function of the Cl^- dependent regulatory network? At least one function must be essential to growth since growth of *H. halophilus* is strictly Cl^--dependent. One has to keep in mind that one essential function of moderate halophiles is to sense external salinity and to respond to it on a transcriptional, translational and enzyme activity level to adjust the intracellular pool size of the compatible solutes. Only little is know about salt sensing mechanisms in moderate halophiles. How do microorganisms sense "osmolality" and how is this signal mediated through the cell as far as gene expression and enzyme activation? There have been model studies with the slightly halotolerant bacteria *Escherichia coli* and *Bacillus subtilis* which can adapt to NaCl concentrations up to 1 M (Altendorf et al., 1998; Csonka et al., 1996; Kempf and Bremer, 1998). These studies led to the conclusion that the cells sense the turgor. One

of the attractive scenarios is that membrane-bound sensors will record a change in turgor by recognizing alterations in transmembrane helix-helix-interactions (Jung et al., 1997; van der Heide and Poolman, 2000; Wood, 1999). Recent studies using purified transporters for compatible solutes and a membrane bound osmosensor involved in potassium uptake in *E. coli* confirmed this hypothesis but also demonstrated that a certain ionic environment on the "right" side of the membrane is required in addition (Jung et al., 2000; van der Heide and Poolman, 2000). Therefore, the question how salt is sensed by these paradigms is still open, but the answer to this question might be different in different organisms.

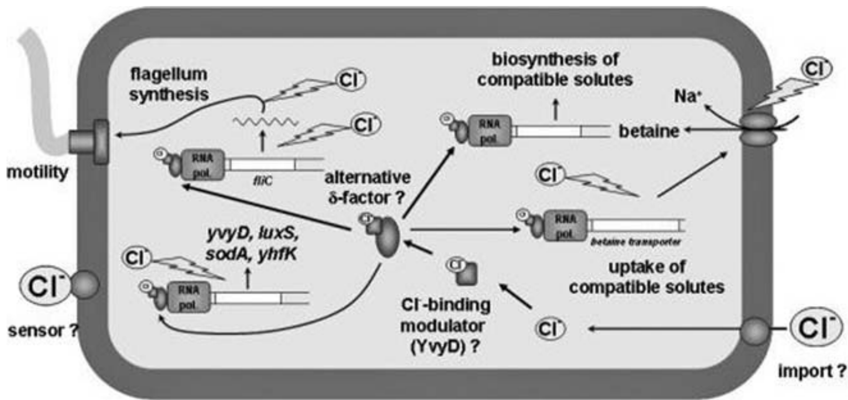

Figure 1. Hypothetical model of the function of chloride in *Halobacillus halophilus*.

Unlike the enteric bacterium *E. coli* moderately halophiles mostly encounter changing salinities, not osmolarity in general. Therefore, it was hypothesized that chloride, one component of common salt, is used as a signal molecule for external salinity in *H. halophilus* (Roeßler and Müller, 2002).

H. halophilus takes up glycine betaine from the medium and accumulates it in the cytoplasm to counterbalance external salt. The energetics of uptake was studied using whole cells. Inhibitor studies suggest that glycine betaine transport was most likely catalyzed by a primary transporter. Most important, uptake of glycine betaine was strictly dependent on the Cl^- concentration. Only Br^- and NO_3^- were able to substitute Cl^- and restored the full activity of glycine betaine transport. But both anions are of no physiological relevance in the natural habitat of *H. halophilus* as already mentioned above. It should be noted that for glycine betaine transport not only the type of anion is important but also the cation. In addition, glycine betaine transport after an osmotic upshock was impaired in the absence of Cl^- but restored by addition of Cl^- (Roeßler and Müller, 2001). Since there was no evidence that Cl^- was transported along with glycine betaine, it was suggested that Cl^- acts as a modulator (activator) of the glycine betaine

transporter. These experiments clearly revealed the first Cl^- dependent osmolyte transporter in prokaryotes. Furthermore, these experiments corroborated the idea that the essential function of the chloride regulon is to sense external salt and to induce / activate systems involved in accumulation of compatible solutes. Unfortunately, the transporter(s) catalyzing Cl^--dependent glycine betaine transport have not been identified yet.

H. halophilus uses a pool of different solutes in a given medium and different solutes in different media. These solutes consist mainly of amino acids, such as glutamine, glutamate, proline and alanine but also of amino acid derivatives such as N-acetyllysine, N-acetylornithine, glycine betaine and ectoine. Remarkably, ectoine could only be found in complex and mineral medium, but not in peptone medium. Almost the same is true for glycine betaine, which could only be identified in complex medium, but not in mineral medium or in peptone medium (Severin, 1993). Experiments are now under way to define the role of Cl^- in the accumulation of compatible solutes in *H. halophilus*. As a prerequisite for these studies, biosynthetic pathways for the solutes synthesized have to be established and transporters involved in accumulation must be identified. These experiments are under way and facilitated by the ongoing genome analysis of the model halophile.

7. Chloride signaling

A Cl^--dependent signal transduction chain does not necessarily require transport of Cl^- into the cell but could involve membrane-bound sensors. Although this question has not been settled, it was shown that the intracellular Cl^- concentration (Cl^-_i) increases with the external Cl^- concentration (Cl^-_e) (Roeßler and Müller, 1998). At suboptimal Cl^-_e concentrations Cl^-_i is ten times lower than Cl^-_e, at 0.5 M Cl^-_e the Cl^-_i concentration was 0.08 M, and in the range of 0.8 to 2.0 M the Cl^-_e/ Cl^-_i gradient decreased to a nearly constant value of 1.5-2. Thermodynamic calculations indicate an active transport of Cl^- which could argue for an intracellular receptor. However, additional experiments are required to define a potential Cl^- receptor.

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BIOSYNTHESIS OF THE COMPATIBLE SOLUTE MANNOSYLGLYCERATE FROM HYPERTHERMOPHILES TO MESOPHILES

Genes, Enzymes and Evolutionary Perspectives

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1. Introduction

Many thermophilic and hyperthermophilic organisms originate from inland geothermal areas where the salinity of the water is very low, but increasingly, the isolation and description of novel microorganisms has shifted to marine geothermal areas where the levels of NaCl can reach those of seawater. These organisms accumulate compatible solutes under osmotic stress that are, in most cases, different from those encountered in mesophilic organisms. Ectoine, hydroxyectoine, glycine betaine and polyols, which are very common in mesophilic Bacteria and Archaea, fungi and algae, are not generally found in thermophilic and hyperthermophilic prokaryotes. Instead, compatible solutes such as di-*myo*-inositol-phosphate (DIP), diglycerol phosphate (DGP), mannosylglycerate (MG) and mannosylglyceramide (MGA), along with some common compatible solutes of mesophiles, namely glutamate and trehalose, have been identified in these organisms (da Costa et al., 1998; Santos and da Costa, 2002).

The accumulation of compatible solutes was initially associated with osmotic stress (Brown, 1976). However, a much more diversified range of activities has recently been attributed to some of these solutes (Santos and da Costa, 2002). Trehalose, for example, while showing the behaviour of a typical compatible solute in some organisms, is the classical example of the universal protector molecule (Elbein et al., 2003). Multiple roles in cellular physiology have been assigned to this disaccharide which has been demonstrated to protect cells and cellular components against the harmful effects of osmotic stress, desiccation, oxygen deprivation, chemical stresses such as copper and ethanol, heat, starvation, oxidative stress and freezing (Elbein et al., 2003; Silva et al., 2003). Di-*myo*-inositol-phosphate, a compatible solute produced by several hyperthermophilic prokaryotes, seems to be involved in the protection of cellular components against heat denaturation since it accumulates primarily at supraoptimal growth temperatures, and under *in vitro* conditions (Martins and Santos, 1995; Santos and da Costa, 2002).

2. Accumulation of Mannosylglycerate in Prokaryotes

Mannosylglycerate was identified in the thermophilic Bacteria *Thermus thermophilus* and *Rhodothermus marinus* in 1995 where it behaved as a compatible solute under salt stress (Nunes et al., 1995), although it had been discovered in red algae, in very low levels many years before (Bouveng et al., 1955). Later it was detected in the Archaea of the genera *Pyrococcus* (Martins and Santos, 1995), *Thermococcus* (Lamosa et al., 1998), *Aeropyrum* and in some strains of *Archaeoglobus* (Santos and da Costa, 2002). The accumulation of MG in these prokaryotes increased concomitantly with the salinity of the culture media behaving therefore as a compatible solute in osmoadaptation (Fig. 1). These Bacteria and Archaea share a thermophilic or hyperthermophilic lifestyle and for that reason, MG was believed to be an archetypal compatible solute of prokaryotes living near or at the highest growth temperatures for life, even though it is also present in some red algae where it is a minor compatible solute which may or may not be involved in osmotic adjustment (Karsten et al., 2003; Santos and da Costa, 2002). Recently, a gene for the synthesis of MG was identified in the mesophilic bacterium *Dehalococcoides ethenogenes* (Empadinhas et al., 2004). Moreover, additional MG genes have been identified in DNA fragments belonging to yet uncultured groups of Archaea, originating from forest soil and also from cold deep sea sediments (Hallam et al., 2004; Quaiser et al., 2002; Treusch et al., 2004). These data seem to argue against the previous assumption that MG is associated with (hyper)thermophilic prokaryotes. It is premature to bestow functions for MG other than osmotic protection; however, an additional role on the protection of enzymes against thermal denaturation was assigned to this solute (Borges et al., 2002; Ramos et al., 1997).

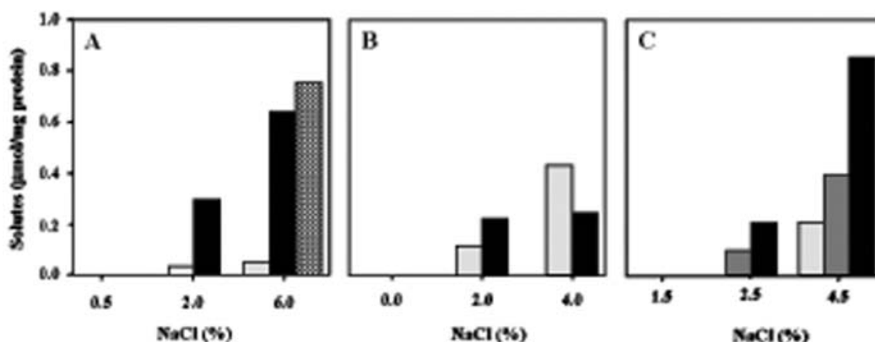

Figure 1. Effect of the NaCl concentration in the growth medium on the accumulation of compatible solutes: in (A) *R. marinus*, in (B) *T. thermophilus* HB-8 and in (C) *P. horikoshii*. ■, mannosylglycerate; ▨, mannosylglyceramide; □, trehalose; ■, di-*myo*-inositol-phosphate; □, glutamate.

Mannosylglycerate shows the typical behaviour of a compatible solute in the thermophilic and slightly halophilic bacterium *R. marinus*, which also accumulates low

levels of trehalose and glutamate, when the organism is grown at supraoptimal growth temperatures (Fig. 1) (Nunes et al., 1995). Interestingly, a MG derivative, mannosylglyceramide (MGA), accumulates in *R. marinus* under salt stress at, or below the optimum temperature for growth (Silva et al., 1999). This is the only organism, to date, where MGA has been found. In *T. thermophilus*, MG accumulates under low level salt stress, while trehalose is the major compatible solute at higher salt concentration (Nunes et al., 1995; Silva et al., 2003). In the Archaea *Pyrococcus horikoshii* and *P. furiosus*, *Thermococcus litoralis*, *T. celer* and *T. stetteri* MG is the major osmolyte, accumulating concomitantly with the increasing levels of NaCl in the growth medium (Empadinhas et al., 2001; Lamosa et al., 1998; Martins and Santos, 1995).

3. Biosynthesis of Mannosylglycerate

3.1. SINGLE-STEP PATHWAY

The first pathway found for MG synthesis involved a novel glycosyltransferase designated mannosylglycerate synthase (MGS) catalyzing the direct condensation of GDP-mannose and D-glycerate into MG (Fig. 2), and encoded by the gene *mgs* (Martins et al., 1999). The utilization of a non-phosphorylated acceptor is not unprecedented for sugar-derived molecules. Sucrose, for example, is directly synthesized in some organisms from an NDP-glucose and a non-phosphorylated fructose acceptor by a sucrose synthase (Lunn, 2002). However, reactions using non-phosphorylated acceptors are not a common feature among glycosyltransferases (Coutinho et al., 2003).

3.2. TWO-STEP PATHWAY

To date, MGS has only been found in the thermophilic bacterium *R. marinus* while most prokaryotes that synthesize MG use an alternative two-step pathway (Fig. 2). This two-step pathway is also present in *R. marinus* (Borges et al., 2004; Martins et al., 1999). The existence of two pathways for the synthesis of a compatible solute in *R. marinus* is not uncommon since trehalose is, for example, produced by two or even three pathways in several Bacteria (De Smet et al., 2000; Silva et al., 2003; Wolf et al., 2003), and two pathways exist for the synthesis of sucrose in higher plants, in some green algae and in cyanobacteria (Lunn, 2002). The existence of two pathways for the synthesis of MG by *R. marinus* reflects not only a higher flexibility for solute pool regulation, but the preference of a cell for a specific molecule. The implications of pathway multiplicity are not completely understood, but undeniably reflect one or several important physiological roles for those solutes in a cell (De Smet et al., 2000).

Mannosylglycerate is synthesized by all other prokaryotes through a pathway involving a phosphorylated intermediate, as is common for the synthesis of sugar-derived compatible solutes such as trehalose, glucosylglycerol, and sucrose (Elbein et al., 2003; Hagemann et al., 2001; Lunn, 2002). The two-step pathway for the synthesis of MG leads to the formation of mannosyl-3-phosphoglycerate (MPG), which is

synthesized from GDP-mannose and D-3-phosphoglycerate. MPG is then dephosphorylated to yield MG (Fig. 2).

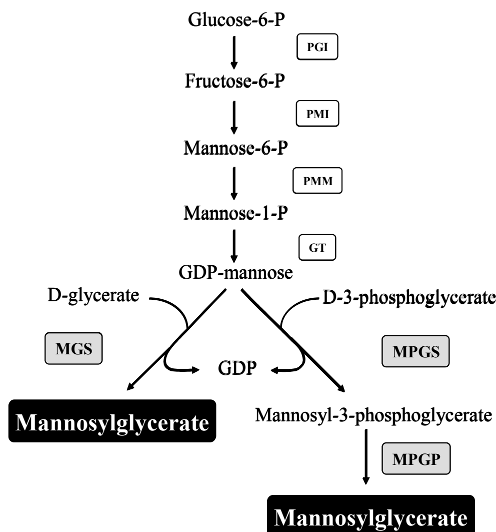

Figure 2. Pathways for the synthesis of MG in prokaryotes. MGS, mannosylglycerate synthase; MPGS, mannosyl-3-phosphoglycerate synthase; MPGP, mannosyl-3-phosphoglycerate phosphatase; PGI, phosphoglucoseisomerase; PMI, phosphomannoseisomerase; PMM, phosphomannosemutase; GT, mannose-1-phosphate guanylyltransferase.

This pathway involves mannosyl-3-phosphoglycerate synthase (MPGS) and mannosyl-3-phosphoglycerate phosphatase (MPGP), encoded by two consecutive genes in most organisms examined, designated *mpgs* and *mpgp*, respectively. However, different gene organizations have been encountered in some genomes (Fig. 3).

Two metagenomes of uncultured mesophilic crenarchaeotes derived from soil samples in Germany possess isolated *mpgs*-like genes (Quaiser et al., 2002; Treusch et al., 2004). Although these gene products have only been partially characterized, this finding foreshadows physiological and evolutionary novelties since, to date, the synthesis of MG has never been detected in mesophilic Archaea (Costa et al., unpublished). These sequences lack the contiguous *mpgp* gene encoding the enzyme that dephosphorylates MPG. However, *mpgp* may be located elsewhere in the genome or the dephosphorylation of MPG may be catalyzed by a different phosphatase. A similar organization was detected for the synthesis of glucosylglycerol in *Synechocystis* spp. PCC6803, where the genes *ggpS* and *stpA*, encoding the glucosylglycerol-phosphate synthase (GGPS) and glucosylglycerol-phosphate phosphatase, respectively, are under the control of individual promoters (Hagemann et al., 1997, 2001). It is also

possible that, in these organisms, MPG is an intermediate in the synthesis of an unknown low molecular weight compound or of a macromolecule.

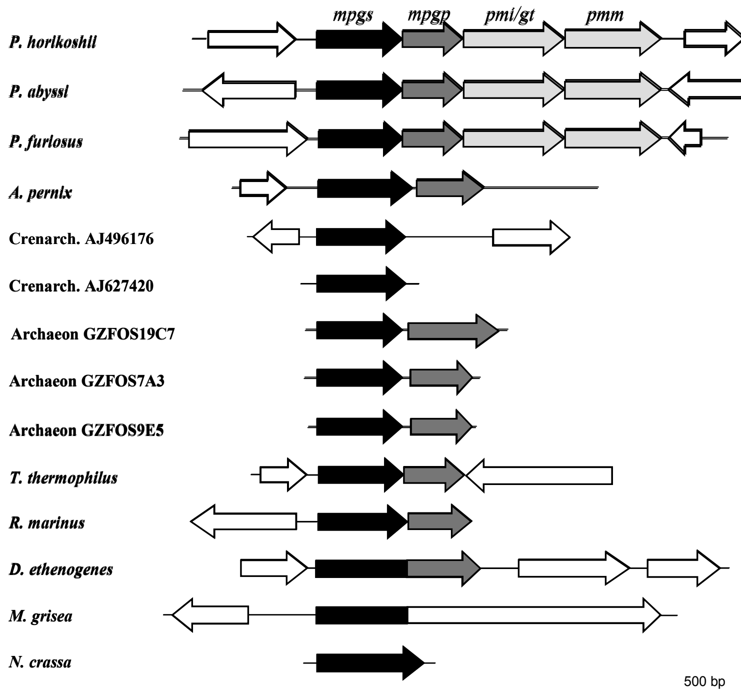

Figure 3. Organization of the genes for the synthesis of MG via the two-step pathway in different organisms. Black and dark grey arrows indicate *mpgs* and *mpgp* genes, respectively. Light grey arrows indicate genes for the synthesis of GDP-mannose. White arrows are unidentified genes. *mpgs*, mannosyl-3-phosphoglycerate synthase; *mpgp*, mannosyl-3-phosphoglycerate phosphatase; *pmi/gt*, phosphomannose isomerase/mannose-1-phosphate guanylyltransferase; *pmm*, phosphomannomutase.

More surprising was the *mpgs*-homologues found in the genomes of two fungi, *Magnaporthe grisea* and *Neurospora crassa* (Fig. 3). This was unexpected since, to date, there are no reports of MG in Eukarya other than red algae. The *M. grisea* MPGS showed unexpectedly higher similarity with *T. thermophilus* and *Pyrococcus* spp. sequences than with the *N. crassa* homologue. While in *N. crassa* the sequence was a single intronless gene, the *mpgs* gene of *M. grisea* corresponded to the first of ten exons encoding a 1718 amino acid protein (Fig. 3). The lack of an MPGP in these organisms could indicate that they do not synthesize MG. It is however possible that MPGP in this organism is highly divergent, that MPG could be hydrolyzed by a non-specific phosphatase or that MPG is involved in the synthesis of an unknown compound cannot be excluded. Moreover, MG has never been detected in these organisms which

accumulate polyols for osmotic adaptation (da Costa et al., 1998; Dixon et al., 1999). Nevertheless, the accumulation of MG by a recombinant *S. cerevisiae* expressing the *mpgs*-like exon from *M. grisea* provides irrefutable evidence for the true nature of the "gene" (Empadinhas et al., unpublished). Although MPG dephosphorylation *in vivo* could not be assigned to a specific *S. cerevisiae* enzyme, MG synthesis from the *mpgs*-like exon argues for a similar activity in the native organism.

The genes responsible for the synthesis of MG in *Pyrococcus* spp. are, like those in the bacterial counterparts, sequentially arranged in the genome. However, *mpgs* and *mpgp* are part of an operon-like structure which comprises two additional genes that lead to the synthesis of GDP-mannose from fructose-6-phosphate (Fig. 3) (Empadinhas et al., 2001). The significance of an operon-like organization of functionally related genes lies in an efficient and economically coordinated co-expression of the enzymes involved in this pathway (Xie et al., 2003).

Another organism using the *mpgs*/*mpgp* pathway for MG synthesis is *Aeropyrum pernix* and the genetic organization closely resembles that found in *T. thermophilus* and *R. marinus* (Fig. 3). However, the *A. pernix* genes are separated by an intergenic region of about 20 nucleotides and are the most divergent of all known *mpgs* and *mpgp* genes. Experimental evidence for the physiological relevance of this pathway is still missing, but the complete genome sequence of this MG-accumulating organism reveals no other candidate genes for MG synthesis (Kawarabayasi et al., 1999). A similar arrangement of *mpgs* and *mpgp* genes has been detected in archaeal DNA fragments isolated from deep-sea sediments (Hallam et al., 2004). Like the genetic organization in *A. pernix*, the *mpgs* and *mpgp* genes are separated by intergenic regions of about 30 to 60 nucleotides.

An unusual gene fusion between *mpgs* and *mpgp* was found in the genome of the mesophilic bacterium *Dehalococcoides ethenogenes* (Fig. 3), an organism, which is unrelated to any (hyper)thermophile (Empadinhas et al., 2004), and which grows so poorly that sufficient biomass has not been available to determine intracellular low molecular weight solutes. The presence of this fusion gene, designated *mgsD*, in *D. ethenogenes*, was the first hint that MG synthesis occurred in mesophilic prokaryotes. The fusion between genes encoding enzymes that catalyze consecutive steps in a specific pathway is not unknown. Cyanobacteria also possess sucrose-phosphate synthase/phosphatase gene fusions to produce sucrose (Lunn, 2002). The activity of the bifunctional mannosylglycerate synthase from *D. ethenogenes* (MGSD) was confirmed by its expression in *E. coli* and *S. cerevisiae*. The recombinant enzyme produced in *E. coli* had only a fraction of the specific activity of the (hyper)thermophilic monofunctional counterparts (Empadinhas et al., 2004), but the expression of *mgsD* in *S. cerevisiae* in an attempt to produce an enzyme with higher specific activity, led to the accumulation of MG by the recombinant yeast. This remarkable result showed that MGSD synthesizes MG *in vivo* and it argues for a role in the osmotic adaptation of *D. ethenogenes* to salt stress.

3.3. CATALYTIC PROPERTIES OF MPGS

Mannosyl-3-phosphoglycerate synthase (MPGS) is the key-enzyme for the synthesis of the phosphorylated intermediate in the two-step pathway for MG. The MPGSs from several organisms have been characterized in detail, to gain insights on the biosynthesis

of MG at different temperatures (Borges et al., 2004; Empadinhas et al., 2001, 2003, 2004). The biochemical and kinetic properties of MPGSs are summarized in Table 1, which includes for comparison, the properties of other sugar-phosphate synthases from bacterial sources, namely trehalose-6-phosphate synthase (TPS), sucrose-6-phosphate synthase (SPS) and glucosylglycerol-phosphate synthase (GGPS). All MPGSs showed identical substrate specificity, optimal pH, and comparable K_M values for the substrates GDP-mannose and D-3-phosphoglycerate.

TABLE 1. Biochemical properties and kinetic parameters of MPGS and other osmolyte-phosphate synthases

		MPGS				TPS	GGPS
		Archaea	Bacteria				
		Hyperthermophilic	Thermophilic		Mesophilic	Thermophilic	Mesophilic
Property		*P. horikoshii*[a]	*T.thermophilus*[b]	*R.marinus*[c]	*D.ethenogenes**[d]	*T.thermophilus*[e]	*Synechocystis* PCC6803[f]
# Amino acids		394	391	427	694	449	499
Mol.mass (kDa)		46	45	49	78	52	57
Opt. temp. (°C)		90-100	80-90	80	40-50	98	40
Opt. pH		~7.6	~7	~7.6	7	~6	7.5
K_m (mM)	GDPman	0.17	0.3	0.5	0.70	UDPglc: 0.5	ADPglc:0.24
	3PG	0.14	0.13	0.6	2.18	Glc6P: 0.8	Gly3P: 0.58
V_{max} (U/mg)		189	122	~15	~3	204	182.5**

*Bifunctional MPGS/MPGP. **Sp. activity expressed in nKat/mg protein. References: [a]Empadinhas et al., 2001; [b]Empadinhas et al., 2003; [c]Borges et al., 2004; [d]Empadinhas et al., 2004; [e]Silva et al., 2004; [f]Hagemann et al., 2001. GDPman, GDP-mannose; 3PG, D-3-phosphoglycerate; UDPglc, UDP-glucose; ADPglc, ADP-glucose; Glc6P, glucose-6-phosphat ; Gly3P, glycerol-3-phosphate.

As expected, the bifunctional enzyme from *D. ethenogenes* has a much lower activity range than the (hyper)thermophilic counterparts. Another major difference between the archaeal and the bacterial MPGS is related to the cation dependency: the bacterial MPGSs are absolutely dependent on the presence of divalent cations for activity, while the *P. horikoshii* MPGS, as well as other osmolyte-phosphate synthases like the TPS from *T. thermophilus* and the GGPS from *Synechocystis* PCC6803 only require cations for maximal activity (Curatti et al., 1998; Hagemann et al., 2001).

4. Evolution of Mannosylglycerate Biosynthesis

The accumulation of MG in response salt stress by unrelated (hyper)thermophilic Bacteria and Archaea, and mesophilic red algae, as well as the identification of genes distributed throughout the tree of life (Fig. 4), namely in *D. ethenogenes*, in the fungus *M. grisea*, and in uncultured Archaea from soil ecosystems and deep-sea sediments, raises questions about the evolution of MG synthesis in these organisms.

Mannosylglycerate synthesis could be an ancient characteristic retained in only scattered groups of Archaea, Bacteria and Eukarya (Santos and da Costa, 2002). However, the considerable sequence conservation among MPGSs, even among distant phylogenetic groups and across domains, calls for more insightful explanations for the biosynthesis of MG in such distantly related organisms.

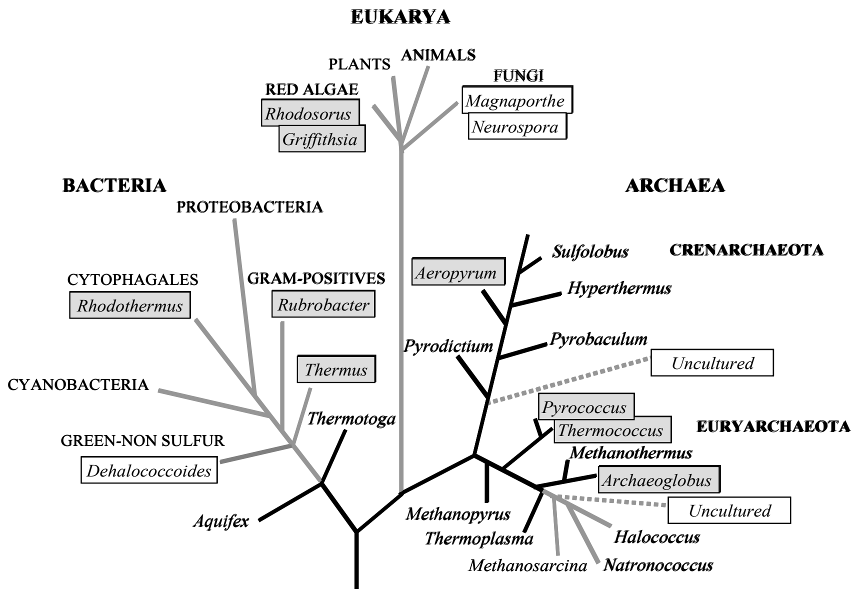

Figure 4. Schematic representation of the phylogenetic tree of life. Shaded genera are those where MG has been detected. Unshaded genera or metagenomes are those where *mpgs* genes have been identified. Dark lines indicate hyperthermophilic lineages.

The synthesis and accumulation of MG in response to salt stress primarily by (hyper)thermophiles could reflect its loss during evolution towards mesophiliy (if one assumes a hot origin for life) or the advantage of other compatible solutes over MG at low growth temperatures. We should, however, be cautious about the hypothetical loss of the ability to synthesize MG during evolution towards mesophily, because recent

findings indicate that the synthesis of MG at low temperatures might be much more prevalent than expected. Hyperthermophiles might have retained more ancestral characters, since their macromolecules have evolved more slowly because of structural constraints related to environmental adaptation (Matte-Tailliez et al., 2002). The prevalence of MG accumulation or the presence of genes for the synthesis of MG in euryarchaeotes and crenarchaeotes could be explained to have occurred primarily by vertical inheritance.

Lateral gene transfer (LGT) appears to be a common phenomenon (Aravind et al., 1998) that can occur even across domains. The presence of *mgsD* (*mpgs*/*mpgp*) in *D. ethenogenes* can been explained by LGT from an (hyper)thermophile (Empadinhas et al., 2004). This assumption has some supporting evidence, namely the codon and nucleotide usage, in *mgsD* and the presence of "Archaeal-like" genes surrounding this fused gene. However, the phylogenetic tree based on the deduced amino acid sequence fails to unambiguously confirm LGT for these genes because bacterial, archaeal and eukaryotic since MPGSs cluster within their respective domains (Fig. 5).

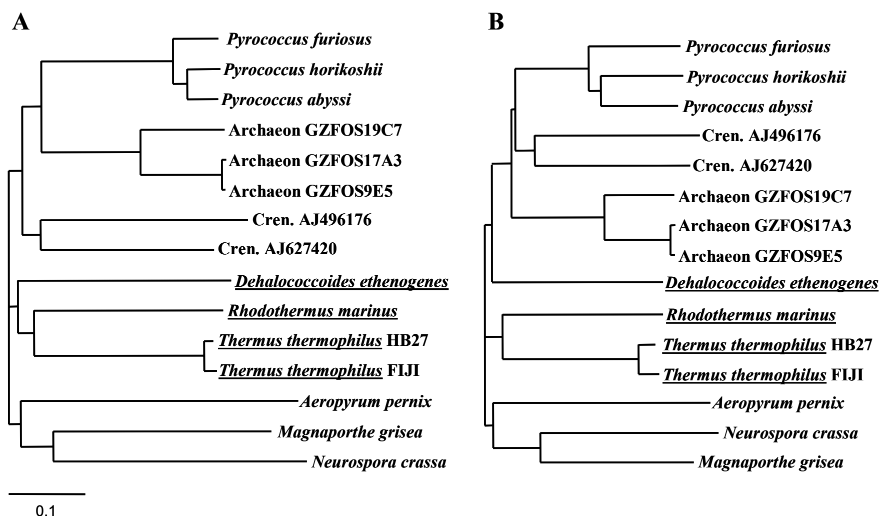

Figure 5. Phylogenetic trees based on available *mpgs* and MPGS sequences. A, protein sequences: B, gene sequences. Sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters. Bacterial representatives are underlined. Scale bar corresponds to 10 % estimated sequence divergence.

The phylogenetic analysis based on the nucleotide sequences indicates that the *D. ethenogenes* gene groups with the archaeal sequences. This clustering tends to support the LGT hypothesis from Archaea (Empadinhas et al., 2004). The implications of these

observations remain unclear and further studies with additional sequences will provide deeper understanding of the evolution of *mpgs*.

The presence of MG genes in *T. thermophilus* and *R. marinus* might be the result of gene transfers from Archaea, where genes for the synthesis of this compatible solute are more abundant, in shared environments (Aravind et al., 1998). The original transfer could have occurred to only one of those lineages, later between both or independently of each other. The slightly halophilic and thermophilic bacterium *Thermonema rossianum* is closely related to *R. marinus* (Tenreiro et al., 1997), but does not accumulate MG, nor does it appear to have the genes for the synthesis of MG (unpublished results), indicating that MG synthesis in *R. marinus* may have been acquired by LGT, unless we assume that these genes were present in a common ancestor but lost in *T. rossianum*.

The family *Thermaceae* comprises species of the genera *Meiothermus*, *Thermus*, *Vulcanithermus*, *Marinithermus* and *Oceanithermus* (da Costa et al., 2001; Miroshnichenko et al., 2003a, 2003b; Nobre and da Costa, 2001; Sako et al., 2003). The latter three genera are composed of four slightly halophilic species isolated from deep-sea hydrothermal vents. We examined the accumulation of compatible solutes in *V. mediatlanticus* but this organism does not accumulate MG and does not appear to possess the genes for the synthesis of this solute (unpublished results). The five species of *Meiothermus* do not grow in media containing more than 1% NaCl. Of the eight species of the genus *Thermus*, only the strains of *T. thermophilus* are halotolerant, accumulate trehalose and MG, and possess the genes for MG synthesis (Alarico et al., unpublished). We are, therefore, led to envision that *T. thermophilus* recently acquired the genes for the synthesis of MG by LGT.

The most striking finding concerning MG synthesis in Bacteria occurs in the ancient extremely radiation-resistant actinobacteria (high G+C Gram positive Bacteria) of the genus *Rubrobacter* (Ferreira et al., 1999). The thermophilic *R. xylanophilus* and the slightly thermophilic *R. radiotolerans*, accumulate MG in response to salt stress. Preliminary results indicate that MG is synthesized by a completely novel enzyme belonging to a new family of MPGSs (Empadinhas et al., unpublished). Moreover, no gene with high homology with known MG genes (*mpgs*, *mpgp* or *mgs*) was found in the recently sequenced genome of *R. xylanophilus*. This MPG-synthesizing enzyme may have evolved independently from the conventional homo-functional enzymes. Future studies will clarify whether a highly divergent MPGS or an analogous enzyme accounts for MG synthesis in *Rubrobacter* spp., casting additional interest in the origin and the evolutionary aspects of MG biosynthesis.

5. Concluding Remarks

Mannosylglycerate continues to be a rare compatible solute, but there is now evidence that it may be much more common than expected, in specific groups of Archaea, Bacteria and Eukarya. Until now MG has only been found in slightly halophilic or halotolerant organisms of marine origin and in this respect, mannosylglycerate like glutamate seems to be important in low level osmotic adaptation. However, in the

future, MG may be found to play a role in the osmotic adaptation of halophilic and extremely halophilic organisms.

The biotechnological potential of MG for the preservation of biological products will drive research into the properties of this compatible solute, but there is no doubt that mannosylglycerate, being found in hyperthermophiles, represents by itself, a very interesting molecule that deserves further study.

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Biodata of **Yuri Trotsenko**, author of ***"Genes and Enzymes of Ectoine Biosynthesis in the Haloalkaliphilic Obligate Methanotroph "Methylomicrobium alcaliphilum 20Z"***

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GENES AND ENZYMES OF ECTOINE BIOSYNTHESIS IN THE HALOALKALIPHILIC OBLIGATE METHANOTROPH "METHYLOMICROBIUM ALCALIPHILUM 20Z" *

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1. Introduction

Most halophilic and halotolerant bacteria maintain their internal osmolality and turgor by accumulation of organic compounds, the so-called compatible solutes. These vital compounds do not disturb cell metabolism even at high intracytoplasmic concentrations. Since its discovery by Galinski et al. (1985), ectoine (1,4.5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) was found to be a widely distributed compatible solute in halophilic/halotolerant phototrophic and aerobic chemoheterotrophic eubacteria representing a predominant class of organic osmolytes (Galinski, 1995; Grant, 2004; Severin et al., 1992; Ventosa et al., 1998).

Biosynthesis of ectoine has been recently investigated in several moderate halophiles. It was shown to proceed through the pathway that is common to ectoine, lysine, threonine, and methionine biosynthesis. In this pathway aspartate is initially converted into β-aspartyl phosphate by the action of aspartokinase (Fig. 1). Aspartate semialdehyde dehydrogenase then converts β-aspartyl phosphate to L-aspartate-β-semialdehyde, which may be funneled into the pathway specific for ectoine synthesis.

In all moderately halophilic bacteria investigated, the pathway for ectoine biosynthesis utilizes the products of the *ectA, ectB* and *ectC* genes as follows: L-aspartate-β-semialdehyde is converted to L-2,4-diaminobutyric acid (DABA) by the enzyme DABA transaminase encoded by the *ectB* gene. DABA is acetylated to N^{γ}–acetyl-L-2,4-DABA by the product of *ectA* gene, DABA acetyltransferase. Finally, ectoine is synthesized by ectoine synthase, the protein product of *ectC*. The genes coding for these enzymes have been characterized in several bacterial species and were shown to be organized in a three-gene cluster *ectABC*. Data obtained suggest that this pathway is evolutionary well conserved with respect to the genes and enzymes involved (Kuhlmann and Bremer, 2002).

* Dedicated to the 70th birthday of Professor Gerhard Gottschalk who inspired this study.

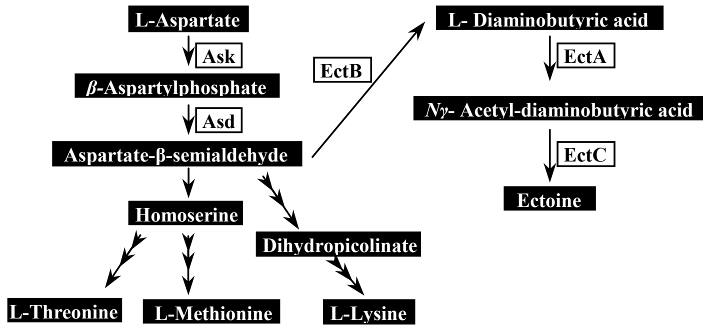

Figure 1. Biosynthetic pathway of ectoine. Ask – aspartokinase, Asd – aspartate-β-semialdehyde dehydrogenase, EctA - diaminobutyric acid acetyltransferase, EctB - diaminobutyric acid transaminase, EctC - ectoine synthase.

Obligate methanotrophs are a highly specialized group of bacteria utilizing methane as the carbon and energy source. A large number of different nonhalophilic neutrophilic methanotrophs have been isolated from soil and freshwater environments (Hanson and Hanson, 1996).

Halophilic, neutrophilic as well as alkaliphilic methanotrophic bacteria have been isolated from Siberian soda lakes, Crimean saline basins and marine sediments. These isolates were tentatively classified as several new species of the genus *Methylomicrobium* (Fuse et al., 1998; Kalyuzhnaya et al., 2001; Khmelenina et al., 1997; Sorokin et al., 2000). Also, two strains of halophilic methanotrophs belonging to the new genera *Methylosphaera* and *Methylohalobius* were recently described (Bowman et al., 1997; Heyer et al., 2005).

We have found that the ability of the halototerant methanotrophs "Methylomicrobium alcaliphilum 20Z", "M. modestohalophilum" and *M. buryatense* to grow at relatively high external salinity is due to de novo synthesis of low-molecular-weight organic solutes, mainly ectoine, sucrose and glutamate (Khmelenina et al., 1999, 2000; Trotsenko and Khmelenina, 2002a, 2002b). Here, we report the identification and sequencing of the genes as well as an initial characterization of some enzymes involved in ectoine biosynthesis in the haloalkaliphilic methanotroph "Methylomicrobium alcaliphilum 20Z".

2. Characterization of Ectoine Biosynthesis Enzymes and their Genes from "Methylomicrobium alcaliphilum 20Z"

"M. alcaliphilum 20Z" grown at different salinities accumulated ectoine, sucrose and glutamate. The total pool of the compatible solutes increased according to the external salinity (Fig. 2). Ectoine was found to be a major organic osmolyte, comprising about

60% of the total osmolyte pool in cells grown in the presence of 1.5 M NaCl (1470 nmol ectoine per mg of dry cell weight).

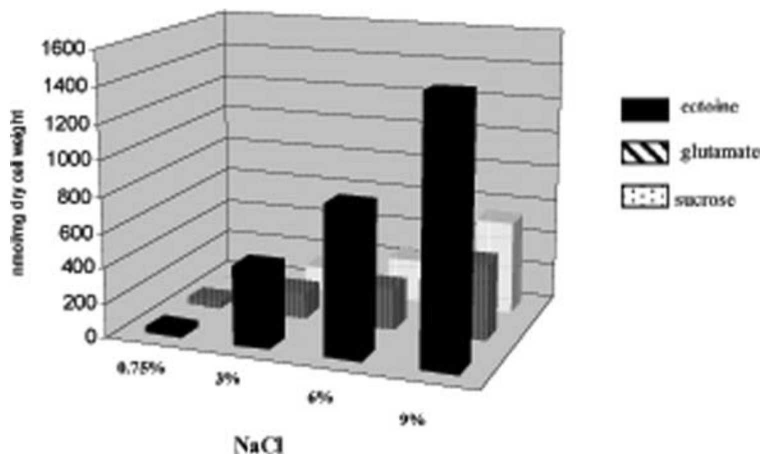

Figure 2. Intracellular solute pools of "M. alcaliphilum 20Z" as measured by analysis of ^{1}H-NMR spectra of methanol extracts of cells grown under methane at different salinities.

TABLE 1. Effect of NaCl in the growth medium on the activity (nmol min^{-1} mg protein^{-1}) of some enzymes involved in ectoine biosynthesis in *M. alcaliphilum* 20Z

Enzyme	- NaCl	+1 M NaCl
Aspartokinase	10	105
Aspartate-semialdehyde dehydrogenase	11	70
DABA transaminase	8	35
DABA acetyltransferase	16	250
Ectoine synthase	5	60

The enzymic analysis of *M. alcaliphilum* 20Z cell-free extracts revealed the activities of L-aspartokinase, L-aspartyl-β-semialdehyde dehydrogenase, DABA transaminase, DABA acetyltransferase and L-ectoine synthase. These activities were several times higher in cells grown in the saline medium (1 M NaCl) in comparison to those grown in the absence of added NaCl (Table 1), indicating their involvement in ectoine biosynthesis via the pathway proposed for *Halomonas elongata* and *Marinococcus halophilus* (Peters et al., 1990).

To identify the putative *ectABC* gene cluster from "M. alcaliphilum 20Z", a PCR strategy was used (Reshetnikov et al., 2004). The primers PCR were designed based on

the conservative motives within the Ect protein sequences published and present in the database. Analysis of the PCR-amplified sequences using the DNAStar package program and inverse PCR revealed the presence of four open reading frames (ORFs) oriented in the same direction (Fig. 3). These ORFs encoded proteins with predicted molecular mass of 18.8 kDa (172 amino acids, EctA), 47.8 kDa (443 amino acids, EctB), 15.2 kDa (134 amino acids, EctC) as well as 53.3 kDa (480 amino acids). The product of the fourth ORF showed a 50% of sequence identity to that of other microbial aspartokinases. Therefore, the four-gene cluster *ectABCask* for ectoine biosynthetic genes may be proposed for “M. alcaliphilum 20Z”.

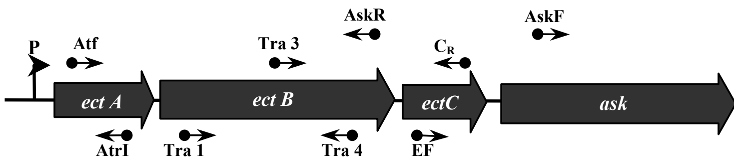

Figure. 3. Genetic organization of the ectoine gene cluster of *Methylomicrobium alcaliphilum* 20Z and position of the primers used for PCR. The degenerate oligonucleotide primers Atf, Tra 1, Tra 4, Tra3 and CR were designed on the basis of the searches of well-conserved amino acid sequences within the Ect proteins of *Marinococcus halophilus* DSM 20408, *Halomonas elongata* DSM 2581, *Sporosarcina pasteurii* and *Vibrio cholerae*. Primers AtrI, EF, AskR and AskF were homologous to the respective DNA fragments of “M. alcaliphilum 20Z” and were used for inverse PCR. A new incomplete ORF downstream to *ectC* was revealed. The product of the new ORF showed 50% sequence identity to microbial aspartokinases. *ask* – a putative gene of aspartokinase.

Phylogenetic analysis revealed a significant degree of sequence identities of EctA, EctB and EctC with the enzymes known to be involved in ectoine biosynthesis in halotolerant bacteria (Table 2): *Marinococcus halophilus* (Louis and Galinski, 1997), *Sporosarcina pasteurii* (Kuhlmann and Bremer, 2002), *Halomonas elongata* (Göller et al., 1998) and *Chromohalobacter salexigens* (formerly *H. elongata* DSM 3034) (Canovas et al., 1998). These database searches also revealed *ectABC* gene clusters in whole genomes of *Vibrio cholerae* (Heidelberg et al., 2000; Pflughoeft et al., 2003), *V. parahaemolyticus* (accession number AP005079) and *Oceanobacillus iheyensis* (AP004594), as well as the incomplete genome sequence of *Streptomyces coelicolor* (Al591322).

Among heterotrophic bacteria, *V. cholerae* showed the highest degree of amino acid sequence identity to “M. alcaliphilum 20Z” (44, 56 and 54% of amino acid sequence identities for EctA, EctB and EctC, respectively) (Fig. 4). Interestingly, database searches also showed the occurrence of a gene presumably encoding aspartokinase in the *V. cholerae* chromosome downstream of *ectABC* (50% of amino acid sequence identity). This halotolerant bacterium may therefore also contain the four-gene cluster for ectoine biosynthesis. Thus, variation in genetic organization of ectoine biosynthesis pathway may occur in various bacteria.

TABLE 2. Sequence identities (%) between the DABA acetyltransferase (EctA), DABA aminotransferase (EctB), ectoine synthase (EctC) proteins of *M. alcaliphilum* 20Z and other halophilic/tolerant bacteria. The BLAST network service was used as the database searches.

Bacteria	Protein		
	EctA	EctB	EctC
"M. alcaliphilum 20Z"	100	100	100
Methylomicrobium sp. AMO1	n.d.	89	95
Vibrio cholerae	44	56	54
Vibro parahaemolyticus	38	56	53
Chromohalobacter salexigens	41	56	49
Bacillus halodurans	36	55	50
Sporosarcina pasteurii	30	55	46
Marinococcus halophilus	39	54	46
Streptomyces coelicolor	32	54	43
Halomonas elongata	38	56	53
Oceanobacillus iheyensis	35	55	43

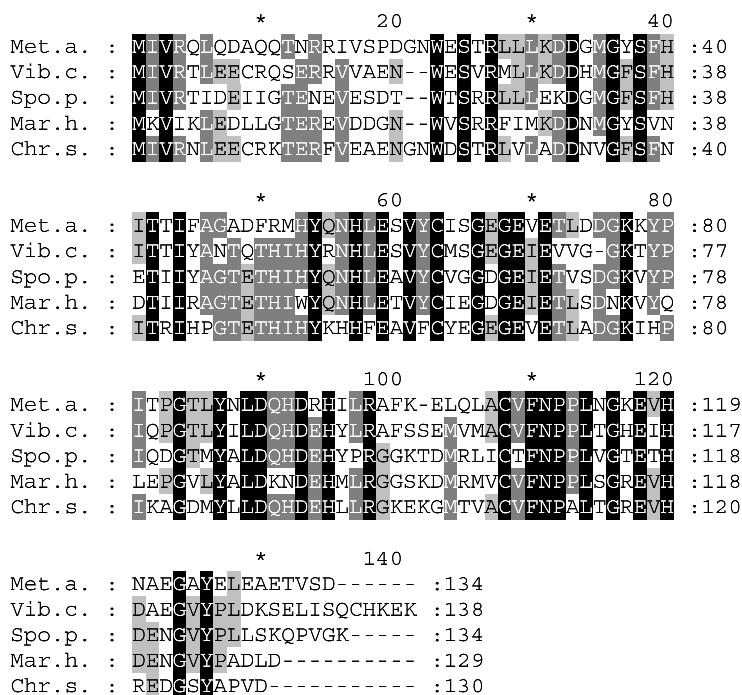

Figure 4. Alignment of the deduced amino acid sequence of reading frame *ectC* from "M. alcaliphilum 20Z" (Met.a.) with the ectoine synthase sequences of *Vibrio cholerae* (Vib.c.), *Sporosarcina pasteurii* (Spo.p.), *Marinococcus halophilus* (Mar.h.) and *Chromohalobacter salexigens* (Chr.s.).

The 2.6-kb DNA fragment from "M. alcaliphilum 20Z" carrying the *ectABC* genes with putative promoter region of 290 bp upstream of the *ectA,* was recovered by PCR and ligated into pHSG575 vector obtained from Dr. E.A. Galinski. Transformation of the resulting recombinant plasmid pHSG*ectABC* into *Escherichia coli* XL1-Blue cells, endowed this non-ectoine producer with the ability to synthesize ectoine. The recombinant strain of *E. coli* grew in minimal medium containing 5% NaCl and synthesized ectoine (425 μmol per g of dry cells) at a level comparable to that in *M. alcaliphilum* 20Z.

The *ectA*, *ectB* and *ectC* genes from chromosomal DNA of *M. alcaliphilum* 20Z were cloned into pET22b vectors and expressed in *E. coli*. Electrophoretically homogenous preparations of DABA transaminase corresponding to a hexamer by gel filtration (M = 300 kDa), dimeric DABA acetyltransferase (M_r = 40 kDa) and dimeric L-ectoine synthase (M_r = 32 kDa) were obtained. Consequently, *M. alcaliphilum* 20Z DABA transaminase showed a large molecular mass similar to that of the *H. elongata* enzyme (Ono et al., 1999).

By using metal-chelating affinity chromatography on Ni^{2+}-NTA-agarose, a homogenous preparation of EctA with six additional His residues on the C-end and having an activity of 200 U per mg of protein was obtained. The enzyme was shown to be stable and most active in the presence of 0.2 M NaCl or KCl. The other molecular properties of the "M. alcaliphilum 20Z" DABA acetyltransferase are presented in Table 3.

TABLE 3. Some molecular properties of recombinant DABA acetyltransferase from "Methylomicrobium alcaliphilum 20Z" and *Halomonas elongata* OUT30018.

Characteristics	*M. alcaliphilum* 20Z	*H. elongata* *OUT30018
Optimum pH	9.5	8.2
Optimum temperature	20 °C	20 °C
Molecular weight (SDS-PAGE)	20 kDa	nd
Molecular weight (gel-filtration)	40 kDa	45 kDa
$K_{m\ (DAB)}$	0.460 mM	nd
$K_{m\ (AcCoA)}$	0.0367 mM	nd
Inhibitors (1 mM)	Zn^{2+}, Cd^{2+}, ATP	nd
Optimum KCl	0.25 M	0.4 M
Optimum NaCl	0.1-0.2 M	0.4 M
Stability (during one month)	at 4 °C or -70 °C	Unstable

nd – not determined; * data from Ono et al., 1999

The results of our analysis of the substrate specifity of DABA acetyltransferase showed that the enzyme was highly specific for DABA and acetyl-CoA. ATP (1-10 mM) slightly inhibited the reaction *in vitro*, thus suggesting its possible involvement in the regulation of ectoine biosynthesis *in vivo*. At the same time, ADP, $NAD(P)^+$ and NAD(P)H did not influence the enzyme activity. Some common features of the enzyme from "M. alcaliphilum 20Z" and *H. elongata* were observed such as requirement for an alkaline pH (optimum 9.5 and 8.2, respectively), an optimum temperature of 20 °C and optimal activity in the presence of 0.2 to 0.4 M NaCl or KCl. The salt concentration

optimum for the enzyme activity is well correlated with the intracellular concentration of K^+ ions in “M. alcaliphilum 20Z” (0.2-0.4 M in cells growing in saline medium) (Khmelenina et al., 1999).

Hence, our results imply that although the ectoine biosynthesis pathway being evolutionary well conserved with respect to the genes and enzymes involved, some differences in their organization occur in various halophilic prokaryotes. Further work will focus on the regulatory aspects of ectoine biosynthesis in aerobic halophilic methanotrophs.

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HALOPHILIC ARCHAEA AND BACTERIA AS A SOURCE OF EXTRACELLULAR HYDROLYTIC ENZYMES

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1. Introduction

Halophiles constitute an important group of extremophilic microorganisms adapted to live and thrive in diverse hypersaline niches such as solar salterns, brines, hypersaline soils and lakes, as well as in salty foods (Horikoshi and Grant, 1998). Thanks to the application of novel phylogenetic and taxonomic approaches, the number of recognized species of halophilic microorganisms has increased tremendously during the past years (Kamekura, 1998; Madigan and Oren, 1999; Oren, 2002). The predominant groups of halophilic microorganisms inhabiting these hypersaline environments are moderately halophilic and extremely halophilic Bacteria and Archaea. The extreme halophiles show optimal growth in media containing 15 to 30% NaCl and are represented mainly by the halophilic aerobic Archaea or halobacteria (Grant et al., 2001), although some extremely halophilic Bacteria have been described as well, including some phototrophic species and the novel bacterium *Salinibacter ruber* (Antón et al., 2002). Moderately halophilic bacteria constitute a heterogeneous physiological group including a variety of Gram-positive and Gram-negative bacteria, which grow optimally in media containing 3 to 15% NaCl, although they can also grow above and below this range of salt concentrations (Ventosa et al., 1998).

Halophiles have developed two different adaptive strategies to cope with the osmotic pressure induced by the high NaCl concentration of the medium (Madigan and Oren, 1999; Oren, 2002). The halobacteria and some extremely halophilic Bacteria accumulate inorganic ions in the cytoplasm (K^+, Na^+, Cl^-) to balance the osmotic pressure of the medium, and they have developed specific proteins that are stable and active in the presence of salts. In contrast, moderate halophiles accumulate in the cytoplasm high amounts of specific organic osmolytes, which function as osmoprotectants, providing osmotic balance without interfering with the normal metabolism of the cell (Nieto and Vargas, 2002).

During the recent years, halophilic microorganisms have been explored for their biotechnological potential in different fields (Mellado and Ventosa, 2003). The applications range from the use of different products such as compatible solutes, biopolymers or carotenoids in a variety of industries or the use of these microorganisms in environmental bioremediation processes. Besides, being intrinsically stable and active at high salt concentrations, halophilic enzymes offer important potential

biotechnological applications, such as food processing, environmental bioremediation and biosynthetic processes.

To understand the molecular adaptation to extreme salinity of these enzymes is essential for the development of novel and more efficient protein engineering strategies. These molecular mechanisms have been most extensively studied in enzymes isolated from extremely halophilic Archaea (Madern et al., 2000; Mevarech et al., 2000). However, the enzymes from moderate halophiles present also a great biotechnological interest, since they are stable without salt and active in the presence of high salt concentrations (Ventosa et al., 1998).

The determination of the 3-D structure of some proteins from extreme halophiles has allowed the performance of comparative analyses between halophilic proteins and their non-halophilic homologs in order to understand the relationships between structure and particular biochemical and biophysical properties (Bieger et al., 2003; Dym et al., 1995; Frolow et al., 1996; Pieper et al., 1998; Richard et al., 2000; Yamada et al., 2002). Halophilic proteins, in general, present a significant excess of acidic amino acid residues over basic residues located mainly at the protein surface (Christian and Waltho, 1962; Danson and Hough, 1997; Ginzburg et al., 1970). These surface-exposed acidic residues have been proposed to help in organizing the solvation shell which surrounds the protein (Fukuchi et al., 2003).

The interest in these salt-adapted enzymes has increased considerably in the last few years, and a variety of extracellular enzymes from halophilic microorganisms have been isolated and characterized, aiming at expansion of their commercial applications. Moreover, the application of novel approaches to search for enzymes from natural environments without first isolating the organisms producing them is opening a new perspective for discovering industrial enzymes from hypersaline sources (Rees et al., 2003a, 2003b; Streit and Schmitz, 2004).

In this review, we will describe the properties of a number of these extracellular enzymes produced by halophilic microorganisms, and we will consider some aspects related to their potential biotechnological applications.

2. Halobacteria

Halobacteria (haloarchaea) constitute a well-defined group of Archaea. The main peculiarity of these microoorganisms is their ability to grow in hypersaline environments (>10% NaCl). They play an important role in the ecology of such extreme environments, representing and excellent example of adaptation to habitats with high salinities.

Halobacteria are included in the family Halobacteriaceae and at present are represented by more than 60 species grouped in 18 different genera: *Halobacterium*, *Haloarcula*, *Halobaculum*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halorhabdus*, *Halorubrum*, *Halosimplex*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas* and *Natronorubrum*.

From the non-halophilic group of Archaea, several extracellular enzymes have been isolated and characterized. However, due to the fact that the common protein separation methods are ineffective at molar salt concentrations, extracellular hydrolases from halophilic Archaea have been less investigated and not many have been purified,

characterized and their genes cloned. These enzymes function under extremely high salts conditions, and they often lose their activities at low ionic strength (Madern et al., 2000). The purification methods of halophilic proteases produced by halobacteria were reviewed by Kamekura (1995).

Several early studies demonstrated the production of extracellular enzymes by halobacteria, such as proteases (Norberg and Hofsten, 1969) or lipases (Gonzalez and Gutierrez, 1970). Good and Hartman (1970) described the properties of an amylase produced by *Halobacterium salinarum* (*halobium*). The optimal activity of the enzyme was determined at 1% NaCl, but it remained active up to 23% NaCl. Hydrolysis products from amylose were maltose, maltotriose and glucose. However, further studies concerning the purification of this enzyme were not carried out.

The first extracellular hydrolase that was purified from an extremely halophilic archaeon, *Halobacterium salinarum* (*halobium*), was a serine protease. The protein was characterized by the irreversible loss of activity at NaCl concentrations lower than 2 M. This protease consists of one polypeptide chain with a molecular mass of 41 kDa and characteristically enriched in the acidic amino acids aspartic acid and glutamic acid (Izotova et al., 1983).

Another early study was carried out by Oren (1983) who described an extracellular thermophilic amyloglucosidase that degraded starch to glucose, produced by *Halorubrum sodomense*, an archaeon isolated from the Dead Sea. This enzyme showed optimal activity at pH 7.5 and 65ºC in the presence of 1.4 M NaCl, and 75ºC in the presence of 3.9 M NaCl. Besides it required high salt concentrations for activity (at least 0.5 M NaCl and up to 4 M NaCl) (Oren, 1983). The enzyme was purified and it was determined that it was a dimer with two different subunits of 72 and 82 kDa, respectively (Chaga and Porath, 1993).

Of the three extracellular proteases produced by the archaeon *Natrialba asiatica* (formerly designated strain 172 P1), one was purified and studied in detail. This enzyme is a serine protease, named halolysin 172P1. The enzyme is a thermophilic and halophilic protease, showing optimal activity at 75-80ºC and 25% NaCl. Its optimal pH is 10.7. Its molecular mass was estimated as 44-460 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Kamekura and Seno, 1990). Further studies allowed the molecular cloning and sequencing of the gene encoding halolysin 172P1 (Kamekura et al., 1992). The deduced amino acid sequence showed that this halolysin consists of 411 amino acids and its molecular mass is 41,963 Da. The highest homology was found with a thermitase from *Thermoactinomyces vulgaris*. It is interesting to note that this halolysin has a long C-terminal extension of approximately 120 amino acids, which was not previously found in any other extracellular subtilisin type serine proteases. The construction of a shuttle vector permitted expression of the protease gene *hly* in another halophilic archaeon, *Haloferax volcanii* (Kamekura et al., 1992).

Species of the genus *Natronoccoccus* are haloalkaliphilic Archaea that require an alkaline pH as well as a high salt concentration for growth. Yu (1991) described the features of a protease produced by *Natronobacterium* sp. strain A2. The maximal activity of the purified enzyme was at pH 9.0, 50ºC and in the presence of 1 M NaCl. *Natronoccoccus* sp. strain Ah-36, lately named as *Natronococcus amylolyticus*, produced an extracellular amylase when grown in a medium containing starch. The addition of 0.1% glucose to the medium completely inhibited the production of the

enzyme (Kobayashi et al., 1992). This amylase was purified following several steps: ethanol precipitation, hydroxylapatite chromatography, hydrophobic chromatography and gel filtration using Sephacryl S-200. Its molecular mass, as estimated by SDS-PAGE, was 74 kDa. The amylase was active in the range of pH 6.0 to 8.6, with only 30% loss of activity at pH 10.5. The enzyme shows optimal activity at 55ºC and 2.5 M NaCl; no activity was detected below 1.0 M. The gene encoding this protein has been cloned; it is 1,512 bp long, and the encoded protein includes a signal peptide of 43 amino acids. The amylase produced by *Natronococcus* sp. shows 30% identical amino acids with other amylases. The gene has been expressed in *Haloferax volcanii* (Kobayashi et al., 1994).

A serine protease secreted by *Haloferax mediterranei* was studied by Stepanov and co-workers in 1992. Its activity increases linearly with NaCl concentrations over the range 2-5 M. The enzyme has a molecular mass of 41 kDa and a pI of 7.5. The N-terminal sequence revealed that it belongs to the subtilisin family. *Haloferax mediterranei* also produced the halolysin R4. The gene encoding this halophilic protein has been cloned and expressed in *Haloferax volcanii*. The deduced amino acid sequence (403 amino acids) showed the highest similarity to the halolysin 172P1 produced by *Natrialba asiatica*. It is remarkable that both halolysins have long C-terminal extensions of 117 and 123 amino acids. This region is essential for the proteinase activity, since the removal of this tail region from halolysin R4 abolished proteinase activity (Kamekura et al., 1996).

Ryu and co-workers (1994) isolated and partially purified an extracellular protease from the extreme halophile *Halobacterium salinarum* presenting a molecular mass of 66 kDa. A dramatic decrease of the enzyme activity was observed when the NaCl concentration was lowered from 4 to 0 M.

Studies of gene expression in haloarchaea have been greatly hindered by the lack of a convenient reporter gene. As a first step in the development of a reporter system for gene expression in halophilic Archaea, a β-galactosidase was purified from *Haloferax lucentense* (Holmes et al., 1997). The enzyme was optimally active at 4 M NaCl. It cleaves several β-galactosidase substrates such as ONP-Gal, X-Gal and lactulose. The entire β-galactosidase gene (designated *bgaH*) was cloned, corresponding to a protein of 74.6 kDa. This gene, *bgaH*, has been used as a reporter gene for promoter analyses in *Halobacterium salinarum* (Patenge et al., 2000).

An extracellular protease from *Natrialba magadii* was purified from stationary-phase cultures. The native molecular mass of the enzyme determined by gel filtration was 45 kDa. The enzyme was purified using ethanol precipitation, affinity chromatography (Bacitracin-Sepharose 4B) and gel filtration (Sephacryl S-200). The highest enzyme activity was obtained at 60ºC, but it was not stable under these conditions, so 45ºC was determined as the optimum temperature. Maximal protease activity was measured at concentrations of 1-1.5 M NaCl or KCl and at pH values between pH 8 and 10. This protease degraded large proteins such as gelatine and casein. The enzyme was activated by thiol-reducing agents such as DTT and 2-mercaptoethanol. However, it was inhibited by SDS, urea and guanidine-HCl (Giménez et al., 2000).

A preliminary characterization of secreted proteolytic enzymes occurring in the haloalkaliphile *Natronococcus occultus*, which lives in environments with 3-4 M NaCl and pH values of 10-11, was reported by Studdert and co-workers (1997). A serine

protease, designated EP, was purified from *N. occultus* stationary phase culture medium (328-fold, yield 19%), and characterized at the biochemical level (Studdert et al., 2001). The protease was purified using ethanol precipitation and a bacitracin-Sepharose 4B affinity column. The maximal azocaseinolytic activity was obtained at 1 M NaCl or KCl. The protease EP retained 100% of its activity for at least 7 days in 3 M NaCl or KCl at 4ºC; however, at concentrations lower than 0.5 M the enzyme loses its activity (Studdert et al., 2001).

An α-amylase produced by the halophilic archaeon *Haloferax mediterranei* has been purified and characterized (Pérez-Pomares et al., 2003). This microorganism is able to grow in a minimal medium containing ammonium acetate as carbon and nitrogen source. When the medium is enriched with starch, α-amylase is excreted. The enzyme is monomeric with a molecular mass of around 58 kDa, as determined by SDS-PAGE. The optimal salt concentration for activity was 3 M NaCl, and very close activities were also reached at higher salt concentrations. No activity was measured in the absence of salt; however, the presence of a NaCl concentration of 1 M was enough to observe activity. The stability increased at higher salt concentrations, reaching half-life times of 83 days at 4 M NaCl (Pérez-Pomares et al., 2003).

Two enzymes, β-xylanase and β-xylosidase, are produced when the extremely halophilic archaeon *Halorhabdus utahensis* was cultured aerobically at 30ºC. β-Xylanase activity was nearly equally stable at 0.05% NaCl and 27% NaCl at 30ºC, whereas β-xylosidase activity was equally stable at 0.5% NaCl and 25% NaCl (Wainø and Ingvorsen, 2003).

Table 1 summarizes the properties of the extracellular enzymes produced by halobacteria that have been studied and characterized in detail.

TABLE 1. Selected extracellular hydrolytic enzymes produced by extremely halophilic Archaea.

Enzyme	Microorganism	NaCl for optimal activity	Molecular mass (kDa)	Reference
Amylase	*Halobacterium salinarum*	1%	ND	Good and Hartman,1970
Serine protease	*Halobacterium salinarum*	-	ND	Izotova et al., 1983
Amyloglucosidase	*Halorubrum sodomense*	7.5%	72 82	Oren, 1983; Chaga and Porath, 1993
Serine protease	*Natrialba asiatica*	10-15%	41.9	Kamekura and Seno, 1990; Kamekura et al., 1992
Protease	*Natronobacterium* sp.	5,5%	ND	Yu, 1991
Amylase	*Natronococcus amylolyticus*	15%	74	Kobayashi et al., 1992, 1994
Protease	*Haloferax mediterranei*	-	41	Stepanov et al., 1992
Protease	*Halobacterium salinarum*	23%	ND	Ryu et al., 1994
β-galactosidase	*Haloferax lucentense*	23%	180	Holmes et al., 1997
Serine protease	*Natrialba magadii*	6-9%	45	Giménez et al., 2000
Serine protease	*Natronococcus occultus*	6%	130	Studdert et al., 2001
Amylase	*Haloferax mediterranei*	17%	58	Pérez-Pomares et al., 2003
β-Xylanase	*Halorhabdus utahensis*	5-15%	45	Wainø and Ingvorsen, 2003
β-Xylosidase	*Halorhabdus utahensis*	5%	67	Wainø and Ingvorsen, 2003

ND, not determined.

3. Moderately Halophilic Bacteria

Several authors have previously reviewed the production of extracellular enzymes by moderately halophilic bacteria (Kamekura, 1986; Mellado et al., 2004; Sánchez-Porro et al., 2004; Ventosa et al., 1998). In this review we will follow a chronological order and describe the main features of the currently reported extracellular hydrolases produced by moderately halophilic bacteria.

The pioneering studies on extracellular enzymes produced by moderately halophilic bacteria were carried out by Onishi and Kamekura in Japan. They were intrigued by the fact that enzymes from such bacteria showed activity in media with high salt concentrations and that most of them were inactive in the absence of salt (Kushner, 1968). During the early studies in the 1970s several extracellular enzymes were described, although they were not studied at the molecular level.

Onishi (1972) reported the production of a halophilic amylase by a moderately halophilic bacterium obtained from unrefined salt, designated as *Micrococcus* sp. 28-3 (ATCC 21727). A more detailed taxonomic study showed that this bacterium constituted a new species, and it was described as *Micrococcus halobius* (Onishi and Kamekura, 1972), and lately was transferred to a new genus as *Nesterenkonia halobia* (Stackebrandt et al., 1995). This organism was able to grow optimally in complex media with 1 to 3 M NaCl, but it did not grow without added NaCl. The highest amount of amylase was produced in media containing starch with 1 to 2 M NaCl after incubation for 2 days. Amylase production was markedly repressed by the addition of glucose. Maximal activity of the crude enzyme was observed at pH 6 to 7 in 1.4 to 2 M NaCl or KCl at 50ºC; in the absence of high concentrations of NaCl or KCl, the enzyme was denatured. It was depended on a divalent metal ion such as Ca^{2+} for activity and stability (Onishi, 1972). Several years later this amylase was purified from culture filtrate to an electrophoretically homogeneous state by glycogen-complex formation, diethylaminoethyl-cellulose chromatography and Bio-Gel P-200 gel filtration (Onishi and Sonoda, 1979). The purified enzyme was maximally active at pH 6 to 7 in 0.25 M NaCl or 0.75 M KCl at 50 to 55ºC. The activity was lost by dialysis against distilled water. The molecular mass of the amylase was estimated to be 89 kDa. Hydrolysis of amylose, soluble starch, and glycogen produced maltose, maltotriose and maltotetraose, with lesser amount of glucose (Onishi and Sonoda, 1979).

Another interesting early study was carried out by Kamekura and Onishi (1974a), who described the properties of a halophilic nuclease (nuclease H) produced by the moderately halophilic bacterium *Micrococcus varians* subsp. *halophilus*, isolated from soy sauce mash. Similarly to *Nesterenkonia halobia*, this organism was unable to grow without NaCl and grew optimally in media with 1 to 3 M NaCl. Maximum nuclease production was obtained between 2 and 4 M NaCl. It is interesting to note that this enzyme was the first hydrolase produced by a moderately halophilic bacterium that was purified biochemically. The enzyme showed both deoxyribonuclease and ribonuclease activities, but phosphomonoesterase and phosphodiesterase activities were not detected. The nuclease activity of the purified enzyme was maximal at 2.9 M NaCl or 2.1 M KCl, pH 8.0 and 43ºC. The enzyme was inactivated by dialysis against low salt buffer. The molecular mass of the enzyme was 99 kDa and it had a high excess of acidic amino acids over basic amino acids (Kamekura and Onishi, 1978a). The production of the extracellular nuclease was completely inhibited by addition of more than 40 mM

$MgSO_4$ to a complex medium with 2 M NaCl. This inhibition of enzyme production was accompanied by flocculation of the cells (Kamekura and Onishi, 1976), and the extracellular enzymes produced were fully adsorbed on the surface of the flocculated cells (Kamekura and Onishi, 1978b).

A simple medium for the commercial production of nuclease H in a fermentor has been described (Kamekura and Onishi, 1979). The medium is composed of 0.7% ammonium sulfate, 1% glucose, minerals, three vitamins, and 2 M NaCl at a pH of 7.5-8.0. Since this enzyme degraded RNA and DNA to produce 5′-mononucleotides exonucleolytically, it was suggested that it could be used for the production of 5'-guanylic acid (5′-GMP) and 5′-inosinic acid (5′-IMP), flavouring agents which are produced commercially by enzymatic degradation of RNA or direct fermentation. Kamekura et al. (1982) reported the production of 5′-GMP from RNA extracted from commercial dry yeast, by using supernatants from broth cultures of *M. varians* subsp. *halophilus*. Kamekura and Onishi (1983) examined the effects of anions and cations on nuclease H production and the stability of the enzyme in the presence of different salts. Based on these studies, a bioreactor with a column of flocculated cells of *M. varians* subsp. *halophilus* was designed for the production of 5′-nucleotides from RNA, based on immobilization of the nuclease H on the surface (Onishi et al., 1988, 1991).

A second extracellular enzyme produced by *M. varians* subsp. *halophilus*, a halophilic 5′-nucleotidase, has been partially purified (Onishi et al., 1984). This enzyme showed maximal activity at 2 M NaCl or 2.5 M KCl and 0.1 mM Co^{2+} or 0.1 mM Mn^{2+}. Finally, an amylase produced by this moderately halophilic bacterium has been purified and characterized (Kobayashi et al., 1986). The production of this amylase was highest in medium with 2 M NaCl with maltose as an inducer. The enzyme had two components with molecular masses of 86 and 60 kDa, and showed optimal activity at pH 6-7, 55ºC (in absence of $CaCl_2$) or 60ºC (with 50 mM $CaCl_2$) and 0.75 to 1 M NaCl or KCl (Kobayashi et al., 1986).

Other early studies focused on the production of a protease by the moderately halophilic *Bacillus* sp. no. 21-1 isolated from unrefined salt (Kamekura and Onishi, 1974b) and of an amylase produced by *Acinetobacter* sp. isolated from sea sand (Onishi and Hidaka, 1978). *Bacillus* sp. no. 21-1 grew optimally in media with 1-2 M NaCl, while the production of the enzyme was optimal at 1 M NaCl; addition of 2 M KCl markedly depressed protease formation. Maximal enzyme activity was obtained at 0.5 M NaCl and 0.75 M KCl, and almost no activity was observed at 3 M NaCl (Kamekura and Onishi, 1974b). No further studies concerning the purification and properties of this protease have been reported. Two different amylases were purified from the culture filtrate of *Acinetobacter* sp. to an electrophoretically homogeneous state by glycogen-complex formation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. Both amylases showed maximal activity in 0.2 to 0.6 M NaCl or KCl, at pH 7.0 and 50-55ºC. The end products of starch hydrolysis were maltose and maltotriose (Onishi and Hidaka, 1978).

The first extracellular protease that was studied at the biochemical level was produced by an unidentified moderately halophilic bacterium, designated *Pseudomonas* sp. strain A-14. The purified enzyme had a molecular mass of 12,000 Da and showed optimal activity at 18% NaCl and pH 8.0 (Van Qua et al., 1981).

Onishi and coworkers (1983) reported the production of another extracellular nuclease by a moderate halophile, *Bacillus* sp. N23-2 (ATCC 49085), isolated from

rotting wood on the seashore in Nauru. This bacterium was lately classified as a new *Bacillus* species, *B. halophilus* (Ventosa et al., 1989). Nuclease was produced when cultivated aerobically in media containing 1 to 2 M NaCl. The enzyme was purified from the culture filtrate to an electrophoretically homogeneous state by ethanol precipitation, DEAE-Sephadex A-50 column chromatography, and Sephadex G-200 gel filtration. The purified enzyme consisted of two isomers of the same molecular mass (138 kDa), but with different charges, and showed both RNase and DNase activities. Its maximal activity was in the presence of 1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl. The activity was lost by dialysis against water and low-salt buffer, but it was protected when 10 mM Ca^{2+} was added to the dialysis buffer. Besides, the enzyme exhibited maximal activity at pH 8.5 and at 50ºC on DNA and at 60ºC on RNA. It attacked RNA and DNA exonucleolytically, producing 5´-mononucleotides (Onishi et al., 1983). This nuclease shows similar characteristics to the nuclease H produced by *Micrococcus varians* subsp. *halophilus* (Kamekura and Onishi, 1974a, 1978a), especially with respect to halophilic properties and the mode of action on RNA and DNA; however, the *B. halophilus* nuclease required 3.5 M NaCl or 10 mM Ca^{2+} for stability and Mg^{2+} and Ca^{2+} (10:1) for maximal activity, whereas the nuclease H produced by *M. varians* subsp. *halophilus* required 2 M NaCl, 10 mM Mg^{2+}, or Mn^{2+}, but not Ca^{2+}, for stability (Kamekura and Onishi, 1978a; Onishi et al., 1983).

Khire (1994) described the production of an halophilic amylase by *Micrococcus* sp. strain 4 that showed optimal activity at pH 7.5, 50ºC and 1 M NaCl.

Coronado and coworkers (2000a; 2000b) studied an extracellular amylase designated as amylase H, produced by *Halomonas meridiana* DSM 5425. Maximal amylase production was achieved toward the end of the exponential phase in a medium with 0.5% starch and 5% total salts, in the absence of glucose. Activity was optimal at 10% NaCl, pH 7.0 (being also relatively stable under alkaline conditions) and at 37ºC. However, considerable amylase activity was detected even at 30% salts. The amylase hydrolyzed starch to form maltose and maltotriose as major products; maltose was not hydrolyzed. These data indicated an α-amylase activity (Coronado et al., 2000a). The gene encoding this amylase, *amyH*, was cloned by functional complementation of a *Tn1732*-induced mutant deficient in extracellular amylase activity. It encodes a 457-amino acid residue protein with a deduced molecular mass of 50 kDa which shows high sequence similarity to α-amylases from Gram-positive and Gram-negative bacteria. Besides, the amyH protein contains the four regions highly conserved in amylases. *H. meridiana* amylase H is a very acidic protein, similar to many other enzymes produced by halophilic microorganisms (Coronado et al., 2000b).

The study of Coronado et al. (2000b) was the first to clone a gene encoding for an extracellular enzyme produced by a moderate halophile. The *amyH* gene was found to be functional in another moderately halophilic bacterium, *Halomonas elongata*, and, when cloned in a multicopy vector, also in *Escherichia coli.* This is thus the first enzyme from a moderately halophilic bacterium that has been cloned and expressed in a non-halophilic host, opening new possibilities from a biotechnological point of view. Coronado and coworkers also achieved the heterologous expression of the *Bacillus licheniformis* thermostable α-amylase gene in two moderately halophilic bacteria: *Halomonas meridiana* and *Halomonas elongata*. This indicated that the secretion machinery of both halophiles was able to recognize and properly cleave the signal

peptide of the *Bacillus* amylase, and to secrete the enzyme to the extracellular medium (Coronado et al., 2000b).

An interesting study has been reported on the cloning, sequencing and expression of an α-amylase gene, *amyA*, from the thermophilic, moderately halophilic, anaerobic bacterium *Halothermothrix orenii* (Mijts and Patel, 2002). The gene encoded a 515 residue protein composed of a 25 amino acid putative signal peptide and a 490 amino acid mature protein. Greatest homology was to the *Bacillus megaterium* group of α-amylases. The *amyA* gene was expressed in *E. coli* as a hexahistidine-tagged enzyme and was purified and biochemically characterized. The purified recombinant enzyme was optimally active in 5% NaCl (showing a significant activity up to 25% NaCl) at pH 7.5 and 65ºC. It required NaCl and $CaCl_2$ for optimum activity and thermostability. However, the relative high proportion of acidic amino acids typically observed for many enzymes from halophiles was absent in this enzyme (Mijts and Patel, 2002). This amylase was the first extracellular enzyme produced by a moderately halophilic bacterium that has been studied crystallographically. The recombinant amyA protein crystallizes in the orthorhombic space (Li et al., 2002). A second α-amylase was found to be produced by *H. orenii*, and its structure has been determined by X-ray crystallography (Tan et al., 2003). The amylase amyB is a 599-residue protein, and is active up to 10% NaCl. The purified recombinant amyB protein crystallizes in the monoclinic space (Tan et al., 2003).

Recently, another extracellular amylase has been described, produced by the moderately halophilic *Halobacillus* sp. strain MA-2, isolated from saline soil in Iran (Amoozegar et al., 2003a). This organism has been proposed as a new species and named *Halobacillus karajensis* (Amoozegar et al., 2003b). Maximum amylase production was exhibited on starch media when 15% Na_2SO_4 or 10% NaCl were added. Optimal amylase activity was obtained in medium containing 5% NaCl at pH 7.5-8.5 and 50ºC (Amoozegar et al., 2003a). Further characterization of this enzyme at the molecular level has not yet been reported.

In order to isolate moderately halophilic bacteria producing extracellular hydrolytic enzymes, a screening was carried out in different hypersaline environments in the south of Spain. A total of 122 moderately halophilic bacteria were isolated. In contrast to culture collection strains that belong to previously described species that show very low hydrolase activities, environmental isolates produced a great variety of hydrolases such as amylases, DNases, lipases, proteases and pullulanases. These strains were identified as members of the genera: *Salinivibrio*, *Halomonas*, *Chromohalobacter*, *Bacillus-Salibacillus*, *Salinicoccus* and *Marinococcus* (Sánchez-Porro et al., 2003a).

A protease, designated as protease CP1, produced by *Pseudoalteromonas ruthenica* CP76, isolated in the screening, has been purified and characterized, and the gene encoding the protein has been cloned and expressed. This enzyme showed optimal activity at 55ºC and pH 8.5, and tolerated a wide range of NaCl concentrations (0 to 4 M NaCl). The most interesting features of this enzyme are its moderate thermoactivity (optimal activity at 55ºC), its activity over a wide range of pH values (6-10), and, specially, its salt tolerance (optimal activity at 7.5% total salt). The protease was purified using Q-Sepharose column chromatography and Superdex S-200 gel filtration. The purified protease has a molecular mass of 38 kDa by SDS-PAGE (Sánchez-Porro et al., 2003b). The gene has been recently cloned by inverse PCR, and shows a 69%

similarity to metalloprotease I of *Alteromonas* sp. O-7 (Sánchez-Porro et al., unpublished results).

Another enzyme under study that was selected during the preliminary screening is an extracellular lipase, designated SL1, produced by an isolate that was classified as a new species of the genus *Marinobacter*, *M. lipolyticus* (Martín et al., 2003). Different methods of purification have been used in order to purify this enzyme. Best results were obtained using octyl gel as hydrophobic support, but purification is as yet incomplete. We have constructed a gene library that allowed us to isolate the gene encoding this enzyme. This gene shows high similarity with α/β-hydrolase genes (Martín et al., unpublished results). These two extracellular enzymes are still under study; the currently available data suggest that they could have future biotechnological applications.

Table 2 includes the features of the extracellular enzymes produced by moderately halophilic bacteria that have been investigated up to date.

TABLE 2. Extracellular enzymes produced by moderately halophilic microorganisms with potential biotechnological applications.

Enzyme	Microorganism	NaCl for optimal activity	Molecular mass (kDa)	References
Amylase	*Nesterenkonia halobia*	0.25 M	89	Onishi,1972; Onishi and Sonoda, 1979
Nuclease H	*Micrococcus varians* subsp. *halophilus*	2.9 M	99	Kamekura and Onishi, 1974a, 1978a
5´-Nucleotidase	*Micrococcus varians* subsp. *halophilus*	2 M	ND	Onishi et al., 1984
Amylase	*Micrococcus varians* subsp. *halophilus*	0.75-1 M	86 60	Kabayashi et al., 1986
Protease	*Bacillus* sp.	0.5 M	ND	Kamekura and Onishi, 1974b
Amylase	*Acinetobacter* sp.	0.2-0.6 M	55 65	Onishi and Hidaka, 1978
Protease	*Pseudomonas* sp.	18%	12	Van Qua et al., 1981
Nuclease	*Bacillus halophilus*	1.4-3.2 M	138	Onishi et al., 1983
Amylase	*Micrococcus* sp.	1 M	ND	Khire, 1994
Amylase H	*Halomonas meridiana*	10%	50	Coronado et al., 2000a, 2000b
Amylase A	*Halothermothrix orenii*	5%	ND	Mijts and Patel, 2002
Amylase	*Halobacillus karajensis*	5 %	ND	Amoozegar et al., 2003a
Serine protease	*Pseudoalteromonas ruthenica*	10%		Sánchez-Porro et al., 2003b
Lipase	*Marinobacter lipolyticus*	ND	ND	Martín et al., 2003; This study

ND, not determined.

4. Uncultured Microorganisms - Enzymes from Environmental DNA Libraries

A large fraction of the microorganisms present in the environment has not been cultured due to difficulties in enriching and isolating them, and these are not accessible for biotechnology or basic research. It is estimated that less than 1% of the prokaryotic species have been cultivated and characterized (Amann et al., 1995). A new technology

called "metagenomics" has been developed trying to overcome this bottleneck in the biotechnological industry. Metagenomics is a new field which tries to assess and exploit the complex genomes of microorganisms found in nature (Knietsch et al., 2003; Streit and Schmitz 2004). The technique is based on the isolation and purification of DNA directly from environmental samples, followed by the construction of DNA libraries in suitable cloning vectors and host strains (Streit and Schmitz, 2004). This approach has yielded an increasing number of DNA sequences which can be used for novel biotechnological and pharmaceutical applications. A variety of enzyme classes have been isolated from cloned environmental DNA including hydrolases such as lipases, proteases, amylases, cellulases and others; nevertheless the list of enzyme activities studied by this method is still rather small (Healy et al., 1995; Henne et al., 2000; Richardson et al., 2002). The classical methodology to isolate new enzymes by enrichment and screening of a wide variety of microorganisms for the desired activity is still currently widely used, although the biodiversity of an enrichment culture will not be as rich as the original environmental source because of the nonculturability of many organisms (Rees et al., 2003b).

The biomass that can be retrieved from environmental samples is generally low, and large amounts of samples are needed to obtain sufficient DNA for cloning (Béjà et al., 2000). *E. coli* is still the preferred host for cloning and expression of any metagenome-derived genes, although other Gram-negative host are being used as well. Recently, large clone libraries have been screened and hundreds of thousands of clones have been analyzed to detect active ones (Henne et al., 2000; Majernik et al., 2001). It is expected that in the future the number of genes identified by this technique will exceed the number of those identified by the classical approach.

Genomic DNA libraries were made from DNA isolated directly from the Kenyan soda lakes, Lake Elmenteita and Crater Lake. Crater Lake clones expressing a cellulase activity and Lake Elmentetia clones expressing a lipase/esterase activity were identified and sequenced (Rees et al., 2003a). More recently, two genomic DNA libraries were made from enrichments obtained from lake sediment and soda soil obtained from the extremely saline and alkaline lakes of the Wadi el Natrun in the Libyan Desert (Egypt). A novel cellulase activity was identified in this environment and characterized. The lake cellulase ORF encoded a protein of 1,118 amino acids. It showed a high similarity with other bacterial endoglucanases, being most similar to a glucanase from *Xanthomonas campestris*. The soda soil cellulase was most closely related to an endo-glucanase from *Fibrobacter succinogenes* (Grant et al., 2004). It is noteworthy that the enrichment cultures obtained in this study showed presence of a wide diversity of species, often most closely related to as yet uncultured microorganisms. This could demonstrate that DNA from enrichment cultures, as well as DNA extracted directly from environmental samples, can be a valuable source of novel enzymes and biologically active compounds that would be very important for future biotechnological applications (Grant et al., 2004).

5. Summary and Future Perspectives

In this chapter we reviewed the properties of a number of extracellular enzymes produced by extremely halophilic Archaea and moderately halophilic Bacteria. These

include amylases, proteases, nucleases, lipases, etc. Besides, new enzymes from genomic DNA obtained from environmental samples have also been reported. In contrast to other extremophilic microorganisms such as the thermophiles or hyperthermophiles, the number of studies on extracellular enzymes from halophiles is very small. Very few enzymes have been studied at the molecular level, and only two have been crystallized and studied by X-ray crystallography. In the future we must expect more extensive studies focused on the biochemical and molecular features of new enzymes produced by halophiles, as well as on other enzymes that have not been previously reported such as xylanases, cellulases or pullulanases that could show novel features with respect to those produced by non-halophilic microorganisms. The extensive data on the complete genomes of microorganisms will help to understand and compare the features of these enzymes.

One interesting aspect that requires special attention is the study of the molecular mechanisms that enable the adaptation of moderately halophilic microorganisms which are able to grow in hypersaline environments as well as in media with low salt concentrations and the adaptation of their enzymes to function in a wide range of salt concentrations. The biotechnological application of enzymes from halophiles would require an in-depth study concerning their features, activity and stability under extreme conditions. Further molecular studies should enable their cloning and expression in non-halophilic hosts. Our studies showed that genes encoding enzymes from moderately halophilic bacteria can be successfully cloned and expressed in *E. coli.* Besides, enzymes from other microorganisms have been also expressed in halophiles (Coronado et al., 2000b; Frillingos et al., 2000). One important aspect that will require extensive studies and that constitutes a bottleneck is the secretion of the enzymes to the extracellular medium at high rates as to warrant an important production level.

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BIOPOLYESTER PRODUCTION: HALOPHILIC MICROORGANISMS AS AN ATTRACTIVE SOURCE

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1. Introduction

The modern society has become highly dependent on the use of plastics in various forms ranging from commodity products like packing material to finer materials such as surgical implants. These plastics are often synthetic polymers that are derived from petroleum e.g. polyethylene, polypropylene, and polyvinylchloride. The major problem associated with the use of commodity plastics is that much of it is discarded as waste on landfills once they are no longer in use. These materials persist in the environment without getting degraded, thus giving rise to a variety of ecological and environmental problems. In order to reduce the amount of plastic waste, programs for recycling of used-plastic materials have been started world-wide. On the other hand, there has also been a great interest in biodegradable polymers with material properties similar to those of conventional synthetic plastics. Among such polymers are the biopolyesters that can be obtained by biotechnological synthesis from renewable raw materials.

Biopolyesters, also known as polyhydroxyalkanoates (PHAs), are accumulated intracellularly by microorganisms, usually under nutrient deficient conditions and excess carbon source (Lee, 1996a; Steinbüchel and Füchtenbush, 1998). PHAs can be synthesized from renewable carbon sources derived from agriculture products or industrial wastes (Lee, 1996a). In addition, PHAs with different properties can be produced and can be commercially exploited in various areas such us packing films, containers and paper coatings (Reddy et al., 2003). Moreover applications such as biodegradable carriers for drugs, hormones, insecticides and herbicides have also been suggested (Reddy et al., 2003; Zinn et al., 2001). The commercial production of PHAs has so far been achieved by microbial fermentations, although the development of transgenic PHA accumulating plants has reached significant progress and its industrialization may also be feasible (Valentin et al., 1999).

The present chapter provides an overview of the area of biopolyesters – including the *in vivo* synthesis of poly(3-hydroxybutyrate) (PHB) - the most studied PHA, the physiological implications of PHB in microorganisms, and the characteristics of other

different kinds of polyesters synthesized by microorganisms. Finally, production of PHA by halophiles is described.

2. Poly(3HB) Accumulation and Function in Microorganisms

The accumulation of PHB was first reported in the Gram-positive bacterium *Bacillus megaterium* (Lemoigne, 1926, 1927). Subsequently, PHB was also found in Gram-negative bacteria (Forsyth et al., 1958), and is now known to be a common reserve material in several bacterial species (Steinbüchel and Füchtenbush, 1998) as well as in Archaea. The latter group includes primarily extreme halophiles (Fernandez-Castillo et al., 1986; Hezayen et al., 2000).

The role of PHB in microorganisms has attracted the attention of scientists since its discovery. In early reports it was considered to be an intracellular reserve carbon and energy source (Doudoroff and Stanier, 1959). Subsequently, PHB accumulation was further suggested to be a survival strategy in most microorganisms (Anderson and Dawes, 1990). Under conditions of nutrient deficiency, the accumulated PHB can retard the degradation of cellular components such as RNA and proteins without significantly affecting the osmotic pressure in various cells (Anderson and Dawes, 1990).

As PHB is frequently synthesized during the stationary phase of growth, it can also have various physiological roles, depending upon the bacterial species. One example of the utilization of the energy stored in PHB is the formation of cysts, resting cells resistant to deleterious agents, in *Azotobacter vinelandii* (Stevenson and Socolofsky, 1966). In *Bacillus* species, PHB accumulation is directly related to spore formation and also prevents the generation of acidic products of cell metabolism (Slepecky and Law, 1960). Yet PHB seems to be involved in the symbiotic nitrogen fixation carried out between bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium* and leguminous plants, whereby the biopolymer is an alternative source of electrons under oxygen deficient conditions (Anderson and Dawes, 1990; Karr et al., 1984; McDermott et al., 1989).

3. Synthesis and Characteristics of Biopolyesters

3.1. BIOLOGICAL PATHWAYS AND ENZYMOLOGY OF PHB SYNTHESIS

Regardless of the type of microorganism, PHB synthesis has acetyl-CoA as starting point followed by three or more steps catalyzed by different enzymes. As shown in Fig. 1 (Pathway I), the first step is catalyzed by 3-keto-thiolase (EC 2.3.1.9), which reversibly links two acetyl-CoA moieties to acetoacetyl-CoA. In the second step the conversion of acetoacetyl-CoA into D-(-)-3-hydroxbutyryl-CoA can be mediated by a NADPH dependent reductase, and the polymerization is accomplished in the last step by a PHB synthase (Anderson and Dawes, 1990; Steinbüchel and Füchtenbush, 1998). In *Rhodospirillum rubrum*, *Methylobacterium rhodesianum* and *Aeromonas punctata* a modification to this pathway (Pathway II) occurs - including the formation of L-(+)-3-hydroxybutyryl-CoA by a NADPH-dependent acetoacetyl-CoA reductase and

conversion of the L-(+)stereoiosomer to the R-(-)-stereoisomer by enoyl-CoA hydratases (Fig. 1) (Doi et al., 1995; Steinbüchel and Füchtenbush, 1998; Steinbüchel et al., 1995).

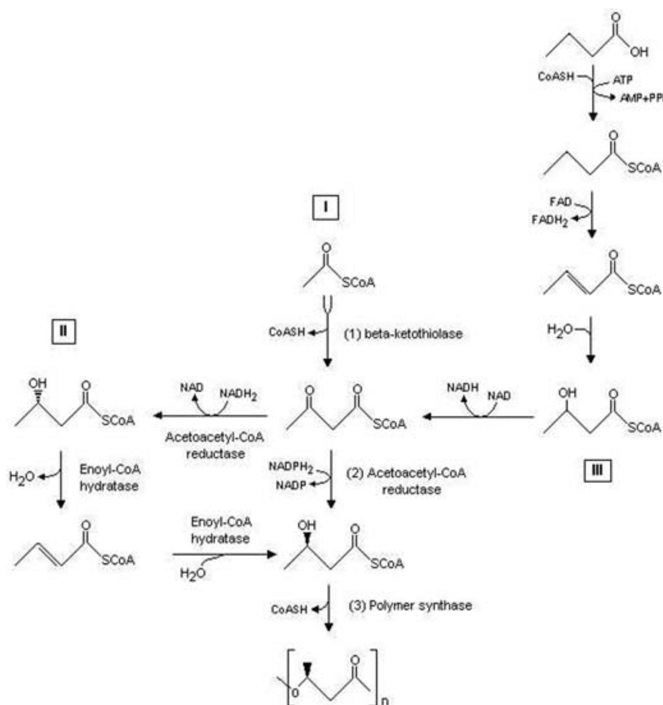

Figure 1. Pathways for the biosynthesis of PHB: [I] from acetyl CoA; [II] by an alternative pathway to [I] using steps catalyzed by via enoyl-CoA hydratases; [III] from butyric acid via acetoacetyl CoA and continuation of pathway [I]. The scheme was adapted from previous reports (Braunegg et al., 1998; Steinbüchel and Füchtenbush, 1998).

The metabolic pathways I, II depicted in Fig. 1 imply that the synthesis of PHB depends on the level of the cellular assimilation of the precursor to be converted into acetyl-CoA. Thus carbohydrates, lipids and fatty acids are, in general, the most utilized sources for PHB production (Lee, 1996a), although methanol, carbon dioxide, and methane can also serve as substrates (Nishioka et al., 2001; Suzuki et al., 1986; Wendlandt et al., 2001). An exception, however, is found when butyric acid is supplied in culture media of *Wautersia eutropha* leading to a third pathway (Doi et al., 1988) (Fig. 1). The incorporation of butyric acid in PHB does not require the decomposition of its structure into acetyl-CoA (Fig. 1) (Doi et al., 1988), thus avoiding the utilization of β-ketotiolase by the organism. The biosynthesis of other polyesters in natural isolates, recombinant bacteria and in higher organisms, is reviewed in detail by Madison and Huisman (1999).

3.2. DIFFERENT PHAs PRODUCED BY MICROORGANISMS

The composition of the PHA produced, and thus its physical properties, depend both on the microorganism and carbon source used. The molecular masses of polymers are in the range of $2x10^5$ to $3x10^6$ dalton (Lee 1996a). More than 100 different monomer units have been detected as constituents of PHAs in various bacteria (Lee 1996a, 1996b). Among these are the 3-hydroxyalkanoates (3-HAs) of 3-14 carbon atoms with a large variety of saturated or unsaturated and straight or branched chains containing aliphatic or aromatic side groups (Steinbüchel and Valentin, 1995). Short chain length (scl) PHAs consist of 3-5 carbon atoms, and medium chain length (mcl) PHAs consist of 6-14 carbon atoms. Although not frequently encountered, blends of short-chain- and medium-chain-length PHAs are also known to occur in some organisms (Kato et al., 1996; Ramsay et al., 1992).

The ability of microorganisms to synthesize a particular form of PHA is mainly due to the substrate specificity of PHA synthases that can only accept 3-hydroxyalkanoates (3-HAs) of a certain range of carbon lengths (Madison and Huisman, 1999). For instance the enzyme allows the formation of a homopolymer of 3-hydroxyvalerate in *Chromobacterium violaceum* from valeric acid (Steinbüchel et al., 1993) or the utilization of C_2 to C_5 substrates in *Wautersia eutropha* to produce a copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [poly(3HB-*co*-3HV)] (Doi et al., 1988; Haywood et al., 1988). The latter was indeed the first biopolyester found with a chemical structure different from that of PHB (Wallen and Rohwedder, 1974).

PHB and poly(3HB-*co*-3HV) have become the most studied short-chain-length PHAs. Several bacteria can join 3HV units to PHB from propionic or valeric acid (Anderson and Dawes, 1990), resulting in copolymers with up to 99 mol% 3HV (Valentin and Dennis, 1996). Nevertheless due to its toxicity, propionic acid at elevated concentrations can hinder the PHA production. As a result, poly(3HB-*co*-3HV) containing only 20 mol% 3HV has been produced in large scale (Kim and Lenz, 2001). On the other hand, *Rhodococcus* species are among the few examples able to produce PHAs containing 3HV in the absence of the typical 3HV precursors (Doi et al., 1987; Kim and Lenz, 2001; Valentin and Dennis, 1996). Moreover terpolymers with 5-hydroxyvalerate poly(3HB-*co*-3HV-*co*-5HV) (Doi et al., 1987) and with 4-hydroxybutyrate poly(3HB-*co*-4HB-*co*-3HV) (Kunioka et al., 1988) were also synthesized, depending on the carbon source supplied in the medium.

3.3. SPECIAL FEATURES OF POLYHYDROXYALKANOATES

PHAs possess the common features of being non-toxic, biocompatible, biodegradable thermoplastics that can be synthesized from renewable resources (Lee, 1996a; Reddy et al., 2003). They also have a high degree of polymerization, are optically active, piezoelectric and insoluble in water. These characteristics confer to PHA an unquestionable advantage over polypropylene or other petroleum derived plastics (Reddy et al., 2003). However, the main drawback to the industrialization of PHAs is the high costs involved in the production and purification of the polymers.

PHB is a right-handed helix with a two-fold screw axis and a melting point near 180 °C, similar to that of polypropylene (Anderson and Dawes, 1990). Furthermore, PHB and polypropylene display similar degrees of crystallinity (Table 1), but PHB can

be more easily dissolved in non-polar organic solvents, and is more brittle and less flexible than polypropylene (Anderson and Dawes, 1990; Steinbüchel and Füchtenbush, 1998).

Physical properties of scl-PHAs and mcl-PHAs are distinct; while the former have a high degree of crystallinity and are brittle and stiff, the latter are elastomers with low crystallinity and low glass transition temperature, and have been drawing much interest because of their flexible properties for a wide range of applications (Holmes, 1988; Preusting et al., 1990). Table 1 shows the effect of modification of PHB with different monomers on the polymer properties. Increase in the content of higher monomer size results in decrease in crystallinity and melting points that in turn leads to a decrease in rigidity and increase in toughness of the polymer, which are more desirable properties for applications. Certainly, the synthesis of new types of bioplastics with superior features to those of petroleum derived polymers would turn PHAs as a highly competitive product group. Nonetheless the yields of the polymers in organisms researched so far have yet to be improved (Lee, 1996b). The maximum polymer contents have been attained in a few bacterial species, the most studied being *Wautersia eutropha* (Lee, 1996b). An adequate PHA production is possible to be attained by both genetic modifications of the polymer synthases (Taguchi and Doi, 2004) and/or bacterial species able to assimilate various carbon sources under simple culture conditions.

TABLE 1. Comparison of thermal and mechanical properties of different PHAs with polypropylene. Data were derived from Doi et al. (1995, 1990), Scandola et al. (1990), and Tsuge (2002).

Polyester	Melting temperature (°C)	Crystallinity (%)	Tensile strength (MPa)	Extension to break (%)
Poly(3HB)	177	60	43	5
Poly(3HB-*co*-20 mol% 3HV)	145	56	20	50
Poly(3HB-*co*-3 mol% 4HB)	177	60	28	45
Poly(3HB-*co*-16 mol% 4HB)	150	45	26	444
Poly(3HB-*co*-10 mol% 3HHx)	127	34	21	400
Poly(3HB-*co*-17 mol% 3HHx)	120	26	20	850
Polypropylene	176	50-70	38	400

4. PHB Production by Extremely Halophilic Archaea

PHB accumulation by extremely halophilic Archaea was reported for the first time in the species *Haloarcula marismortui* and *Haloferax mediterranei* (Fernandez-Castillo et al., 1986; Kirk and Ginzburg, 1972). Although some other extremely halophilic organisms are also able to produce PHB (Fernandez-Castillo et al., 1986), *Hfx.*

mediterranei accumulates high amounts of the polymer - about 65% of the cell dry weight from starch or glucose, with optimum salts concentration of 25% (w/v) and under phosphate limitation conditions in batch cultivations, and up to 46% of PHB in continuous cultivations (Lillo and Rodriguez-Valera, 1990; Rodriguez-Valera and Lillo, 1992). The high level of polymer accumulation and cell density of *Hfx. mediterranei* in a chemostat might indicate production of PHB to be associated with cell growth. PHA production in continuous cultivation is not feasible with majority of microorganisms since they accumulate PHAs during their stationary phase of growth (Section 2) (Babel et al., 2001). Two examples of non-halophiles able to support growth-associated PHB production are a mutant strain of *Azotobacter vinelandii* and an *Alcaligenes latus* strain that has a deficiency in substrate assimilation (Babel et al., 2001).

Besides having the advantage of producing high amounts of PHB from a cheap carbon source, *Hfx. mediterranei*, like most members of the family *Halobacteriaceae*, can be easily lysed in presence of water, implying a simple procedure for recovery of the polymer (Rodriguez-Valera and Lillo, 1992). This species is also able to produce the copolymer poly(3HB-*co*-3HV) from starch without an additional precursor (Rodriguez-Valera and Lillo, 1992), a characteristic not usually found in microorganisms. The high salt concentration required for optimal polymer production allows the cultivation to be performed under conditions that are not strictly sterile. On the other hand, the large amounts of salt required for cultivations could present a drawback since the cost of the salts can approximately equal the price of the carbon source (Rodriguez-Valera and Lillo, 1992) and can further accelerate the corrosion of the commonly used stainless steel fermentors (Chisti, 1992; Park et al., 2002). Also *Hfx. mediterranei* produces exopolysaccharides associated with the PHB synthesis, that could both interfere with the purification of the polyester and hinder the fermentation process due to the high viscosity at high concentrations of the polysaccharide.

More recently, an extremely halophilic archaeon designated strain 56 was also reported to produce PHB with excess carbon sources and reduced amounts of yeast extract in the medium (Hezayen et al., 2000). When cultivated in a corrosion resistant bioreactor with butyric acid and sodium acetate as carbon source, the organism could store up to 53 % PHB of its cell dry weight. However the time period for PHB accumulation was as long as 11 days, which led to a considerably reduced volumetric productivity of the polymer (Hezayen et al., 2000).

PHB synthase of strain 56 was further characterized (Hezayen et al., 2002), and like some enzymes produced by members of the family *Halobacteriaceae*, it showed a high thermostability, but its residual activity remained invariable in the presence or absence of NaCl (Hezayen et al., 2002). However, unlike polymer synthases from non-halophilic bacteria, strain 56 enzyme possessed a very narrow substrate specificity and was not even able to incorporate 3-hydroxyvaleryl in the polymer (Hezayen et al., 2002).

5. Poly(3HB) Accumulation in Moderately Halophilic Bacteria

So far, polymer production by moderately halophilic Bacteria has been reported to be in the form of biosurfactants and exopolysaccharides (Margesin and Schinner, 2001;

Ventosa et al., 1998). During the taxonomic characterization of various members of the family *Halomonadaceae* it was found that several of them produce PHA (Mata et al., 2002). Table 2 presents some of the *Halomonas* spp. known to produce PHA, their capability to utilize various carbohydrates and growth characteristics.

TABLE 2. Culture conditions and carbon source utilization by some of type strains of the genus *Halomonas* able to accumulate PHA. Strains: 1, *H. boliviensis* DSM 15516^T; 2, *H. aquamarina* ATCC 14400^T; 3, *H. variabilis* DSM 3051^T; 4, *H. meridiana* DSM 4225^T; 5, *H. cupida* CECT 5001^T; 6, *H. elongata* CECT 4279^T; 7, *H. pantelleriensis* DSM 9661^T; 8, *H. eurihalina* ATCC 49336^T; 9, *H. salina* CECT 5288^T; 10, *H. ventosae* DSM 15911^T. Data were derived from previous taxonomic descriptions of the bacterial species by Martinez-Cánovas et al. (2004), Mata et al. (2004), and Quillaguamán et al. (2004c).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Growth:										
Optimum salt %(w/v)	5	8	10	1-3	8	3-8	10	7.5	5	3-9
Temperature range °C	0-45	15-37	15-37	4-45	15-37	4-45	10-45	4-45	4-45	6-10
pH range	6-11	5-10	6-9	5-10	5-10	5-10	6-11	5-10	5-10	15-50
Carbon source utilization:										
Starch	-	+	-	-	+	-	+	-	-	-
Fructose	+	-	+	-	+	+	-	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	-	+	-	+	-	+	+	+	ND
D-Xylose	+	-	-	-	-	+	-	+	+	ND
Cellobiose	-	-	+	-	-	+	+	+	+	-
Maltose	+	-	+	+	+	-	+	+	+	+
Lactose	-	+	+	-	-	+	-	+	-	ND
Acetate	+	+	+	-	-	+	-	+	+	+
Propionic acid	ND	+	+	-	-	+	-	+	-	+

Only 10 of the more than 30 type species of the genus *Halomonas* are shown in the table. Nevertheless the ability of these species to utilize different carbon sources originating from agricultural residues or by-products of food industry is evident, substrates that can potentially serve as raw material for PHA production.

5.1. PHB PRODUCTION BY *HALOMONAS BOLIVIENSIS*

Halomonas boliviensis is a moderate halophile that has been isolated from the shores of an hypersaline lake located in Bolivia (Quillaguamán et al., 2004c). This bacterium grows optimally with 5% NaCl in the culture medium, in a wide range of pH (6-11)

and temperature (0-45°C), and is able to grow on various carbohydrates as carbon source (Quillaguamán et al., 2004c).

We have originally noted that *H. boliviensis* starts accumulating PHB when yeast extract is the limiting nutrient in the medium and in the presence of excess of carbon source comprising a mixture of butyric acid and sodium acetate (Quillaguamán et al., 2004a). Studies on the influence of NaCl concentration in the culture medium on PHB accumulation done in shake flasks showed that variation in the salt concentration does not have appreciable effect on the final PHB yield (53 wt% with respect to the cell dry weight). However, at higher concentrations (10-15% w/v) the initiation of PHB synthesis is delayed and cell growth is drastically affected (Quillaguamán et al., 2004a). When *H. boliviensis* is cultivated in a fermentor under controlled conditions, a final polymer content of 88 wt% is reached in 30 hours. This is similar to the highest yields reported so far in organisms such as *Alcaligenes latus*, *Wautersia eutropha*, *Azotobacter vinelandii*, pseudomonads, and recombinant *Escherichia coli* strains, few of the strains regarded as having potential industrial applications (Lee, 1996a; Steinbüchel and Füchtenbush, 1998). Some other carbon sources such us glucose, sucrose, and the combination of sucrose and sodium acetate also serve as PHB precursors. In all cases, *H. boliviensis* cells stored about 53 wt% PHB (Quillaguamán et al., 2004a).

The organism is, however, unable to grow with starch as sole carbon source. Hence, starch was partially hydrolyzed using a recombinant maltooligosaccharide forming amylase from the halotolerant bacterium *Bacillus halodurans* LBK 34 (Quillaguamán et al., 2004b), and the starch hydrolysate was supplemented to the culture medium of *H. boliviensis* as the carbon source for the production of PHB. Use of such a hydrolysate showed that the organism uses preferentially maltose for PHB formation. It is also able to hydrolyze higher sugars if no other simpler carbon source is available but with significantly lower yields of the polymer. Figure 2 depicts a common profile of PHB accumulation found under such cultivation conditions. The versatility of *H. boliviensis* in the selection of the carbon source might hence provide an attractive alternative for the utilization of starch-derived resources.

Although PHA production has only recently been studied in *H. boliviensis*, other members of the family *Halomonadaceae* could become an attractive group of microorganisms to be investigated for PHA production. The moderate salt concentrations required for the optimal growth of these microorganisms are enough to inhibit the development of non-halophiles, hence allowing cultivations under relatively non-sterile conditions. Moreover, the drawbacks associated with cultivation of extreme halophiles, i.e. high costs and corrosion of bioreactors, are considerably reduced.

6. Concluding Remarks

It is by now established that biopolyesters constitute interesting “green” alternatives to some of the petrochemistry based plastics. Although many microbes are capable of producing these bioplastics, much can be achieved by searching for an optimal producer of the polymer. In this line, the halophilic microorganisms constitute an important group to investigate further.

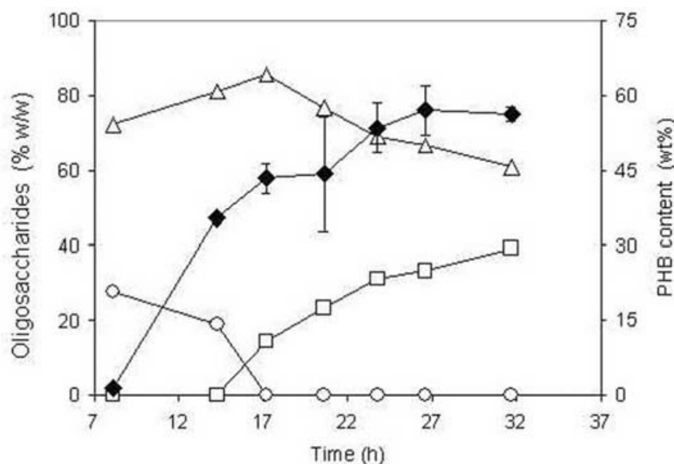

Figure 2. Profiles of PHB and oligosaccharide levels during cultivation of *H. boliviensis* LC1 in shake flasks using starch hydrolysate as carbon source. The concentration of recombinant *B. halodurans* amylase used for starch hydrolysis for 4 h was 6.4 U ml^{-1}. Symbols: (◆), PHB content wt% and (△) G4; (□) G3; (○) G2 % (w/w) of the total sugars in the medium.

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Section IV. Fungi

Relation of halotolerance to human pathogenicity in the fungal tree of life: an overview of ecology and evolution under stress
G. Sybren de Hoog, Polona Zalar, Bert Gerrits van den Ende and Nina Gunde-Cimerman

Halotolerant and halophilic fungi from coastal environments in the Arctics
Nina Gunde-Cimerman, Lorena Butinar, Silva Sonjak, Martina Turk, Viktor Uršič, Polona Zalar and Ana Plemenitaš

Halotolerant and halophilic fungi and their extrolite production
Jens C. Frisvad

Introducing *Debaryomyces hansenii*, a salt-loving yeast
José Ramos

Cellular responses in the halophilic black yeast *Hortaea werneckii* to high environmental salinity
Ana Plemenitaš and Nina Gunde-Cimerman

Halotolerance of lichen symbioses
Martin Grube and Juliane Blaha

Biodata of **Sybren de Hoog**, author of *"**Relation of Halotolerance to Human-Pathogenicity in the Fungal Tree of Life: An Overview of Ecology and Evolution under Stress**"*

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RELATION OF HALOTOLERANCE TO HUMAN-PATHOGENICITY IN THE FUNGAL TREE OF LIFE: AN OVERVIEW OF ECOLOGY AND EVOLUTION UNDER STRESS

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1. Introduction

It is a general belief in mycology that fungi growing on substrates with low water activity display a general xerophylic phenotype (Northolt et al., 1995), determined primarily by the water potential of the medium rather than by the chemical nature of the solute (Hocking, 1993; Pitt and Hocking, 1997). Therefore fungi are considered xerophilic if they grow well at water activity (a_w) of 0.85 or less, corresponding to 17% NaCl or 50% glucose added to the growth medium. In contrast to obligate halophilic bacteria, only a few species of food-borne fungi (*Basipetospora halophila*, *Polypaecilum pisce* and *Hortaea werneckii*) were known to be stimulated by NaCl but without any obligate requirement (Andrews and Pitt, 1987; Pitt and Hocking, 1997). Therefore Pitt and Hocking (1997) concluded that there was no evidence of halophily in fungi. This conviction remained unchallenged, until fungi as well as bacteria, able to adapt to a wide range of salinites, were isolated from natural hypersaline environments (Gunde-Cimerman et al., 2000; Oren, 2002) and the question how to define a halophile was reopened.

Over the years, several definitions of halophily have been proposed. Kushner (1978) divided microorganisms into four groups, based on the salt concentrations optimal for their growth. Lanyi (1978) suggested the requirement for salt as the basis of halophily, while Brock (1994) accommodated also marine microorganisms among halophiles, as long as they required sodium ions. In his recent monograph, Oren (2002) included in his list of halophilic microorganisms those which are able to grow above 100 g l^{-1} salts, even if their salinity optimum is lower.

Fungi display a halophilic behaviour different from that of the majority of halophilic prokaryotes. Although halophilic fungi do not require salt for viability, they are able to grow and adjust to the whole salinity range, from freshwater to almost saturated NaCl solutions. This flexibility enables halophilic fungi to survive periods of extreme environmental stress in a resting state. When conditions improve, they immediately respond with increased metabolic activity, growth and propagation. Therefore Gunde-

Cimerman et al. (2000, 2005) considered fungi isolated from hypersaline environments as halophilic if they were isolated with high frequency on selective saline media from environments at salinities above 10% and able to grow *in vitro* with 17% NaCl. They were regarded as halotolerant if they were isolated from water with lower salinities but nevertheless able to grow *in vitro* with 17% NaCl.

Although halotolerance and halophily according to these definitions are uncommon amongst fungi, truly halophilic species do exist. On the other hand, xerotolerance is observed more frequently. Fungi able to grow with little available water are rare in lower fungi, in the divisions *Chytridiomycota* and *Zygomycota*. For example, thraustochytrids and labirinthulid non-filamentous fungi (*Labirinthuloides minuta*, *Schizochytrium* sp. and *Thraustocytrium* sp.) have been isolated from a sandy beach of Great Salt Lake, Utah (Amon, 1978). In the *Zygomycota*, *Mucor hiemalis*, *M. racemosus*, *M. circinelloides*, *Rhizopus nigricans*, *R. oryzae* and *R. stolonifer* have occasionally been isolated from saline soil and *Mucor circinelloides* and *M. hiemalis* from salterns (Abdel-Fattah et al., 1977; Abdel-Hafez, 1982; Guiraud et al., 1995; Grishkan et al., 2003; Mahdy et al., 1996; Steiman et al., 1995, 1997). In addition, *Mucor circinelloides*, *M. racemosus*, *M. spinulosus* and *Rhizopus stolonifer* are known as food-borne fungi.

Halophily is scattered amongst the higher fungi and occurrs in several orders of the *Basidiomycota* and *Ascomycota* (Fig. 1, Table 1). In any particular order, growth at decreased water activity is in most cases limited to a few species or a single genus of an order. In the orders *Wallemiales*, *Eurotiales* and *Dothideales*, however, halophily is expressed in several groups of the same order that are not each others nearest phylogenetic neighbours. This suggests that this otherwise rare property either is an evolutionary ancient trait (plesiomorph) within these orders or that different species of genera independently have adapted to halidic conditions. If its origin is evolutionary old, there may be a general constitutional and physiological cellular mechanism underlying this behaviour. In the following we will review how xerotolerance, halotolerance and halophily is expressed in individual species.

Despite the known phylogenetic diversity of the xerotolerant fungi, we have made a remarkable observation when we compared the distribution of xerotolerance in the fungal kingdom with that of fungi able to invade warm-blooded animals. Table 1 compares the occurrence of xerotolerance with opportunism in humans and animals in orders of *Asco-* and *Basidiomycota*. At present, a total of 106 orders of fungi are known (Kirk et al., 2001). Tolerance of low water-activity is apparent in only ten of these (Table 2). Pathogenicity and consistent opportunism (BioSafety Levels 2 or 3; de Hoog et al., 2000) are also found in ten orders. A further twelve orders include species with low or insignificant pathogenicity, shown in occasional infections and therefore regarded as BSL-1 (de Hoog et al., 2000). Table 2 lists the orders with species proven to belong to BSL-2 or 3. Both properties, i.e. consistent xerotolerance as well as consistent invasive ability, are uncommon in the fungal kingdom. Nonetheless, the two lists show total overlap: eight orders with xerotolerance also contain opportunistic fungi of BSL 2-3, while the remaining three contain occasional opportunists (BSL-1). This strongly suggests that the genetic backbone of each of these eight orders encodes properties that are useful for both life strategies. Focusing on individual species, we notice, however, a dual tendency. With only a few exceptions discussed below, species exhibiting xerotolerance have no BSL attribution at all or belong to BSL-1 (Table 1). BSL-1 species have either never been encountered in medical mycology, or morbidity was

insignificant, coincidental or extremely rare). Thus, the eight orders of fungi including xerotolerant and opportunistic species respectively strongly coincide. However, the individual species within these orders nearly always have only one of the two properties. Thus, at the species level, xerotolerance and pathogenicity seem to be mutually exclusive.

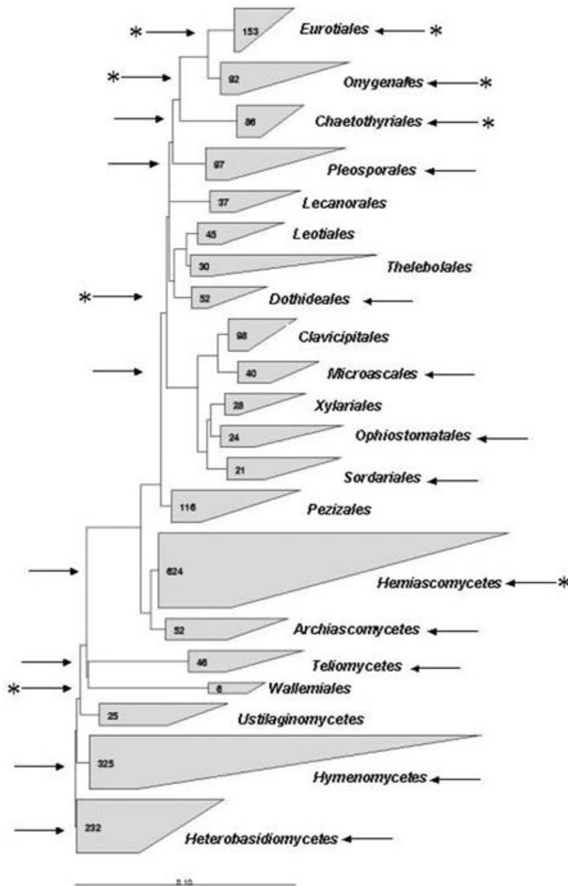

Figure 1. Phylogenetic tree of the higher fungi (*Basidiomycota* and *Ascomycota*) based on 2229 near-complete SSU sequences, aligned with reference to *Saccharomyces cerevisiae*. The tree was constructed with 1514 positions using the Neighbour Joining algorithm. Each flag displays the number of sequences it contains. Arrows at the left indicate the presence of X_T or H_T species, additional asterisks indicate the presence of X_P or H_P species. Arrows at the right indicate the presence of species with BioSafety Levels 1-2, additional asterisks indicate the presence of species with BSL-3. *Saccharomycetales* are under *Hemiascomycetes*, *Filobasidiales* are under *Heterobasidiomycetes*, *Polyporales* are under *Hymenomycetes*, and *Sporidiales* are under *Teliomycetes*.

TABLE 2. Approximate overview of xerotolerant, xerophilic, halotolerant and halophilic species in the fungal kingdom and their BSL.

	Xero-/halo-tolerance	BSL		Xero-/halo-tolerance	BSL
Wallemiales			***Eurotyales*** (continued)		
Wallemia ichthyophaga	H_P	-	*Penicillium corylophilum*	X_T	1
Wallemia muriae	X_P	-	*Penicillium chrysogenum*	X_T	1
Wallemia sebi	X_T	1	*Penicillium citrinum*	X_T	1
			Penicillium crustosum	X_T	-
Filobasidiales			*Penicillium cyclopium*	X_T	-
Trichosporon mucoides	X_T	2	*Penicillium digitatum*	X_T	-
Trichosporonoides nigrescens	X_T	-	*Penicillium duclauxii*	X_T	-
Trichosporonoides oedocephalis	X_T	-	*Penicillium expansum*	X_T	-
			Penicillium fagi	X_T	-
Sporidiales			*Penicillium fellutanum*	X_T	-
Rhodosporidium sphaerocarpum	X_T	1	*Penicillium flavigenum*	X_T	-
Rhodotorula babjevae	X_T	-	*Penicillium freii*	X_T	-
Rhodotorula laryngis	X_T	-	*Penicillium frequentans*	X_T	-
			Penicillium funiculosum	X_T	-
Polyporales			*Penicillium glabrum*	X_T	-
Phanerochaete chrysosporium	X_T	1	*Penicillium herquei*	X_T	-
			Penicillium implicatum	X_T	-
Dothideales			*Penicillium islandicum*	X_T	-
Aureobasidium pullulans	X_T	1	*Penicillium janczewskii*	X_T	-
Cladosporium sphaerospermum	X_T	1	*Penicillium janthinellum*	X_T	-
Cladosporium herbarum	X_T	1	*Penicillium lanosum*	X_T	-
Cladosporium cladosporioides	X_T	1	*Penicillium manginii*	X_T	-
Hortaea werneckii	H_P	1	*Penicillium miczynskii*	X_T	-
Phaeotheca triangularis	H_P	-	*Penicillium melinii*	X_T	-
Trimmatostroma salinum	H_P	-	*Penicillium montanense*	X_T	-
			Penicillium nordium	X_T	-
Eurotiales			*Penicillium oxalicum*	X_T	-
Aspergillus caespitosus	X_T	-	*Penicillium polonicum*	X_T	-
Aspergillus candidus	X_T	1	*Penicillium purpurogenum*	X_T	1
Aspergillus carneus	X_T	1	*Penicillium restrictum*	X_T	-
Aspergillus caesiellus	X_T	1	*Penicillium rugulosum*	X_T	1
Aspergillus clavatus	X_T	1	*Penicillium roqueforti*	X_T	-
Aspergillus conicus	X_T	1	*Penicillium simplicissimum*	X_T	-
Aspergillus elegans	X_T	-	*Penicillium sizovae*	X_T	-
Aspergillus egyptiacus	X_T	-	*Penicillium steckii*	X_T	-
Aspergillus flavipes	X_T	1	*Penicillium solitum*	X_T	-
Aspergillus flavus	X_T	2	*Penicillium spinulosum*	X_T	-
Aspergillus fumigatus	X_T	2	*Penicillium sumatrense*	X_T	-
Aspergillus heteromorphus	X_T	-	*Penicillium variabile*	X_T	-
Aspergillus homomorphus	X_T	-	*Penicillium verrucosum*	X_T	-
Aspergillus melleus	X_T	-	*Penicillium viridicatum*	X_T	-
Aspergillus niger	X_T	1	*Penicillium waksmanii*	X_T	-
Aspergillus niveus	X_T	1	*Penicillium westlingii*	X_T	-
Aspergillus ochraceus	X_T	1	*Polypaecilum pisce*	H_P	-
Aspergillus oryzae	X_T	1	*Talaromyces flavus*	X_T	-
Aspergillus parasiticus	X_T	-	*Talaromyces dupontii*	X_T	-

Aspergillus penicillioides	X_T	-	*Talaromyces stipitatus*	X_T	-
Aspergillus proliferans	X_T	-	*Talaromyces wortmanii*	X_T	-
Aspergillus puniceus	X_T	-	*Xeromyces bisporus*	X_P	-
Aspergillus restrictus	X_T	1			
Aspergillus sclerotiorum	X_T	1	***Chaetothyriales***		
Aspergillus sydowii	X_T	1	*Exophiala dermatitidis*	X_T	2
Aspergillus sulphureus	X_T	-	*Exophiala pisciphila*	X_T	1
Aspergiluus tamarii	X_T	1			
Aspergillus terreus	X_T	2	***Microascales***		
Aspergillus tubingensis	X_T	-	*Pseudallescheria boydii*	X_T	2
Aspergillus ustus	X_T	1			
Aspergillus versicolor	X_T	1	***Onygenales***		
Aspergillus wentii	X_T	-	*Chrysosporium farinicola*	X_T	-
Basipetospora halophila	H_P	-	*Chrysosporium fastidium*	X_T	-
Emericella nidulans	X_T	1	*Chrysosporium inops*	X_T	1
Emericella purpurea	X_T	-	*Coccidioides immitis*	X_T	3
Emericella rugulosa	X_T	-	*Coccidioides posadasii*	X_T	3
Emericella xerophila	X_P	-	*Gymnascella marismortui*	H_T	-
Eremascus albus	X_T	-			
Eremascus fertilis	X_T	-	***Pleosporales***		
Eupenicillium crustaceum	X_T	-	*Alternaria mouchaccae*	X_T	-
Eupenicillium egyptiacum	X_T	-	*Dendryphiella salina*	H_T	-
Eupenicillium sinaicum	X_T	-	*Ulocladium chlamydosporum*	X_T	-
Eurotium amstelodami	X_T	1			
Eurotium chevalieri	X_T	1	***Saccharomycetales***		
Eurotium desertorum	X_P	-	*Candida famata*	H_P	1
Eurotium echinulatum	X_T	-	*Candida magnoliae*	X_T	-
Eurotium halophilicum	X_T	-	*Candida parapsilosis*	X_T	1
Eurotium herbariorum	X_T	1	*Candida scottii*	X_T	-
Eurotium medium	X_T	-	*Candida versatilis*	X_T	-
Eurotium minor	X_T	-	*Metschnikowia bicuspidata*	X_T	-
Eurotium intermedium	X_T	-	*Pichia farinosa*	X_T	-
Eurotium repens	X_T	1	*Pichia membranifaciens*	X_T	-
Eurotium rubrum	X_T	-	*Pichia sorbitophila*	X_T	-
Eurotium umbrosum	X_T	-	*Pichia guilliermondii*	X_T	1
Penicillium adametzi	X_T	-	*Schizosaccharomyces pombe*	X_T	-
Penicillium albocoremium	X_T	-	*Zygosaccharomyces rouxii*	X_P	-
Penicillium antarcticum	X_T	-	*Zygosaccharomyces lentis*	X_P	-
Penicillium aurantiogriseum	X_T	1	*Yarrowia lipolytica*	X_T	1
Penicillium brevicompactum	X_T	1			
Penicillium charlesii	X_T	-			

X_T = Xerotolerant; X_P = Xerophilic; H_T = Halotolerant; H_P = Halophilic. NB: most species are attributed to any of these categories on the basis of their general ecology, as growth curves at different levels of water activity / salinity have not been established.

2. Basidiomycota

2.1. *FILOBASIDIALES*, *TREMELLALES*, *TRICHOSPORONALES*

The small genus *Trichosporonoides* and its relative genus *Moniliella* comprise species (de Hoog, 1979) associated with industrial production of lipids and acids (Dakin and

Stolk, 1968). *Trichosporonoides* contains one species inhabiting pollen (Haskins and Spencer, 1967), and one from jelly (Hocking and Pitt, 1981). The genus was recently revised by de Hoog and Smith (1998), but the exact phylogenetic relation between *Trichosporonoides* and *Moniliella* has not yet been established. Members of the genus *Trichosporonoides* are too rare, both in nature and in the human-dominated environment, to speculate on any evolutionary tendencies. A relationship between these two genera and *Cryptococcus* (*Filobasidiales / Tremellales*) may be surmised. In *Cryptococcus* several species are known to occur in the osmotic environments of e.g. flower petioles (Sriburee et al., 2004) and in seawater (Nagahama et al., 2003b). Several species of *Cryptococcus* are known from the Antarctic (Vishniac and Onofri, 2003) and have been isolated with high frequency from the ice of polythermal glaciers in the Arctic (Gunde-Cimerman et al., 2003). Pathogenic potential is particularly found in the neurotropic *Cryptococcus neoformans* complex, which has an environmental niche in, among others, dried bird faeces (Gokulshankar et al., 2004).

TABLE 2. Correspondence of ability to tolerate low water activity* and invasive potential in humans among the 106 currently recognized[1] orders of the fungal kingdom.

Halo- / xerotolerant:	Minimum A_w[2]:	Medically significant:	Highest BSL level[3]
Wallemiales	0.77	*Wallemiales*	1
Filobasidiales	0.90	*Filobasidiales*	2
Sporidiales	0.90	*Sporidiales*	1
Polyporales	0.90	*Polyporales*	1
Dothideales	0.80	*Dothideales*	1
Eurotiales	0.80	*Eurotiales*	3
Onygenales	0.90	*Onygenales*	3
Saccharomycetales	0.80	*Saccharomycetales*	2
Microascales	?	*Microascales*	2
Chaetothyriales	?	*Chaetothyriales*	3
Pleosporales	?	*Pleosporales*	2
Sordariales	?	*Sordariales*	2
Hypocreales	?	*Hypocreales*	2
Ophiostomatales	?	*Ophiostomatales*	2

*Most species are attributed to any of these categories on the basis of their general ecology, as growth curves at different levels of water activity / salinity have not been established.
[1]According to Kirk et al. (2001).
[2]Estimate, adapted from Pitt and Hocking (1997).
[3]Adapted from de Hoog et al. (2000).

The arthroconidial yeast *Trichosporon mucoides* was frequently isolated from hypersaline water of the salterns and the Dead sea (Butinar et al., 2005b). *Trichosporon* was recently segregated from the *Filobasidiales / Tremellales* on the basis of 26S rRNA gene phylogeny to be classified in the new separate order *Trichosporonales* (Scorzetti et al., 2002). *Trichosporon* contains mainly soil-borne species and human opportunists. Interestingly, several species are the exclusive agents of human white piedra, referring to

fungal colonization of mainly pubic and axillary hairs (Guého et al., 1992), which can be regarded to be a low a_w environment due to drying transpiration.

2.2. *POLYPORALES*

This order contains the single xerotolerant species *Sporotrichum pruinosum*, previously referred to as *Chrysosporium xerophilum* (Boekhout et al., 1989). The species is occasionally found on foodstuffs (Kinderlerer, 1997). It is the anamorph of *Phanerochaete chrysosporium*, which is one of the very few filamentous basidiomycetes with some invasive potential in warm-blooded animals (de Hoog et al., 2000).

2.3. *SPORIDIALES*

Rhodosporidium, *Leucosporidium* and *Sporidiobolus* are teleomorphs of several species of *Rhodotorula* and *Sporobolomyces*. Frequently isolated red yeast species are several *Rhodotorula* species from the deep sea and marine sediments (Nagahama et al., 2003a). The *Rhodotorula glutinis* and *R. minuta* species complexes are occasionally found in a clinical setting, but their virulence is low (de Hoog et al., 2000). *Rhodosporidium sphaerocarpum*, *R. babjevae* and *Rhodotorula laryngis* were repeatedly isolated from hypersaline water of salterns (Butinar et al., 2004b). *Leucosporidium* is consistently isolated from Antarctic marine waters (Fell et al., 1998).

2.4. *WALLEMIALES*

This order was recently introduced to accommodate the single genus *Wallemia*, a phylogenetic maverick in the *Basidiomycota* (Zalar et al., 2005). The genus contains only three species, all frequently involved in food spoilage. Members can be isolated from sweet (fruits, jams, cakes, pure sugar), salty (fish, meat, peanuts), or dried food (Samson et al., 2002). In nature they inhabit desiccated fruits and sea fish, and are common in hypersaline evaporation ponds at the Mediterranean, the Caribbean, as well as from the Dead sea (Wasser et al., 2003; Zalar et al., 2005). It is remarkable that the entire genus *Wallemia*, and therefore the entire order *Wallemiales*, are xerophilic or xerotolerant. Because of having their growth optimum in media with additional solutes and showing no growth without them, two out of three *Wallemia* species are considered to be xerophilic rather than xerotolerant. *Wallemia ichthyophaga* still shows growth at a water activity of 0.77, equivalent to 32% NaCl. It grows better with salt than with other solutes. *Wallemia ichthyophaga* is one of the most halophilic fungi known to date.

The combination of xerotolerance with human-opportunism is questionable in *Wallemiales*. *Wallemia sebi* infection (= 'hemisporiosis', named after the synonymous species *Hemispora stellata*), has only been reported in older literature, before the fifties in the previous century. Infections were reported to be cutaneous or subcutaneous, without specific clinial features. Indeed, the identity of one of these strains, CBS 196.56, has never been verified as *W. sebi* (Zalar et al., 2005). It is remarkable, however, that no modern case of any *Wallemia* infection has been reported after 1950. The cases all occurred well before the emergence of immunocompromised patients. We therefore doubt whether these cases were really caused by *W. sebi* as primary etiologic agent, and prefer to omit the *Wallemiales* as an order with dual behaviour. The group is

characteristic in its preference of environments with low water activity, but it is unlikely to have an opportunistic potential.

3. *Ascomycota*

3.1. *DOTHIDEALES*

Remarkably, nearly all dothideaceous species growing at low water activity are halotolerant or halophilic (Table 1), whereas outside the *Dothideales* this property is rare. The natural habitat of the species concerned is the hypersaline coastal salterns worldwide (Butinar et al., 2005c). These fungi are practically unknown in non-natural environments – not even on salted fish such as their halophilic counterparts in *Eurotiales* and *Wallemiales*. Apparently the ecology of these fungi is not solely based on tolerance of high salt concentrations, and this is further evidenced by the properties of *Hortaea werneckii* (Petrovič et al., 2002). This species is the dominant fungal halophile in hypersaline evaporation ponds (Gunde-Cimerman et al., 2000; Zalar et al., 1999), and the only one causing human tinea nigra, a characteristic type of colonization of human hands (Severo et al., 1994). This disorder is caused by non-invasive colonization of the salty, dead keratin-layers of the hands of hyperhydrotic individuals after prolonged desiccation in the sun at the beach (Göttlich et al., 1995). These patients placed their exceptionally salty hands in seawater and particularly the hypersaline evaporation ponds around the world (Zalar et al., 1999). *Hortaea werneckii* seems to have taken accidental advantage of the environment of the human skin which is very different from its natural habitat, but resembles it in one essential regard, high salinity. This dependence of crucial factors enables a fungus to make an enormous ecological leap which might be the starting point for a process of sympatric speciation. However, the tinea strains do not show any further transmission and thus this new niche is an evolutionary dead end. The fungus is easily removed with ointments dissolving dead keratinous material (Rippon, 1988) and thus should not be regarded as a clinical problem. Therefore, it has been recommended to lower the biosafety level of this fungus to BSL-1 (de Hoog et al., 2000). Remarkably, two related species of *H. werneckii* with very similar ecology, *viz. Phaetotheca triangularis* and *Trimmatostroma salinum*, are not known as etiologic agents of human tinea nigra. Perhaps *H. werneckii* is exceptionally versatile, as it is widely dispersed in seawater-related environments and is also found on salty hands and in house-dust (Zalar et al., 1999).

The order *Dothideales* has a xerotolerant tendency as it contains a large number of extremotolerant species growing as epilithic or cryptoendolithic species at high temperature, such as on Mediterranean marble (Wollenzien et al., 1996), at low temperature in Antarctic sandstone (Selbmann et al., 2005) and Arctic glacier ice (Gunde-Cimerman et al., 2003). Nearly all species are heavily melanized, have thick cell walls, and frequently exhibit a meristematic, isodiametric type of thallus expansion, leading to highly resistant cell clumps (Sterflinger, 1998). *Aureobasidium pullulans* is an ubiquitous xerotolerant fungus on honeydew, and at lower environmental salinities in saltern evaporation ponds. It is common as well in the artificial environment on low-nutrient, moist surfaces such as glass where it exhibits its xerotolerant, meristematic ecotype (Schabereiter-Gurtner et al., 2001). *Aureobasidium pullulans* is a typical

opportunist in causing insignificant, cutaneous infections after trauma in otherwise healthy patients. It very rarely shows systemic dissemination and only if the patient is severely immunocompromised (de Hoog et al., 2000). This species occurs commonly on damp inert surfaces such as stone, glass and metal including medical instruments.

Most members of the genus *Cladosporium* (section *Cladosporium*; David, 1997) have a cosmopolitan distribution as ubiquitous decomposers of dead plant material (David, 1997), therefore they are commonly found in indoor and outdoor air. Contrary to the ubiquitous distribution of saprobic Cladosporia, some species were consistently isolated from specific, extreme environments of salterns and salty lakes worldwide and they represented the most common and frequent fungal taxa in various hypersaline environments (1000-3600 CFU l^{-1} for hypersaline saltern water) (Gunde-Cimerman et al., 2000). Cladosporia were reported from wood immersed in hypersaline water of Great Salt Lake in Utah, they were isolated from saline soils and salt marshes, the phylloplane of Mediterranean plants and the rhizosphere of halophytic plants (references after Gunde-Cimerman et al., 2004). The halotolerant *Cladosporium* saltern community was found to be composed of mainly two round-spored species, *C. sphaerospermum*, and a possibly new, yet undescribed species (Zalar et al., 2001). Common, frequently air-borne saprobic species, such as *C. herbarum* and *C. cladosporioides* were also detected, but showed only mild halotolerance, expressed as an ability to grow in the presence of 5% additional NaCl. Human *Cladosporium* infections are extremely rare, and all species reported from clinical cases are listed as BSL-1.

3.2. *EUROTIALES*

Tolerance for high salt concentrations is a property found in many members of *Eurotiales*. When a survey was made of the NaCl tolerance of 975 species of terrestrial fungi selected from the major taxonomic classes, species of *Penicillium* and *Aspergillus* were notably the most resistant, with the majority of their species able to grow in the presence of 20% or more of NaCl. Over 70% of the Penicillia could tolerate 20% NaCl and more than half survived at 25% or greater concentrations. The aspergilli appeared to be somewhat less tolerant; nevertheless, about 70% could tolerate 20% NaCl and nearly half survived at 25% (Tresner and Hayes, 1971). The teleomorphs in *Emericella*, *Eupenicillium*, *Eurotium* and *Talaromyces* were all represented in saline soil, arid areas and salt marshes, in the ectorhizosphere-rhizoplane zone of different halophytic plants (El-Morsy, 1999).

In the *Eurotiales* xero- and halotolerance are recurrent phenomena (Table 1). To date, eleven species are known to be xerotolerant, three xerophilic, and two show better growth with salt than with any other solute (Butinar et al., in press). The species listed are located in remote clades within the order. Thus it is likely that growth and survival of low levels of water activity is a main, plesiomorphic trait shared by the entire order. Indeed, although the natural ecology has not been established for every individual species, we note that numerous taxa originate from desert soil, brines, desiccated fruits in arid climates, dry bird and bat guano, and similar substrates. In the human-dominated environment they are among the main spoilage organisms of food products (Pitt and Hocking, 1997). Since, however, foodstuffs are basically derived from nature, despite the addition of salts and sugars for presevation no dramatic shift in ecology is apparent. But the *Eurotiales* are also among the predominant indoor fungi as highly successful

colonizers of surfaces after slight moistening, and are able to inhabit a wide variety of toxic compounds such as paint, tannins and industrial fluids. Under such conditions they are frequently encountered as near-monocultures, underlining their successful occupation of these new habitats. It thus can be stated that the human-dominated environment has provided an enormous potential for growth and dispersal of such fungi. They have emerged with clonal offshoots, while related species that still inhabit their original stressed habitats mostly exhibit their teleomorphs (Grishkan et al., 2003).

The result of the introduction of new environments for rapid reproduction may explain the repeated loss of teleomorphs in some groups of eurotialian fungi (LoBuglio et al., 1993). The sudden preponderance of clonality has certainly led to natural selection of genotypes that are more suitable for survival in the indoor environment. Therefore, in *Eurotiales* accelerated evolution is expected in those species that are successful colonizers of the indoor environment.

The genus *Eurotium* is the teleomorph genus for *Aspergillus* sections *Aspergillus* and *Restricti*, that have a xero- and halophilic preponderance (Pitt and Hocking, 1997). These species are common in a diversity of substrates with low water activity including saline environments. Members of the genus have been reported to live in concentrated salt or sugar solutions at a_w as low as 0.75 (Martín et al., 1998). The genus *Eurotium* has been reported both in arid and saline soils. Six different species of the known teleomorphic food-borne xerophilic genus *Eurotium* were repeatedly isolated in a mycodiversity study of hypersaline waters. At salinities above 17% NaCl, *E. amstelodami* was detected most consistently, followed by *E. repens* and *E. herbariorum.* Spatiotemporal frequency of occurrence and *in vitro* determined adaptive ability of propagules to survive a prolonged exposure to hypersaline conditions indicate that *E. amstelodami*, *E. herbariorum*, and *E. repens* contribute to the indigenous fungal community in hypersaline water environments (Butinar et al., 2005a).

The genus *Aspergillus* and its teleomorphs currently contains 254 accepted species (Pitt et al., 2000). It can be divided into three main groups (Peterson, 2000b). The first group contains species that are particularly xerotolerant and associated to the teleomorphic genera *Eurotium*, *Chaetosartorya*, *Petromyces*, *Neopetromyces* and *Fennellia*. Species in *Chaetosartorya* are also highly xerotolerant, but it is only the anamorphic taxon *Aspergillus wentii* that has been repeatedly reported from low water activity environments. The second group comprises the ascomycete genera *Emericella* and *Sclerocleista* and the third contains species associated to the ascomycete genera *Neosartorya* and *Neocarpenteles*.

Species in the genera *Petromyces* (anamorph *Aspergillus* section *Flavi*), *Neopetromyces* (anamorph *Aspergillus* section *Circumdati* or formerly the *Aspergillus ochraceus* group) (Frisvad and Samson, 2000) and *Aspergillus* section *Nigri* are all common at low water activity conditions, especially in warmer climates. Species associated with the genus *Fennellia* (anamorphs *Aspergillus* sections *Flavipedes*, *Terrei*, *Candidi* and *Cervini*) are less frequent in dry habitats, but species such as *A. flavipes*, *A. terreus* and *A. candidus* are quite common in haline environments.

Species in *Emericella* and its anamorphs *Aspergillus* sections *Nidulantes*, *Versicolores* and *Usti* are common in marine environments and in dry foods and other low water activity environments. *Emericella nidulans*, *A. versicolor* and *A. sydowii* are particularly common in salterns. The thermophilic genus *Neosartorya* with the

anamorph *Aspergillus* section *Fumigati* is common in self-heated plant waste and also in desert soil. *A. fumigatus* is found repeatedly in salterns (Tepšič et al., 1997).

The genus *Penicillium* is associated to two teleomorph genera, *Eupenicillum* and *Talaromyces*. *Eupenicillium* is rather closely related to *Aspergillus* in a phylogenetic sense, while *Talaromyces* with its associated anamorphs in *Penicillium* subgenus *Biverticillium* may be a distinct genus closer to *Byssochlamys* and *Thermoascus* (Peterson, 2000a; Pitt, 1979; Stolk and Samson, 1972). *Eupenicillium* is uncommon in saline environments, but its *Penicillium* anamorphs are frequently food-borne (Frisvad et al., 2000) and soil-borne (Christensen et al., 2000). Many species in subgenus *Penicillium* grow well in salted foods, especially species in series *Chrysogena*, *Olsonii*, *Viridicata*, *Verrucosa*, *Camemberti*, *Expansa*, *Solita* and *Urticicola* (Frisvad and Samson, 2004). Many members of the soil-borne *Penicillium* subgenus *Furcatum* are also recovered from saline environments.

Species in the teleomorph genus *Talaromyces* in general thrive at higher water activities than *Eupenicillium* (Andersen and Frisvad, 2002; Pitt and Hocking, 1997). However, some species that have been recovered from haline environments are classified in *Talaromyces* and associated Biverticillia: *T. flavus*, *P. funiculosum*, *P. islandicum*, *P. purpurogenum* and *P. variabile*.

The *Eurotiales* is also one of the most significant orders with respect to pathogenicity to humans. It contains the biverticillate species *Penicillium marneffei*, which is a BSL-3 pathogen emerging at very high frequency in the AIDS-patients of Southeast-Asia (Ukarapol et al., 1998). It produces a unique pathogenic phase, with arthroconidia actively reproducing within phagocytes and subsequent dormancy in healthy bamboo rats (Chariyalertsak et al., 1996). The infection kinetics of *P. marneffei*, with endogenous reactivation at the moment of impairment of acquired cellular immunity, is identical to that of established systemic pathogens such as *Coccidioides immitis* and *Histoplasma capsulatum* (Schaffner, 1989). Its natural environmental niche is hitherto unknown with certainty, because all attempts to isolate the fungus from directly soil or air have failed, although PCR-based tests are positive (Vanittanakom et al., 2002). *Penicillium* subgenus *Biverticillium* contains a large number of thermophilic species from dry, heated soil. Outside *P. marneffei* more species are encountered that have a pathogenic potential for humans and animal (G.S. de Hoog and R.A. Samson, unpublished data). The origin of AIDS-association in *Biverticillium* is hitherto not understood. In the second main group of potential human-invaders, the *Aspergillus* section *Fumigatii* with its *Neosartorya* teleomorphs, a compromised innate immune system is a necessary condition for infection. Hence, such species are associated with e.g. leukemic patients and transplant recipients, and are rarely observed in AIDS patients (de Hoog et al., 2000). In contrary *A. fumigatus* is extremely common during self-heated phases of composting of plant material (Göttlich, 1995) and is ubiquitous in outside air. In susceptible humans it causes a typical inhalation mycosis, whereby colonization and invasion are usually accompanied by an allergic response from the host. *P. marneffei* and *A. fumigatus* / *A. flavus* thus are ecologically fundamentally different.

3.3. *CHAETOTHYRIALES*

This small order of black yeast-like fungi and their teleomorphs (genus *Capronia*) is exceptional in the fungal kingdom in comprising a wide diversity of opportunistic human

pathogens. Slight xerotolerance is expressed in the fact that particularly the species with capsulate yeast cells (*Exophiala dermatitidis* and *E. spinifera*) are regularly encountered on sweet surfaces of fruits and flowers (G.S. de Hoog, unpublished data), and thus exhibit a dual tendency as observed in *Cryptococcus* above. Numerous species are oligotrophic, living e.g. in drinking water, while some are particularly found in sea water and are opportunists of fish. *Exophiala dermatitidis* is associated with human cystic fibrosis (Haase et al., 1991; Horré et al., 2004), a genetic disorder in the transmembrane conductance regulator gene (CFTR) leading to a high degree of salt excretion in lungs, intestines and on the skin. The mucous lungs of a significant percentage of CF patients is asymptomatically colonized with this otherwise uncommon fungus. Its natural niche probably is in association with tropical frugivorous birds and bats, while the fungus goes through a habitat shift in the human environment by occurrence in a high frequency in human bathing facilities (Matos et al., 2002). The frequencies of two main genotypes in natural and artificial environments are different (M. Sudhadham and G.S. de Hoog, unpublished data). CF patients probably acquire their infection from this pre-selected set of strains in the steam bath or ordinary bathrooms. Humans are colonized by a genotype that is rarely encountered in nature. This selection must be based on strains being differentially predisposed to survival in the human environment, and thus may be accompanied by changes in infectious ability. The trigger for this speciation process may be linked to the species' halotolerance.

3.4. *MICROASCALES*

The order contains a single species exhibiting halotolerance, *Pseudallescheria boydii*. *In vitro* it tolerates 5% additional NaCl (de Hoog et al., 1994). This property enables the fungus to reside in somewhat osmotic environments such as dry bat faeces (Ajello et al., 1977) and bird guano at their roosting sites (Brandsberg et al., 1969). It occurs in brackish and salt water, such as submerged wood in estuaria (Kirk, 1967), tide-washed areas and marine soil (Dabrowna et al., 1964; Pawar et al., 1963). This natural ecology predisposes the fungus for colonization of the mucous lungs of patients with cystic fibrosis (Cimon et al., 2000), very similar to the black yeast *Exophiala dermatitidis* mentioned above. Also *P. boydii* passes through a selective step during the transition from nature to the human-dominated environment, which is agricultural soil and polluted ponds (de Hoog et al., 1994). It is presently one of the important opportunistic, potentially fatal agents in the immonocompromised patients. It causes death with a frequency of 1:1000 in transplant recipients (Nucci, 2003) and 4:1000 in patients with hematological malignancies (Pagano et al., 2001). Its invasive potential is not emerging with this recent hospitalized patient population, but the fungus always has been a major cause of traumatic infection in otherwise healthy hosts, e.g. in mycetoma (Kemper, 2000) and arthritis (Ginter et al., 1995).

3.5. *ONYGENALES*

Several *Chrysosporium* species grow on dry foodstuffs, particularly *C. farinicola* having an optimum near a_w 0.93. They grow poorly on media in which the controlling solute is something else than sugars. Species of the genus *Gymnascella,* such as *G. dankaliensis* and *G. hyalospora* naturally inhabit dung and soil including saline and desert soil

(Deshmukh, 2002). *G. marismortui* is found in water of the Dead Sea and was never recorded on other localities. It grows optimally at NaCl concentration between 3 and 10% NaCl (Buchalo et al., 1998).

The order *Onygenales* comprises the the main families of human-pathogenic fungi: viz. the *Arthrodermataceae* with the dermatophytes on humans and other mammals, and the *Onygenaceae* with the systemic pathogens such as *Histoplasma* and *Coccidioides*. Most species, cutaneous as well as systemic, are viewed upon as true pathogens, since they are poorly affected by the intact innate immune system but are typically controlled by acquired cellular immunity (de Hoog et al., 2000). The systemic species show endogenous reactivation when the acquired immune system is impaired, such as in the case of AIDS (Schaffner, 1989). The systemic species have a double life cycle, involving an invasive phase in an animal vector, and an environmental phase. The xerotolerant environmental phase resides in habitats that are hostile to most fungi, namely dried bat dung and guano in the case of *Histoplasma* (Lyon et al., 2004) and salty, alkaline desert soil in the case of *Coccidioides* (Maddy, 1957). Both presently recognized *Coccidioides* species tolerate 8% NaCl (Fisher et al., 2002).

3.6. *PLEOSPORALES*

Xero- and halotolerance in this order is moderate. *Dendryphiella salina* is a marine hyphomycete (Genilloud et al., 1994), although it was also recovered on driftwood immersed in hypersaline water of the Adriatic salterns, but failed to grow at concentrations higher than 17% NaCl in the medium (N. Gunde-Cimerman, unpublished data). It can utilize different nitrogen compounds, has a broad pH growth range, particularly at alkaline values and a broad temperature profile (Clipson et al., 1989, 1990; Galpin et al., 1977). Several species of *Alternaria* and *Ulocladium* are specialized to live in salt marsh soils in arid deserts (Ranzoni, 1968; Simmons, 1981), all having a preponderantly meristematic phenotype.

The pathogenic potential of the *Pleosporales* is also moderate. Two saprophytic *Alternaria* species, *A. alternata* and *A. infectoria*, are frequent cutaneous opportunists in immunocompromised humans (de Hoog and Horré, 2002). Several species causing the subcutaneous disorder mycetoma are also members of the *Pleosporales* (de Hoog et al., 2004).

3.7. *SACCHAROMYCETALES*

This order of ascomycetous yeasts contains numerous species associated with sugary plant saps and exudates. Osmotolerant yeast taxa are found mainly in the families *Saccharomycetaceae* and *Metschnikowiaceae*. They are known from honey, pollens, molasses, fresh and dried fruit (Prada and Pagnocca, 1997), and are associated with sugary plant saps and exudates (Hocking and Pitt, 1997). Tolerance to high concentrations of sugars is found in *Zygosaccharomyces rouxii*, *Z. bailii* and *Schizosaccharomyces pombe*. Osmophilic *Z. rouxii* and *Candida versatilis* are used for soya sauce fermentation. Yeasts preferring reduced a_w for growth, described as osmophilic, were known primarily as contaminants in the food industry, but they remained largely unknown in natural low water activity habitats. Yeasts isolated from hypersaline waters, having hemiascomycetous affinities, belong to the genera *Candida*,

Debaryomyces, *Metschnikowia* and *Pichia*. Different species of the genus *Pichia* are frequent saline aquatic isolates, with *P. membranifaciens* being the most common (Soares et al., 1997).

Candida famata is a halotolerant food-borne yeast and a model organisms for fungal halotolerant adaptation (Prista et al., 1997) has been found mainly in cold sea water (Norkrans, 1966) and recently in natural hypersaline habitats, particularly salterns at the Namibian Skeleton coast and Great Salt Lake, both exposed to low seasonal temperatures. Xerotolerant strains usually belong to the variety *famata*, which is a common contaminant of foodstuffs (Butinar et al., 2005b). The variety *flareri* is more thermotolerant and known as an opportunistic pathogen on humans (de Hoog et al., 2000).

In the waters of the Dead Sea diverse opportunistic pathogenic species of the genus *Candida* were found. These included *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*, occasionally known from the more polluted intertidal estuarine water and sediments (Soares et al., 1997), but isolated for the first time from hypersaline water of the Dead Sea (Butinar et al., 2005b). Among these only *C. parapsilosis* was known previously as a food-borne.

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4. Discussion

The main purpose of this review is to reveal a possible link of xerotolerance with other ecophenotypes in the evolutionary history of fungi. This idea was motivated by the observation that xerotolerance and the ability to cause infection in humans – either having impaired immunity or otherwise healthy – show a similar distribution at the ordinal level in the fungal tree of life. Thus all orders including xerophilic species also comprised human pathogens and vice versa, despite the fact that each trait is fairly rare amongst fungal species and orders. Remarkably, however, at the species level the two properties seem to be nearly mutually exclusive. Pronounced xerotolerance thus does not seem to be directly associated or beneficial for pathogenicity, while lower levels of xerotolerance are promotive. We conclude that a moderate degree of xerostress-tolerance is likely be a plesiomorphic (ancestral) character in the orders with dual behaviour. Better survival of environmental stress resulting from the development of mechanisms for xerotolerance may have enhanced evolutionary fitness of environmental pathogens with low competitive ability such as *Coccidioides*. Cellular mechanisms put in action by xerotolerance are also helpful to overcome various other hostile conditions.

Also survival from phagocytosis and subsequent killing by oxygen-radicals of the innate immune system, cytotoxic activity from complement and killer cells, low pH, and iron deprivation may be promoted by the dramatic response by a complex gene expression underlying osmotic shock (Crespo et al., 2001; Gash et al., 2000). The few fungal orders where appreciable xerotolerance is encountered share other types of stress tolerance that are equally uncommon elsewhere in the fungal kingdom, suggesting that a general type of stress-response underlies both types of ecology.

Immediate responses to an increase in osmolarity involves shrinking of the cell, a loss of cell polarity, disassembly of the actin cytoskeleton, changes in the permeability of the plasma membrane, accumulation of polyols and a temporal arrest of the synthesis of the cell wall (Hernandez-Saavedra et al., 1995). As a consequence, shrinkage of the cytoplasm and local redistribution of periplasmic and/or matrix cell wall material takes place, while osmotic stress does not affect the synthesis of cell wall constituents. Cells become more isodiametric in shape. Similar processes take place in the human-invasive forms of *Coccidioides*, *Exophiala* and *Fonsecaea* as they establish themselves in the mammal host (Kurappayil and Szaniszlo, 1997; Sun et al., 1986), triggering a change from longitudinal to isodiametric cellular expansion. Their invasive forms consist of isodiametrically expanding spherules and muriform cells, respectively.

A key factor in stress in clinical fungi is the ability to grow at elevated temperatures. This property is enhanced by mechanisms such as regulation of membrane fluidity which are also involved in xerotolerance. Changes in membrane composition and its properties represent an important factor in the adaptation to high salinity (Russell, 1989). It seems that salt-tolerant fungi show a broad spectrum of alterations in lipid composition as the response to increased external salinity. Sterols and phospholipids are the two major lipid constituents of eukaryotic membranes. Overall changes in phospholipid to sterol ratio, changes in the degree of unsaturation of fatty acids, changes in phospholipids as well as in membrane fluidity, have been demonstrated in salt-tolerant yeasts when exposed to higher salinities and compared to salt-sensitive *Saccharomyces cerevisiae* (Turk et al., 2004). At elevated salinities an increase in unsaturation of phospholipid-esterified fatty acids was shown in halotolerant black yeast *Aureobasidium pullulans*, although changes in the membrane fluidity over the tested salinity range resembled to that of salt-sensitive *S. cerevisiae.* In both cases the membrane fluidity decreased with the rise in NaCl concentration, while in halophilic fungi, such as *Hortaea werneckii*, fluidity is maintained over increasing salt levels. Therefore it was concluded that higher membrane fluidity is of crucial importance for halophily (Turk et al., 2004). On the other hand it seems that at elevated temperatures changes in the membrane composition have an oposite affect, by primarily decreasing the membrane fluidity, although very few studies have been performed (Hazel and Williams, 1990).

Extracellular glycoproteins have been isolated from many fungi grown in media containing elevated concentrations of salt. Glycoproteins produced under salt stress can differ (Breierova et al., 1997a). These glycoproteins are known to be associated with the ability of binding water and protection of water regime in the cell (Breierova et al., 1997b). Capsular polysaccharides of *Cryptococcus* delay drying processes in environments with extremely low humidity (Aksenov et al., 1973). Extracellular polysaccharides (EPS) of *Cryptococcus* have also been proven to be a major virulence factor using animal experiments (Chang and Kwon-Chung, 1994). Capsular glucuronoxylomannan (GXM) down-regulates cell-mediated protective immune response

by reducing the production of pro-inflammatory cytokines (Andrade et al., 2002). In the black yeast genus *Exophiala*, the two most pathogenic species are the capsulate ones, *E. dermatitidis* and *E. spinifera* (Yurlova and de Hoog, 2002). Remarkably, both genera *Exophiala* and *Cryptococcus* exhibit an association with occurrence on fruits and flowers as well as with dry bird and bat guano (Lopez-Martinez and Castanon-Olivares, 1995; Matos et al., 2002), the common factor being decreased water activity.

Melanin is a further, additive virulence factor, in that melanin-deficient mutants are less virulent in *Cryptococcus* (Noverr et al., 2004) as well as in *Exophiala* (Dixon et al., 1992; Peltroche-Llacsahuanga et al., 2003). Members of the *Dothideales* enhance their stress-tolerance with increased melanization (Figueras et al., 1996) combined with thick cell walls and meristematic growth. Ultrastructural studies of the halophilic black yeasts *Hortaea werneckii*, *Phaeotheca triangularis* and *Trimmatostroma salinum* cell walls showed that the organisation of melanin granules is dependent on the concentration of salt in the medium (Kogej et al., 2001; Ravishankar et al., 1995). The granules in the outer part of the cell walls were loosely organised in medium without salt, but became more densely packed as the salt concentration in the medium increased. This kind of granular reorganisation at increased salinity was absent in xerotolerant *A. pullulans*. These results suggest a potential osmoprotective role of melanin in the cell wall of halophilic black yeasts, and a higher degree of specialization of the halophilic compared to xerotolerant species.

The EPS-producing black yeast *Aureobasidium pullulans* is very common in indoor and human-made environments, while the natural habitat predominantly is found on plant surfaces covered with honeydew. Similarly, many *Penicillium* species have shifted from natural to indoor environments and thus have made a considerable ecological leap. *Exophiala dermatitidis* is rarely found in nature but has a striking prevalence in hot steam baths (Matos et al., 2002). In all these fungi a high degree of fitness is reached in the new environment, and transmission is efficient. This shift is likely to be accompanied by a step of natural selection, as not all genotypes may be equally suited for growth and dispersal in the new environment.

The phenomenon of habitat-shift is pronounced in *Exophiala dermatitidis* and *Pseudallescheria boydii*. These species combine a (low) degree of halotolerance with an obvious clinical potential. In *E. dermatitidis* a jump from frugivorous animal faeces in the tropical rain forest to human-made steam baths is supposed (M. Sudhadham and G.S. de Hoog, unpublished data); in *P. boydii* the leap possibly concerns estuarine brackish muds to agricultural effluents (de Hoog et al., 1994). The two species have a marked association with the salty mucus of patients with cystic fibrosis (Haase et al., 1991; Horré et al., 2004). Accelerated evolution through an enforced process of natural selection may be expected in these species, but the direction of evolution is unpredictable. The selective processes take place in the human-made environment rather than in the human host. Changes in virulence of the organism, either up or down, will be coincidental. The result of the process may nevertheless lead to an organism that is better equipped to infect the human host. If selection for temperature tolerance takes place, an increase in virulence may be expected.

Fungi growing indoors have an increased probability to be traumatically introduced into humans. However, since there is no further transmission, human infections are evolutionary dead ends and are unlikely to have any impact on the evolution of the fungus. The selective process apparently has led to a preponderance of genotypes that

are coincidentally more predisposed to cause human infection, e.g. by more pronounced thermotolerance, but that are not adaptive.

In this connection *Candida famata* is particularly interesting because the species comprises two varieties, var. *famata* and var. *flareri*, that show a predilection for dry foodstuffs and for human patients, respectively. It is tempting to speculate that in this species two evolutionary sympatric lines have developed by either a selection in dry environments, or in warm environments and thus leading to an entity that is better suited for human infection.

It seems that the truly halophilic / xerophilic species, such as *Trimmatostroma salinum* and *Wallemia muriae* are already too specialized for life in extreme ecological niches to be able to successfully survive an ecological shift, as described above for xerotolerant taxa. They seem to have adapted well to the special stress provided by their highly osmotic environmental niches. Species tolerating very low-water activities (X_P and H_P; Table 2) are consistent in their ecology, both in natural and in artificial environments. They are unflexible from an evolutionary perspective.

Habitat-shifts can be dramatic when an environment is accidentally colonized that is very different from the natural habitat – provided that further transmission takes place and fitness is not impaired. The evolutionary processes accompanying sudden leaps in ecology are conveniently monitored in artificial, human-made environments, such as indoor environments, because these are non-existent in nature. Any fungus that is overabundant in a fragmented artificial environment is subjected to dramatic natural selection leading to accelerated evolution (Vandergast et al., 2004). Species with less pronounced xero- or halotolerance (X_T and H_T; Table 2) may be more prone to evolution in other directions.

Many X_T and H_T species, particularly members of *Eurotiales*, are found on foodstuffs, such as dry food, dry meat or sea fish. These habitats basically consists of materials that occur as well in nature, the main difference being that it is now being treated for preservion and subsequently eaten by humans. The occurrence of pronounced natural selection is thus less likely in food-borne fungi. Accelerated evolution thus is more likely to happen as a result of natural selection after an environmntal shift. In the case of the systemic *Onygenales* such as *Coccidioides* the shift was accompanied by a plesiomorphic pathogenic potential, and thus has led to a process of adaptive, sympatric evolution, whereas the possibly enhanced virulence of black yeast-like fungi is more likely to be coincidental. The systemic *Onygenales* seem to have reached a high degree of adaptation, with an eleborate ecological strategy using rodents as vectors for dispersal, whereas the process of natural selection in indoor fungi has just begun after the introduction of suitable habitats by humans.

5. Conclusion

If xerotolerance is regarded as a general condition to cope with general stress, presence of properties underlying this ability provides the fungus with an armament to survive types of stressful conditions other than decreased water activity. Low degrees of xerotolerance therefore may mark different starting points of subsequent evolution, either into a direction of an even higher degree of stress tolerance (e.g., *Wallemia muriae*), or in another direction, leading to disruptive selection (e.g., *Exophiala dermatitidis*) or to

adaptive sympatric speciation (e.g., *Coccidioides immitis*). A diagrammatic representation of these evolutionary options is given in Fig. 2. Thus, xerotolerance is likely to be the closest evolutionary origin of dual capacities observed in the fungal orders of Table 2.

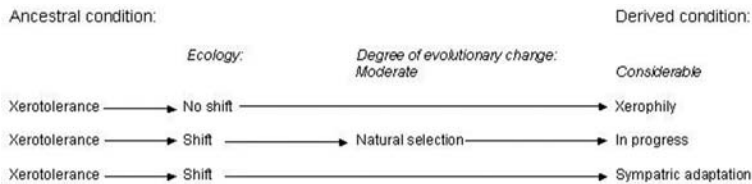

Figure 2. Diagram of hypothetical evolutionary options, with xerotolerance as a starting point.

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HALOTOLERANT AND HALOPHILIC FUNGI FROM COASTAL ENVIRONMENTS IN THE ARCTICS

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1. Introduction

Extreme environments on Earth have fascinated microbiologist since the discovery of extremophilic microorganisms, thriving in niches previously thought to be abiotic. The initial focus of attention was directed to unravelling the biodiversity of microorganisms inhabiting them. This may lead to the identification of new interesting extremophilic model organisms, enabling in-depth molecular studies (Aguilar, 1996).

This exciting field of extremophilic microbiology has so far been primarily dedicated to the study of prokaryotic microorganisms, due to the general belief that eukaryotes are unable to inhabit such environments. Contrary to these expectations, recent studies have revealed the presence of a diversity of eukaryotic microorganisms in extreme habitats. Amongst them fungi reaffirmed themselves as one of the ecologically most successful eukaryotic lineages, as they have been isolated from hypersaline waters (Gunde-Cimerman et al., 2000), at 10 km depth below the surface of the oceans (Nagahama et al., 2001), from extremely acidic mine waters (Hölker et al., 2004), and from the surface of rocks in arid and cold climates (Gorbushina et al., 1996; Sterflinger and Krumbein, 1997; Sterflinger et al., 1999). However, fungal diversity in many of these habitats is mostly unknown.

2. Halotolerant and Halophilic Fungi

So far, the best studied fungal extremophiles are halophilic and halotolerant fungi, inhabiting hypersaline waters of salt lakes and salterns around the world (Buchalo et al., 1998; Casamayor et al., 2002; Gunde-Cimerman et al., 2000; Petrovič et al., 2002). Through these studies it became evident that halophilic fungi use adaptive strategies different from the majority of halophilic prokaryotes. Although truly halophilic fungi do exist, the majority of fungi growing on hypersaline substrates displays a general xerophylic phenotype (Filtenborg et al., 2000; Northolt et al., 1995), reflected by their ability to grow at low water activity (a_w), unregarding the chemical nature of the solute

(Hocking, 1993; Pitt and Hocking, 1985a, 1985b). Fungal species are considered xerophilic if they grow well at water activities of 0.85 or less, corresponding to 17% NaCl or 50% glucose in the growth medium. According to the recent definition of halophily in fungi, those species which are isolated regularly with high frequency on selective saline media from environments at salinities above 10%, and are able to grow *in vitro* on media with at least 17% NaCl are considered halophilic (Gunde-Cimerman et al., 2004, 2005). Although halophilic fungi do not require salt for viability, they are, contrary to many halophilic prokaryotes, able to grow and adjust to the whole salinity range, from freshwater to solutions saturated with NaCl. They can adapt to this broad ecological amplitude by surviving periods of extreme environmental stress in a resting state, but when conditions change, they can use the available water immediately and respond with increased metabolic activity, growth and propagation. This adaptive halophilic behaviour, named poikilophilic halophily, enables continuous colonization of the hypersaline environments (Butinar et al., 2005a).

3. Fungi in Polar Regions

Cold polar regions are extreme environments, in which the majority of studies have been oriented towards psychrophilic bacteria, while the occurrence and diversity of psychrophilic fungi remained largely unknown.

So far fungi have been reported primarily in connection with sub-Arctic vegetation and soil in polar regions. Mainly basidiomycetous yeasts were isolated from berries, flowers, vegetation of the littoral zone, soils, forest trees, grasses (Babjeva and Reshetova, 1998) and Antarctic mosses (Tosil et al., 2002). Recently, fungi belonging to the Ascomycota and Basidiomycota, many of them new, were discovered in abundance below snow-covered tundra (Pennisi, 2003; Schadt et al., 2003). Although much of the water in tundra regions for most of the year is not biologically available, the peak in fungal activity was detected during winter, while in spring and summer bacteria prevailed (Hodkinson et al., 1999; Schadt et al., 2003).

Few studies exist on the biodiversity of fungi in Antarctic soils (Vishniac and Onofri, 2003). Such soils represent an interesting habitat for xerophilic fungi, since they exhibit extraordinary aridity, with a correspondingly low a_w, as well as a relatively high salt content (Vishniac and Onofri, 2003). The major soluble salts in Antarctic soils are sulfates, chlorides, nitrates of Na, K, Mg and Ca. Besides, microbial life is exposed to low temperatures, low nutrient availability, seasonally increased UV radiation, and geographic isolation (Onofri et al., 2004). In contrast to the mycobiota present in mesophilic soils, dominated primarily by diverse ascomycetous filamentous fungi (Domsch et al., 1980), diverse basidiomycetous yeasts prevail in Antarctic soils. The dominant yeast genera are *Candida*, *Cryptococcus* and *Leucosporidium* (Vishniac and Klinger, 1986; Vishniac and Onofri, 2003). In all cases their diversity was low and dominated by a few highly specialised and often endemic taxa (Abyzov, 1993; De Wit et al., 2003). The highest halotolerance for yeasts isolated from Antarctic soil was recorded for *Cr. albidus* and *Cr. himalayensis* (9% NaCl) (Onofri et al., 2004), although basidiomycetous yeasts in general show low salt tolerance and inability to grow on media with low a_w.

Viable yeast and fungi were isolated sporadically also from Siberian permafrost sediments, firmly fixed by ice. They were maintained in a frozen state for extended periods, but upon thawing they were nevertheless able to resume their metabolic activity (Takano, 2004). The most common yeast genera were again *Cryptococcus* with the most frequently encountered species being halotolerant *Cr. albidus*, followed by *Sporobolomyces*, *Rhodotorula* and *Cystofilobasidium.* They were found with the highest frequency of occurrence in the youngest layers, less than 10.000 years old, although they were also detected in three millions years old Pliocene samples. In all cases the share of the yeasts represented 25% of all aerobic heterotrophs, independent of the organic matter content (Dmitriev et al., 1997a, 1997b; Gerday et al., 1998; Rivkina et al., 2000).

The occurrence of fungi in polar aquatic habitats has even been less investigated. Yeasts and fungi were isolated from fresh water samples, benthic microbial mats and biofilms on pebbles beneath the ice of Antarctic lakes (Baublis et al., 1991; De Wit et al., 2003).

From polar offshore sea waters mainly basidiomycetous yeasts of the genera *Leucosporidium*, *Rhodosporidium* and *Sporobolomyces* were isolated (Jones, 1976). Sequences belonging to Eumycota were detected in up to 3000 m deep Antarctic polar front waters (Lopez-Garcia et al., 2001). There is no report on the isolation of fungi from sea ice, although their characteristic small subunit rRNA gene sequences were present in DNA extracted from diverse Antarctic and one Arctic sea ice sample (Brown and Bowman, 2001). Fungi were detected as well in the hypersaline Antarctic Lake Wanda (Kriss et al., 1976).

The presence of fungi was least investigated in polar glaciers. Filamentous fungi and yeasts were found in the microbial cryoconite holes that probably serve as biological refuges during extreme cold (Margesin et al., 2002; Reeve et al., 2002). Viable filamentous fungi and yeasts have been isolated from 10,000-13,000 years old Greenland ice (Ma et al., 1999a, 1999b), 12,000 years old Antarctic Vostok ice core sections (Christner et al., 2000, 2002), and even from Antarctic ice layers up to 38,600 years old (Abyzov, 1993). In all these cases the isolated fungi were filamentous and their numbers were low, while viable yeasts of the genera *Cryptococcus* and *Rhodotorula* have been found only in the upper, younger ice-sheet horizons and surface layers of ice and snow. The oldest yeasts were isolated from horizons 700-3,250 years old (Abyzov, 1993).

By PCR amplification of fragments of the eukaryotic 18S rRNA gene, a diversity of fungi was identified in 2000-4000 years old ice-core samples from North Greenland. They were not tested for viability (Price, 2000). All findings of fungi in glacier ice were interpreted as the result of coincidental Aeolian deposits of spores or mycelium into the ice during its geological history.

4. Potential Ecological Niches for Halophilic/Halotolerant Fungi in the Coastal Arctic Environment

Due to the adaptive fungal behaviour at low a_w, we have assumed that coastal Arctic environments, in particular diverse type of ice, could represent a potential ecological habitat for halotolerant and halophilic fungi. High concentrations of NaCl create both ionic and osmotic stress, while high concentrations of sugars and drought cause osmotic stress. There is great similarity between osmotic stress and matric water stress since in

both cases the water activity is low. As water becomes ice it is not biologically available, and diverse types of ice can therefore be characterized as well as low a_w environments. Additionally, freezing leads to cellular dehydration due to reduced water absorption, while high salinity causes the same effects due to osmotic imbalances.

4.1. SEA ICE

Sea ice is dominated by strong gradients in temperature, salinity, space, and light. Frozen seawater forms a semisolid matrix, permeated by a network of channels and pores, filled with brine formed from expelled salts as the ice crystals freeze together. The salinity of sea ice brines within channels and cracks of the sea ice (formed when salt is ejected during freezing) can rise as high as 200‰, while the salinity can drop to below 10‰ as the sea ice melts (Brown and Bowman, 2001). Sea ice is also an extreme environment regarding temperatures, which can range from -1°C to as low as -50°C in winter (Thomas and Dieckmann, 2002). Brine inclusions, in general ranging from several μm to cm in size, become increasingly disconnected at lower temperatures, although they remain liquid down to -35°C. Microorganisms inhabiting sea ice have to combine freezing tolerance with tolerance to dehydration caused by the lack of free water at low temperatures and/or high salinities. Changing water activity is therefore the dominant factor in the sea ice external chemistry (Deming, 2002; Junge et al., 2002; Krembs et al., 2002).

The evaporating ponds on the surface of sea ice, formed when spring surface melting occurs, form an additional potential habitat for halotolerant fungi. These melt-water ponds with temperature near 0°C are characterized by increased salinity, and they are occasionally nutrient rich due to ornithogenic contributions.

4.2. GLACIAL ICE

Atmospheric circulation over polar regions provides air-mass exchange with lower latitudes. As a result, microorganisms from air-borne terrestrial dust may become embedded in ice formed from snow. This microbial diversity is thus represented by taxa that are probably endemic to the polar regions as well as exotic species from temperate and tropical regions. These can originate from ocean mist, wind-borne pollen and soil particles, infected plant surfaces, and many other sources. They may have been transported and deposited by the action of waves, wind, rain, snow, animals, or by other means (Abyzov, 1993; Ma et al., 1999a, 1999b). Glacial ice thus provides a unique global source of microorganisms, enabling the study of both contemporary and ancient microbial diversity.

Glacial ice is known as an extremely stable, frigid and static environment. Microorganisms or their spores are protected from UV irradiation, and because they become desiccated, DNA damage is minimized. Viable microorganisms, randomly entrapped in ice even for thousands of years, are destined to be released during glacial melts or after the calving of icebergs into the ocean (Ma et al., 1999a, 1999b). The few existing studies of fungi in glacier ice have provided evidence on the viability and diversity of the randomly entrapped mycobiota.

Recent investigations have shown that glaciers are much more dynamic than previously assumed on the micro scale as well as on the geomorphological level. Ice in

temperate glaciers is permeated by a continuous network of aqueous veins, formed at the linear junctions of three ice crystals. They are formed due to sea salts deposited as aerosols, that are essentially insoluble in ice crystals. These liquid veins can have high ionic strength, and due to the percolation of salts from the top of the glacier to its bottom, salts can be accumulated to relatively high concentrations in the bottom parts of polythermal glaciers (Price, 2000). Besides, due to quick seismic shifts (Ekström et al., 2003; Fahnestock, 2003) and cryokarst phenomena in connection with massive surface ablations, liquid water can temporarily appear as ponds or streamlets on the surface of the glacier and as caves or interglacial lakes, artesian fountains and moulins within the glaciers (Christner et al., 2000). These supraglacial waters can also reach the glacier bed and mix with groundwater and basal meltwater generated by frictional and geothermal melting of ice at the glacier base. These liquid waters interact with rocks and sediments, and hence contain high solute and suspended sediment concentrations. When frozen onto the basal glacier ice, they can be transported to the glacier margins, where it can be aseptically sampled. Until recently subglacial environments were thought to be abiotic, but lately viable bacteria have been found (Foght et al., 2004; Gaidos et al., 2004; Lanoil, 2004; Priscu et al., 1998; Siegert et al., 2001; Skidmore et al., 2000). In all these cases, there were no reports on the presence of fungi.

5. Isolation of Fungi from Arctic Coastal Environments

A study involving isolation of halotolerant and halophilic fungi from an Arctic coastal environment was performed in Kongsfjorden, one of the largest fjords found at the western coast of Spitsbergen, Svalbard, located at 79°N, 12°E. It runs from ESE to WNW, joins with Krosfjorden at the mouth and continues into the Greenland Sea. The fjord has a narrow, long shape, is 26 km long and 8 km wide, with several small islands, a steep coastal line, and a deep (140 m) water body. The tide is diurnal with a range of ca. 2 m. Fjord water is in contact with the overlying atmosphere through its surface all year. The water in the fjord is rather immobile, but is occasionally stirred by the wind. It is warmer and less salty than open sea at the same latitude, with an annual mean temperature around -5°C and spring/summer temperatures which can be as high as 3.8°C. The salinity decreases as the season progresses. The fjord is filled with Atlantic deep water at 34.95 psu at the end of the winter. Fresh water is added in summer due to the ablation of glaciers, and this reduces the mean salinity to a minimum of 34.00 psu (Ito and Koduh, 1997). The fjord remains mainly unfrozen despite its location at high latitude, as only the coastal part of the fjord develops a stable sea ice cover in most years. The majority of the drainage basin is covered by glaciers, and most of these reach the sea. Therefore many small pieces of glacier ice are found on the surface of the fjord during most of the year (Ito and Koduh, 1997).

Isolation conditions were designed to accommodate xerotolerant/halotolerant fungi by using media with high concentrations of salt or sugar, and thus low a_w, such as previously used for the isolation of halotolerant/xerotolerant fungi from temperate hypersaline environments (Gunde-Cimerman et al., 2000). These media should give a selective advantage to cultivable microorganisms adapted to ice, thereby possibly enabling the isolation of higher fungal colony forming units (CFU) numbers than previously reported (Gunde-Cimerman et al., 2000, 2003).

Melanized yeast-like fungi were identified by their morphology, their physiology, and by sequencing of ITS rRNA to the species level (Zalar et al., 1999). Isolates of filamentous fungi were identified to the species level by morphology, physiology, and in most cases also by secondary metabolite profiles using HPLC-DAD (Smedsgaard, 1997; Sonjak et al., 2005). The identification methods for non-melanized yeasts followed those described by Yarrow (1998), and the strains were examined as well by sequence analyses in the D1/D2 region at the 5' end of the large subunit rRNA.

All isolates are maintained in a genetically stable way in the Culture Collection of the National Institute of Chemistry (MZKI) (Slovenia) and in the EXF Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia). Most penicillia are preserved as well in the fungal collection (IBT) at the Centre for Microbial Biotechnology (BioCentrum-DTU), Denmark, while yeast-like strains are preserved in CBS, Utrecht, The Netherlands.

5.1. PHYSICO-CHEMICAL CHARACTERISTICS

The pH of the water from which isolates were obtained varied between 7.1 and 7.4 in all samples. The highest cation concentrations were determined for sea water and the lowest for glacial ice, where they ranged from 5 to 340 mg sodium kg^{-1}, from 20 to 310 mg potassium kg^{-1} and 70 to 550 mg magnesium kg^{-1}, respectively. The highest phosphorus content was determined in glacier-ice samples and the lowest in the sea water (<1.00) (Gunde-Cimerman et al., 2003).

5.2. FREQUENCY OF OCCURRENCE

In Kongsfjorden sea water the highest CFU of fungi were determined on a general-purpose medium, with a_w 1.0, at 10°C incubation temperature. The CFU was as high as $3x10^3$ CFU l^{-1}, while it was considerably lower on medium with a_w lowered to 0.946 (Fig. 1). Almost all detected fungi were non-melanized yeasts and diverse penicillia, with few melanized yeasts. The frequency of occurrence was considerably higher than reported in polar offshore waters, usually in the range of 1-12 CFU l^{-1} and only occasionally up to$2x10^2$ CFU l^{-1} (Jones, 1976).

It was assumed that higher fungal CFU numbers than previously reported will be obtained from diverse types of ice on media with lowered water activity, due to the prevention of osmotic imbalances. In accordance, the total number of fungal CFU isolated from sea-ice on medium with a_w 1.0 was only up to $2x10^2$ CFU l^{-1}, but when using medium with a_w lowered to 0.946, it increased up to $7x10^3$ l^{-1} for sea ice and up to $1x10^4$ l^{-1} for molten glacier ice (Fig. 1) (Gunde-Cimerman et al., 2003).

In both types of ice non melanized yeasts predominated, although in samples of glacier ice with gypsum inclusions, melanized yeast-like fungi prevailed, with CFU as high as $6x10^5$ l^{-1} (Fig. 2). There are no reports in the literature on the frequency of occurrence of fungi in sea or glacier ice, apart the infrequent sporadic isolations of individual fungal species, deposited by wind or snowfall into the ice (Abyzov, 1993; Christner et al., 2003; Ma et al., 1999a, 1999b; Price, 2000).

The frequency of occurrence of fungi in sea water and glacial ice enumerated on salt-based media is presented in Fig. 3. Isolations from sea ice are not presented, since they were performed only using 17% NaCl medium, on which primarily *Penicillium* and black

yeast-like fungi were isolated with low frequencies (up to 25 CFU l^{-1}). On selective saline media the highest fungal CFU was detected on medium with 5% NaCl added. Counts occasionally increased up to $1.3x10^4$ l^{-1} for glacier-ice samples. With increasing salinity, the number of fungal CFU decreased. Therefore, the upper salinity range for the detection of fungi was 24% NaCl with CFU values up to 5 CFU l^{-1} only.

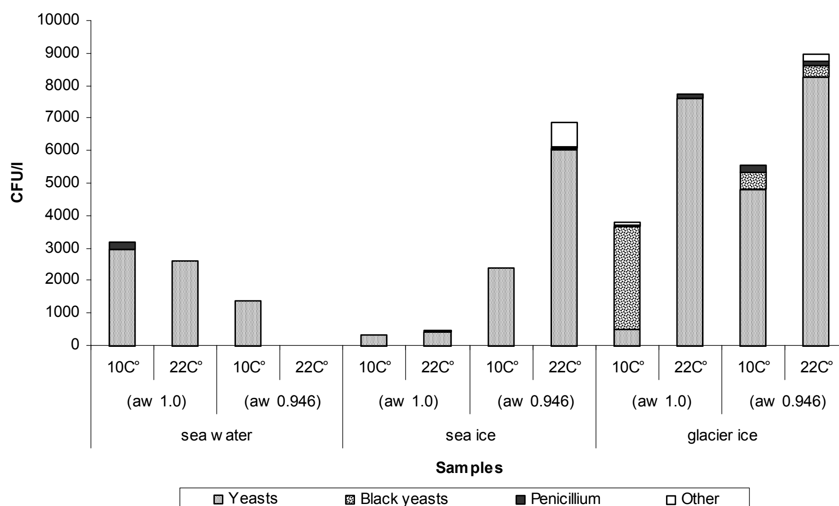

Figure 1. Colony forming units (CFU l^{-1}) of fungi on enumeration media.

Figure 2. Sample of glacier ice with gypsum inclusions.

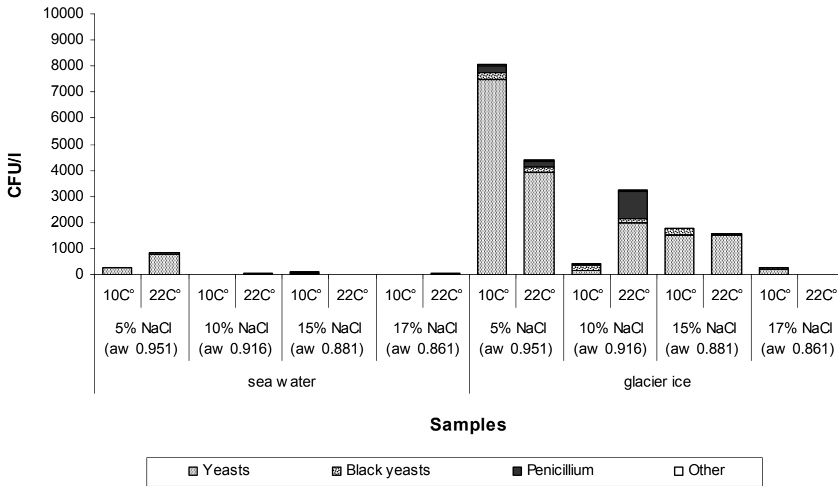

Figure 3. Colony forming units (CFU l^{-1}) of fungi on malt extract agar medium with various NaCl concentrations added (5, 10, 15, 17%).

At lower salinities non-melanized yeasts dominated, but with increasing salinity the proportions changed in favour of melanized fungi and *Penicillium* spp., with *P. crustosum* being the most frequently isolated species (Fig. 4 and 5).

Figure 4. Non-melanized yeasts on 5% NaCl medium after filtration of 0.1 ml of molten glacial water.

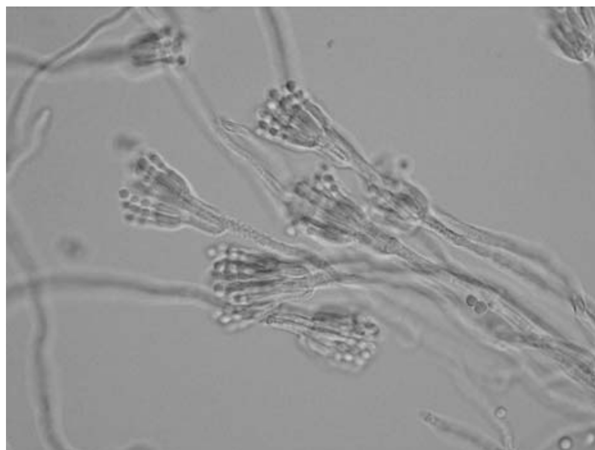

Figure 5. *Penicillium crustosum*, the *Penicillium* species most frequently isolated from glacial ice.

6. Diversity of Fungi

A list of fungal genera isolated in diverse ecological niches in Kongsfjorden is presented in Table 1.

The main taxa of halotolerant/halophilic fungi isolated in Kongsfjorden appear as well in hypersaline environment of solar salterns and hypersaline lakes worldwide. These isolates include melanized fungi that were mainly represented by the oligotrophic genus *Cladosporium*, taxonomically and phylogenetically closely related to the black yeast-like halotolerant genus *Aureobasidium*. Among melanized fungi the genera *Alternaria* and *Phoma* occur both in the ice and in the solar salterns, although in both cases with low frequencies. Filamentous fungi, isolated with high frequency from both environments, were represented by the cosmopolitan anamorphic genera *Aspergillus* and *Penicillium*, together with the teleomorphic form *Eurotium* (Butinar et al., 2005a, 2005b), although in the polar environment *Penicillium* prevailed over *Aspergillus* and *Eurotium*. Other filamentous genera that appeared in both habitats with low frequency were *Mucor* and *Trichoderma* (Gunde-Cimerman et al., 2001).

Non-melanized yeasts that were detected recently in hypersaline waters around the world as well as in Konggsfjorden include *Cryptococcus*, *Debaryomyces*, *Filobasidium*, *Metschnikowia*, *Pichia*, *Rhodosporidium*, *Rhodotorula* and *Trichosporon* (Butinar et al., 2005c). Genera that have been isolated only in the Arctic niches, but not in the hypersaline waters of the salterns include *Cadophora*, *Geomyces* and *Phialophora* (data not published). When strains of these genera were tested for halotolerance, they could all grow on media with at least 10% NaCl. The halophilic black yeast-like species *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and the halophilic genus *Wallemia*, fungi that dominate in hypersaline waters of solar salterns, were not detected in the Arctic environment (Gunde-Cimerman et al., 2000; Zalar et al., 2005).

The diversity of species was highest in sea water but decreased in sea ice, where fewer detected species were represented in higher numbers. Species diversity in glacier ice depended considerably on the occurrence of mineral inclusions. In samples with visible sediments penicillia prevailed, and their species diversity was high, while in samples of clear ice the species diversity was low, although individual species of non-melanized yeasts occurred with high frequency (Gunde-Cimerman et al., 2003).

TABLE 1. List of fungal genera isolated from diverse ecological niches in Kongsfjorden.

Species	Seawater	Sea ice	Ponds on sea ice	Snow/ice in tidal zone	Glacier ice	Glacier melt water	Antarctic ice (literature data)
Acremonium				x			x
Alternaria		x					
Ameolosidium						x	
Aspergillus		x	x		x		x
Aureobasidium	x	x	x	x	x	x	
Cadophora			x		x		x
Cladosporium	x	x	x	x	x	x	x
Cryptococcus	x	x	x		x	x	x
Debaryomyces	x				x	x	
Eurotium	x	x	x	x	x		x
Filobasidium	x	x	x		x		
Geomyces					x		x
Metschnikowia	x	x					
Mucor		x	x		x		x
Penicillium	x	x	x	x	x	x	x
Phialophora				x			x
Phoma		x	x			x	
Pichia	x				x		
Rhodosporidium					x		
Rhodotorula	x	x			x	x	x
Tricellula				x			
Trichoderma			x			x	
Trichosporon					x		

Aureobasidium

Aureobasidium is a genus of xerotolerant fungi, primarily inhabiting oligotrophic environments. The most ubiquitous species is the halotolerant *A. pullulans*, which is regularly detected in salterns, where it represents one of the core species of saltern mycobiota at lower salinities (up to 10% NaCl). The salinity range of growth for *A. pullulans*, defined *in vitro*, was from 0-18% NaCl, while it grew optimally on medium without NaCl (Butinar et al., 2005a). Besides salterns and sea water its ecological niches are damp inert surfaces such as glass, stone or the phylloplane of plants. It is also known for its ability to grow at high levels of radioactive gamma contamination, since some strains have been isolated even from the walls of the Chernobyl reactor. In polar environments *A. pullulans* has been previously isolated from continental Antarctic soil samples (Onofri et al., 2004).

The dominant genus of black yeast-like fungi isolated from the coastal Artic environment with high abundance was *Aureobasidum*. Based on ITS sequencing, isolates

were identified as diverse genotypes of *A. pullulans* (data not published). Although strains were isolated from sea ice and ponds on its surface, they prevailed in the bottom ice of polythermal glaciers containing crystal gypsum inclusions (Fig. 2). *A. pullulans* strains were primarily isolated on media with 5% NaCl after one week of incubation at 22°C, or two weeks at 10°C.

Cladosporium

The genus *Cladosporium* is taxonomically and phylogenetically closely related to black yeasts belonging to the order *Dothideales* (De Hoog et al., 1999; Sterflinger et al., 1999). It comprises about 500 plant-pathogenic and saprophytic species, many of which are among the most airborne species in the fungal kingdom.

The species *C. cladosporioides*, *C. herbarum*, *C. sphaerospermum*, *C. tenuissimum*, and *C. oxysporum* are often isolated from salty/sugary food (Samson et al., 2000) and other environments with low water activity such as saline (coastal) soils and salt marshes, the phylloplane of Mediterranean plants, and the rhizosphere of halophytic plants (Abdel-Hafez et al., 1978). During the study on the occurrence of fungi in hypersaline environmnts, strains from this genus were isolated from thalassohaline and athalassohaline hypersaline waters worldwide. They were in general among the most abundant and most consistently detected fungi in all natural hypersaline waters sampled, at environmental salinities between 15-25% (Butinar et al., 2005a; Gunde-Cimerman et al., 2005; Zalar et al., 2001). The majority of isolates from the salterns belong to the species *C. sphaerospermum.* Less abundant were isolates belonging to *C. cladosporoides*, *C. oxysporum* and *C. herbarum*.

The majority of cladosporia isolated from Kongsfjorden comprise the same species as most frequently isolated from Antarctic soil and hypersaline waters in the salterns: *C. cladosporioides*, *C. herbarum*, *C. sphaerospermum* (Fig. 6), although several yet unidentified strains were isolated as well (Abyzov, 1993; Gunde-Cimerman et al., 2005; Ma et al., 1999a, 1999b), amongst them a small-spored *Cladosporium* sp. These *Cladosporium* species were primarily isolated from glacial ice samples and considerably less frequently from sea water and sea ice. The highest frequency of occurrence was obtained on media with 50% glucose added at 10°C, although all tested strains grew well on 17% NaCl media.

The genus *Cladosporium* is known for its psychrotolerance. The reported temperature range for growth of *C. cladosporoides* is from 0-32°C, with slight growth possible at -3°C and even at -10°C. Some isolates of *C. herbarum* were shown to grow even at -6°C. All three species can grow at high levels of radioactive gamma irradiation (Onofri et al., 2004).

Non-melanized yeasts

There have been only few studies on the occurrence of halotolerant/halophilic yeasts in natural environments. Most available information on the biodiversity of the few known xerotolerant yeasts is thus derived from food contaminations. Among xerotolerant food-borne yeasts ascomycetous genera prevail. They include *Candida* (*C. krusei* = *Issatchenkia orientalis* [teleomorphic state], *C. lambica*), *Hansenula* (*H. anomala*), *Debaryomyces* (*D. hansenii*), *Pichia* (*P. ohmeri*, *P. guilliermondii*) and *Zygosaccharomyces* (*Z. rouxii*, *Z. bisporus*). The only known halotolerant food-borne basidomycetous yeast genus is *Rhodotorula* (*R. glutinis*).

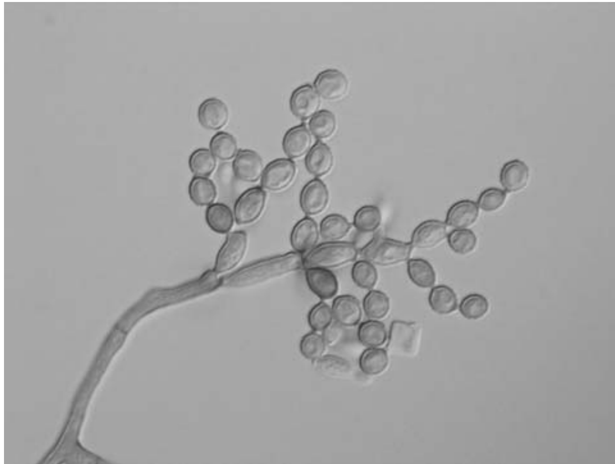

Figure 6. *Cladosporium sphaerospermum* from glacial ice.

Halotolerant yeasts in natural habitats were mainly investigated in sea water, where basidiomycetous yeasts from the genera *Rhodotorula* (*R. mucilaginosa*, *R. glutinis*), *Rhodosporidium*, *Trichosporon* and *Cryptococcus* (*Cr. laurentii*, *Cr. albidus*) prevail (Jones, 1976). Although ascomycetous yeasts are most abundant on food preserved with high concentrations of salt or sugar, they are not common in sea water. The most important ascomycetous marine genera are *Debaryomyces* represented by *D. hansenii* and *Metschnikowia*, although species of *Candida*, *Hansenula* and *Pichia* have been occasionally reported as well. Among these, *M. bicuspidata* var. *bicuspidata*, a parasite of the brine shrimp, is the only species in which a real dependency on salt has been so far discovered (Butinar et al., 2005c; Jones, 1976; Javor, 1989).

Contrary to the belief that hypersaline waters do not harbour any yeast population (Hernandez-Saavedra et al., 1995), halotolerant yeasts were recently isolated from such environments (Butinar et al., 2005c). The frequency and occurrence of halotolerant yeast species in different hypersaline environments was highly inconsistent. They were represented by diverse ascomycetous (*Pichia guilliermondii*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida parapsilosis*, *C. glabrata*-like, *Metschnikowia bicuspidata*) and basidiomycetous species (*Rhodosporidium sphaerocarpum*, *R. babjevae*, *Rhodotorula laryngis*, *Trichosporon mucoides*). Notably, the species that had the highest temporal frequency of occurrence were *P. guilliermondii*, *C. parapsilosis* and *T. mucoides*. These species could be indigenous and they probably represent a stable core of fungal hypersaline communities.

The diversity of yeast in the extremely cold polar environments was before our study mainly investigated in Antarctic soil and Siberian permafrost, where basidiomycetous genera represented the dominant psychrophilic microorganisms (Abyzov, 1993; Babjeva and Reshetova, 1998; Deegenaars and Watson, 1998; Golubev, 1998; Rivkina et al., 2000; Vishniac and Onofri, 2003). The isolated species were not known or tested for

their halotolerance. There were no investigations performed on the occurrence of (halotolerant/xerotolerant) yeasts in sea ice or glacial ice.

In our study, non-melanized yeasts were isolated primarily from the ice at the bottom of polythermic glaciers. Their frequency of occurrence was surprisingly high, reaching up to 3.10^6 CFU l^{-1}. Although to our knowledge ascomycetous yeasts were hardly ever isolated from the extremely cold polar regions, the majority of yeasts isolated in our study on media with low water activity were ascomycetous, while on mesophilic media basidiomycetous species prevailed.

Most of the isolated yeast genera were the same as described from temperate hypersaline environments: *Debaryomyces*, *Metschnikowia*, *Pichia*, while the prevailing basidiomycetous genera were *Cryptococcus*, *Filobasidium*, *Rhodotorula*, *Rhodosporidium* and *Trichosporon*. After identification to the species level (Yarrow, 1998), representative strains of the isolated species were tested for their halotolerance by inoculating on media with 10% and 17% NaCl. The basidiomycetous species *Cryptococcus albidus*, *Cr. liquefaciens*, *Cr. victoriae*, *Rhodosporidium diobovatum*, *Rhodotorula mucilaginosa*, *Rh. laryngis*, and *Trichosporon mucoides* and the ascomycetous yeasts *Debaryomyces hansenii*, *Pichia guilliermondii* and *Metschnikowia zobellii* were all able to grow on media with 10% NaCl added, while only *Rh. mucilaginosa*, *D. hansenii* and *P. guilliermondii* could grow at 17% NaCl (Table 2).

Cryptococcus albidus was the dominant species isolated from glacier ice, followed by *Cr. liquefaciens*. Strains in the Albidus clade are widely distributed in nature, both in natural and man-made environments (Chand-Goyal and Spotts, 1996; Middelhoven, 1997; Roostita and Fleet, 1996; Slavikova and Vadkertiova, 1997), and *Cr. albidus* is also reported as an infectious agent in humans (Horowitz et al., 1993; Loison et al., 1996). Many species have been collected from soil and plants in the polar regions (Onofri et al., 2004; Vishniac and Onofri, 2003).

The *Sporidiobolus* lineage includes species of the teliosporic genera *Rhodosporidium* and *Sporidiobolus* and their anamorphic counterparts in the genera *Rhodotorula* and *Sporobolomyces* (Fonseca et al., 2000). From clade Glutinis the halotolerant species include *Rhodosporidium diobovatum* and from clade Sphaerocarpum *Rhodotorula mucilaginosa.* The latter was obtained from sea water, sea and glacier ice. In hypersaline waste water ponds in Israel it represeted one of the two dominant microorganisms (Lahav et al., 2002). Otherwise it is rather common in diverse aquatic environments. The species *Rhodotorula laryngis* from clade Occultifur of the *Erythrobasidium* lineage was recovered in this study from glacial ice, but it was otherwise found in aquatic environments of glacial origin in Patagonia (Libkind et al., 2004), as well as in the hypersaline waters of the Dead Sea (Butinar et al., 2005c).

From the genus *Trichosporon*, the major taxon of the Trichosporonales, *Trichosporon mucoides* was the isolated halotolerant species from the clade Cutaneum. The isolates of *T. mucoides* are otherwise mostly of human origin (Kurtzman and Fell, 1998).

D. hanseni, a known halotolerant food-borne yeast has been found together with its imperfect form *Torulopsis famata* as in this study mainly in cold sea water (Norkrans, 1966). It has been isolated also from natural hypersaline habitats that are seasonally exposed to low temperatures (Butinar et al., 2005c).

TABLE 2. Occurence of non-melanized yeast in diverse ecological niches in Kongsfjorden.

Halotolerant yeast species	*Sea water*	*Sea ice*	*Ponds on sea ice*	*Snow/ ice in tidal zone*	*Glacier ice*	*Glacier ice melt-water*	*Isolation on media with low a_w*	*Growth on 10% NaCl*	*Growth on 17% NaCl*	*Growth at 10°C*	*Temperate hypersaline environments*
Cryptococcus albidus			+		+	+	+	+	-	+	-
Cr. liquefaciens					+		+	+	-	+	-
Cr. victoriae	+				+		+	+	-	+	-
Debaryomyces hansenii	+				+	+	+	+	+	+	+
Metschnikowia zobellii	+	+			+		+	+	-	+	-
Pichia guilliermondii	+				+		+	+	+	+	+
Rhodotorula laryngis					+		-	w	-	+	+
Rh. mucilaginosa	+	+			+	+	+	+	+ (25°C)	+	+
Rhodosporidium diobovatum					+		+	+	-	+	-
Trichosporon mucoides					+		+	+	-	+	+

Different species of the genus *Pichia* are frequently isolated from sea water, with *P. membranefaciens* being the most common (Soares et al., 1997). *P. guilliermondii*, identified as one of the two dominant microorganisms in hypersaline waste water in Israel (Lahav et al., 2002), was isolated from sea water and glacier ice of Kongsfjorden, although it has not been so far reported from polar regions.

The genera *Aspergillus* and *Penicillium* and their teleomorphs

Tolerance to high salt concentrations is a property found in many species of *Aspergillus* and *Penicillium.* In a survey of the NaCl tolerance encompassing 975 species of terrestrial fungi selected from the major taxonomic classes, the penicillia and aspergilli were notably the most resistant, with the majority of species able to grow in the presence of 20% NaCl or more (Tresner and Hayes, 1971). In nature their occurrence was primarily investigated in saline soil. Species belonging to the genera *Aspergillus* and *Penicillium* isolated from the natural hypersaline environments have mainly been known as contaminants of low water activity food and similar substrata (Filtenborg et al., 2000). When they were first isolated from the salterns they were considered as common airborne occurrences, able to survive prolonged exposure in hypersaline water in a dormant state. However recent results indicate that spores and mycelium of some species are able to survive prolonged suspension in brines (Butinar et al., 2005b; Kis-Papo et al., 2003). These species probably represent part of the core of indigenous hypersaline mycobiota. It seems that in saline environments with higher environmental temperatures *Aspergillus* isolates prevail, while in colder environments *Penicillium* isolates are more abundant (Samson et al., 2000).

Penicillium

The genus *Penicillium* is only associated to two teleomorphic states *Eupenicillum* and *Talaromyces*. *Eupenicillium* and its associated anamorphs *Penicillium* subgenus *Aspergilloides*, *Furcatum* and *Penicillium* are rather closely related to *Aspergillus* in a phylogenetic sense, while *Talaromyces* with its associated anamorphs in the *Penicillium* subgenus *Biverticillium* is a distinct genus closer to *Byssochlamys* and *Thermoascus* (Peterson, 2000; Pitt, 1979; Stolk and Samson, 1972). According to Pitt et al. (2000) there are 166 species in *Eupenicillium* and its related anamorphs, while 59 species are accepted in *Talaromyces* and its *Biverticillium* anamorphs. The genus *Eupenicillium* has not been found very often in saline environments, but its *Penicillium* anamorphs are common, both the food-borne forms (Frisvad et al., 2000) and the soil-borne forms (Christensen et al., 2000). Species in the genus *Talaromyces* in general thrive at higher water activities than *Eupenicillium* (Pitt and Hocking, 1997).

The *Penicillium* species isolated most frequently from salterns were *P. antarcticum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. cyclopium*, *P. glabrum*, *P. solitum*, *P. manginii*, *P. miczynskii*, *P. neodoratum* and *P. westlingii* (data not published).

All isolated halotolerant penicillia are also psychrotolerant, and they are very efficient producers of a large number of families of extrolites (Frisvad, 2004; Frisvad et al., 2000; Sonjak et al., 2005). The ubiquitous halotolerant species *P. chrysogenum*, *P. crustosum* and *P. brevicompactum* isolated during this study as well as from the Antarctic (mainly from soil) can even grow at -2°C.

The dominant *Penicillium* species in glacier ice was the halotolerant *P. crustosum* (Sonjak et al., 2005), not reported from Antarctic ice (Table 3; Fig. 5). *P. chrysogenum*,

known to be xerophilic and resistant to radioactivity, survived in Antarctic ice approximately 8000 years, and was also amongst the species most frequently isolated from Kongsfjorden.

TABLE 3. List of halotolerant species of *Penicillium* isolated from diverse polar ecological niches, their occurrence in hypersaline water of the salterns and *in vitro* growth on media with 10 or 17% NaCl added.

Halotolerant *Penicillium* spp.	Sea water	Sea ice	Ponds on sea ice	Coastal snow	Glacier ice	Glacier Melt water	Isolation on media with NaCl	10% NaCl	17% NaCl	Salterns	10°C
"P. arcticum"	x	x			x	x	x	x	x		x
P. bialowiezense	x							x	x		
P. brevicompactum					x			x	x	x	
P. chrysogenum	x	x	x	x	x		x	x	x	x	x
P. corylophilum					x			x	x	x	x
P. crustosum	x	x	x	x	x	x	x	x	x	x	x
P. decumbens	x		x				x	x	x		
P. echinulatum			x		x		x	x	x		x
P. olsonii					x			x	x		x
P. polonicum					x		x	x	x		x
P. resedanum			x				x	x	x		
P. solitum		x		x	x		x	x	x	x	x
"P. svalbardense"					x		x	x	x		x

"P. arcticum" and "P. svalbardense" are new species, so far undescribed.

Halotolerant *P. antarcticum* and P. *aurantiogriseum* that survived in the central Antarctic sheet for 10,000 years (Onofri et al., 2004) were not isolated during our study.

Compared to the fungi found in glacier ice, melted ice and sea water from Svalbard, the mycobiota found in Antarctica are not very different. McRae (1999) found in Antarctica the following halotolerant penicillia: *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. echinulatum*, and *P. solitum*, in addition to the Aspergilloides and Furcatum species *P. corylophilum* and *P. waksmanii*. Most seem to be panglobal except for *P. svalbardense* and *P. bialowiezense* (Frisvad, 2004).

Aspergillus

The genus *Aspergillus* and its teleomorphic states contains 254 broadly accepted species (Pitt et al., 2000). Many of these are known for their xerotolerance and frequent occurrence as contaminants of food preserved with high concentrations of salt or sugar, primarily in warmer climates. Amongst the species of the genus *Aspergillus* most frequently isolated from the salterns are *A. candidus*, *A. fumigatus*, *A. melleus*, *A. niger*, *A. ochraceus*, *A. sydowii*, *A. terreus*, *A. sclerotiorum*, *A. versicolor* and *A. wentii* (data not published). Amongst these *A. sydowii*, *A. terreus* and *A. versicolor* were frequently detected in the Dead Sea as well (Wasser et al., 2003).

In spite of their ubiquitious nature and preference for low water activity, only few halotolerant *Aspergillus* species were isolated from Kongsfjorden: *A. niger*, *A. tubigensis*, *A. sydowii* and *A. versicolor*. *A. niger* and *A. sydowii* have been repored world-wide from sand dunes, salt marshes, estuaries, mangrove mud and other marine environments

(Gunde-Cimerman et al., 2005). *A. sydowii* spores survived prolonged suspension in Dead Sea water (Kis-Papo et al., 2003). This species is also one of the few filamentous fungi reported to have survived in the Antarctic ice sheet for 10,000 years or more. *A. versicolor*, isolated most frequently from diverse ice samples in Kongsfjorden, was repeatedly isolated as well from Antarctic soils, and is also common in diverse low a_w environments in temperate zones. It belongs to the most xerophilic Antarctic species, able also to grow at high levels of radioactive contamination (Onofri et al., 2004).

Eurotium

The genus *Eurotium* is the holomorphic ascomycete genus for *Aspergillus* sections *Aspergillus* and *Restricti*. Species in this genus are particularly xero- and halophilic (Pitt and Hocking, 1985a, 1985b). Its representatives have been reported to live in concentrated salt or sugar solutions at a_w as low as 0.7 (Martín et al., 1998; Wheeler and Hocking, 1993). Different species of the teleomorphic genera *Eurotium* were often isolated from natural hypersaline water environments such as Dead Sea water and hypersaline waters of salterns around the globe. *Eurotium amstelodami* was isolated most frequently, followed by *E. herbariorum* and *E. repens*, *E. rubrum* and *E. chevalieri* were isolated with lower frequency (Butinar et al., 2005b).

In Kongsfjorden *Eurotium* species were rarely isolated, although more frequently than *Aspergillus* spp. They were primarily derived from glacial and sea ice. The most frequent isolate was *E. repens*, followed by *E. amstelodami* and *E. rubrum*. *E. repens* and *E. rubrum* were previously isolated from 8,150 years old ice and Antarctic soil (Onofri et al., 2004).

Alternaria

The most frequently isolated species from hypersaline environments is *Alternaria alternata.* It was repeatedly isolated from water in salterns on three different continents, as well as from Dead Sea water, salt marshes, desert soil and low a_w food (Gunde-Cimerman et al., 2005).

Besides our isolations from sea ice, sea water and glacial ice, *A. alternata* was reported from maritime Antarctica, Alaska and Spitsbergen. It can grow at least at 0°C, at high levels of radioactive contamination, and is UV resistant (Onofri et al., 2004).

Phoma

Phoma species are common coelomycetous soil fungi, which are relatively often obtained from deserts. *Phoma herbarum*, reported from maritime Antarctica, Alaska and Spitsbergen, was isolated from sea ice and ponds on its surface. It has been reported as one of the xerotolerant Antarctic species, and is able to grow at 0°C (Onofri et al., 2004).

7. Cellular Responses to High Salinity and Low Temperatures

Studies performed thus far on the presence of fungi in extreme environments have shown that several species are well adapted to stressful conditions (Onofri et al., 2004). Fungi can typically grow at much lower minimal a_w than prokaryotes, and they also appear to be able to survive strong fluctuations in osmotic and thermal conditions, low environmental temperatures, high UV irradiance and low nutrient availability.

Black multi-cellular thick-walled spores and meristematic endoconidia are probably the most stress-tolerant fungal structures known, but even the hyphae of dematiceous fungi show considerable resistance. In polar regions, thick cell walls and surrounding polysaccharides help overcome the stress associated with low temperatures and consequential water loss, causing increased intracellular solute concentrations, decreased cell size, weakening of the cell membrane, and physical cell rupture which can be caused by freezing and thawing (Christner et al., 2000; Krembs et al., 2002). Adaptations experienced at low temperatures can be related to other environmental challenges such as salt stress, and may therefore reveal new links between psychrophily and halophily (Deming, 2002).

Extracellular freezing leads to cellular dehydration, and chilling temperatures reduce water absorption (Zhu, 2001). Dehydration caused by high salinities and low a_w, is therefore a major stress for ice-trapped organisms. Conversely, when the ice melts, the released organisms are suddenly exposed to hyposaline conditions close to freshwater values (Thomas and Dieckmann, 2002), giving advantage to halotolerant organisms with a wide range of amplitude. Although salt, water and cold stresses are clearly different from each other in their physical nature, each eliciting specific responses, they also activate some common reactions. Salinity creates both ionic and osmotic stress, and drought and cold cause osmotic and oxidative stress. Accordingly, they can induce some common responses. At very low temperatures one of the threats represents ice formation within the cell, which might lead to cell lysis because of the volume increase on expansion of water as ice is formed. The second is increased salinity outside the cell, as ice formation leads to the separation of salt out of pure water (as ice) and a corresponding increase in salt concentration, leading to an osmotic gradient across the cell membrane. It has been shown that mechanisms of freezing and dehydration tolerance in plants and arthropods may involve common gene products and cell signalling and regulatory pathways, and it is likely that these occur in other organisms too. These diverse stresses often activate the production of stress proteins (Cheng, 1998), up-regulation of anti-oxidants and accumulation of compatible solutes, which are at the same time osmolytes and cryoprotectants (Wang et al., 2003). Since protection against dehydration damage is correlated with intracellular accumulation of compatible solutes, an increase in the amount of unfreezable water may be achieved by their production (British Antarctic Survey, 1995; Mindock et al., 2001). Glycerol acts as the primary compatible solute in response to high osmolarity and low temperature in fungi (Gunde-Cimerman et al., 2005), while glycine betaine is synthesized by diverse organisms, including several ascomycetous and basidiomycetous fungi, under conditions of environmental stress such as drought, low temperatures and high salinity (Blackwell et al., 2001).

Water transport through the plasma membrane is important in determining freeze tolerance and halotolerance. Aquaporins, channel membrane proteins with transmembrane domains, are involved in the transport of water and/or small neutral solutes such as glycerol. The precise physiological functions of the fungal aquaporins (and others) have remained unknown so far, although in *S. cerevisiae* a correlation was found between resistance to freezing and the presence of aquaporin genes. Overexpression improved freeze tolerance, while deletion had the opposite effect. A rapid osmotically driven efflux of water during the freezing process reduced intracellular ice crystal formation and the resulting cell damage (Rodriguez-Vargas et al., 2002; Tanghe et al., 2002). On the other hand, osmotic stress induced a decrease of water in the

cytoplasm by efflux of water through aquaporins, with a resultant increase in intracellular ion concentrations (Allakhverdiev et al., 2000).

The plasma membrane is the primary structure through which the cell maintains contact with its environment. Changes in its composition therefore affect its integrity and stability. Membrane characteristics most important for cellular functions are passive and active permeability, nutrient uptake, electron transport, environmental sensing, and recognition (Georlette et al., 2004). Since extreme environmental conditions such as high salinity and low temperatures influence membrane properties, several studies on their adaptations have been performed.

So far the response of the membrane lipid composition of psycrophilic bacteria to changes in temperature has been well documented. In studies of the properties of bacterial membranes at low temperatures, especially alterations in fatty acid composition have been observed. A decrease in temperature led to one or more changes, including a decrease in average fatty acid chain length, an increase in fatty acid unsaturation, an increase in methyl branching, and an increase in the ratio of *anteiso*-branching relative to *iso*-branching. This termal effect was mediated via temperature-dependent changes in the activity of the enzymes involved in fatty acid and lipid biosynthesis (Chattopadhyay and Jagannadham, 2001; Russell, 1997). The fatty acid composition of membrane phospholipids regulates the membrane fluidity; hence, shorter and more unsaturated fatty acids including polyunsaturated fatty acids (PUFAs) are central in retaining membrane fluidity at low temperature. A novel enzyme family, the polyketide synthases, which are active at low temperatures and are required for the biosynthesis of PUFAs, have recently been found in several psychrophilic bacteria isolated from sea ice (Thomas and Dieckmann, 2002).

Only few such studies have yet been performed with fungal species. In the few studies dealing with the membrane adaptation of psychrophilic/psychrotolerant fungi, such as *Mortierella antarctica* and *Cadophora fastigiata* isolated in the Antarctic, it was observed that at low temperatures the amounts of linoleic and arachidonic acid increased respectively. In *Cryptococcus albidus*, *Cr. laurentii*, and *Rhodotorula mucilaginosa*, so far only known for their psychrotolerance, at lower temperatures unsaturated fatty acids predominated in the membranes (Zlatanov et al., 2001).

Studies on the adaptation of membranes of halophilic black yeast-like fungi at high salinity have only recently been performed. Before that the effect of salt stress on lipid composition and membrane fluidity had been investigated only in a restricted group of salt-sensitive fungi. The study on the influence of increased NaCl concentration on membrane lipid composition and fluidity in the halophilic *H. werneckii* and *P. triangularis* and the halotolerant *A. pullulans* showed an increase in the phospholipid-esterified fatty acid unsaturation and the maintenance of a low sterol-to-phospholipid ratio compared to the salt-sensitive *S. cerevisiae*. The total sterol content, including ergosterol as the major sterol, did not change significantly in response to raised NaCl concentrations in the halophilic/halotolerant melanized fungi studied. The major classes of phospholipids were phosphatidylcholine and phosphatidylethanolamine, followed by anionic phospholipids. The most abundant fatty acids in the phospholipids were 16- and 18-carbon fatty acids with a high percentage of $C18:2^{\Delta 9,12}$. These data agreed with electron paramagnetic resonance spectroscopy measurements, which showed that the membranes of the halophilic/halotolerant fungi remained fluid over a much broader range of salinities than membranes of salt sensitive fungi (Turk et al., 2004).

When adaptations to low temperature and high salinity were compared, similar mechanisms of membrane fluidity regulation could be observed. A reduction in temperature slowed down most physiological processes, reduced membrane fluidity, and caused an increased viscosity of water. Thus, tight regulation of membrane fluidity and ion channels permeability are needed, as shown earlier in halophilic organisms (Georlette et al., 2004; Turk et al., 2004). At low temperatures and high salinities, the microbe's ability to modulate the fluidity of its membranes is crucial for its survival. The interrelationship of low temperature and salt stress has been already shown in cyanobacterial cells, in which unsaturated fatty acids in membrane lipids play important roles in the tolerance to salt stress as well as to cold stress. In response to a decrease in ambient temperature or to an increase in salt concentration, psychrophiles or halophiles increase the degree of unsaturation of their fatty acids, thus maintaining the appropriate fluidity of their membrane lipids.

Further investigations are needed to unravel and compare the mechanisms which enable life in some of the most extreme environments on our planet, perhaps on others as well: the hypersaline and extremely cold areas.

8. Conclusions

Fungi, one of the ecologically most successful eukaryotic lineages, have been discovered in diverse extreme environments on Earth. Halotolerant and halophilic fungi represent an integral part of the microbial communities inhabiting hypersaline waters of salterns and hypersaline lakes worldwide, while psychrotolerant and psychrophilic fungi in extremely cold polar areas play important roles in geomicrobiological processes.

Ice, whether in the form of tundra, glacier, snow, lake- or sea ice, presents a special environment for microbial life. Ice combines several stressful factors such as freezing, desiccation, occasional increased salinities, low nutrient availability, thawing and solar irradiation. The occurrence of microorganisms in diverse types of ice has not much been investigated. Occasional fungal isolates from ice were interpreted as random deposits that in some cases survived prolonged periods in the frozen state. However, recent studies have revealed the existence of microbial communities able to retain their viability through dynamic processes of ice melting, followed by micobial enrichments and refreezing. Such studies have so far unraveled only the presence of Bacteria and Archaea, but the presence and role of fungi and other eukaryotic microorganisms have not been investigated.

Certain species of fungi display a general xerophylic phenotype, determined primarily by the low water activity (a_w) potential, which could represent an advantage in ice polar environments. Particularly for halotolerant/xerotolerant and halophilic/xerophilic fungi, adapted to ionic, osmotic or matric water stress, ice could therefore represent a potential habitat. Although halophilic fungi dominant in hypersaline environments (black yeast-like species and *Wallemia*) were not detected in ice, the main taxa of halotolerant fungi isolated from hypersaline environments were isolated either from diverse types of ice or its meltwater. These fungi include the non-melanized basidiomycetous yeast genera *Cryptococcus*, *Rhodosporidium*, *Rhodotorula* and *Trichosporon*, as well as the ascomycetous yeast genera *Debaryomyces*, *Filobasidium*, *Metschnikowia* and *Pichia.* Filamentous fungi prevailing in both habitats belong to the

cosmopolitan anamorphic *Penicillium*, *Cladosporium* and the phylogenetically related *Aureobasidium pullulans*. The genera *Alternaria*, *Phoma* and *Aspergillus* were present to a lesser extent, together with *Eurotium* – the teleomorphic form of *Aspergillus*, as well as *Mucor* and *Trichoderma*.

It seems that fungal adaptations to low temperatures can be related to salt stress, revealing new links between psychrophily and halophily. Both extracellular freezing and hypersaline stress lead to cellular dehydration, and both can therefore activate some common responses. These include among others the level of compatible solutes, ion transport across membranes, regulation of water efflux, the composition and fluidity of cell membranes responsible for cellular functions such as passive and active permeability, nutrient uptake, electron transport, environmental sensing, and recognition.

These salt- and cold-adapted fungi represent a new world of eukaryotic extremophiles, a world of organisms that have developed strategies different from those previously investigated. The ability of fungi to adapt to changing water activity conditions, whether due to low temperatures or high salinities, is crucial for their successful survival in some of the harshest extreme environments on our planet.

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HALOTOLERANT AND HALOPHILIC FUNGI AND THEIR EXTROLITE PRODUCTION

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1. Introduction

New lead compounds from fungi are often expected to be found in unusual habitats, for example in dung, in plants (as endotrophs), associated to other groups of living organisms, or in marine environments, even though a substantial part of potential new lead compounds are still found in soil. There are less data on the possibility to find new extrolite producers in extreme environments, the general opinion being that extremophiles may need a smaller number of bioactive molecules to compete with fellow extremophiles. On the other hand, many bioactive extrolites are known from lichens, often regarded as extremophiles

Many groups of organisms are able to produce extrolites (Frisvad and Samson, 2004), which include secondary metabolites (= special metabolites = natural products), extracellular enzymes, acids etc. (Béhal, 2003; Margesin and Schinner, 2001), but actinomycetes, filamentous fungi and plants are especially efficient producers of natural products (Bull et al., 1992; Cordell, 2000; Cragg et al., 1997; Eisner, 1989; Kurtböke and Swings, 2004; Lancini and Lorenzetti, 1994; Porter and Fox, 1993; Rouhi, 2003; Wildman, 2003; Wink, 2003). Some filamentous fungi are unusually halotolerant (Reed, 1986; Trüper and Galinski, 1986) and among the filamentous fungi, a large number of species produce several extrolites (Bills et al., 2002; Concepcion et al., 2001; Dreyfuss and Chapela, 1994; Hyde, 2001; Monaghan et al., 1995; Wildman, 1995, 1997). Some of these fungi are found in marine environments (Bernan et al., 1997; Ireland et al., 1988, 1993; Kobayashi and Ishibashi, 1993), while other unusual extrolites are found in endotrophs of plants (Schulz et al., 2002; Strobel et al., 2004), the latter having low concentrations of salts. It has often been recommended to screen unusual or extreme habitats for producers of novel interesting extrolites, rather than screen soil organisms. However soil and food-borne fungi such as *Penicillium* and *Aspergillus* continue to surprise by their chemical inventiveness (Frisvad et al., 2004a).

In this review halotolerant and halophilic *Penicillium* and *Aspergillus* species are compared to less halotolerant fungi to examine whether halophilic species show less pronounced chemical inventiveness than species from less extreme habitats.

2. Halotolerant and Halophilic Fungi and their Extrolites

Halophilic and halotolerant fungi (Hooley et al., 2003; Tresner and Hayes, 1971; Trüper and Galinski, 1986) include the basidiomycetous genus *Wallemia* (*W. sebi*) and the ascomycetous genera *Debaryomyces* (*D. hansenii*), *Pichia* (*P. guilliermondii*), *Rhodotorula* (*R. mucilaginosa*), *Hortea* (*H. werneckii*), *Basipetospora* (*B. halophila*), *Polypaecilum* (*P. pisce*), *Scopulariopsis* (*S. brevicaulis*), *Cladosporium* (*C. cladosporioides*) and in particular *Aspergillus* and *Penicillium* species and their perfect states (Lahav et al., 2002; Pitt and Hocking, 1997). In many of those genera only a few species are halophilic. For example in *Monascus*, some species are halophilic (*Basipetospora halophila*), while others are xerophilic (*M. bisporus*) or acidophilic (*M. ruber*) (Pitt and Hocking, 1997; Stchigel et al., 2004). Of all these halotolerant and halophilic fungi only few are known for their production of extrolites, except species related to the genera *Penicillium* and *Aspergillus*. Over 75% of all 273 strains of *Penicillium* could tolerate 20% NaCl and more than half of these survived 25% NaCl (Tresner and Hayes, 1971). Similarly, over 70% of 196 strains of *Aspergillus* could tolerate 20% NaCl and nearly half survived at 25% NaCl (Tresner and Hayes, 1971). Except for the latter two ubiquitous genera, only *Wallemia* has been screened systematically for extrolites, and only walleminol and wallemia A & B have been structure elucidated (Cole and Cox, 1981; Cole and Schweikert, 2003a, 2003b; Cole et al., 2003; Turner, 1971; Turner and Aldridge, 1983).

3. The Subgenus *Biverticillium* and its Teleomorphic state *Talaromyces* (62 Taxa)

Penicillium subgenus *Biverticillium* belongs to the phylogenetically unique ascomycete genus *Talaromyces* (LoBuglio et al., 1993; Peterson, 2000a), and is very different from the genus *Eupenicillium* and its associated anamorphs. Species of *Talaromyces* and its associated anamorphs are not considered to be particularly halotolerant or xerotolerant (Andersen and Frisvad, 2002; Pitt, 1979), but Steiman et al. (2004) obtained many isolates of *Talaromyces wortmannii* and *Penicillium duclauxii* from a hypersaline lake, so these species should be tested on media with NaCl to be sure they are generally less halotolerant than members of *Eupenicillium*. El-Morsy (2000) found *P. purpurogenum* from the endorhizosphere of halophytic plants from the Red Sea coast, and Buchalo et al. (1999) found *P. variabile* in the Dead Sea, showing that *Biverticillium* species can be recovered from haline environments. Tresner and Hayes (1971) found that 7 out of 20 strains of *Biverticillium* and *Talaromyces* ("Biverticillata") could grow at conditions with more than 25% NaCl. Some of these isolates could have been *P. herquei*, now belonging to subgenus *Furcatum* (Frisvad and Filtenborg, 1990; Pitt, 1979) and *P. olsonii*, now belonging to subgenus *Penicillium* (Frisvad and Samson, 2004; Pitt, 1979), which are moderate halophiles (see below). *P. herquei* and *P. olsonii* was placed by Raper and Thom (1949) in their Symmetrica-Biverticillata, now subgenus *Biverticillium* and the genus *Talaromyces*, but correctly placed as a part of *Eupenicillium* and its anamorphic states by Pitt (1979).

4. The Subgenus *Penicillium* and its Teleomorphic state *Eupenicillium* (64 Taxa)

Penicillium is the genus name given to imperfect states of the ascomycete genus *Eupenicillium* (Samson et al., 2004a). Many species of *Eupenicillium* and its associated anamorphs are salt-tolerant, even though there are major differences between species (Frisvad and Samson, 2004; Pitt, 1979; Pitt and Hocking, 1997). For example, the species series *Italica* (*P. italicum* and *P. ulaiense*) and *Digitata* (*P. digitatum*), that cause citrus fruit rot, are very sensitive to salt (Frisvad and Samson, 2004). The same is the case for dung-associated fungi in the series *Claviformia* (Frisvad and Samson, 2004). Other species that are less tolerant to NaCl are found in the series *Aethiopica*, *Persicina*, *Roqueforti* and *Urticicolae*.

The most halotolerant species in *Penicillium* are concentrated in the following series in subgenus *Penicillium*: *Viridicata*, *Verrucosa*, *Corymbifera*, *Solita*, *Camemberti*, *Chrysogena*, *Mononematosa* and *Olsonii* (Table 1, Frisvad and Samson, 2004). According to this way of measuring halotolerance (the ratio of colony diameters at 25ºC after one week of growth between Czapek Yeast Autolysate (CYA) and CYA with 5% NaCl (CYAS), *P. thymicola* is the species in subgenus *Penicillium* that is stimulated the most by 5% NaCl. The CYA/CYAS ratio may not be a good predictor of the ability to grow at higher NaCl concentrations, however, and has to be examined for all the 58 species in subgenus *Penicillium*. It is characteristic for most species in those series, mostly comprising food-borne fungi, that they grow better and sporulate better on CYA agar with 5% NaCl than on CYA agar. They can thus be called moderate halophiles according to the definition of Kushner (1978). Many species in those series grow well on salted foods. *P. chrysogenum* and *P. nalgiovense* are even used as starter cultures on mold-fermented salami (Frisvad and Samson, 2004). *P. chrysogenum* is among the most halotolerant species of *Penicillium* (Kis-Papo et al., 2001; Steiman et al., 2004; Tresner and Hayes, 1971). *P. camemberti*, used on white mold fermented cheese and *P. caseifulvum* are much more NaCl tolerant than *P. roqueforti*, while the latter species has adapted to the more acidic, less salty and more CO_2 containing central parts of blue mold cheeses. Other species contaminate salamis or cheeses and may produce mycotoxins on those products, especially *Penicillium nordicum*, a major ochratoxin A producer (Frisvad and Samson, 2004). However, species occurring both on salted products and dry cereals have approximately the same tolerance to NaCl, (Frisvad and Samson, 2004), so indications are that the NaCl tolerance is species specific, rather than being an adaptation to salty products and habitats. On the other hand it has been shown that *Emericella nidulans* can adapt to gradually higher concentrations of NaCl (Redkar et al., 1998). Steiman et al. (2004) found *P. chrysogenum* (reported as its synonym *P. griseoroseum*) and *P. aurantiogriseum* in the hypersaline Mono Lake in California.

TABLE 1. Inhibition or stimulation by NaCl on growth of the terverticillate Penicillia (Frisvad and Samson, 2004). The diameter on CYA is divided by the diameter on CYAS, after one weeks of incubation at 25ºC, to obtain the CYA/CYAS ratio.

Section	Series	Species	CYA/CYAS (average and range)
Coronata	*Olsonii*	*P. bialowiezense*	0.8 (0.5-1.3)
		P. brevicompactum	0.9 (0.8-0.9)
		P. olsonii	0.8 (0.6-0.9)
Roqueforti	*Roqueforti*	*P. carneum*	1.2 (1.1-1.2)
		P. paneum	2.0 (1.4-2.6)
		P. roqueforti	1.3 (0.6-1.6)
Chrysogena	*Chrysogena*	*P. chrysogenum*	0.9 (0.8-1.1)
		P. dipodomyis	0.9 (0.8-1.0)
		P. flavigenum	0.9 (0.7-1.1)
		P. nalgiovense	0.8 (0.6-0.9)
	Mononematosa	*P. confertum*	0.9
		P. mononematosum	1.0 (0.7-1.4)
	Persicina	*P. persicinum*	1.4 (1.3-1.5)
	Aethiopica	*P. aethiopicum*	1.4 (1.1-1.7)
Penicillium	*Expansa*	*P. expansum*	1.1 (0.7-1.6)
		P. marinum	1.0 (0.8-1.2)
		P. sclerotigenum	2.0 (1.5-2.6)
	Claviformia	*P. clavigerum*	6.3 (3.0-11.5)
		P. concentricum	1.1 (0.8-1.3)
		P. coprobium	1.2 (0.9-1.3)
		P. coprophilum	1.0 (0.9-1.2)
		P. formosanum	15.5
		P. glandicola	0.9 (0.8-1.0)
		P. vulpinum	0.9 (0.8-1.0)
	Urticicolae	*P. dipodomyicola*	1.1 (1.0-1.3)
		P. griseofulvum	1.5 (1.3-1.7)
	Italica	*P. italicum*	5.4 (2.2-11)
		P. ulaiense	6.6 (2.0-11)
	Gladioli	*P. gladioli*	0.8 (0.7-0.9)
Digitata	*Digitata*	*P. digitatum*	No growth on CYAS
Viridicata	*Viricicata*	*P. aurantiogriseum*	0.7 (0.6-1.0)
		P. cyclopium	0.9 (0.8-1.1)
		P. freii	0.6 (0.5-0.9)
		P. melanoconidium	0.7 (0.6-0.7)
		P. neoechinulatum	1.0 (0.8-1.1)
		P. polonicum	0.8 (0.7-1.0)
		P. tricolor	1.2 (1.0-1.3)
		P. viridicatum	0.8 (0.7-0.9)
	Corymbifera	*P. albocoremium*	0.9 (0.8-1.0)
		P. allii	0.8 (0.8-0.9)
		P. hirsutuim	0.9 (0.5-1.1)
		P. hordei	0.9 (0.7-1.0)
		P. radicicola	0.8 (0.7-1.0)
		P. tulipae	1.1 (1.0-1.3)
		P. venetum	0.8 (0.7-1.0)
	Verrucosa	*P. nordicum*	0.8 (0.4-1.0)
		P. thymicola	0.6 (0.4-0.7)
		P. verrucosum	0.7 (0.6-0.9)
	Camemberti	*P. atramentosum*	1.0 (0.8-1.2)
		P. camemberti	1.0 (0.8-1.2)
		P. caseifulvum	0.8 (0.7-1.0)
		P. commune	1.0 (0.6-1.4)
		P. crustosum	1.3 (1.1-1.5)
		P. palitans	0.9 (0.7-1.1)

Solita	*P. cavernicola*	0.8 (0.8-0.9)
	P. discolor	0.9 (0.7-1.1)
	P. echinulatum	0.8 (0.6-1.0)
	P. solitum	0.8 (0.7-0.9)

Both halotolerant and halointolerant species of *Penicillium* produce a large number of families of extrolites. One of the most halotolerant Penicillia is *P. chrysogenum*. This species can produce a large number of extrolites (Table 2). It has not been investigated whether these extrolites are inhibited by NaCl in the production medium.

TABLE 2. Extrolites produced by *Penicillium chrysogenum* (Frisvad et al., 2004a).

Extrolite biosynthetic family	**Biosynthetic group**
Secalonic acids	Polyketides
Sorbicillins and sorrentanone	Polyketides
Emodic acids	Polyketides
PR-toxin	Terpenes
Penicillins	Amino acid derived
Roquefortines and meleagrin	Amino acid derived + terpene unit
Xanthocillins	Shikimic acid pathway
Chrysogines	Amino acid derived
Questiomycins	Amino acid derived
Penitrinic acid	?
Chrysogenin	?
Negapillin	?
Notatin	Protein (glucose oxidase)
PAF	Protein
Fungisporin	Peptide

Another halotolerant fungus *P. olsonii* is also able to produce several extrolites (Table 3). In common with the two other members of series *Olsonii*, *P. brevicompactum* and *P. bialowiezense*, it produces a large number of biosynthetically very different extrolites.

TABLE 3. Extrolites produced by *Penicillium olsonii* (Frisvad et al., 2004a)

Extrolite biosynthetic family	**Biosynthetic group**
Verrucolones, pestalotins	Polyketides
2-(4-hydroxyphenyl)-2-oxo acetaldehyde oxime	Polyketide?
Bis(2-ethylhexyl)phthalate	Polyketide?
Xanthoepocin	Polyketide
Breviones	Terpenes
Asperphenamate	Amino acid derivative
OL3 (new chromophore type)	Amino acid derivative
LU3 (new chromophore type)	?
Olnacin (new chromophore type)	Amino acid derivative
Olsonin (new chromophore type)	?

The species that appear to be most stimulation by NaCl in subgenus *Penicillium* is *P. thymicola*. This species also produce an impressive profile of extrolites (Table 4). Thus in none of the species listed above, the extrolite production is less diverse than in less halotolerant species (see Frisvad et al., 2004a).

TABLE 4. Extrolites produced by *Penicillium thymicola* (Frisvad et al., 2004a).

Extrolite biosynthetic family	**Biosynthetic group**
Verrucolones, pestalotins	Polyketides
Daldinine D	Polyketide
Fumiquinazolones	Amino acid derivative
Alantrypinone, serantrypinone	Amino acid derivatives
Anacines	Amino acid derivatives
2-methyl isoborneol	Terpene
Dipodazin	Amino acid derivative
Penigequinolone	Amino acid derivative
FLAVO (new chromophore type)	?
YEL (new chromophore type)	?

5. The Subgenera *Aspergilloides* and *Furcatum* and their Teleomorphic State *Eupenicillium* (104 Taxa)

The majority of the mostly soil-borne species in the subgenera *Aspergilloides* and *Furcatum* have not been examined specifically for NaCl tolerance. *Penicillium westlingii* has been recovered from the Dead Sea (Buchalo et al., 1998), but this identification is doubtful, as the identification was referred to Pitt (1979) and Ramirez (1982), and none of these authors accept *P. westlingii*, referring it to *P. waksmanii*. Later Frisvad and Filtenborg (1990) accepted *P. westlingii*, but this species sporulates poorly, even at low NaCl conditions, in contrast to the data given in Buchalo et al. (1998). It remains to be seen whether this is a new species of *Penicillium*. Steiman et al. (2004), however, found *P. waksmanii* in the hypersaline Mono Lake in California, so this fungus may be the same as that found by Buchalo et al. (1998). *P. citrinum* was another species in *Furcatum* reported from Mono Lake and the Dead Sea (Kis-Papo et al., 2001; Steiman et al., 2004). Furthermore Kis-Papo et al. (2001) found *P. fellutanum*, *P. implicatum* and *P. restrictum* in the samples from the Dead Sea. Even though these identifications cannot be checked, other reports have indicated that *P. fellutanum* is very halotolerant (Park and Gander, 1998). *P. herquei* (teleomorphic state *Eupenicillium malachiteum*) is also very NaCl tolerant (Tresner and Hayes, 1971).

An example of a *Penicillium* species from subgenus *Furcatum* that is found in marine environments and is halotolerant is *P. antarcticum*. This species was officially described by McRae et al. in 1999 from soil in Antarctica near a Southern Fulmar nest. Long before its description, *P. antarcticum* was found in a salami factory in Denmark, on salami from Germany, from margarine in Denmark and from indoor air in Denmark. This unequal distribution in Antarctica and Denmark was later shown to be caused by the marine origin of this species. It has now been found on seaweed on a beach in Denmark, in a water bottling plant in Atlantic Ocean, on seaweed in the Philippines, in

sea sand from Bondeena Beach near Sydney in Australia, and in beach sand in New Zealand. Thus this species could be a true marine *Penicillium* species, maybe transported by gulls or via seawater. Recently it has been found in the salterns in Secovlje in Slovenia. *P. antarcticum* produces many different extrolites, known and unknown, and thus in this case is as chemically inventive as other *Penicillium* species (Table 5). The geography or NaCl content of the marine or terrestrial environment did not influence the type of extrolites being produced (Frisvad, unpublished observations).

TABLE 5. Extrolites produced by strains of *Penicillium antarcticum.*

Extrolite biosynthetic family	**Biosynthetic group**
Andrastins	Terpenes
Asperentins	Polyketides
Atrovenetins	Polyketides
Chrysogines	Amino acid derived
Deacetoxyfructigenins	Amino acid derived + terpene unit
Patulins	Polyketides
Penitrems	Amino acid derived
SEL (new chromophore type)	?
SPA (new chromophore type)	?
SEA (unknoen chromophore type)	?

In contrast to *P. antarcticum*, *P. spathulatum* has never been found in a marine or NaCl containing environment. Nevertheless this fungus is growing very slowly on ordinary identification media such as CYA agar. When 5% NaCl is added to this medium the fungus grows much faster and sporulates better. The fungus has been found on a chestnut in France, in soil under *Pinus* and *Nothofagus* in Chile, on honey dew on *Poa annua* in Denmark, in air in a cake factory in Denmark, in basic soil in a limestone quarry in Denmark, in soil under a *Raphia* palm in Costa Rica and in inland ice in Greenland (Frisvad, unpublished observations). Thus, like *P. antarcticum*, this fungus is geographically widely dispersed. This halotolerant fungus also produces an impressive profile of extrolites (Table 6).

6. The Genus *Aspergillus* and its Teleomorphic States (263 Taxa)

The genus *Aspergillus* has been divided by Raper and Fennell (1965) into 18 groups and was later officially subdivided into 6 subgenera and 17 sections (Gams et al., 1985). This latter classification has later largely been supported by DNA sequence data (Peterson, 2000b), even though some sections have been revised or placed in synonomy. The teleomorphic states of *Aspergillus* and the sections accepted currently are listed in Table 7.

TABLE 6. Production of extrolites by *Penicillium spathulatum*.

Extrolite biosynthetic family	**Biosynthetic group**
Anthraquinones	Polyketides
Asperphenamate	Amino acid derived
Breviones	Terpenes
Benzomalvins	Amino acid derived
Kotanins	Polyketides
Quinolactacins	Amino acid derived
HIMS (new chromophore type)	?
HUKO (new chromophore type)	?
PYR (new chromophore type)	?
PYROK (new chromophore type)	?
senT (new chromophore type)	?
SPUT (new chromophore type)	?

TABLE 7. The sectional subdivision of the genus *Aspergillus* and associated teleomorphic states.

Ascomycete genus	***Aspergillus* section(s)**	**Approximate number of species with and without a teleomorphic state**
Chaetosartorya	*Wentii* (syn *Cremei*)	3 + 14
Emericella	*Nidulantes*, Sparsi *Sydowii*, *Usti*, *Versicolores*	61 + 59
Eurotium	*Aspergillus*, *Cervini*, *Restricti*	36 + 8
Fennellia	*Candidi*, *Flavipedes*, *Terrei*	3 + 30
Hemicarpenteles	"Paradoxi"	2
Neocarpenteles	*Clavati*	1 + 10
Neopetromyces	*Circumdati*	1 + 25
Neosartorya	*Fumigati*	35 + 14
Petromyces	*Flavi*	2 + 26
Saitoa?	*Nigri*	0? + 16
Sclerocleista	*Ornati*	2
(*Hemisartorya*)	"Maritima"	1
(*Warcupiella*)	"Spinulosa"	1

The most halotolerant species in *Aspergillus* include species in the associated ascomycete genera *Eurotium* (including *Aspergillus* section *Restricti*), *Chaetosartorya*, and *Neopetromyces*, while *Aspergillus* species associated to the ascomycete genera *Petromyces*, *Fennellia* and *Emericella* are also halotolerant to halophilic. *Neosartorya*, *Neosarpenteles*, *Sclerocleista* and *Aspergillus* section *Cervini* are among the less halotolerant species (Tresner and Hayes, 1971). Nevertheless, *A. fumigatus* associated to *Neosartorya* has been found repeatedly in salterns (Tepsič et al., 1998). Other species of *Aspergillus* and teleomophs found in haline environments include *Eurotium* (Butinar et al., 2005), *Eurotium herbariorum*, *A. caespitosus*, *A. ustus*, *A. sydowii*, *A. versicolor* and *Emericella nidulans* (the latter five species associated to *Emericella*), *A. carneus* and *A. terreus* associated to *Fennellia*, *A. niger* and *A. fumigatus* associated to

Neosartorya were isolated from the Dead Sea (Buchalo et al., 1999, Kis-Papo et al., 2001; Molitoris et al., 2000). *A. carneus* (associated to *Fennellia*), *A. niger* and *A. sulphureus* (associated to *Neopetromyces*) were recovered from the endorhizosphere of halophytic plants from the Red Sea coast (El-Morsy, 2000). Steiman et al. (2004) found *A. niger* and *A. fumigatus* in hypersaline soil from Mono Lake in California. Even though these fungi can grow at high NaCl concentrations in vitro, the conidia of these fungi are not necessarily surviving in brine. For example *E. nidulans* conidia died quickly when incubated in undiluted Dead Sea water (Kis-Papo et al., 2003).

Presence of 5-15% of NaCl delayed conidium germination in *A. niger*, *A. terreus*, *A. flavus* and *Emericella nidulans*, but addition of 4-5% NaCl to the growth medium enhanced the growth of *A. niger*, *A. ochraceus* and *A. flavus* (Ramaswamy and Krishnamurti, 1979). While 15% NaCl did not inhibit *A. terreus* or *A. flavus*, more than 11% of NaCl inhibited *E. nidulans*. Furthermore, citric acid production by *A. niger* was enhanced by 5% NaCl (Ramaswamy and Krishnamurti, 1979). In *A. flavus* and *A.parasiticus*, 5% NaCl slightly increased growth and aflatoxin production, while higher concentrations inhibited both (Khattap et al., 1996). Ochratoxin production by *A. ochraceus* was optimal at 5% NaCl and aflatoxin production by *A. parasiticus* was optimal at 3% NaCl according to Kheiralla et al. (1996). An increase in NaCl concentration to 3-5% in fungal growth media will often increase extrolite production (Masuma et al., 2001).

In general *Aspergillus* species produce a very high number of extrolites (Cole and Cox, 1981; Cole and Schweikert, 2003a, 2003b; Cole et al., 2003; Samson et al., 2004b; Turner, 1971; Turner and Aldridge, 1983) and no particular *Aspergillus* species of those investigated yet produce few extrolites. *Eurotium* species all produce echinulins, neoechinulins and cryptoechinulins, while most species produce physcion, emodin, flavoglaucin, auroglaucin, dihydroauroglaucin and tetrahydroauroglaucin (Butinar et al., 2005), but several more extrolites are known (Turner and Aldridge, 1983). Some of these metabolites are also produced by *Chaetosartorya*, another moderate to extreme halophile, but both *Eurotium* and *Chaetosartorya* species have unique metabolites only found in that genus. The moderate halophile *A. westerdijkiae* form *Aspergillus* subgenus *Circumdati* (the *Aspergillus ochracus* group) has recently been described. This fungus consistently produces a large number of bioactive extrolites (Table 8).

TABLE 8. Production of extrolites by *Aspergillus westerdijkiae* (Frisvad et al., 2004b).

Extrolite biosynthetic family	**Biosynthetic group**
Aspyrones	Polyketides
Penicillic acids	Polyketides
Melleins	Polyketides
Xanthomegnins	Polyketides
Ochratoxins	Polyketides + amino acid
Circumdatins = asperloxins	Amino acid derivatives
Aspergamides = avrainvillamides = stephacidins	Amino acid derivatives
L-657,398	Amino acid derivative
NB1 (new chromophore type)	?

A representative for the Aspergilli less tolerant to NaCl is *Aspergillus clavatus*. This coprophilic fungus is alos an efficient producer of extrolites (Table 9). A comparison of the extreme halophile *Eurotium*, the moderate halophile *A. ochraceus* and the slight halophile *A. clavatus* shows that all groups of fungi produce large number of diverse extrolites.

TABLE 9. Production of extrolites by *Aspergillus clavatus* (Turner and Aldridge, 1983).

Extrolite biosynthetic family	Biosynthetic group
Xanthocillins	Shikimic acid derivative + amino acid
Patulins (including ascladiol)	Polyketides
Clavatol	Polyketide
Antafumicins	Polyketides
Kotanin	Polyketides
Pseurotins	Polyketides + amino acid
Tryptoquivalins, tryptoquivalons	Amino acid derived + terpene unit
Cytochalasins (E and K)	Amino acid derived + terpene unit
Sarcins, clavin	Peptides
Expansolide	Terpene

7. Conclusions

Species of *Penicillium* and *Aspergillus* are often halotolerant: slight, moderate or extreme halophiles, even though some species cannot tolerate even low concentration of sodium chloride (for example the citrus rotting organism *Penicillium digitatum*). All species in general produce a large number of both polyketide, amino acid derived extrolites and terpenes and combinations of those biosynthetic types also occur. Thus producers of new lead compounds can be found in both extreme and non-extreme habitats, including saline and non-saline habitats. The hypothesis that moderate and extreme halophiles may need a smaller number of bioactive extrolites to compete with fellow inhabitants of saline and hypersaline has to be rejected. On the other hand extrolites are often produced in highest diversity and amounts at conditions from 0-5% NaCl, and will decline in habitats with higher concentrations than 5%. Maybe more biosynthetic energy is used for avoiding stress in saline habitats than for production of bioactive extrolites at such conditions.

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INTRODUCING *DEBARYOMYCES HANSENII*, A SALT LOVING YEAST

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1. Introduction

The physiology and molecular biology of halophilic prokaryotic microbes (Bacteria and Archaea) has been studied in detail during several decades, and a large number of species have been used in those studies (Oren, 1999; Sørensen et al., 2004; Ventosa et al., 1998). However, this is not the case for eukaryotic microorganisms, much less information is available and only a few genera have been studied. In fact even the concept of a halophilic eukaryotic microbe is not completely established and, for example, the existence of real halophilic yeasts is still controversial (Silva-Graça et al., 2003), in spite of the fact that one *Candida* species has been named *C. halophila* (Kurtzman and Fell, 2000). In any case, algae such as *Dunaliella* (Fisher et al., 1997; Katz et al., 1989), filamentous fungi such as *Penicillium* or *Aspergillus* (Cuppers et al., 1997; Mert and Ekmekci, 1987), yeasts such as *Debaryomyces* (Norkrans, 1966) or yeast-like fungal species such as *Hortaea* (Gunde-Cimerman et al., 2000; Petrovič et al., 2002) can be found in salty environments, and these are attractive organisms to study salt tolerance mechanisms and salt stress responses in eukaryotic cells.

Debaryomyces hansenii is an ascomycetous yeast (Class Hemiascomycetes, Order Saccharomycetales, usually included in the Saccharomycetaceae family although its family affiliation is uncertain). Some *Pichia* species have phylogenetical relationships to *Debaryomyces* species (Kurtzman and Fell, 2000). In addition *D. hansenii* is also closely related to *Candida albicans* and, although it is considered as non-pathogenic, clinical samples identified as *D. hansenii* have been isolated from superficial infections (Nishikawa et al., 1996). *D. hansenii* tolerates high salinity levels (Lages et al., 1999; Prista et al., in press; Ramos, 1999), and it can be isolated from sea water, salty food or brines (Norkrans, 1966; Seiler and Busse, 1990). *D. hansenii* is also the most common species found in cheeses and provides enzymatic activities during cheese ripening (Fadda et al., 2004). Some strains have also been isolated from high sugar food (marzipan) (Casas et al., 1999).

The yeast group in the University of Göteborg has studied different aspects of salt tolerance in *D. hansenii* and they provided us some solid basis in the study of those processes (André et al., 1988; Larsson et al., 1990; Norkrans, 1966; Norkrans and Kylin, 1969). They found that among several marine occurring yeast, *D. hansenii* was the one least affected by high NaCl concentrations (Norkrans, 1966; Norkrans and Kylin, 1969). Since then *D. hansenii* has been defined as a halotolerant (Alba-Lois et

al., 2004), halophilic (González-Hernandez et al., 2004), or even salt-loving yeast (Silva-Graça et al., 2003), a concept that I will also use in this chapter.

Due to the great interest of *D. hansenii*, the consortium Génolevures recently selected this yeast among those to sequence and annotate its genome (Dujon et al., 2004; http://cbi.labri.fr/Genolevures/).

2. *Debaryomyces hansenii* is a Salt-Loving Yeast

In the following, I am summarizing the available information reported by different research groups indicating that *D. hansenii* is a salt-loving yeast. The discussion about its halotolerant or halophilic nature is outside the scope of this chapter.

Several groups have shown that sodium is not toxic for *D. hansenii*. The group of C. Lucas studied the effect of Na^+ and K^+ on several growth parameters and on respiration and fermentation rates and they did not find significant differences between both ions (Neves et al., 1997). The same year we reported the effect of increasing concentrations of different alkali cations on the specific growth rate of *Saccharomyces cerevisiae* and *D. hansenii*. While *S. cerevisiae* was inhibited by Li^+ or Na^+, *D. hansenii* was inhibited by Li^+ but not by Na^+. In addition, relatively high concentrations of NaCl (0.5 M) slightly improved the specific growth rate of *Debaryomyces* (Prista et al., 1997). Higher concentrations of NaCl or KCl (1.5-2 M) decreased the growth rate, but since K^+ is not a toxic cation, this effect was most probably due to a non specific osmotic effect (Prista et al., 1997). Similar conclusions were reached by the group of Peña. In a series of two papers they reported that *D. hansenii* grows optimally in 0.6 M of either NaCl or KCl (González-Hernandez et al., 2004), and that both lag phase and doubling time were reduced by high NaCl or KCl (Thomé-Ortiz et al., 1998). Moreover, the measurement of ATP levels showed that the levels of this metabolite were higher for cells grown in 1 M NaCl than for cells grown in 1 M KCl (Thomé-Ortiz et al., 1998). An additional proof of the salt-loving character of *D. hansenii* is its capacity to accumulate high levels of internal sodium without becoming intoxicated. For that reason this yeast has been defined as a Na^+ includer organism (Prista et al., 1997). Although the different research groups that have measured internal ion concentrations in this yeast do not concur from a quantitative point of view, all of them are agreed with the idea that in the presence of salt *D. hansenii* grows better and accumulates more Na^+ than *S. cerevisiae* (González-Hernández et al., 2004; Neves et al., 1997; Prista et al., 1997). Prista et al. (1997) showed that under cation concentrations comparable to those of sea water (low K^+ and high Na^+) *D. hansenii* grew at rates close to the optimum, while the growth of *S. cerevisiae* was completely inhibited.

Finally, we have shown that Na^+ somehow protects *D. hansenii* cells against additional stress factors (Almagro et al., 2000). Figure 1 is a comparison of the effects of different stress factors on growth of *S. cerevisiae* (strain DBY746) and *D. hansenii* (strain PYCC2968). While *S. cerevisiae* behaved better at acidic pH or at high temperature (panels C and E), *D. hansenii* showed a good performance at high pH (panel B) and in the presence of high salt (panel D). Results in panel F show that the presence of high amounts of NaCl in the growth medium favors growth at limiting temperatures. Similar results were also found when growth under extreme pH values,

acidic or alkaline, was studied in the presence or in the absence of salt (Almagro et al., 2000).

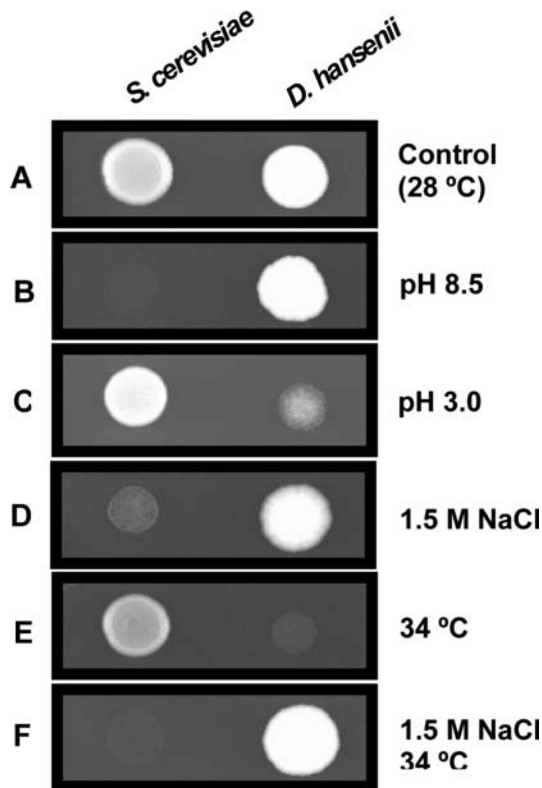

Figure 1. Comparison of the effects of different stress factors on growth of *Saccharomyces cerevisiae* and *Debaryomyces hansenii*. Strains DBY746 (*S. cerevisiae*) and PYCC2968 (*D. hansenii*) were grown overnight in liquid complex medium (Yeast extract 1%, Peptone 2%, Dextrose 2%; YPD). Then, 20×10^3 cells were deposited on YPD plates under the indicated conditions. Growth was monitored after 48 h (panels A and E) or after 72 h (panels B, C, D and F).

3. Salt Tolerance Determinants

Why is *D. hansenii* a salt loving yeast? Why is growth under saline conditions not inhibited by internal Na^+? Which are the physiological and molecular reasons for this behavior? Unfortunately our information is limited, and these are still open questions without a definitive answer. However the information already available provides us important clues to understand these problems.

Are the enzymes from *D. hansenii* especially resistant to high salt? The determination of the effect of salt on the activity of different enzymes is an approach used by several researchers. Two examples indicate that answers are not so obvious. The activity of the purified glycerolphosphate dehydrogenase from *D. hansenii* is stimulated twice by high Na^+. However, this stimulation is weaker than the one observed in *S. cerevisiae* (André et al., 1991). More recently, the activity of NADP-dependent glutamate dehydrogenase has been found to be more sensitive to ionic strength than the corresponding isoenzymes in *S. cerevisiae* (Alba-Lois et al, 2004). Certainly, not all enzymes of interest have been studied. In addition, to compare *in vitro* activities with cell physiology *in vivo* is not always possible. Much more work is required to obtain conclusions, but the two mentioned examples indicate that this is not an easy task and that it is extremely important to select the right enzymes to be studied and the most appropriate working conditions.

Glycerol is the main compatible solute accumulated by *D. hansenii* under conditions of osmotic stress. In fact it has been recently shown that this yeast accumulates more glycerol than trehalose under saline stress (2.0 and 3.0 M salts) (González-Hernandez et al., 2005). Therefore glycerol synthesis, transport and accumulation are important events to function under salty conditions, as has been proposed in the marine black yeast *Hortaea werneckii* for which hypersaline waters of salterns are the primary environmental ecological niche (Petrovič et al., 2002). Thus, Lages et al (1999) reported that in *D. hansenii* accumulation of glycerol is stimulated in the presence of high Na^+. By following a biochemical and physiological approach, the same group has also proposed the existence of a Na^+/glycerol symporter. Apparently this transporter is not present in other salt sensitive yeasts such as *S. cerevisiae,* and it could therefore play an essential role in salt tolerance (Lucas et al., 1990). It is also interesting to note that a search in the Genolevures database indicates that the main *S. cerevisiae* glycerol extrusion channel, coded by the *FPS1* gene, is not present in *D. hansenii* (Prista et al., in press).

The most important salt tolerance determinant in *S. cerevisiae* is probably the sodium extrusion pump coded by *ENA* genes. Deletion of these genes causes hypersensitivity to Na^+ in this yeast (Benito et al., 2002; Haro et al., 1991). Two *ENA* genes have been found in *D. hansenii,* they have been heterologously expressed in a *S. cerevisiae* mutant lacking its own efflux systems and, as a consequence, the mutant recovered salt tolerance up to the level of the wild type *Saccharomyces*. Northern analysis showed that the expression of *DhENA* is increased by high Na^+ and high pH, conditions found in sea water (Almagro et al., 2001). From all these results it was concluded that Ena is a Na^+ pump that regulates ionic homeostasis in *D. hansenii.* However, this protein does not seem to be more efficient than the corresponding ScEna system (Almagro et al., 2001). In addition, biochemical data indicate the existence of a low affinity Na^+ and K^+ channel in *D. hansenii*, but neither the molecular nature of the channel nor its contribution to the whole salt tolerance process have been elucidated (González-Hernández et al., 2004).

Because in *S. cerevisiae* the capacity to transport K^+ and to discriminate between K^+ and Na^+ is an important determinant of halotolerance (Bertl et al., 2003; Gómez et al., 1996) it is of interest to determine the situation in *Debaryomyces*. Two aspects are of special relevance. On the one hand it has been shown that at least under certain external conditions Na^+ stimulates K^+ uptake rather than inhibiting it (Prista et al.,

1997). On the other hand, while two *TRK* genes involved in K^+ uptake are present in the sensitive yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* (Calero and Ramos 2003), one *TRK* gene and one *HAK* gene have been identified in *D. occidentalis* (previously named *Schwanniomyces occidentalis*) (Banuelos et al., 2000) and in *D. hansenii* (Prista and Loureiro-Dias, unpublished results). It has been proposed that Hak proteins function as high affinity K^+ transporters, and therefore their contribution to salt tolerance could be considerable.

4. Hal2, Nhx1 and Gzf3: Three Proteins Involved in Tolerance to High Salt and High pH

Our research is mainly focused on the study of three *D. hansenii* genes involved in tolerance to high salt and high pH. Genes orthologous to *DhHAL2, DhNHX1* and *DhGZF3* have been found in other organisms (Fig. 2), and we intend to characterize their functions in *D. hansenii*.

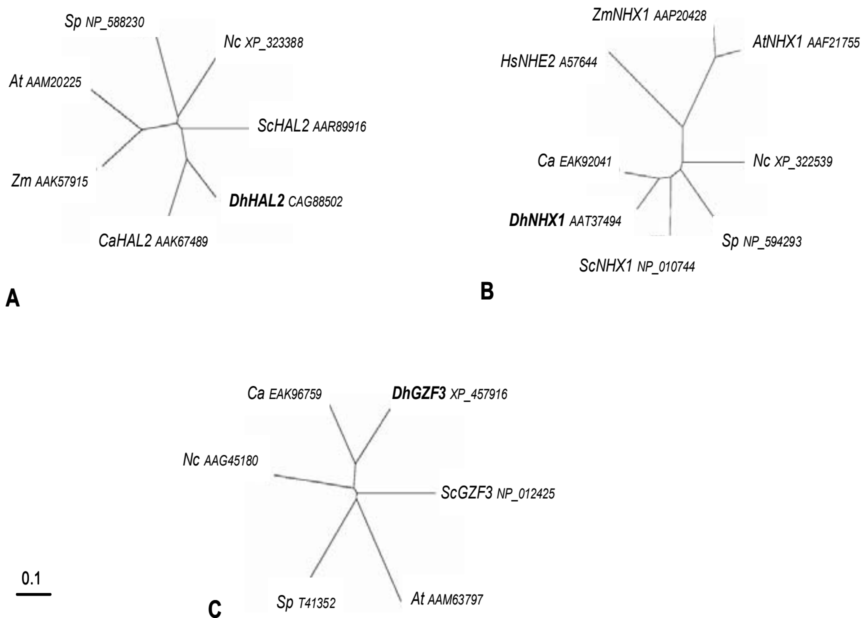

Figure 2. Phylogenetic tree of *HAL2* (A), *NHX1* (B) and *GZF3* (C), three genes involved in tolerance to high salt and high pH in *Debaryomyces hansenii*. The tree was constructed using the CLUSTAL X program. The scale bar corresponds to a distance of 10 changes per 100 amino acid positions. Accession numbers are shown. *Sc*, *Saccharomyces cerevisiae*; *Nc*, *Neurospora crassa*; *Sp*, *Schizosaccharomyces pombe*; *Zm*, *Zea mays*; *Hs*, *Homo sapiens*; *At*, *Arabidopsis thaliana*; *Ca*, *Candida albicans*; *Dh*, *Debaryomyces hansenii*.

Hal2 (for Halotolerance) is the only important target of salt toxicity so far identified in *S. cerevisiae.* Deletion of *ScHAL2* did not affect the capacity to grow in salt, but its overexpression significantly increased salt tolerance. Hal2p is a nucleotidase that hydrolyzes 3′-phosphoadenosine-5′-phosphate (PAP) to AMP to recycle adenosine, and it is also involved in methionine biosynthesis (Gläser et al., 1993). Genes orthologous to *HAL2* are present in other yeasts and in higher plants (Fig. 2A). An orthologue of *HAL2* was recently identified in *D. hansenii* (Dujon et al., 2004). Our preliminary results suggest that, *in vitro*, Hal2p activity from *D. hansenii* is not affected by Na^+ concentrations that inhibit ScHal2. Even more, much higher concentrations of Li^+ are required to inhibit the function of DhHal2 than to inhibit the corresponding protein from *S. cerevisiae* (Montiel and Ramos, unpublished results). Therefore, Hal2 seems to be a good candidate to explain some aspects related to the salt-loving character of *D. hansenii*.

I have mentioned above that *D. hansenii* is a Na^+ includer organism. One possibility to explain tolerance to high internal Na^+ would be the sequestration of the cation into intracellular organelles keeping, in that way, low levels of cytoplasmic Na^+. In fact, a protein in charge of driving Na^+ into the vacuolar and/or prevacuolar compartments is present in different eukaryotic organisms (Fig. 2B). The protein is coded by *NHX1*gene and it has been reported that both in yeast (Nass and Rao, 1999) and in higher plants (Apse et al., 1999). Nhx1 is involved in salt tolerance, in the regulation of the internal distribution of cations, and probably also in the regulation of internal pH. We have identified, cloned and sequenced an orthologue to *NHX1* in *D. hansenii* (GenBank database, Accession No. AAT37494). Therefore it is reasonable to assume that the protein plays a similar function in this yeast. However, it is important to note that when the cytoplasmic cation content was determined both in *D. hansenii* and in *S. cerevisiae* grown under the same conditions, we observed that *Debaryomyces* grew better and kept significantly higher cytoplasmic Na^+ levels than *S. cerevisiae*, indicating that the existence of *DhNHX1* by itself cannot explain the differences found between both yeasts (Montiel and Ramos, unpublished results).

In our search to understand the reasons why *D. hansenii* grows well under conditions present in sea water (high salt and high pH), we used a gene library from *D. hansenii* (Prista et al., 2002) to transform a wild type *S. cerevisiae* strain. I commented above that *D. hansenii* is more tolerant to high pH than *S. cerevisiae* (Fig. 1, panel B). Therefore we looked for *S. cerevisiae* transformants able to grow at alkaline pH. We selected several clones and we ended up with an orthologue to *GZF3* (Fig. 2C) which in *S. cerevisiae* codes for a GATA-type zinc finger transcription factor playing a complex role in Nitrogen Catabolite Repression (NCR). Multiple NCR genes have been identified and studied in *Saccharomyces* and the functioning of ScGzf3 has been indirectly related to the salt tolerance processes (Crespo et al., 2001; Soussi-Boudekou et al., 1997). However no information is available on the possible role of Gzf3 in relation to the capacity to grow at high pH in yeasts. Although the *Debaryomyces* protein is quite similar to the one in *S. cerevisiae* in reference to the predicted size (mass 59.1 and 60.5 kDa; number of amino acids 543 and 551 in *Debaryomyces* and *Saccharomyces* respectively), *DhGZF3* is closest to the one in *Candida albicans* from a phylogenetical point of view (Fig. 2C).

5. Perspectives

Our knowledge about the salt-loving character of *D. hansenii* can be compared to a puzzle (Fig. 3). Pieces of information that I have summarized in this chapter are available but we lack a global picture of the process. Hopefully, the recent sequencing of *D. hansenii* genome is going to be a powerful tool. Much work and the development of genetic tools in *Debaryomyces* are still required, but it looks like that understanding *D. hansenii* will help in the next future to elucidate the mechanisms underlying salt tolerance, not only in yeasts but also in other eukaryotic cells.

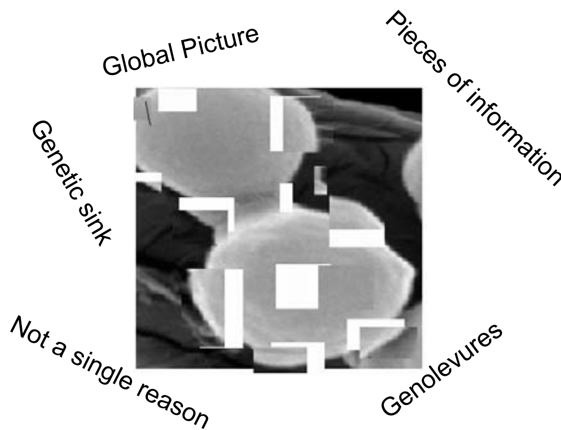

Figure 3. Salt tolerance in *Debaryomyces hansenii* as a puzzle. Several pieces of information about the salt-loving character of *D. hansenii* are available and apparently there is not a single reason to explain this behaviour. The sequencing of the genome is already finished and hopefully the genome will provide us with information on many new genes involved in salt- and pH tolerance, to establish, in the near future, a global picture of the whole process.

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CELLULAR RESPONSES IN THE HALOPHILIC BLACK YEAST *HORTAEA WERNECKII* TO HIGH ENVIRONMENTAL SALINITY

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1. Introduction

Biodiversity of microorganisms in extreme habitats attracts attention of researchers, since harsh environment stress influences the adaptations of organisms and provides unique opportunities for unraveling the genetic basis of adaptive convergent evolution. Solar salterns, as other similar hypersaline environments, are extreme habitats that prevent growth of all except very few organisms. It was long believed that such environments are populated only by halophilic and halotolerant bacterial species and some rare algae, but never by fungi. In contrast to obligately and facultatively halophilic bacteria, fungi were not considered truly halophilic, since growth of the few species of halotolerant food-borne fungi (*Basipetospora halophila*, *Polypaecilum pisce* and *Hortaea werneckii*) was stimulated by NaCl without any obligate requirement (Andrews and Pitt, 1987). This conviction remained unchallenged until halophilic fungi were isolated from natural hypersaline environments. During the last few years, a surprisingly rich diversity of long neglected mycobiota was discovered in the hypersaline waters of salterns and salt lakes world-wide (Butinar et al., 2005; Kis-Papo et al., 2001; Wasser et al., 2003). These fungi belong to a limited number of genera, but they display a surprisingly high diversity. Certain species occur consistently in different natural saline environments, representing stable communities in interaction with prokaryotic extremophiles and randomly occurring microorganisms.

According to a new selective criterion introduced by Gunde-Cimerman, fungi isolated from solar salterns are considered halophilic if they are isolated from waters with 17 to 32% NaCl, primarily on selective saline media, and if they are able to grow *in vitro* on 17% NaCl. Strains are classified as halotolerant if they are isolated from water with lower salinities, but are able to grow *in vitro* up to 17% NaCl (Gunde-Cimerman et al., 2000, 2005). The ability to grow in axenic culture at a_w below 0.85 was acknowledged as a good indicator of competence in natural hypersaline habitats.

Among the saltern mycobiota community, melanized fungi, so called “black yeasts” from the ordo *Dothideales* display some distinct features that help them to adapt to high as well as to low salt concentrations. They are able to propagate at optimal salinities and to survive periods of extreme environmental stress in a resting state. When conditions again change they respond immediately with increased metabolic activity, growth and propagation. Their pleomorphism and adaptive halophilic behaviour enables a

continuous colonization of salterns in which NaCl concentrations may vary considerably during the season (Gunde-Cimerman et al., 2005). Based on their metabolic activity at extremely high saline conditions, pronounced adaptive ability, and *in vitro* physiological experiments of growth at different salinities, some melanized yeast-like fungi can be considered truly halophilic (Gunde-Cimerman et al., 2005).

Molecular mechanisms of adaptation that allow halophilic organisms to survive in hypersaline environments have been extensively studied in prokaryotes. Since fungi have been isolated from these environments only recently, little is known about their molecular mechanisms of adaptation.

In this chapter we will explore, at the molecular level, the mechanisms of adaptation of the halophilic black yeast *Hortaea werneckii*, a species dominant in waters of solar salterns. Since *H. werneckii* can grow from 0 up to 32% NaCl in the natural environments as well as *in vitro* under laboratory conditions, it represents a suitable model organism.

2. *Hortaea werneckii*, a Halophilic "Black Yeast"

Hortaea werneckii belongs to those yeast-like fungi, so-called black yeasts, able to produce dark pigment inside and outside the cell wall. Black yeasts are characterized by slowly expanding colonies. They reproduce by isodiametric enlargement of subdividing cells (Sterflinger et al., 1999; Wollenzien et al., 1995). Their main features are polymorphism, meristematic growth, endoconidiation, frequently muriform cells, which develop by conversion from undifferentiated hyphae, and thick, melanized cell walls (de Hoog, 1993; Zalar et al., 1999b). This morphological ecotype is important for their survival in different extreme environments. Several genera and species of black yeasts from the order *Dothideales* represent a group of rare but poorly described extremophilic eukaryotic microorganisms. They have been isolated from arid and UV-stressed atmosphere-exposed inorganic and organic surfaces (Butin et al., 1996; Krumbein et al., 1996), Arctic glacial ice (Gunde-Cimerman et al., 2003), extremely low pH media (Hölker et al., 2005) and hypersaline waters of the salterns (Gunde-Cimerman et al., 2000). They were described as a new group of eukaryotic halophiles, represented by *Hortaea werneckii* (Horta) Nishimura and Miyaji, *Phaeotheca triangularis* De Hoog and Beguin, *Trimmatostroma salinum* Zalar, De Hoog and Gunde-Cimerman, *Aureobasidium pullulans* (de Bary) Arnaud, together with the phylogenetically closely related *Cladosporium* spp. (De Hoog et al., 1999; Gunde-Cimerman et al., 2000; Sterflinger et al., 1999).

The genus *Hortaea* contains a single species, *H. werneckii* (Horta) Nishimura and Miyaji, with no known sexual stage (Zalar et al., 1999a). *H. werneckii* has received many designations: *Cladosporium werneckii*, *Exophila werneckii*, *Pullularia werneckii*, *Aureobasidium werneckii*, *A. mansonii*, *Sarcinomyces crustaceus* and *Pheoannellomyces werneckii.*

H. werneckii was known primarily as the etiologic agent of human tinea nigra, a superficial infection of the human hand, in which the fungus is strictly limited to the stratum corneum and does not invade living tissue. Göttlich and co-workers proved that the fungus is not keratinolytic but lipolytic, limiting the infection to the grease on the skin and therefore representing only a cosmetic problem (Göttlich et al., 1995). On the basis

of ecophysiological studies it was suggested that salt might be the decisive factor in the etiology of tinea nigra and *H. werneckii* ecology. In addition to human skin, the fungus has been isolated from seawater (Iwatsu and Udagawa, 1988), marine fish (Todaro et al., 1983), salted freshwater fish (Mok et al., 1981) and beach soil (de Hoog and Gueho, 1998). Nevertheless, its primary environmental ecological niche remained was not known. It was speculated that it might grow intermittently in drying salty ponds at the seaside (de Hoog and Gerrits van den Ende, 1992). Indeed, Gunde-Cimerman and co-workers isolated *H. werneckii* initially from the crystallisation ponds of Adriatic salterns in Slovenia during the season of salt production (Gunde-Cimerman et al., 2000). Later, its distribution was followed in five different evaporitic ponds in the Adriatic salterns, covering the entire salinity range (3-32% NaCl), throughout the year. Its presence was confirmed as well in hypersaline waters of six salterns on three continents (Butinar et al., 2005).

H. werneckii was the dominant black yeast in the hypersaline water of all the sampled salterns and the prevailing species at environmental salinities above 20%. It was also isolated from the surface and interior of wood submerged in brine, from biofilms on the surface of hypersaline water, and from dry ponds and microbial mats (Butinar et al., 2005; Zalar et al., submitted). The salinity range of growth for *H. werneckii*, defined *in vitro*, was from 0% to saturation (32%) NaCl with a broad optimum from 6-14% NaCl.

Ecophysiological observations and morphological studies under saline conditions *in vitro* indicate that *H. werneckii* exhibits a complicated, polymorphic life cycle, with hydrophilic yeast cells, hydrophobic hyphae and meristematic growth, apparently suited for survival in the changing extreme environment of a saltern (Fig. 1).

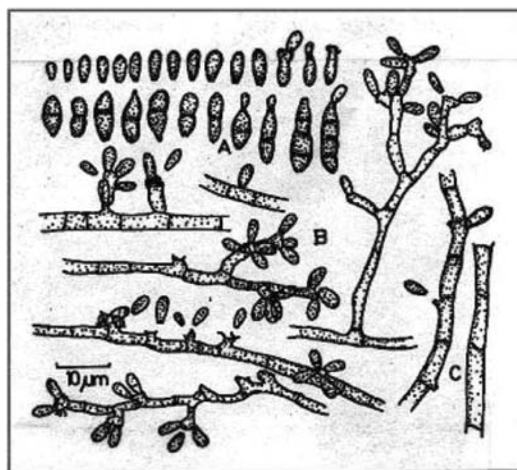

Figure 1. Morphological forms of *H. werneckii*. A- hydrophilic yeast form, B- budding cells, C- hydrophobic hyphae.

If sufficient nutrients are available, the hydrophilic yeast phase rapidly colonizes hypersaline water environments. Yeast cells respond to increasing salinity with meristematic development of budding cells and finally dormant meristematic sclerotial bodies with endogenous conidiation. Under conditions of drought, with no water in the ponds, the fungus changes into an aerophilic, hydrophobic hyphal stage with the production of conidia, which can be dispersed by air currents.

3. Cellular Responses of *H. werneckii* to High Salt Concentration

Molecular mechanisms of adaptation in halophilic Bacteria and Archaea revealed unique strategies which enable them to survive in extremely salty environments (Oren, 1999; Ventosa, 2004). In the absence of appropriate model organisms, molecular mechanisms of salt tolerance have so far not been studied in halophilic fungi. Instead studies have involved salt-sensitive *Saccharomyces cerevisiae* (Blomberg and Adler, 1992; Blomberg et al., 2000; Hohmann, 2002; Hohmann and Mager, 2003) and halotolerant filamentous fungi (e.g. *Aspergillus nidulans*), and yeasts (e.g. *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Candida versatilis*, *Rhodotorula mucilaginosa* and *Pichia guillermondii* (Lahav et al., 2002; Ramos, 1999; Silva-Graça and Lucas, 2003; Silva-Graça et al., 2003). These studies demonstrated differences in cellular responses between salt sensitive *S. cerevisiae* and the above mentioned salt-tolerant fungi. *S. cerevisiae* cells respond vigorously to high salt concentration in their environment. DNA chip technology has revealed that exposure to high concentration of salt causes dramatic effects on the genomic expression program in this salt-sensitive species (Crespo et al., 2001; Gash et al., 2000).

While we understand at least some of the mechanisms of adaptation among fungi that tolerate moderate salt concentrations in their environment, until recently nothing was known about the molecular mechanisms of adaptation of fungi that can survive in environments with NaCl concentrations reaching almost the saturation point, like for example the black yeast *H. werneckii*. According to the two main criteria for halophily proposed by Gunde-Cimerman et al. (2005) and mentioned above ([i] frequent isolation from waters with 17 to 32% NaCl, primarly on selective saline media and [ii] capability of growth *in vitro* on 17% NaCl and above), *H. werneckii* belongs to the group of true halophiles. Based on its growth rate which increases to up to 10% NaCl, is stable between 10 and 17%, and decreases between 20 and 32% NaCl, we have defined *in vitro* conditions for *H. werneckii* as being hyposaline between 0 and 10% NaCl, optimal between 10% and 20% NaCl and hypersaline at NaCl concentrations above 20%.

In our studies of adaptation to a wide range of salinities in *H. werneckii* we have addressed changes in membrane properties, compatible solutes and salt sensing signal transduction pathway(s) at these three sets of conditions. Physiological and biochemical responses were distinct at different salinities and often followed a similar course at both, hypo and hypersaline conditions (Petrovič et al., 1999, 2002). Some parameters, like CO_2 production, enzyme activity of hydroxymethylglutaryl-CoA reductase (HmgR), glycerol production and membrane fluidity, are summarized in Fig. 2, and these will be discussed in more detail in the following sections.

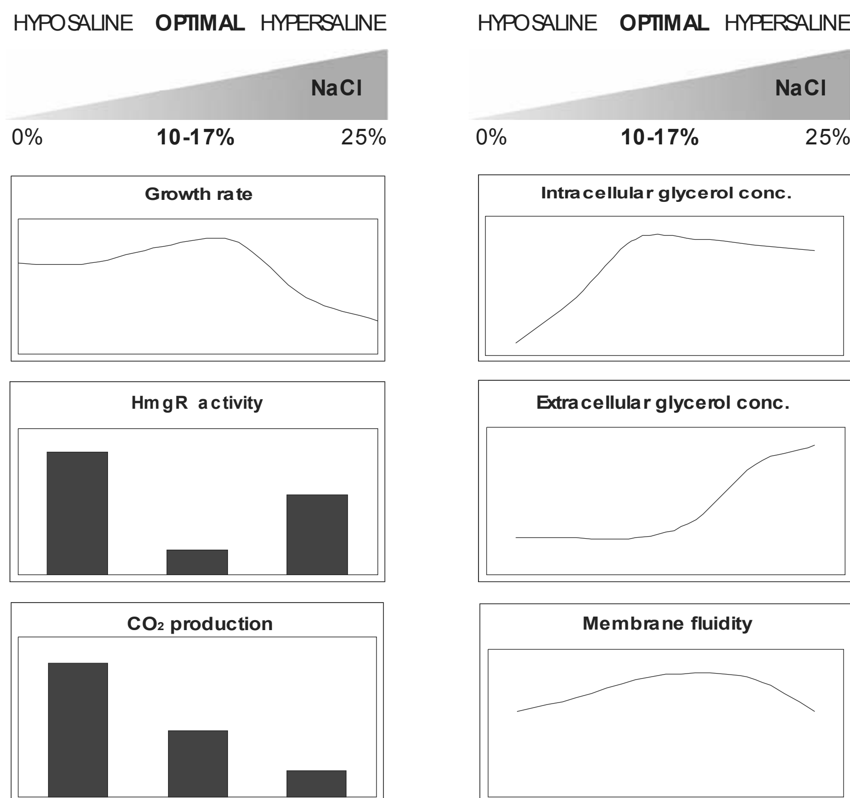

Figure 2. Schematic representation of the growth rate of *Hortaea werneckii* in medium without salt, with 17% NaCl or with 25% NaCl, and the observed changes in membrane fluidity, CO_2 production, glycerol accumulation and enzyme activity of hydroxymethylglutaryl-CoA reductase (HmgR).

The halophilic *H. werneckii* has not beforehand been studied from the point of view of adaptation to strong fluctuations of salt concentrations in its environment at the molecular level. In our initial studies of molecular responses to increased salt concentration, we took a global approach. We first wanted to identify genes that are expressed differentially at different salinities and thus may be involved in the mechanisms of adaptation. Since the transcriptome of *H. werneckii* was unknown, and we wanted to reduce the number of false positive results, we introduced a modified version of restriction fragment - differential display technique. Using such an approach, we obtained 19 expressed sequence tags, and nine of these were unambigously identified as homologs of known genes from data banks (Fig. 3). We named these nine genes *SOL1-SOL9* ("sol" is the Slovenian word for salt) (Petrovič et al., 2002).

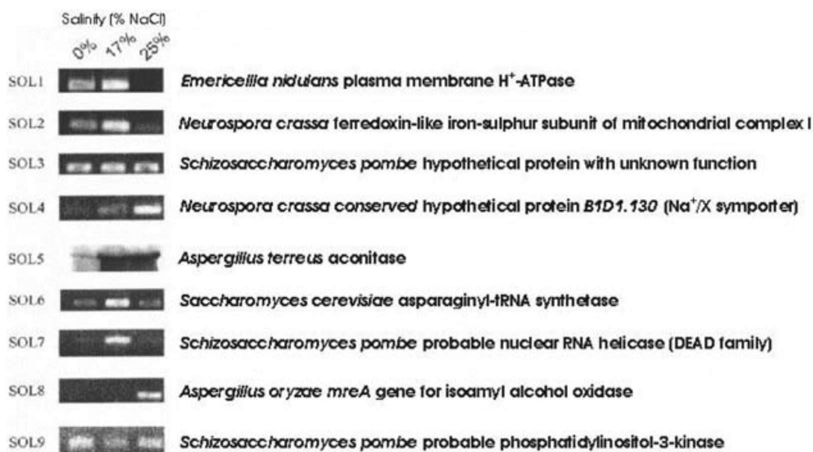

Figure 3. Differentially expressed genes in *H. werneckii* at different salinities

Based on additional studies related to the physiological responses of *H. werneckii* at different salinities, we can by now propose some of their functions. Since glycerol is abundant at higher salinities, we speculated that relatively less metabolic flux remains for the late stages of glycolysis and for the Krebs cycle. This assumption was substantiated by the decreased CO_2 production rates measured at higher salt concentrations (see Fig. 2). Increased expression of *SOL5*, a putative aconitase gene, at increased salinity could also be related to this phenomenon. Putative roles of *SOL4* and *SOL8* are discussed below.

At the level of the proteome, we found by applying 2D PAGE analysis that cellular proteins in *H. werneckii* are more acidic in comparison to salt sensitive *S. cerevisiae* (data from the SWISS 2D-PAGE database) and halotolerant *D. hansenii* (Goeteborg yeast 2D-PAGE) (Petrovič, 2001). As shown in Fig. 4, the isoelectric points of the proteins are clustered between 4.0 and 5.5. Preliminary data on the analysis of proteins from fractionated cell lysates show that plasma membrane proteins contribute importantly to this phenomenon and that cytosolic proteins are less acidic.

Different organisms have developed different strategies to counteract salt stress. The most important are:

- changes in membrane composition and properties.
- efficient transport systems for exclusion of ions as well as the storage systems for ions to reduce their toxic effects in the cytosol .
- accumulation of low molecular weight organic compounds, so called compatible solutes.
- special signal transduction systems, which sense and respond to increased salt concentrations.

In our further studies of cellular responses in *H. werneckii* to high salt concentrations, we adressed these levels.

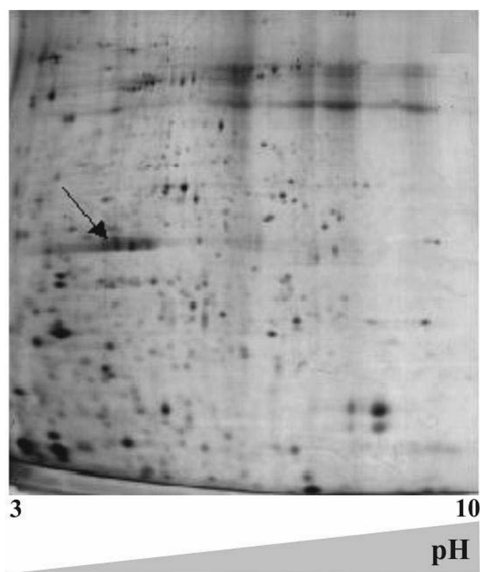

Figure 4. 2D PAGE of cellular proteins from halophilic *H. werneckii*.

3.1. CELL WALL AND MEMBRANES IN *H. WERNECKII*

Shrinking of the cell, loss of cell polarity, disassembly of the actin cytoskeleton and changes in the permeability of the plasma membrane are some of the immediate responses to increased osmolarity described for yeast cells (Slaninova et al., 2000). The cell volume of the salt-tolerant *D. hansenii*, *R. rubra*, *A. pullulans* and *Candida versatilis* was reduced by increase in NaCl (Hernandez-Saavedra, 1995). Deep plasma membrane invaginations were also observed in osmotically stressed cells. These were believed to represent a surface reserve and they deepened or expanded according to actual turgor pressure (Javor, 1989; Slaninova et al., 2000). It has been speculated that hyperosmotic shock induces changes in the organization of cell wall, most probably as a result of displacement of periplasmic and cell wall matrix material into invaginations of the plasma membrane. In *H. werneckii*, such invaginations have not been observed. On the other hand, we observed that melanin, which is responsible for the dark colour of *H. werneckii*, is involved in changes of the cell wall structure. We have demonstrated that *H. werneckii* synthesizes 1,8-dihydroxynaphthalene (DHN) melanin under saline as well as non-saline growth conditions (Kogej et al., 2004a). While the biosynthesis was not salt-dependent, the ultrastructural studies of the *H. werneckii* cell wall showed that the organisation of melanin granules depends on the concentration of salt in the medium. The granules in the outer part of the cell walls are loosely organised in the medium without salt, and are more densely packed as the salt concentration in the medium increases (Fig. 5). This kind of granular reorganization at increased salinity was absent in

salt-tolerant *A. pullulans*. It is not surprising that *H. werneckii* has a heavily melanized cell wall, since melanins are among the most stable and resistant of biochemical materials (Jacobson, 2000), and are known to confer protection to UV-irradiation, temperature extremes (Bell and Wheeler, 1986) and desiccation (Zhdanova et al., 1973, 1990). They also play an osmotic role (Elliot and Henson, 2001; Ravishankar et al., 1995). Our results suggest a potential osmoprotectant role of melanin in the cell wall of the halophilic *H. werneckii*.

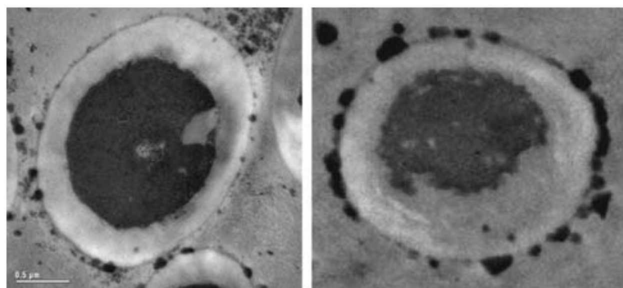

Figure 5. Organization of melanin granules is salt dependent in halophilic *H. werneckii*. Electron micrographs of the cell wall of *H. werneckii* cells grown without salt (left panel) or in the presence of 10% NaCl (right panel).

Changes in the membrane composition and membrane properties have been reported as an important factor in the adaptation to high salinity (Russell, 1989). Such changes proved to be crucial also in the adaptation of *H. werneckii* to hypersaline environments. Sterols and phospholipids are the two major lipid constituents of eukaryotic membranes, in which sterols play an important role in the structural organization of biological membranes. In the maintenance of proper fluidity, several factors are involved: the type of fatty acyl chains (their length and degree of unsaturation), the amount of sterols, and the nature of polar head-groups of phospholipids. The effects of salt stress on lipid composition and membrane fluidity have been investigated for only a limited number of eukaryotic microorganisms, including salt-sensitive *S. cerevisiae* and the salt-tolerant *D. hansenii*, *Z. rouxii*, *Y. lipolytica* and *Candida membranefaciens*. Overall changes in phospholipid to sterol ratio, changes in the degree of unsaturation of fatty acids and phospholipid composition as well as in membrane fluidity were demonstrated in salt-tolerant yeasts when exposed to higher salinities as compared to the salt-sensitive *S. cerevisiae* (Hosono, 1992; Khaware et al., 1995; Ravishankar et al., 1994; Tunblad-Johansson et al., 1987; Watanabe and Takakuwa, 1984). Published data suggest that salt-tolerant fungi show a broad spectrum of alterations in lipid composition in response to changes in the external salinity. Individual organisms modify different lipid groups by which the composition and properties of their membranes reflect their halotolerance.

In the halophilic *H. werneckii*, a high phospholipid to sterol ratio and a high degree of unsaturation of phospholipid fatty acids was demonstrated. While sterol content

varied only slightly at increased salt concentration, phospholipids, especially PE, were enriched at higher salinities. In accordance with the lipid composition, and in contrast to *S. cerevisiae*, increased plasma membrane fluidity over a wide range of NaCl concentration was demonstrated (Turk et al., 2004). These results indicate high intrinsic salt stress tolerance, and they are in good agreement with eco-physiological data and the dominance of *H. werneckii* in hypersaline waters of salterns. In contrast to the halophilic *H. werneckii*, the membrane fluidity of the related halotolerant black yeast *A. pullulans* resembles that of the salt-sensitive *S. cerevisiae* (Turk et al., 2004). Therefore, membrane fluidity appears to be a good indicator of the degree of salt tolerance.

Sterols are important constituents of biological membranes that affect membrane fluidity. Their synthesis is tightly regulated in all organisms. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HmgR) is the main regulatory enzyme in sterol biosynthesis. We have identified two isoforms of HmgR in *H. werneckii*: mitochondrial HwHmg1p and HwHmg2p, localized in the endoplasmatic reticulum (Vaupotič and Plemenitaš, 2004). We found that, in contrast to the salt-tolerant *A. pullulans* and the salt-sensitive *S. cerevisiae* in which the enzyme activities increased with increased salinity, the enzyme activity of HmgR in *H. werneckii* is high under hypo- and hypersaline stress and low at optimal salinities (see Fig. 2). It seems that the activity of HmgR is regulated at the level of transcription, since a significant increase in gene expression at the hypersaline stress was observed at hypersaline stress, and also at the post-translational level (Petrovič et al., 2002). We have demonstrated that salt affects the level of degradation of HwHmg2p by the ubiquitin-proteosomal pathway (Petrovič et al., 1999; Vaupotič and Plemenitaš, 2004). Membrane sterols in *H. werneckii* do not change accordingly, and the relative content of ergosterol, the main sterol in *H. werneckii*, and other sterols varied only slightly in response to increased salt concentrations.

Some novel sterol intermediates of ergosterol biosynthesis, 24-methylcholest-7-en-3β-ol and 24-methylcholesta-7,24(28)-dien-3β-ol were identified in *H. werneckii* as well as in other halophilic and halotolerant black yeasts. Although their content did not change significantly as a response to changes in salt concentration, they can provide a specific signature for these fungi in the environment. They also suggest different mechanism of regulation of sterol biosynthesis (Méjanelle et al., 2000, 2001; Turk et al., 2004). Since the activity of HmgR is extremely salt responsive, and the amount of sterols does not follow this pattern, it is possible that an alternative pathway, downstream of mevalonate, is important in *H. werneckii*. One such possibility is a pathway which includes isopentenoid isoamyl alcohol. If we assume that the product of isoamyl alcohol dehydrogenase, isovaleraldehyde, is transformed to isovaleryl-CoA, this pathway could be involved in the generation of ATP. Coordinated expression of *HMGR* which encodes HmgR2 and *SOL8*, which encodes isoamyl alcohol oxidase (see Fig. 2) supports this idea.

3.2. IONS AND COMPATIBLE SOLUTES IN *H. WERNECKII*

Presence of high concentration of salts in the environment means that the organism is in danger of losing water by osmosis. Halophilic and halotolerant organism thrive in environments with high salt concentration because they accumulate osmotically active compatible solutes which counterbalance the osmotic stress. For most eukaryotic organisms, salts are not appropriate compatible solutes, since high internal concentrations are harmful.

Although a high intracellular salt concentration is toxic for most organisms, some halophilic Archaea accumulate potassium chloride up to the molar concentrations when exposed to high external salinity (Oren, 1999). In contrast, eukaryotic microorganisms can not tolerate high ion concentrations. Data on different halotolerant yeast species like *D. hansenii* (Almagro, 2000; Andre et al., 1988; Larsson et al., 1990; Prista et al., 1997; Ramos, 1999), *C. versatilis* (Silva-Graça et al., 2003), *R. mucilaginosa*, *P. guillermondii* (Lahav et al., 2002) and others show that the maintenance of positive turgor pressure at high salinity in salt tolerant eukaryotes is mainly due to an increased production and accumulation of glycerol, trehalose and other organic compatible solutes.

Studies on salt-tolerant organisms showed that a large amount of ATP was required for glycerol synthesis and active uptake, as well as for the efflux of Na ions from the cells under salt-stressed conditions (Yagi, 1992). The flow of ions across cellular membranes is mediated by ion transporters and channels. P-type ATP-ases that transport H^+, Na^+, K^+ and some other ions are present in the plasma membrane and in intracellular membranes. They are encoded by *ENA* genes present in virtually all fungi, and have been mostly connected to Na^+-efflux. Many of them function also as K^+ pumps. It has been speculated that to maintain the K^+ efflux capacity of the cells as well as a better adaptation to high Na^+ concentration, *ENA* genes duplicated and generated pumps that are more active with Na^+ (Benito et al., 2002). In the salt-tolerant species *D. hansenii* and *Z. rouxii*, *ENA* genes encoding Na^+-ATP-ases were identified and analysed. Their expression is Na^+ dependent, suggesting that ENA genes play an important role in salt-tolerance (Almagro et al., 2001; Watanabe et al., 2002).

In *H. werneckii* we have identified two homologs of *ENA* genes and demonstrated, that expression of only one, *HwENA2*, is salt dependent (Gorjan and Plemenitaš, 2003). No major changes in intracellular ion concentration occur in *H. werneckii* with increased external salinity. Although the Na^+ and K^+ ratio increased in parallel with external salinity, the total intracellular cation concentration was far below the external sodium concentration. Intracellular sodium was kept below 200 nM in *H. werneckii*. On the other hand, in the salt tolerant black yeast *A. pullulans*, higher intracellular ion concentrations, with more intracellular ion fluctuations and an even more profound increase in Na^+ to K^+ ratio after salt stress were observed (Kogej et al., 2004b). These data suggest that true halophiles are better adapted to changes in external ion concentrations, most probably due to their ability to effectively exclude sodium ions. The expression profile of two genes that could be connected to Na^+ transport in *H. werneckii*, *SOL4* coding for a putative Na^+ transporter (Fig. 3) and one of the two homologues of *S. cerevisiae ENA1* in *H. werneckii* (*HwENA2*), support the existence of an effective transport system that helps maintaining low intracellular ion concentrations in *H. werneckii*. RT-PCR analysis revealed that the transcription of the *HwENA* gene, which encodes a putative plasma membrane P-type ATPase, is affected by increased salinity. On the other hand, salt dependent changes of the measured enzyme activity of the plasma membrane P-type ATP-ase do not follow the pattern of the expression profile of Hw*ENA2* (Fig. 6). These results suggest that high expression of *HwENA2* at extremely high salt concentration is probably due to the activation of a signalling pathway (most probably HOG) in this stressful situation. However, enzyme activity is additionally regulated at the post-transcriptional level under these conditions.

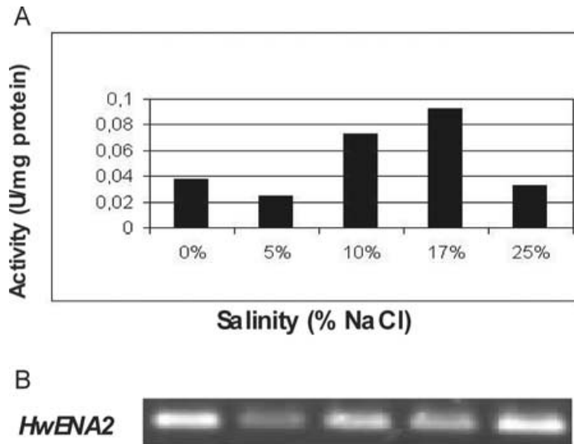

Figure 6. Enzyme activity of plasma membrane P-ATP-ase (A) and expression profile of Hw*ENA1* (B) in *H. werneckii* grown in the medium with different salt concentrations.

When cells are exposed to increased salinity, they balance high external osmotic pressure by accumulating low molecular weight compounds, compatible with cellular functions (Hochachka and Somero, 2002; Oren, 1999, 2002). Polyols are prevalent compatible solutes found in halotolerant and halophilic fungi. Commonly accumulated compounds include glycerol, erythritol, inositol, arabinitol, xylitol and mannitol, with glycerol by far being the most important one (Blomberg and Adler, 1992; Pfyffer et al., 1986). Differences in the mechanism of intracellular glycerol accumulation are distinguished on the species level (Hohman, 2002; Iwaki et al., 2001; Lages and Lucas, 1995; Lages et al., 1999; Lahav et al., 2002; Larson et al., 1990; Lucas et al., 1990; Thome and Trench, 1999), and they are not fully understood. In *H. werneckii* glycerol accumulates intracellularly in the cells when growing in the media with salinities up to 10% NaCl. Its concentration stays virtually unchanged at higher salinities. On the contrary, extracellular glycerol concentration is low between 0% and 17% NaCl in the medium and increases only at concentrations above 17% NaCl (see Fig. 2). One possible interpretation is that glycerol, which is synthesized at increased salt concentrations, is efficiently kept inside at conditions optimal for *H. werneckii*. Increased intracellular glycerol accumulation could result from an increase in endogenous glycerol synthesis and active uptake of the external glycerol. The increase in glycerol synthesis in *H. werneckii* is supported with the identification of two genes coding for putative glycerol-3-phosphate-dehydrogenases, Hw*GPD1* and Hw*GPD2*. According to the expression profile obtained by RT-PCR, only *HwGPD2* is differentially expressed at increased salinity (Gorjan and Plemenitaš, 2003; Petrovič et al., 2002). These results suggest that in *H. werneckii*, as in *S. cerevisiae*, glycerol biosynthesis is regulated at the level of transcription of glycerol-3-phosphate-dehydrogenase. Although our results show that glycerol is the most important compatible solute, the lack of change in intracellular glycerol concentration above 10% NaCl in *H. werneckii* indicates the probable presence

of another, yet unidentified compatible solute(s) in this halophilic black yeast. According to literature, other compatible solutes such as glycine betaine and free amino acids are produced as a response to salt stress in different microorganisms (Hochachka and Somero, 2002). None of such responses have so far been observed in *H. werneckii*.

3.3. SENSING IONS IN THE ENVIRONMENT – THE HOG SIGNALLING PATHWAY IN *H. WERNECKII*

Multiple signalling pathways allow organisms to respond to different extracellular stimuli and to adjust their cellular machinery to the changes in their environments. The most prominent signalling pathway that senses osmotic stress in fungi is the HOG (high osmolarity glycerol) pathaway. Signalling pathways involving protein kinase A, phosphatidylinositol 3,5-biphosphate and increased cytosolic calcium are also known to mediate osmotic stress, but their phisiological significance is less understood (Tamais and Homann, 2003)

The HOG signalling pathway is one of the most investigated and best understood in *S. cerevisiae*. Upon osmotic stress, MAP kinase kinase (MAPKK) Pbs2, which also acts as a scaffold protein, phosphorylates key MAP-kinase Hog1p in this salt-sensitive yeast. This phosphorylation causes translocation of Hog1p from the cytoplasm to the nucleus, where Hog1p phosphorylates transcription factors which triggers transcription of target genes. Among the genes that are affected by Hog1p are also those involved in glycerol production and ion transport (Tamás and Homann, 2003). *S. cerevisiae* homologues of individual kinases from HOG signalling pathway have been identified and their putative physiological role proposed for only few halotolerant microorganisms (Bansal and Mondal, 2000; Kinclova et al., 2001).

We investigated whether a similar signalling pathway exists and plays a role in the adaptation mechanisms of the halophilic *H. werneckii*. So far, homologues of two key kinases of this pathaway in *S. cerevisiae*, HwHog1p and HwPbs2p, were identified. When we used antibodies raised against *S. cerevisiae* Hog1p and Pbs2, we detected in *H. werneckii* their homologous 46 kDa and 80 kDa proteins, respectively. In vitro kinase assay demonstrated that, when compared to *S. cerevisiae* in which Hog1p is activated even at very low salt concentration, the *H. werneckii* homologue of Hog1 kinase is fully active only at extremely high salt concentrations (Turk and Plemenitaš, 2002). We isolated the gene coding HwHog1p, a 359 aa protein with domains characteristic for other known MAP kinases, like a common docking domain at the C-terminal domain, a TGY motif at 171-173, and an aspartate in the active site. HwHog1p was classified into a subgroup of fungal stress-activated protein kinases (SAPKs). To check the functionality of HwHog1p, we introduced the *HwHog1* gene into a *Hog1* deleted mutant strain of *S. cerevisiae*. HwHog1p only partialy complemented Hog1p in *S. cerevisiae*. This result was not surprising, since one would expect that the whole machinery which senses high ion concentrations is different in the halophilic *H. werneckii* from that of salt sensitive organisms. Although the protein structure of the HwHog1 was not very different from Hog1p from *S. cerevisiae*, we observed differences in the activation mechanism: membrane localization of MAP kinase components plays a crucial role in the activation of signalling pathways. In *S. cerevisiae*, Hog1 circles between cytosol and nucleus, whereas Pbs2 is localized at the plasma membrane. In contrast, in *H. werneckii* we demonstrated that both kinases HwHog1 and HwPbs2 are temporarly localized at the

plasma membrane (Plemenitaš et al., 2003; Turk and Plemenitaš, 2002). The different localization of phosphorylated and unphosphorylated forms of HwHog1p, when compared to localization of Hog1p in *S. cerevisiae*, and the prolonged activation of the HOG pathway in *H. werneckii* and the only partial complementation of *S. cerevisiae* Hog1p function by HwHog1p, as demonstrated in our studies, point to a different regulation of the HOG signalling pathway in the halophilic *H. werneckii.*

4. Conclusions

The behaviour of the halophilic *H. werneckii* at different salinities showed the following special features:
• coordinated expression of salt-stress responsive genes, correlated to their putative functions like amongst the activation of HwHog1, expression of *gpd2* and glycerol accumulation.
• salt-dependent expression of the *ENA* gene, related to the enzyme activity of a putative plasma membrane P-ATP-ase.
• different post-translational regulation of enzyme activity.
• the ability to maintain membrane fluidity unchanged over a wide range of salinities.
All these are newly discovered mechanisms that enable *H. werneckii* to thrive at extremely high salt concentration and to adapt to a wide range of NaCl concentrations in its environment. We have found that some genes which are crucial to counteract salt stress are present in *H. werneckii* in two copies, and that expression of one of these is usually highly salt dependent. We speculate that the existence of two sets of genes expressed differentially at different salinities is one of the possible strategies used by *H. werneckii* that enables the successful adaptation to changing salinities in its environment.

We are aware that studies of molecular mechanisms of adaptation to extremely high salt concentrations in *H. werneckii* as a halophilic eukaryotic model organism are still in their infancy. Future molecular studies will enable us a better understanding of halophily in the kingdom of fungi.

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HALOTOLERANCE AND LICHEN SYMBIOSES

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1. Introduction

Lichens form intricate partnerships of at least two different organisms. They are regarded as a stable and self-supporting association between fungi (the mycobionts) and photoautotrophic, algal partners (the photobionts). The usually dominant mycobiont characterizes the phenotype of the symbiotic association and therefore, lichens are traditionally classified as a life form of fungi. The genetic potential to form lichen symbioses evolved apparently in a major phylogenetic radiation in the sister group of the non-lichenized Pezizomycetes (Lutzoni et al., 2004). Being an evolutionary successful form of fungal symbioses, the lichen forming habit is maintained by one fifth of all fungi. This includes more than c. 40% of Ascomycota, but only a few Basidiomycota. Lichens of the Ascomycota are classified in morphologically diverse groups. This includes the Lecanoromycetes, Lichinomycetes, and Arthoniomycetidae, classes and subclasses predominantly containing lichen-forming species, but lichens are also found in Chaetothyriomycetidae (Eurotiomycetes) and Dothideomycetidae (Sordariomycetes), i.e., subclasses that primarily represent other fungal life-styles.

Lichenized fungi are among the organisms that inhabit the harshest environments on Earth, ranging from the continental areas of Antarctica to dry, hot deserts. Among the extreme habitats are also coasts and rocky sea shores. Certain lineages of lichens are abundant in the littoral to supralittoral belts, where they are exposed both to salt and dry stress and, towards the poles, to cold stress as well. Because they often share the habitat with coastal birds, some species are also exposed to high levels of nitrogen due to bird manure.

In this contribution we will present an overview of knowledge about halotolerant lichens, focusing on maritime lichens. We will first present a brief account of the diversity of coastal lichens, further on we will outline some morphological patterns, sketch out the knowledge about ecophysiology of osmotic stress in lichens, and then present preliminary molecular investigations with an outlook to possible future research on halotolerance in lichen symbioses.

2. Diversity

It has since long been recognized that coastal lichens form distinct zones of occurrence (Weddell, 1875). Knowles (1913) distinguished 3 colour belts (dark, orange, grey-

green), a fourth was added by Knowles (1915: white belt), while Fletcher (1973a, 1973b) named the following zones according to an increasing distance to the water line: sublittoral, littoral, supralittoral, and terrestrial. The littoral zone corresponds broadly to the intertidal area, but can extend with the stronger action of waves. This zone corresponds to the black belt and is inhabited by a limited number of species of lichens (16 species in Great Britain; Gilbert, 2000). The dominant species here belong to pyrenocarpous lichens (Santesson 1939), including the subcosmopolitan genera *Lichina* (Lichinomycetes), *Collemopsidium* (Dothideomycetidae) and *Verrucaria* (Chaetothyriomycetidae).

The zone below the littoral belt is known to host a number of marine fungi, yet the contribution of lichens is low: only five lichen-like associations were mentioned by (Kohlmeyer and Kohlmeyer, 1979). One further example, *Halographis runica* (Kohlmeyer and Volkmann-Kohlmeyer, 1988) was described since then, but the placement of this species in the Opegraphales is in need of a modern re-evaluation. How deep lichens may reach below the sea level has not been studied, yet, *Verrucaria serpuloides*, another poorly known species, was reported from c. 30 m below the waterline (Lamb, 1973).

In the zone above, the mesic-supralittoral, orange *Caloplaca* species may dominate, forming the characteristic 'orange belt' by their rich production of anthraquinones as secondary metabolites (e.g. Arup, 1995). On shaded shores their coverage is usually reduced, and replaced by more common white and grey colored species so that the zone becomes more grey in colour. Most species in the littoral and in the supralittoral zones are usually bound to their habitats and might therefore considered halophilic, but compared with the habitats of some halophilic non-lichenized fungi (Gunde-Cimerman et al., 2004), the salinity of the littoral is clearly lower.

The terrestrial “white” zone along the coasts includes more species which can also be found further apart from the influence of the sea. They may even have their optimal habitats under salt-free conditions, but are apparently able to tolerate salt stress. Such species are also encountered on barks of trees in coastal and inland forests, e.g., *Ramalina* species.

While the knowledge about the diversity of the lichen mycobionts is fairly good, we now also beginning to understand more about the involved photosynthetic partners. Recently, photobionts were isolated from a series of maritime lichens by Watanabe et al. (1997) and Takeshita and Harada (2001). These authors found several green algal genera: *Trebouxia* (several species), *Dictyochloropsis*, *Myrmecia*, *Chlorella*, *Palmellococcus*, *Pseudococcomyxa*, *Apatococcus*, and *Dilabifilum.* All of these genera can also be found outside of maritime habitats in lichens. Yet, the species concepts in these genera are still poorly settled, and it is therefore not known whether the coastal strains in these genera are distinct and specifically linked to salt-rich habitats. *Dilabifilum* sp. (probably *D. arthopyreniae*, Ulvophyceae) is the typical photobiont of aquatic and marine *Verrucaria* species, the latter including the most common species *V. maura* (Thüs et al., 2003; Tschermak-Woess, 1976). Apart from symbioses with the mentioned eucaryotic algae, some of the littoral lichens are associated with cyanobacteria. *Lichina* is associated with a photobiont genus resembling *Calothrix* or *Rivularia*, whereas members of the genus *Collemopsidium* have a *Hyella*-like photobiont, except for one finding of *Dilabifilum arthopyreniae* in *C. halodytes* by Vischer (1953). One species of *Collemopsidium*, *C. pelvetiae* thrives on brown algae of

the genus *Pelvetia*, but develops a genuine thallus with a cyanobacterial symbiont. In other *Collemopsidium* species, the photobiont was identified as *Hyella caespitosa* (Mohr et al., 2004; Tschermak-Woess, 1976). So far no detailed phylogenetic investigations of these cyanobacterial photobiont groups are carried out and their precise relationships are not known.

A unique association is known from another *Verrucaria* species. In the intertidal zone, *Verrucaria tavaresiae* is associated with the brown alga *Petroderma maculiforme* (Sanders et al., 2004). Another unusual photobiont, hardly found in other lichens is *Prasiola*, a member of Ulvophyceae. *P. borealis* and *P. crispa* ssp. *antarctica* associates with *Mastodia* (Kohlmeyer et al., 2004). It seems that there is a strong selectivity for only a few algae well-adapted to wetness in littoral environments, while inland lichens are known to associate with various algal species of the same genus.

It should be noted that the morphology of algae is generally modified when they are in lichenized stage, and maritime lichens are no exception. While, for example, a free-living *Hyella caespitosa* is filamentous, it is unicellular in lichenized stage. Similar is true for *Dilabifilum arthopyreniae*, which forms distinctly filamentous thalli when free-living beneath the lichens or in culture, while in the lichens (e.g. *Verrucaria* species) it occurs as isolated, rounded cells or in chains of 2-3 cells (Tschermak-Woess, 1976). Changes induced by lichenization are also observed at the sub-cellular level and involve the shape of chloroplast, pyrenoids and the contents of pigments (see also further below).

3. Morphology

Lichens possess a characteristic and complex vegetative body, the thallus, which is formed by a fungal plectenchyma, and the usually enclosed algal symbiotic partners. In the majority of the lichen species, the thallus has a strict organization with different layers. Typically this includes a surface layer (the upper cortex, usually only consisting of the mycobiont), an algal layer (containing the photobiont and contacting hyphae) and a medullary layer (consisting of the mycobiont). This principal morphological pattern can be modified in the diverse lichen lineages, and sometimes the differentiation into different layers is poorly developed or completely reduced especially in many crustose lichens. Given the diversity of growth forms it is difficult to generalize whether halotolerant and halophilic lichens have shared morphological adaptations to salt-rich habitats. To study such features, investigations need to focus on lineages with similar gross morphology, where comparisons are possible between related species in different habitats. Poelt and Romauch (1977) compared closely related species of crustose and lobate coastal and inland lichens of the order Lecanorales for their thallus anatomy. Interestingly, they observed similar anatomical differences in all lichens investigated. In the coastal relatives, the thalline hyphae are more strongly conglutinated by intercellular gels and more densely arranged. The medullary layer which normally contains large intercellular spaces is densely occupied by hyphae in maritime relatives. Moreover the algal layer is more or less distinctly subdivided by vertically oriented hyphal strands. The functional consequences of these shared morphological similarities are a matter of speculations, but it might well be asked whether compounds of the hyphal walls confer a osmotic buffering function.

Poelt and Romauch (1977) focused in their study on species of the supralittoral belt. In comparison, the species of the littoral belt have different morphologies, but these differences are apparently not only related to the salt-rich habitat. All species, however, have in common that the tissues are rather dense and devoid of air-containing intercellular spaces. *Lichina*, with a brush-like morphology, and containing a cyanobacterial symbiont, has anatomically poorly structured thalli, i.e. without a clear differentiation of internal layers. Only at the surface, dark compounds are produced, which apparently give the thalli some rigidity.

The morphology of *Mastodia* and *Collemopsidium* were recently described in detail by Kohlmeyer et al. (2004), who also clarified the taxonomy of these species. These authors also coined the term "borderline" lichens for these associations and morphologically similar ones between fungi and algae to account for the rather simple organizational level (Lud et al., 2001). Despite its foliose habit, *Mastodia tesselata* produces thalli with very simple organization. Kohlmeyer and Kohlmeyer (1979) described this phenotype previously as a mycophycobiosis or a case of parasitism. Due to the dense formation of hyphae (textura intricata) around the algal cells and the accumulation of hyphal and algal cells it may nevertheless be regarded as a primitive form of a lichen symbiosis.

The thalli of crustose *Collemopsidium,* a recently re-established genus for some former *Pyrenocollema* species (Grube and Ryan, 2002), and *Verrucaria* are also poorly differentiated. In *Verrucaria* species, there are various patterns of melanization of the thalli. For example, *Verrucaria maura* has a strongly melanized basal layer (Fig. 1), which connects with the rocky substrate, whereas *Verrucaria mucosa* is melanized at the upper surface (Fig. 2). Both, *Collemopsidium* and *Verrucaria* have closed, pyrenocarpous fruitbodies, and the peridial layers which enclose the delicate hymenia are to varying degree melanized. In many species a melanized involucrellum is developed, which forms an outer ascomatal cover that may extend to various degree into the surrounding vegetative thallus. Especially in the involucrellum, the melanization can be very strong (which makes it difficult to cut the ascomata for analyses with a razor-blade). On the other hand in these species, as in all species of lichens, the hyphae which connect with the photoautotrophic partner are not melanized. Beside the wall-bound dark compounds, no species of the littoral belt and below do produce typically crystallized lichen compounds, which are common in other lichen lineages of supralittoral belts.

While melanins might play an osmoregulatory role in halophilic black yeasts (Kogej et al., 2004), their function could be complex in lichenized fungi. As the observation of transversal sections of lichen thalli from *Verrucaria* species of the black littoral belt shows, the melanins are deposited only in particular parts of the thalli, and often in a lower interface of the species with the rocky substrate. As melanized fungal plectenchyma are rather rigid, the strong melanization of the fruit-bodies could mediate mechanical properties that make these species suitable for growth in habitats with strong osmotic fluctuations. Under these conditions the delicate cells and intercellular gels in the fruit-bodies will be prone to strong mechanical pressures, and this might impair proper function of the fruit-body unless there is a mechanism to keep their overall shape. This could be mediated by the melanized peripheric layers. It might rather be hypothesized that the resulting increased pressure upon air-drying (e.g., at low tide) may promote efficient ascospore dispersal in intertidal lichens. A further

mechanical role of melanization could be suspected in some *Verrucaria* species with broader ecological amplitude. These develop more strongly melanized thalli when they grow more exposed to the action of waves (H. Thüs, personal communication). Species above the intertidal zone have usually no such distinctly melanized structures in their thalli.

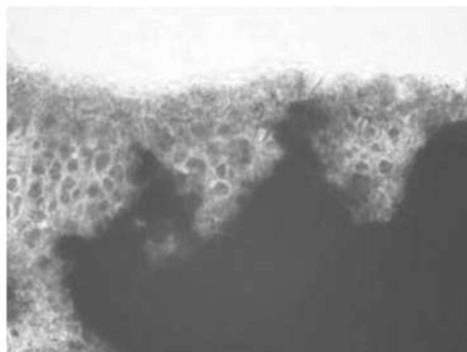

Figure 1. *Verrucaria maura*. Melanin-like pigments occur only in basal layers.

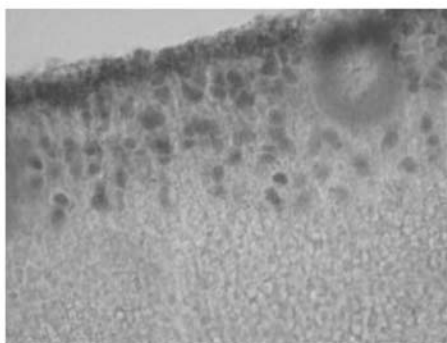

Figure 2. *Verrucaria mucosa*. Melanin-like pigments occur only in the uppermost layer.

4. Ecophysiology

A first review of ecological aspects of coastal lichens was presented by Fletcher (1976). His account was based on field observations and on experimental evidence. Despite the preliminary nature of the experiments, this work is still a rich source of information for future experimental designs. Flechter's studies suggested that species along the coasts are arranged according to their tolerance of wetness in the environment, whereas the salinity of the water had only limited effects.

Similar was also found in more recent experimental studies. Matthes-Sears et al. (1987) studied assimilation rates of the corticolous coastal lichen *Ramalina menziesii* (Fig. 3). Assimilation rates were studied among coastal and inland sites and within these sites, to assess variation patterns. Moreover NaCl treatment experiments were carried out. Neither were differences found in the assimilation rates between coastal and inland sites, nor within sites. Moreover, there was no change in CO_2 assimilation rates after NaCl treatments for 27 days. To summarize, there was no evidence that variations of assimilation rates are caused by salinity levels, thus this species apparently has efficient osmotic regulation mechanisms.

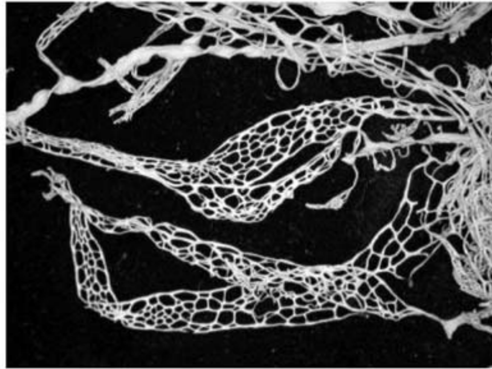

Figure 3. Habitus of *Ramalina menziesii*

Studies of salt tolerance of lichens certainly need to consider results from investigations of desiccation tolerance. Nash et al. (1990) differentiated between the effects of atmospheric desiccation and osmotic stress by using 4 species in their experiments. In all of them dark respiration was clearly more sensitive to atmospheric desiccation than to osmotic dehydration in sorbitol or salt solutions. Interestingly, measurable CO_2 fixation occurred in the coastal *Dendrographa minor* to levels of water potential as low as -38 MPa.

Photosynthesis studies of the coastal Antarctic lichen *Mastodia tesselata* (syn. *Turgidiusculum complicatulum*, see Fig. 4) indicate that it is so well acclimated to saline environments that its activity is reduced when treated with fresh water (Huiskes and Moerdijk-Poortvliet, 2000). In this nitrophilous species CO_2 assimilation rate was not significantly influenced by different salinity levels, whereas NH_4NO_3 depleted net photosynthesis rate at all salinities except at 100% seawater.

While in these above-mentioned studies salinity effects were investigated with whole lichens, other authors attempted to analyze salt tolerance of axenic cultures with separated symbionts. The influence of NaCl concentration on spore germination of lichen mycobionts was studied by Takahagi et al. (2000), using 18 different species. No germination was found in all species at 1.2 M NaCl. Interestingly the reaction of the species in culture did not correlate with their natural habitat. Further experiments were

carried out with regard to salt tolerance of cultured mycelia of mycobionts (Yamamoto et al., 2001). In that study, 77 species including 21 species from marine habitat were sampled. Of these, 37 species tolerated, i.e. grew, at concentrations of 0.6 M NaCl, including all 21 species from maritime habitats. Six maritime species tolerated 1.2 M NaCl. Only one species, *Niebla homalea*, showed enhanced growth at 0.6 and 1.2 M NaCl, although this species is bark-inhabiting and may occur not only close to the coasts.

Figure 4. *Mastodia tesselata*.

To compare salt tolerance properties within a genus comprising maritime and inland species, Takahagi et al. (2002) studied cultured mycobionts and photobionts of *Ramalina*. Six species were included in that study, with 3 maritime and 3 inland species. All mycelia of mycobionts grew at concentrations of 0.8 M NaCl, while all photobionts stopped growth at 0.6 M or lower. The results generally showed no correlation of salt tolerance between originally associated mycobionts and photobionts.

It has recently been shown that antioxidant and photoprotective mechanisms in a lichen are clearly more effective than those in its isolated partners. In the lichenized stage, the photobiont reduces chlorophyll concentrations, while photoprotective pigments are upregulated, as well as the antioxidant tocopherol that plays an important role as free radical scavenger in membranes. The upregulated carotenoids dissipate excess light energy as heat and thus contribute to avoiding formation of reactive oxygen species in the photosynthetic apparatus. On the other hand, the fungal partner is only in lichenized stage able to react rapidly to desiccation, the antioxidant glutathione being essential for counteracting oxidative stress (Kranner et al., 2005). Such mutual benefits may also be expected to confer tolerance to osmotic stress.

Halotolerant organisms generally accumulate solutes to cope with increasing external salinity. Feige (1972, 1973, 1975) detected mannosylmannose in *Lichina confinis* and showed that this solute is rapidly synthesized in *L. pygmaea* (Fig. 5) during exposure to sea water. Feige (1975) suggested that mannosylmannose is produced by the algal partner. This sugar alcohol certainly plays a role in the osmoregulation in this

lichen. It probably has the same function as mannitol has in the red algal genus *Caloglossa*, namely as a compatible solute in the cytoplasm and thereby achieving osmotic acclimation (Karsten et al., 1992, 1994). Some of the enzymes related to mannitol synthesis are known (e.g. Iwamoto et al., 2001), but none of the genes regulating mannosylmannose synthesis have so far been characterized. Except for Feige's (1972, 1973, 1975) research on the carbon fixation of *L. pygmaea*, virtually nothing is known about the patterns of compatible solute production in lichens adaptated to a life in a saline environment. It still needs to be checked in other lichens how levels of the more "typical" lichen polyols, viz. mannitol and D-arabitol, which are converted from green-algal ribitol and erythrol or cyanobacterial glucose (Honegger, 1991), are changed upon salt stress. Moreover the contents of glycerol, a widely occurring osmoregulatory solute in other organisms including free-living fungi, needs to be investigated. Our hypothesis is that it is the total intracellular polyol concentration rather than the concentration of specific polyols, which gives the best correlation with environmental salinity, but this needs still be studied experimentally. It may be hypothesized that production of transportable polyols, a feature of lichenized algae, may generally enable lichens to tolerate varying levels of salt stress. However, it still needs to be tested whether the fungal partner benefits primarily from enhanced production and transfer of algal sugar alcohols under salt stress or whether both symbionts maintain the genuinely produced osmotically active compatible solutes in their cells.

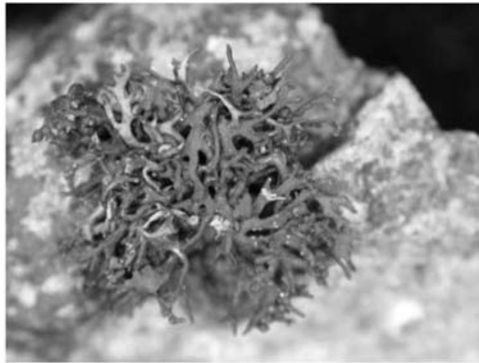

Figure 5. *Lichina pygmaea*

Lichens cease metabolism when desiccated, and this might also be the case under high osmotic stress. However, close to the coast lichens are constantly exposed to high salt load by salt spray from the air, and must be metabolically active even under higher extracellular concentrations of salts. *Roccella*, which develops rather large thalli, is an especially productive example. Notably, some species of *Roccella* were economically important in the past and harvested for production of lichen dyes (orchille) on the Canary Islands. Follmann (1967) suggested that the salt crusts found on species exposed to salt spray may enhance water uptake, but his conclusions were somewhat

ambiguous. Other species, such as members of *Ramalina*, were recently used as "saltfall" monitors (Figueira et al., 1998, 1999), yet metabolic responses to high levels of extra-thalline salt deposits are not known.

One interesting observation shall briefly be appended here. Osmotically stressed mycobiont cultures seem to induce the biosynthesis of secondary metabolites in mycobiont cultures (Hamada, 1988, 1993; Miyagawa et al., 1993; Yamamoto et al., 1985), and Solhaug and Gauslaa (2004) showed that resynthesis of secondary metabolites in acetone-soaked thalli is promoted by exposure to osmolytes. The possible effects of osmotic stress on metabolite production under natural conditions definitely deserve further studies.

5. Preliminary Phylogenetic Investigations of Functional Genes

Lichens fungi are a largely unexplored group for studying the evolution and expression of functional genes. Halotolerance could be an interesting property of lichens for studying any co-adaptation to saline environments at the genetic level. Moreover, lichens growing in saline environments are closely related to species from non-saline environments. This is particularly true for the genus *Verrucaria*, which has numerous marine representatives (e.g., Brodo and Santesson, 1997; Santesson, 1939) and numerous non-marine aquatic representatives (e.g. Thüs, 2002). Thus, this and other ecologically diverse genera are excellent models for analyzing the evolution and expression of functional genes related to salt tolerance. So far, there have been no attempts to find and characterize genes likely involved in salt tolerance in lichenized ascomycetes. A screening for such genes could be the first step, starting with the present knowledge about these genes in non-lichenized fungi. Here we present first examples for phylogenetic studies of newly detected genes.

Polyketide synthase genes for the synthesis of the pentaketide dihydroxynaphthalene (DHN), a precursor of ascomycetous melanin, are known so far only from a few non-lichenized fungi. The presence of dihydroxynaphthalene melanin production might be involved in salt tolerance in non-lichenized halophilic fungi (Kogej et al., 2004). Apart from a polyketide synthase, the production of DHN-melanine involves the action of tetrahydroxynaphthalene reductase, scytalone dehydratase (which also acts as vermelone dehydratase), and trihydroxynaphthalene reductase. The resulting dihydroxynephtalene is then polymerized, possibly by a laccase, to yield melanin. Primers for genes of some of these enzymes have been published recently (Tsuji et al., 2003; Wang et al., 2001). As a pilot study, polyketide synthase (PKS) and scytalone dehydratase (SCD) from members of the lichen order Verrucariales were amplified using LC1 and LC2C (Bingle et al., 1999) for PKS and SD1 and SD2 (Wang et al., 2001) for SCD. The sequences were aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic position of the polyketide synthase in a larger tree as a sister group to a melanin related PKS from *Wangiella dermatitidis* suggests that the detected gene is involved in melanin production (Grube and Blaha, 2003).

To allow a comparison of the phylogenies of the polyketide synthase with those from scytalone dehydratase for congruence of their topology, the trees were rooted with the available melanin pathway genes from *Colletotrichum lagenarium* and subject to a

phylogenetic analysis using MrBayes Version 3.0 (Huelsenbeck and Ronquist, 2001, using the GTR+I+G model of substitutions). The topologies of this analysis (Fig. 6) were largely similar except for the insufficiently supported position of two halophilic *Verrucaria* species at the basis. The finding of scytalone dehydratase suggests that DHN melanin is potentially be produced by these fungi, and the congruence with the phylogeny of the polyketide synthase indicates that this polyketide synthase gene is involved in the assembly of the corresponding pentaketide.

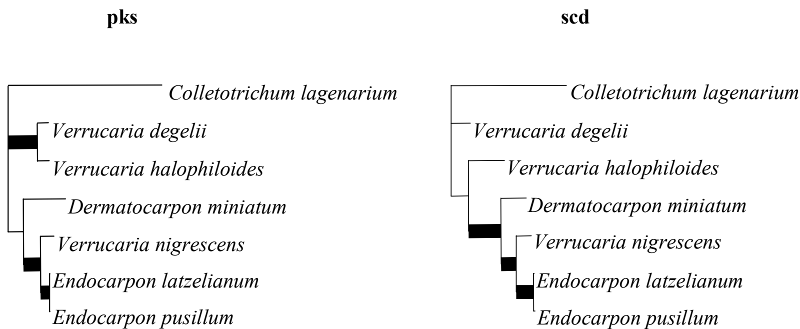

Figure 6. Trees from the ketoacyl synthase domain of a PKS gene (left) and a scytalone dehydratase gene (right). 50% majority rule consensus tree of a Bayesian phylogenetic tree sample. Posterior probabilities ≥95% are indicated by thick branches.

Five mitogen-activated protein kinase (MAPK) signal transduction pathways, which enable yeast cells to regulate various aspects of cellular functions, are known from *Saccharomyces cerevisiae*. Of these, the high-osmolarity glycerol response (HOG) signalling pathway senses and responds to hyperosmotic stress (Bansal and Mondal, 2000; Gustin et al., 1998; Hohmann, 2002; O'Rourke et al., 2002). The key MAP kinase of the HOG pathway in *S. cerevisiae* is HOG1p, which is phosphorylated by Pbs2p upon osmotic stress (Cobb and Goldsmith, 1995). This gene, HwHog1p, is thus activated post-transcriptionally under high salinity. A homologous gene of the *S. cerevisiae* HOG1 gene was recently isolated from the halophilic black yeast *Hortaea werneckii* by Turk and Plemenitaš (2002).

HOG1 is for the first time also detected in lichenized fungi here (Fig. 7 presents a preliminary phylogeny). We have used a heterologous primer approach to detect this gene in members of Verrucariales, including the halotolerant *V. mucosa*, and from Lecanorales (*Lecanora* species). Based on an alignment of known protein kinases involved in osmoregulation we designed the following primers for PCR: MAP1537-5' (5'-TCG AGA CCN YTV GAG AAG C), MAP2190-5' (5'-CCN GAA ATC ATG CTN ACN TGG C), and MAP2748-3' (5'-GAG TAC ATC ATG ACC TTC C) to amplify corresponding fragments from genomic DNA extractions of whole lichen thalli. Sequencing was carried out with MAP2190-5' and MAP2748-3', and the resulting alignment produced by manual adjustment after running ClustalW as implemented in

BioEdit was subjected to a Bayesian analysis as above in the analysis of scytalone dehydratase. Interestingly the terminal branches in the genus *Dermatocarpon* seem to be generally longer than those in the genus *Lecanora* in this tree. It might be tested in future studies whether there are differences in substitution rates of HOG1 among diverse lichen mycobionts lineages.

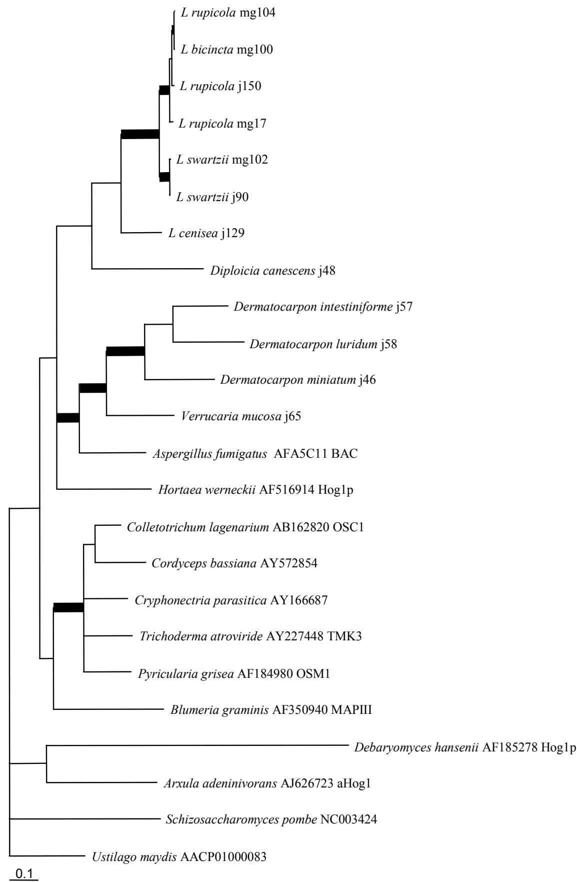

Figure 7. Preliminary phylogenetic analysis of HOG1 sequences (fragment length 524 bp) in several fungi and lichenized species. 50% majority rule consensus tree of a Bayesian phylogenetic tree sample. Posterior probabilities ≥95% are indicated by thick branches.

Clearly, many more genes involved in salt tolerance need to be detected from both lichen symbionts in the future. After this has been achieved, their transcription can be analyzed via reverse transcription assays using specific primers. Alternatively, the

transcriptome of lichens under salt stress can be explored to find genes under transcriptional control. In an approach to detect specific transcriptional responses, 4 selected lichen thalli of *Parmelia subrudecta* at the natural habitat were soaked twice per day with 1 M NaCl solutions. After one week, RNA from these thalli and 4 control thalli without treatment was isolated. The reverse transcribed RNA was subjected to AFLP, and the resulting fragments were separated on an agarose gel (Fig. 8). In 3 of the salt-treated thalli we found a fragment of c. 400 bp length, which was not present in untreated thalli (the fragment was missing, though, in a salt-treated thallus that was likely affected by rainfall during the experimental period). This fragment, which still needs to be characterized by sequencing, could be corresponding to a gene that is involved in salt stress response (Grube et al., in preparation). However, we do not know yet, whether this fragment will be part of the algal or fungal RNA pool, respectively.

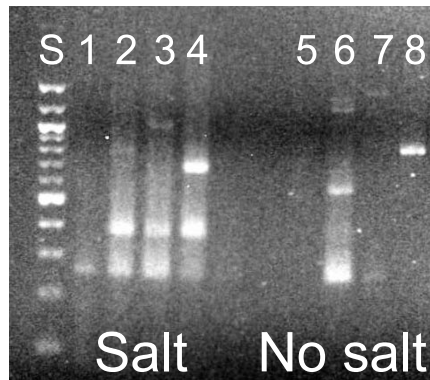

Figure 8. Resulting fragments of reverse transcribed RNA subjected to AFLP on an agarose gel. S. 100 bp ladder, 1-4: salt treated samples of *Parmelia subrudecta*, 5-8: control.

Several genes related to the synthesis of compounds that mediate salt tolerance have been reported from free-living fungi, but are not yet characterized from lichens. For example, the gene *HAL1*, which is involved in the regulation of K^+ transport, was characterized in unicellular fungi (e.g., Gisbert et al., 2000; Serrano and Gaxiola, 1994). The *CNH1* gene from *Candida albicans* (Soong et al., 2000) and the *HAL3* gene in *Saccharomyces cervisiae* (Ferrando et al., 1995) are other salt-tolerance genes. Likewise, genes involved in stress reactions of algae (e.g., Meijer et al., 2001) still need to be found in halotolerant lichen photobionts.

6. Conclusions

In this contribution we mainly discussed species that occur in littoral and supralittoral habitats or those with salt spray exposure, but further inland lichens will as well be

exposed to locally high concentrations of salt. This could apply especially to terrestrial lichens because evaporation increases salt concentrations at the uppermost soil layers. In such environments, *Collema tenax* or other species with broad ecological amplitude will be especially interesting to study.

As is apparent from the experiments with cultured lichen symbionts, the compound lichen seems to react differently to salt stress than the separated partners alone. This agrees with recent results from desiccation tolerance studies. Thus studies of halotolerant lichens need to consider also the interactions of the partners in the symbiosis, as a study of separately cultured symbionts may not adequately reflect the behaviour of the organisms in their natural, symbiotic state. This applies both to physiological studies and studies of the transcriptome.

However, much is still unknown about the genes which are involved in halotolerance of lichens and their evolutionary fates. Considering the high amount of duplicated genes of fungi it would not be surprising to find paralogy in genes that are involved in salt stress response. Therefore results from heterologous primer approaches should be interpreted carefully. Studies need to test for paralogy of the involved genes, and if such exists, clarify whether paralogs are specifically involved in halotolerance. For this purpose it will be useful to analyze well selected model symbioses in greater detail. To conclude, we want to briefly mention a further interesting observation. In samples of *Verrucaria maura*, we repeatedly observed bacterial colonies on top of the non-melanized thalli. These colonies may sometimes occupy substantial portions of the surface layers (Fig. 9). At present it is unclear what these bacteria are and whether they could represent a functionally important component. Bacteria are not yet characterized from coastal lichens and in need of further study, as they might add to the complexity of these fascinating symbioses.

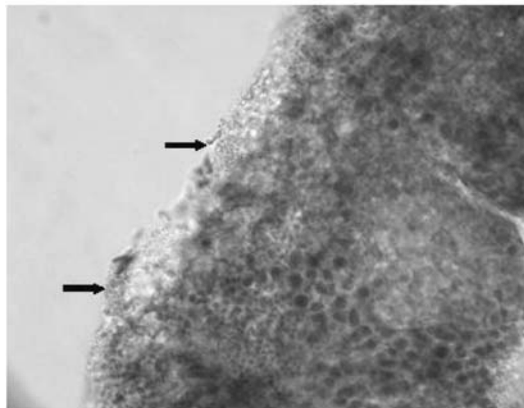

Figure 9. Transversial section of a *Verrucaria maura* thallus, showing bacterial colonies at the upper surface (arrows).

Acknowledgements

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Section V. Algae

A century of *Dunaliella* research: 1905-2005
Aharon Oren

Molecular determinants of protein halotolerance: structural and functional studies of the extremely salt tolerant carbonic anhydrases from *Dunaliella salina*
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A CENTURY OF *DUNALIELLA* RESEARCH: 1905-2005

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1. Introduction

In 1905, exactly hundred years before the publication of this book, two papers appeared in the scientific literature in which *Dunaliella* was described as a new genus of unicellular green algae that inhabit salterns and other hypersaline water bodies. The first article was by Teodoresco from Bucharest, who based the description of two species, *D. salina* and *D. viridis*, on material collected from a Rumanian salt lake (Teodoresco, 1905, 1906). Teodoresco named the genus in honor of Michel Felix Dunal, the French botanist who first observed the brightly red, β-carotene-loaded cells of *D. salina* in saltern evaporation ponds near Montpellier in the south of France in 1838. Figure 1 shows some of Teodoresco's original drawings showing the morphology of *D. salina* and *D. viridis*. With the discovery of the organism Dunal disproved an earlier theory that the red-pink color of saltern crystallizer brines is caused by the brine shrimp *Artemia salina*. The contribution of red Archaea of the family Halobacteriaceae to the coloration of these brines was realized only much later (Oren and Dubinsky, 1994; Oren et al., 1992;).

Dunal had named the organisms *Haematococcus salinus* and *Protococcus salinus* (Dunal, 1838). In the course of the 19th century the organism was sighted again in hypersaline lakes and salterns in Crimea, Algeria, France, and Rumania, and is was described under different names, including *Monas Dunalii* (Joly, 1840), *Diselmis Dunalii* (Dujardin, 1841), *Chlamydomonas Dunalii* (Blanchard, 1891; Bujor, 1900; Cohn, 1865), and *Sphaerella lacustris* var. *Dunalii* (Hansgirg, 1886). The formal description by Teodoresco of the genus *Dunaliella* ended the long confusion on the nomenclature status of the organism.

In the same year in which Teodoresco published his study, a second paper with an in-depth description of the alga appeared in the literature, written by Clara Hamburger from Heidelberg, based on studies of samples from the saltern crystallizer ponds of Cagliari, Sardinia (Hamburger, 1905). Hamburger became aware of Teodoresco's publication at the time when she was finalizing her own studies, as she writes:

> "Anfang März wollte ich an die Ausarbeitung meine zen gehen, als ich am 10. März von Herrn Prof. Lauterborn eine Arbeit von Teodoresco mit dem Titel: "Organisation et développement du *Dunaliella*, nouveau genre de Volvocaceae – Polyblepharidée erhielt, welche als Separatdruck aus dem botanischen Centralblatt soeben versendet war. *Dunaliella* ist der von

> mir untersuchte Organismus, den ich schon als Vertreter einer neuen Gattung erkannt hatte. Unsere Resultaten stimmten in vielen Punkten überein, in anderen müssen meiner Ansicht nach erst weitere Untersuchungen die entgültige Entscheidung bringen. Da jedoch meine Studien, besonders bezüglich des innern Baues eingehender sind (Teodoresco hat nur lebendes Material untersucht) und ich auch einige noch bestehende Lücken ausfüllen kann; da ferner alle meine Resultate unbeinflußt von denen Teodoresco's erhalten wurden, so möchte ich sie dennoch veröffentlichen."
>
> [In the beginning of March [1905] I wanted to start to work out my notes, when on March 10 I received from Prof. Lauterborn a paper by Teodoresco entitled: "Organization and development of *Dunaliella*, a new genus of the Volvocida – Polyblepharidae", which was just sent as offprint from the Botanisches Centralblatt. *Dunaliella* is the organism that I had been investigating, and that I had already recognized as representative of a new genus. Our results corresponded in many respects, while in other respects I am of the opinion that further investigations will have to decide. However, because my studies, especially with respect to the internal structure, are more thorough (Teodoresco had studied only live material) and I also can fill in certain still existing gaps in the knowledge, and also because my results were obtained independently of those of Teodoresco, I still would like to publish them.]

Also Hamburger's article is accompanied by beautiful drawings of the organism, some of them as color plates (Fig. 2). The papers by Teodoresco and Hamburger complement each other, and together they provide in-depth information on the morphology, structure, behavior and reproduction of the alga.

Dunaliella, being the main primary producer in the plankton of hypersaline water bodies, both natural and man-made, has rapidly become a popular model organism for the study of adaptation of eukaryotic cells to high salt concentrations. This chapter commemorates the 100th anniversary of *Dunaliella* as a genus, by reviewing some of the nowadays poorly known early studies, and highlighting those that have since formed the basis of some important concepts such as the biosynthesis of glycerol and its function as a compatible solute, as well as the massive accumulation of β-carotene in some *Dunaliella* species, a process that also has triggered considerable interest in the organism for biotechnological applications. A more extensive historical survey of one century of *Dunaliella* research will be published elsewhere (Oren, 2005). More in-depth treatises on the properties of the alga can be found in the 1987 review by Ginzburg, in the book on "*Dunaliella*: Physiology, Biochemistry, and Biotechnology" edited by Avron and Ben-Amotz (1992), and in my recent monograph on microbial life at high salt concentrations (Oren, 2002).

2. Taxonomy of *Dunaliella*

The genus *Dunaliella* (family Polyblepharidaceae) consists of unicellar algae that lack a rigid cell wall, and reproduce by longitudinal division of the motile cell or by fusion of two motile cells to form a zygote. Teodoresco's studies led to the description of the first two species within the genus: *D. salina* with larger, often red pigmented cells, and *D. viridis* which is smaller and never produces carotenoids in sufficiently large amounts to impart a red color to the cells. Pigmentation of *D. salina* greatly depends on growth conditions. As a result, considerable controversy uncertainty existed in the early years whether *D. viridis* should indeed be considered a separate species or a non-pigmented variant of *D. salina*. Many workers at the time considered the green cells as juvenile stages of the red ones (Hamburger, 1905) or expressed the opinion that the pigmen-

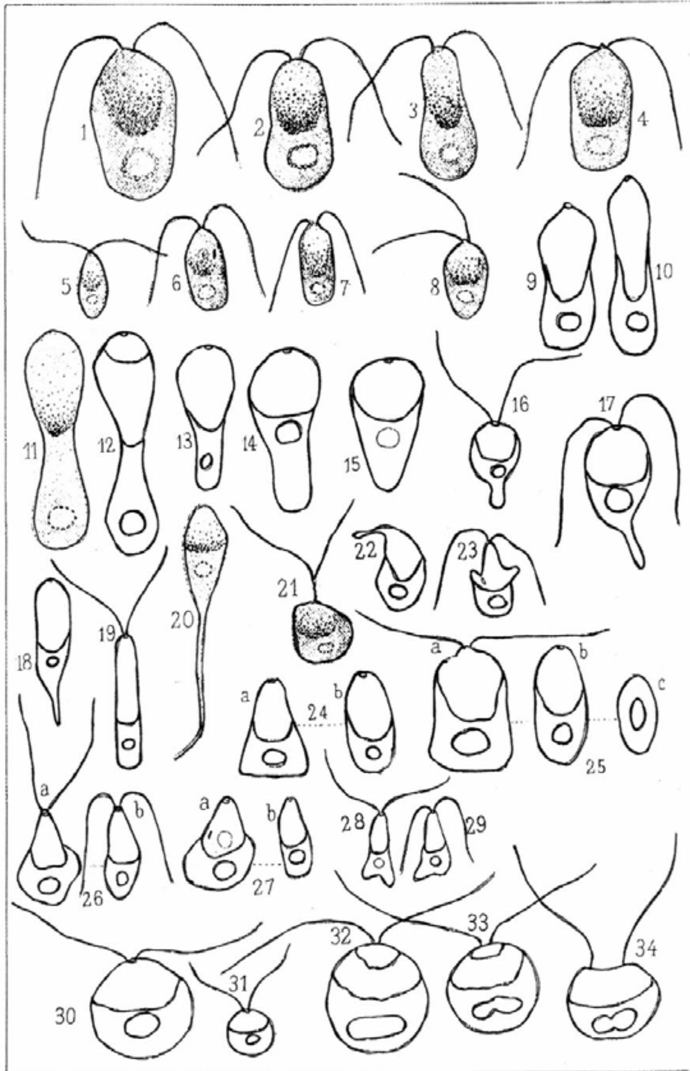

Figure 1. Drawings of *Dunaliella salina* (1-4) and *Dunaliella viridis* (5-8) as given in the original description of the genus by Teodoresco (1905). The figure also shows diverse shapes observed in a drop that becomes more concentrated by evaporation (9-29), spherical forms obtained upon dilution (30-31), and initiation of cell division (32-34).

tation is solely a function of salinity. Thus, Labbé (1921a, 1921b), who studied the algae in the salterns of Le Croisic on the Atlantic coast of France, claimed that the red form originates from a very euryhaline chlorophyll-containing flagellate that at the highest salt concentrations produces red cells that cannot revert to chlorophyll-containing forms. Now we know that those few *Dunaliella* species that can produce massive amounts of β-carotene do so only under certain growth conditions, a combination of high light intensities and nutrient limitation being especially favorable.

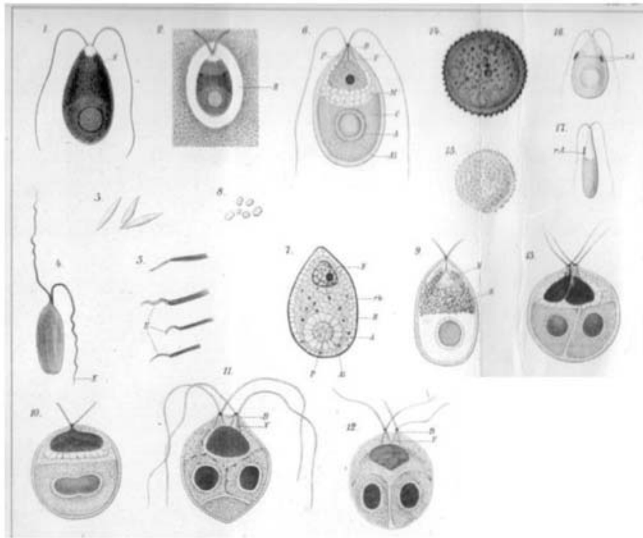

Figure 2. *Dunaliella salina* (1-17) preserved with different fixation techniques, pigment crystals (3), unidentified intracellular granules (8), division stages (10-13), aplanospores (14,15), and green cells (*D. viridis*?) (16-17), as drawn by Hamburger (1905).

Additional species were added to the genus in the course of time. These included *D. peircei* (Nicolai and Baas Becking, 1935), *D. media*, *D. euchlora*, *D. minuta*, and *D. parva* (Lerche, 1937), and *D. primolecta*, *D. tertiolecta*, *D. quartolecta*, and *D. polymorpha* (Butcher, 1959). Some of these are limited to the marine environment and are not found in hypersaline habitats. The taxonomic monograph on the genus *Dunaliella* by Massyuk (1973) listed as many as 28 species, divided over two subgenera: *Dunaliella* (23 species) and *Pascheria* (5 species, all from fresh-water habitats). Some of the new species recognized may possibly be variants of earlier recognized taxa (Preisig, 1992). Another species worth mentioning is *D. acidophila*, found in acidic waters and soils in the Czech Republic and in Italy, and showing optimal growth at pH 0.5-2.0 (Albertano et al., 1981; Gimmler and Weis, 1992; Kalina, 1965).

Some *Dunaliella* species not only reproduce by division of vegetative cells but show sexual reproduction as well. Sexual reproduction was observed in *D. salina* already by

Hamburger (1905) and by Teodoresco (1906). Lerche (1937) provided a very detailed documentation of sexual reproduction in *Dunaliella*, in part based on elegant experiments in which carotenoid-rich red cells were crossed with green cells, so that the fusion process of the parent cells could easily be followed. She reported sexual zygote formation in *D. salina*, *D. parva*, *D. peircei*, *D. euchlora*, and *D. minuta*. The zygote forms a thick outer layer, and then it can withstand unfavorable conditions such as prolonged dryness or exposure to freshwater. The cyst-like structures observed in the Dead Sea at the end of a *Dunaliella* bloom in 1992 (Oren et al., 1995) may possibly have been such zygotes. Asexual formation of resting cysts may be possible as well in some species (Loeblich, 1969; Borowitzka, 1981). Existence of a vegetative palmelloid stage consisting of round non-motile cells was also documented (Brock, 1975; Lerche, 1937).

Molecular phylogenetic studies of the genus *Dunaliella*, based on the 18S rRNA genes and the internal transcribed spacer regions, have been initiated in recent years (González et al., 1999, 2001; Olmos et al., 2000). The outcome of these molecular charcterizations is only poorly correlated with the earlier taxonomic schemes, and it is now clear that many culture collection strains have been misnamed in the past.

3. Ecology of *Dunaliella*

Dunaliella is found worldwide as the main or only primary producer in salt lakes at or approaching NaCl saturation. Therefore it is to some extent surprising that only few quantitative studies have been published on the dynamics of *Dunaliella* populations in hypersaline environments. In the early 1920s, Labbé published quite detailed observations on the development of *Dunaliella* during the annual cycle of operation of the French salterns of Le Croisic. He documented changes in the algal community structure as related to changes in brine salinity, light regime, water temperature and pH (Labbé, 1921a, 1921b, 1922). Based on the incorrect notion that the smaller green cells and the larger red cells are stages in the development of a single organism, he described an annual cycle which starts in the beginning of the winter with a few red and green motile cells. Dilution of the water by winter rains triggers the formation of red cysts, while the green cells develop rapidly and then conjugate. When the salt concentration increases in the summer season, red motile cells start to appear, always accompanied by green cells.

Quantitative studies of *Dunaliella* in natural salt lakes are equally rare. Little is known about the abundance and activity of the alga in Great Salt Lake, Utah, beyond a few isolated enumerations by Post (1977) and primary production measurements in the south arm of the lake (salinity around 135 g l^{-1}) in 1973 (Stephens and Gillespie, 1976). Quantitative estimates of the *Dunaliella* population in the Dead Sea in 1964 showed high numbers – up to $4x10^4$ cells per ml of surface water (Kaplan and Friedmann, 1970). No further data were collected until a systematic monitoring program of the biology of the lake was started in 1980. *Dunaliella* blooms have been observed in the Dead Sea only twice since then: in 1980 and in 1992, in both cases following unusually wet winters that caused a dilution of the upper water layers of the lake (Oren and Shilo, 1982; Oren et al., 1995).

Controlled growth experiments to elucidate the conditions enabling growth of *Dunaliella* have been documented from the 1930s onwards (Baas-Becking, 1931; Lerche, 1937). These studies, supplemented by later reports (Brock, 1975; Gibor, 1956; Johnson et al., 1968; Van Auken and McNulty, 1973) show that the salt concentrations optimal for growth of the different strains are generally much lower than those prevailing in the environments from which those strains had been isolated.

4. Carotenoid Pigments

The “hematochrome” pigment, as it was named in the older literature, of *D. salina* was already very early recognized as a carotenoid (Blanchard, 1888; Lerche, 1937; Ruinen, 1938; Teodoresco, 1906). However, the exact location of this pigment within the cell has been a subject of considerable discussions before the electron microscope showed the location of the β-carotene granules among the thylakoid membranes of the chloroplast (Ben-Amotz et al., 1982, 1988). Some authors were of the opinion that the red pigment is distributed throughout the cells’ cytoplasm (Hamel, 1931; Labbé, 1921a; Teodoresco, 1905, 1906). Hamburger (1905) stated that the red pigment is found as small droplets, but she located these droplets in the outer alveolar layer of the cytoplasm and not in the chloroplast. Baas Becking (1931) and Leche (1937) correctly identified the chloroplast as the organelle that contains the red pigment.

Major biotechnological operations nowadays exploit *D. salina* and *D. bardawil* for the commercial production of β-carotene, using different methods to grow the alga, from extensive cultivation in lagoons to intensive cultivation at high cell densities under carefully controlled conditions (Ben-Amotz, 1980; Ben-Amotz and Avron, 1983; Borowitzka et al., 1984). The biotechnological potential of *Dunaliella* for β-carotene production was realized already early, and the first pilot plant was established in the USSR already in 1966 (Drokova, 1961; Massyuk, 1968).

5. Osmotic Behavior and Intracellular Salt and Solute Concentrations

Dunaliella cells have do not possess a rigid cell wall, and they are surrounded by a thin elastic membrane only. As a result, the cells can change their volume and shape as a reaction to changes in the ambient salt concentration. In his 1905 paper, Teodoresco provided very accurate descriptions of the osmotic behavior of *D. salina* (see also Fig. 1):

> “Ainsi, si nous plaçons une goutte d’eau salée, contenant des zoospores, sur le porte-objet, on constate, au microscope, qu’elles se présentent sous la forme mentionée plus haut. Mais si nous laissons la goutte s’évaporer un peu, on observe que le corps commence à s’allonger et à se difformer … ; si alors nous ajoutons à la préparation une goutte d’eau douce, les zoospores s’arrondissent brusquement …. Cette expérience, que j’ai répétée un trés grand nombre de fois, m’a toujours donné les mêmes résultats. …..
> Si à une goutte d’eau salée on ajoute une goutte plus grande d’eau douce, ce qui amène une abaissement brusque de la concentration, les zoospores non seulement s’arrondissent, mais encore cessent leurs mouvements; le volume du corps augmente et devient parfois deux fois plus grand et à la fin la zoospore éclate. La cause de cet éclatement n’est pas difficile à comprendre:

> c'est l'action méchanique de la pression osmotique trop élevée par rapport à la densité diminuée du milieu ambiant."
>
> [Thus, when we place a drop of salt water that contains zoospores [= motile vegetative cells] on a microscope slide, one detects in the microscope that these present themselves in the above-described form. However, when we let the drop evaporate a little, one observes that the body starts to elongate and to lose its shape. … ; when we then add to the preparation a drop of fresh water, the zoospores suddenly round up. …. This experiment, which I have repeated a great number of times, has always given me the same results. …..
> If to a drop of salt water one adds a larger drop of fresh water, which leads to a sudden drop in concentration, the zoospores not only round up, but in addition cease their movements; the volume of the body increases and sometimes becomes twice as large and finally the zoospore bursts. The cause of this burst is not difficult to understand: it is the mechanical action of the too high osmotic pressure in comparison to the decreased density of the ambient medium.]

Similarly, Lerche (1937) noted that when a drop of *D. salina* cells suspended in 20% salt is flooded with distilled water, many of the cells burst, but some survived the treatment.

The first indications that *Dunaliella* cells growing at high salinity do not maintain a dilute cytoplasm came from measurements of the freezing point of the cytoplasmic fluid of *D. salina:* the apparent intracellular "salt" concentration was higher than the 3.9 M salt in which the cells had been grown (Marrè and Servettaz, 1959). At the time it was believed that the cell membrane is highly permeable, so that NaCl is taken up during salt upshock and movement of water then equalizes the osmotic pressure at both sides of the membrane (Ginzburg, 1969; Marrè and Servettaz, 1959; Trezzi et al., 1965). However, the enzymological studies by Johnson et al. (1968) clearly showed that the salt concentrations within *Dunaliella* cells cannot be high: key enzymes of the algal metabolism such as pentose phosphate isomerase, ribulose bisphosphate carboxylase, glucose-6-phosphate dehydrogenase and phosphohexose isomerase, are all strongly inhibited by NaCl. It is now well established that the intracellular ionic concentrations of *Dunaliella* are very low indeed: intracellular Na^+ concentrations of cells grown in 4 M NaCl do not exceed 100 mM (Katz and Avron, 1985).

The first indications that intracellular accumulation of photosynthetically produced glycerol provides the necessary osmotic equilibrium in *Dunaliella* cells were reported in 1964. Following incubation of *D. tertiolecta* cells in media containing 0.025, 0.5, and 2.5 M NaCl with $^{14}CO_2$ and fractionation and identification of the products, glycerol amounted to 56, 76, and 81% of the radioactivity of the neutral fraction extracted from the cells. Moreover, when the salinity of the medium was increased 100-fold from 0.025 to 2.5 M, 94-fold more radioactivity ended up in the neutral fraction (Craigie and McLachlan, 1964). The presence of molar intracellular concentrations of glycerol within *Dunaliella* cells, serving as a compatible solute, was firmly established during subsequent studies (Ben-Amotz and Avron, 1973; Borowitzka and Brown, 1974; Wegmann, 1971).

6. Current Trends in *Dunaliella* Research

Most of the research on *Dunaliella* in the past few years has centered on the identification of specific proteins that play a role in salt tolerance and to enable adaptation to changes in medium salinity. When *D. salina* cells are exposed to a salt

upshock, two membrane-bound proteins are strongly induced. One is a 60 kDa carbonic anhydrase that apparently helps the cell to take up carbon dioxide in concentrated brines in which the solubility of gases is decreased (Fisher et al., 1996; Sadka et al., 1991). The second is a 150 kDa transferrin-like protein that is involved in the transport of iron into the cell (Fisher et al., 1994, 1997).

Application of state-of-the-art proteomic approaches now allows a more complete picture of the changes that occur within *Dunaliella* cells as a reaction to changes in the extracellular salt concentration (Liska et al., 2004). Comparison of protein patterns of low- and of high-salt-grown cells on two-dimensional gels enabled the identification of 76 salt-induced proteins. These include key enzymes of the Calvin cycle, enzymes involved in starch mobilization and redox energy production, regulatory factors in protein biosynthesis and degradation, as well as a homolog of bacterial Na^+-redox transporters. *Dunaliella* thus responds to transfer to a high salinity by enhancement of photosynthetic CO_2 assimilation and by diversion of carbon and energy resources for glycerol synthesis. With this beautiful study *Dunaliella* research now enters its second century, in which we undoubtedly will obtain a far more in-depth understanding of this fascinating organism.

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MOLECULAR DETERMINANTS OF PROTEIN HALOTOLERANCE: STRUCTURAL AND FUNCTIONAL STUDIES OF THE EXTREMELY SALT TOLERANT CARBONIC ANHYDRASES FROM *DUNALIELLA SALINA*

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1. Introduction

The best studied example for the astounding acclimation power of proteins to salt is provided by proteins from extremely halophilic Archaea that typically require multimolar salt concentrations for proper folding and activity (Lanyi, 1974; Madern et al., 2000; Mevarech et al., 2000). High salt is thought to counteract like-charge repulsion owing to the surplus of surface acidic residues characterizing halophilic proteins and to allow the formation of protective hydration networks in high salinities (Bieger et al., 2003; Dym et al., 1994; Frolow et al., 1996; Madern et al., 2000; Pieper et al., 1998). A different response to salt is displayed by a versatile group of halotolerant proteins from non-halophilic as well as halophilic microbial sources that remain active throughout a broad range of salinities without exhibiting obligatory salt dependence (Ahmad et al., 2001; Deutch, 2002; Madern and Zaccai, 2004; Polosina et al., 2002; Wejse et al., 2003; Yoshimune et al., 2004). The structural principles discriminating halotolerant from mesophilic or halophilic proteins have yet to be elucidated.

Our studies focus on the unicellular green alga *Dunaliella salina* that proliferates in low to nearly saturating salt concentrations and therefore provides a potential source of halotolerant proteins. The alga attains osmotic balance by intracellular accumulation of glycerol (Avron, 1986) and consequently, only extracellular, but not intracellular proteins are expected to be able to cope with the broad spectrum of salinities sustaining algal growth.

Two plasma membrane proteins from *D. salina* identified in our studies that conform to this expectation belong to the α-type carbonic anhydrase (CA) family (EC 4.2.1.1). The first discovered enzyme, dCA I, is a 60 kDa protein consisting of two, ~52% identical, tandemly arranged α-type carbonic anhydrase domains (Bageshwar et al., 2004). The more recently discovered dCA II is a ~30 kDa, single-domain α-type carbonic anhydrase exhibiting ~55% sequence identity to each of the dCA I domains (Premkumar et al., 2003).

Carbonic anhydrases constitute a family of Zn enzymes found in all kingdoms of life. The α-type members of the family are found predominantly in animals, but also occur in some bacteria, green algae and higher plants. By reversibly converting CO_2 and water to bicarbonate and proton ions, carbonic anhydrases fulfill essential roles in a

variety of physiological processes including the transport of CO_2 and bicarbonate, acid-base regulation and water and electrolyte balance (Parkkila, 2000). In photosynthetic organisms, carbonic anhydrases perform crucial roles in CO_2 uptake and concentrating mechanisms conducive to efficient photosynthetic CO_2 fixation. The catalytic mechanism employs a Zn-bound hydroxide in the active site as a nucleophile in CO_2 hydration to bicarbonate, a step followed by regeneration of the Zn-bound hydroxide by inter-molecular proton transfer via a shuttling amino acid residue(s) from the Zn-bound water to a buffer molecule (Lindskog and Silverman, 2000). An important corollary of this mechanism is that carbonic anhydrases are subject to inhibition by a variety of monovalent anions, including chloride and bromide, that displace the catalytically-essential Zn-bound water/hydroxyl and disrupt hydrogen-bonded networks at the active site, as indicated in crystal structures of CA-anion adducts (Jonsson et al., 1993; Liljas et al., 1994). In this context, the capability of the two dCAs to remain active in up to multimolar anion concentrations poses a particularly intriguing problem.

This chapter reviews biochemical and structural studies that provide insights into the structural principles underlying the exceptional halotolerance of the two dCAs. In particular, the determination of the 3D structure of dCA II by X-ray crystallography provides for the first time insights into the structural and electrostatic features that make the algal α-type carbonic anhydrases structurally and functionally halotolerant. Finally, a model for protein halotolerance is offered.

2. Activity, Folding and Stability of dCAs

2.1. EFFECT OF SALT ON THE CATALYTIC ACTIVITES OF dCAS

The salt tolerance of the dCAs was examined in assays of three different CA activities (Bageshwar et al., 2004). The steady-state kinetic analysis of bicarbonate dehydration showed that the catalytic efficiency and substrate affinity of the dCAs remained nearly constant in up to 0.5 M NaCl. In esterase activity, the catalytic efficiency rose continuously with salt concentration, reaching a ~2-fold increase at 2.0 M NaCl, relative to the activity in the absence of salt. In CO_2 hydration assays, the dCAs retained activity to at least 1.5 M NaCl and dCA I, but not dCA II, was activated up to ~2 fold by low to moderate concentrations of monovalent or divalent cations (Na^+, K^+, Mg^{2+}). Although measurements of CO_2 hydration activity at higher salinities were constrained by the interference of salt in the assay, the activities of the two enzymes remained detectable even at 4.0 M NaCl. In contrast, mesophilic homologs such as human CA isozymes (hCA) and the *Chlamydomonas reinhardtii* periplasmic cCA were strongly inhibited by Cl^- in all three CA activities, *e g*., the I_{50} for Cl^- inhibition of CO_2 hydration activity did not exceed 0.2 M NaCl (Table 1).

These results demonstrate that the dCAs are exceptionally salt tolerant and, unlike mesophilic enzymes, largely immune to anion inhibition. The dCAs also differ from halophilic proteins that generally require salt for catalytic activity.

TABLE 1. Comparison of constants for Cl^- inhibition of dCAs and other CAs. The I_{50}, values stand for the concentration of NaCl causing 50 % inhibition of CO_2 hydration activity.

I_{50} (mM) for Cl^- inhibition					
dCA I	dCA II	hCA I	hCA II	hCA IV	cCA
>1.5 X10^3	>1.5 X10^3	6[b]	200[b]	36	56[a]

2.2. EFFECT OF SALT ON THE FOLDING OF dCAs

The heterologous expression of dCAs in *Escherichia coli* yielded functionally active enzymes (Premkumar et al., 2003a, 2003b) that could be stored in a low ionic strength buffer without loss of activity. Furthermore, dCA I refolded correctly after being unfolded in 8.0 M guanidine hydrochloride. These results agree with the conclusion that the dCAs do not require salt for solubility and proper folding and are thus distinguished from halophilic proteins, that often formed inclusion bodies when expressed in *E. coli* and required salt for refolding and storage (Camacho et al., 2002; Pire et al., 2001).

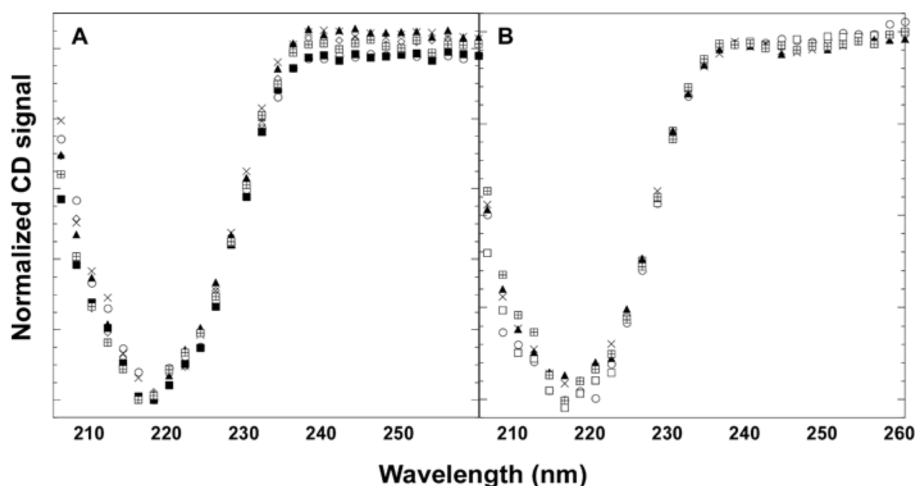

Figure 1. Far UV CD spectra of dCA I (A) and dCA II (B) at different salinities. O, no NaCl; ▲, 0.1 M NaCl; ◇, 0.25 M NaCl; □, 0.5 M NaCl; ■, 1.0 M NaCl; ×, 2.0 M NaCl; ⊞, 3.0 M NaCl.

That the dCAs maintained a native conformation in a broad range of salinities was also reflected in far UV CD spectra (Fig. 1A and 1B) that did not vary significantly between 0 to 3.0 M NaCl, thus excluding salt-dependent conformational transitions. In

this respect, the dCAs differed from the halotolerant enzyme nucleoside diphosphate kinase from the haloalkaliphilic archaeon *Natrialba magadii*, that, while remaining active over a broad range of salinities, underwent salt-dependent transitions in conformation and oligomerization states (Polosina et al., 1998, 2002). The dCAs also differ from halophilic proteins such as dihydrofolate reductase and malate dehydrogenase that were shown to lose their native conformation and/or oligomerization state at low salinity (Bonete et al., 1994; Wright et al., 2002).

2.3. EFFECT OF SALT ON THE THERMOSTABILITY OF dCAs

The experiments described above were all conducted at ambient temperatures, i.e., ~25°. To gain further insights into the effects of salt on the dCAs, their thermostability was monitored at various salinities using esterase activity and intrinsic fluorescence spectra as indicators. Esterase activity was determined in enzymes incubated at 25ºC - 60ºC, in 0 or 1.0 M NaCl. Figures 2A and 2B show that the T_m (the temperature at which activity is decreased by 50% relative to the activity of controls incubated at 25ºC) at 1.0 M NaCl is higher by as much as 10°C when compared to the T_m in the absence of salt. Further, incubating the enzymes at temperatures corresponding to the respective T_m's at 0-3.0 M NaCl indicated a rise in thermostability in up to 1.0 M NaCl and retention of this level in up to 3.0 M salt (Fig. 2C and 2D).

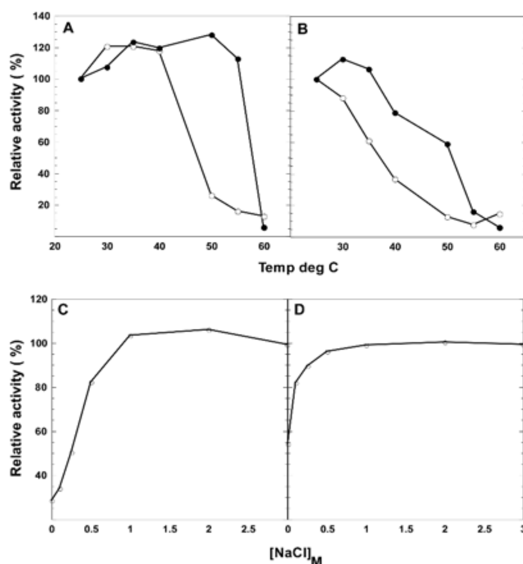

Figure 2. Salt stabilization of dCAs against thermal inactivation as monitored by esterase activity. A and C, dCAI; B and D, dCA II. Thermal treatments in A and B were in the presence of zero (O) or 1.0 M NaCl (●). Thermal treatments in various NaCl concentrations in C and D were at 50ºC and 37ºC, respectively.

To determine whether the thermal loss of catalytic activity is correlated with temperature-induced structural perturbations, intrinsic Tyr fluorescence was determined for dCA I incubated in 0-3.0 M NaCl, at 25ºC or at 50ºC, a temperature corresponding to the T_m in the absence of salt. The spectra for 25ºC (Fig. 3A) are not affected by salt except for mild decreases in fluorescence intensity due to salt-caused quenching. However, spectra for 50ºC (Fig. 3B) are strongly influenced by salt: at the higher NaCl concentrations, i.e., 1.0 to 3.0 M, the spectra resembled those obtained for 25ºC, whereas at lower salt concentrations, i.e., 0 to 0.25 M, large spectral shifts were observed. The large shift in Tyr fluorescence maximum from 309 to 337 nm and the ~2-fold increase in fluorescence intensity indicate structural perturbations that parallel the thermal loss of catalytic activity of dCA I. The presence of 1.0 to 3.0 M NaCl completely protects the enzyme from thermal denaturation at 50ºC.

Although dCA II is stabilized by salt, the difference in T_m values between dCA I and dCA II observed in the absence salt suggests that the internally-duplicated dCA I is inherently more stable than dCA II. Moreover, the shape of the melting curve for dCA I (Fig. 2a) suggests that cooperative interactions, possibly between the two CA domains, are involved in the thermostability of dCA I. It was previously demonstrated that when the Nter and Cter, domains were expressed separately, they showed no detectable esterase activity and exhibited at most 2-10% bicarbonate dehydration activity as compared to the native dCA I (Bageshwar et al., 2004). Together, these results establish that salt-protein interactions enhance the stability of the dCAs and suggest a stabilizing role for Nter-Cter interactions in the native dCA I.

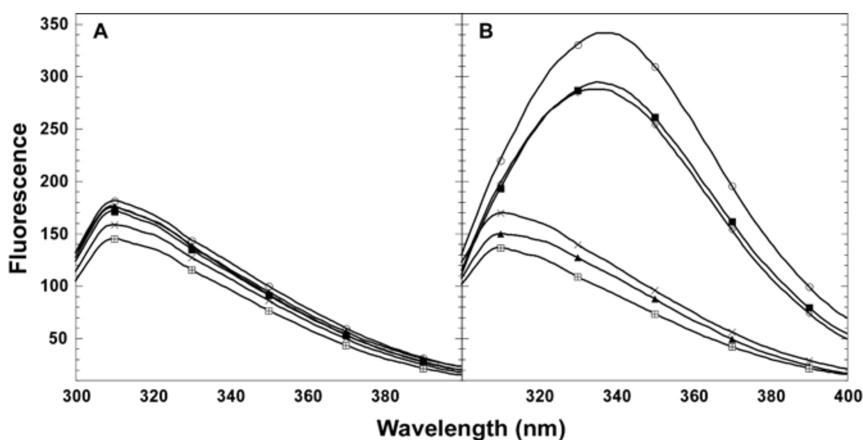

Figure 3. Tyr fluorescence emission spectra of dCA I measured at various NaCl concentrations at 25ºC (A) or 50ºC (B). ○, no NaCl; ■, 0.1 M NaCl; □, 0.5 M NaCl; ▲, 1.0 M NaCl; ✕, 2.0 M NaCl; ⊞, 3.0 M NaCl.

3. Structural Studies of dCAs

3.1. CRYSTAL STRUCTURE OF dCA II

To address the structural basis of the outstanding salt tolerance and anion resistance of the dCAs, the X-ray structure of the recombinant dCA II was determined at 1.86 Å resolution (Premkumar et al., submitted for publication). The crystal structure shared the global fold of mesophilic homologs in containing a central anti-parallel ten-stranded β-sheet, two α-helices, E and G, and a catalytic Zn^{2+} (Fig. 4 and 5). Distinctive features of dCA II include the extensions of the two α-helices, that make them significantly longer than those in mesophilic CAs, and a unique insertion forming a sodium binding loop (Fig. 4 and 5).

The core structure of dCA II is well conserved. Specifically, all the amino acid residues located within ~8 Å from the catalytic Zn are superimposable with those in the mesophilic hCA II (Premkumar et al., submitted for publication). These include the catalytic Zn ligands, active site H-bonding residues and the substrate binding residues (Fig. 5). However, the solvent-accessible surface of dCA II significantly differs from that of the other CAs in possessing a high ratio of acidic over basic amino acid residues (Premkumar et al., submitted for publication).

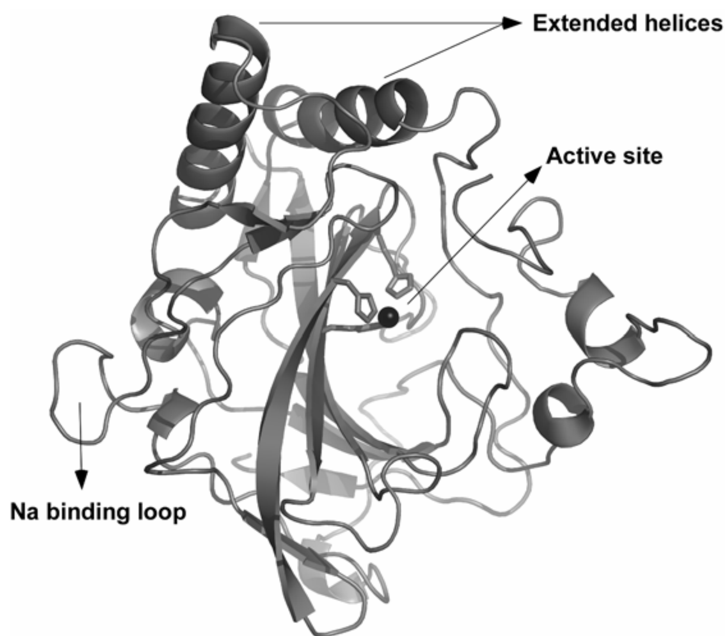

Figure 4. Ribbon diagram of the crystal structure of dCA II.

Despite the structural similarity of dCA II to that of other CAs, it differs in exhibiting a predominantly negative surface electrostatic potential as compared with the uneven surface distribution of neutral, negative and positive potentials displayed by other CAs (Fig. 6). The uniform surface negative potential is reminiscent of halophilic proteins in which the negatively charged surfaces were ascribed with salt protective roles such as the formation of hydrated cation networks (Dym et al., 1994; Frolow et al., 1996; Madern et al., 2000). Nonetheless, the halotolerant dCA II does not exhibit an obligatory requirement for salt.

Figure 5. Structure-based sequence alignment of dCA II and human (hCA) isozymes as well as a CA from *Neisseria gonorrhoeae* (nCA). Helices and strands are shown as black and gray boxes, respectively. Helix E and helix G are indicated. Other designations are: Z, Zn coordinating residues; N, Na binding residues, H, active site H-bonding residues; S, substrate binding residues.

3.2. PROPOSED STRUCTURAL BASIS FOR dCA II SALT TOLERANCE

A number of features of dCA II can be considered as potential determinants of salt tolerance. The predominantly negative surface potential endows the enzyme with the electrostatic properties that, in analogy to halophilic proteins, stabilize the protein in high salt. On the other hand, the lower surface negative charge density of dCA II, close to that of mesophilic homologs, can enable the protein to fold correctly even at low salt. Long-range electrostatic interactions emanating from the acidic surface modify the potential at a site adjacent to the catalytic Zn rendering it less positive and thus less sensitive to Cl^- inhibition. Furthermore, electrostatic repulsion by the negative surface

charges, particularly those close to the active site entrance, are likely to hinder the approach of inhibitory anions to the active site. In addition to its unique electrostatic properties, the two extended surface α-helices and the Na-binding loop probably serve as stabilizing elements in high salt. Extended helices have been implicated in the stability of thermophilic and halophilic proteins (Chakravarty and Varadarajan, 2002; Frolow et al., 1996). The contribution of the Na-binding loop may be similar to the stabilizing effect of monovalent cations bound at specific sites of halophilic proteins (Bieger et al., 2003; Frolow et al., 1996).

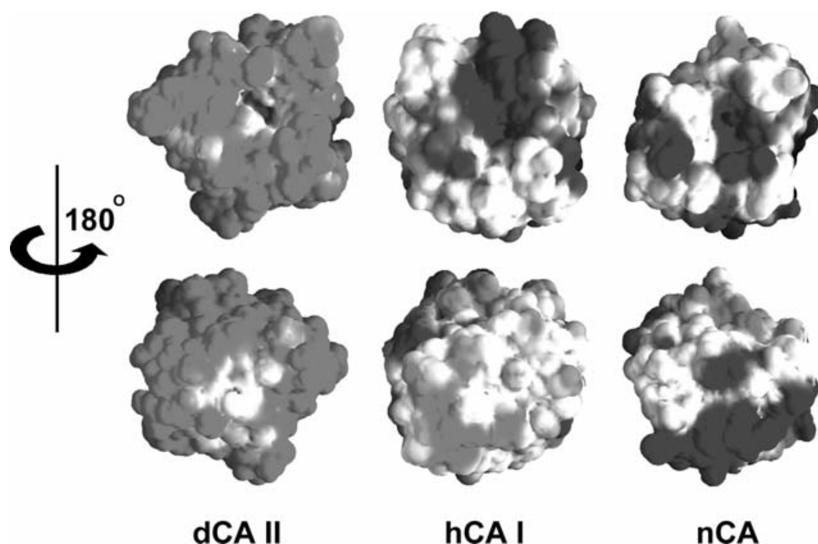

Figure 6. Surface electrostatic potential of dCA II and other CAs. Potentials ate contoured to –2.5 kT/e (Gray) and +2.5 kT/e (black). Upper row, images looking down the active site; lower row, 180º rotated view.

3.3. STRUCTURAL FEATURES OF dCA II ARE SHARED BY dCA I

As dCA II is ~55 % identical in sequence to each of the dCA I domains, it was possible to use the crystal structure of dCA II as a template to build homology models for Nter and Cter. The amino acid sequence insertions that extend the two conserved α-helices and form the Na-binding loop in dCA II are also shared by the Nter and Cter models. Similar to dCA II, the solvent-accessible surfaces of Nter and Cter display a high ratio of acidic over basic amino acid residues compared to mesophilic CAs. Moreover, electrostatic calculations indicated that both Nter and Cter showed a predominantly negative surface potential. The similarities between dCA I and dCA II suggest they shared a common evolutionary origin. The presumed gene duplication yielding the

internally repeated structure of dCA I afforded additional stability and possibly, still unrecognized specialized functions.

It is intriguing to note that the electrostatic features proposed to underlie the halotolerance of the dCAs allowed us to predict, and biochemically confirm, the unanticipated halotolerance of a mammalian CA, the murine CA XIV(Premkumar et al., submitted for publication).

4. Salt Adaptation: Variations on a Theme

For a broader view of protein salt-adaptive strategies, structural and functional characteristics of dCAs were compared with those of malate dehydrogenase (hMDH), a model halophilic protein and their respective mesophilic homologs. Structural comparisons showed that salt adaptation of the two proteins was achieved while keeping the active site architecture and global fold largely unchanged, except for modifications in the solvent-accessible protein surface (Premkumar et al., submitted for publication). Discriminating features between hMDH and dCAs relevant to their salt responses are listed in Table 2. The stabilizing effect of salt against thermal denaturation of the dCAs could be mechanistically related to the conformational stabilization of hMDH at ambient temperatures. As a whole it appears that fine-tuning of surface electrostatic properties plays a critical role in determining the degree and nature of protein salt adaptation.

TABLE 2. Biochemical and structural features of hMDH, dCAs and their mesophilic homologs.

Features		**hMDH**	**eMDH**	**dCAs**	**CAs**
Salt requirement for	Activity	+	-	-	-
	Folding	+	-	-	-
	Conformational stability	+	NA	-	NA
	Thermostability	+	-	+	-
NaCl (M) concentration where activity is observed		> 2.0	0 – 1.0	0 – 4.0	< 0.2
Surface density of acidic residues ($Å^2$ per aa)		236	484	429	453
Surface density of basic residues ($Å^2$ per aa)		746	523	923	522

A schematic illustration of such fine-tuning (Fig. 7) shows the average surface densities, but not specific localizations, of charged residues in the halophilic hMDH, the halotolerant dCAs and their mesophilic homologs. The scheme holds true for a number of halophilic and mesophilic proteins, but whether other halotolerant proteins share the

surface charge distribution characteristic of the halotolerant dCAs must await further structure determinations.

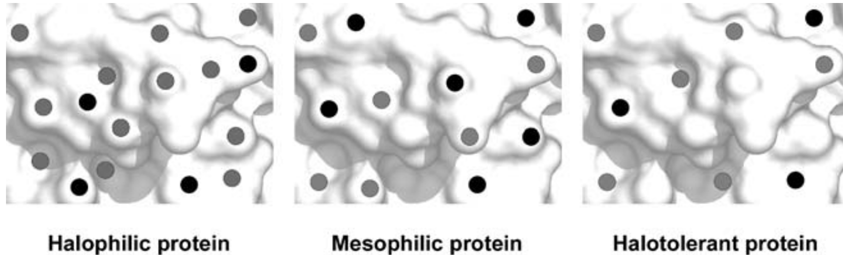

Figure 7. A schematic illustration of the surface charge distribution in hMDH (halophilic) dCAs (halotolerant) and their mesophilic homologs (mesophilic). Gray, acidic residues (Asp. Glu); black, basic residues (Arg, Lys).

Acknowledgements

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SECTION VI. PROTOZOA

Heterotrophic protozoa from hypersaline environments
Gwen Hauer and Andrew Rogerson

Heterotrophic flagellates in hypersaline waters
Byung C. Cho

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HETEROTROPHIC PROTOZOA FROM HYPERSALINE ENVIRONMENTS

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1. Introduction

Most eukaryotes living in extreme salt environments are microbes and a casual review of the literature suggests that the autotrophic protists dominate (Gilmour, 1990). This is especially true of the alga *Dunaliella salina* that grows up to saline saturation and has been reported from numerous hypersaline habitats worldwide (Borowitzka and Borowitzka, 1988). This biased conclusion is, in part, due to problems associated with cultivating eukaryotic extremophiles (Grant, 1991) and the general lack of studies spanning high salt habitats. However, according to Finlay (1990), all water bodies with salinity levels varying from 0.12 g l^{-1}, the mean salt content of world river water, to 332 g l^{-1}, the salt content of the north arm of the Great Salt Lake in Utah and the deep waters of the Dead Sea in Israel, should contain protozoa. A major aim of the present paper is to test this assertion by providing an in depth review of protozoa inhabiting salt environments. This is needed because not all authors are in agreement with Finlay. For example, Ramos-Cormenzana (1991) and Pedrós-Alió et al. (2000) suggest that eukaryotes are unable to adapt to the extreme environments populated by prokaryotes and that at the highest salinities, biota is mainly composed of prokaryotes and one eukaryotic protist, the alga *D. salina.*

Throughout this review, the term heterotrophic protists is used to cover all single-celled eukaryote microbes that prey on preformed organic carbon (usually bacteria and other microbial prey). In light of what we now know about the diversity of protists (see Corliss, 1998), this is a sweeping oversimplification that ignores, for example, the autotrophic protists that can also prey on bacteria and other mixotrophic forms that take up soluble carbon by osmotrophy. Protozoa in this paper encompass only the heterotrophic flagellates, ciliates and naked amoebae. The amoebae are those protists that move by pseudopodia of one form or another and 'naked' refers to the fact that they are not housed within tests or walls with distinct opening for prey ingestion. This sets them apart from the testate amoebae and the foraminiferans that are not considered here. This is unfortunate since at least one recent paper points out that foraminiferans can be common in habitats of elevated salinity (> 10% salt, Brazilian Lagoon, Debenay et al., 2001). Heterotrophic dinoflagellates, with their complex nutritional strategies, are also excluded from this review although several recent studies have reported their presence between 6 and 30% salt (Ayadi et al., 2002; Garcia and Neill, 1993; Laybourn-Parry, 2002; Laybourn-Parry et al., 2002; Patterson and Simpson, 1996).

The interest in protozoa in general has come about because of the paradigm of the microbial loop that has placed great emphasis on the role of microorganisms in the cycling of nutrients and carbon in aquatic ecosystems (Azam et al., 1983). As a result of microbial ecology ‘coming of age’, numerous studies have sought to clarify the ecological role of grazing protozoa, especially the numerically important heterotrophic nanoflagellates (2-20 μm) and the conspicuous ciliates. Less attention has been paid to the naked amoebae although several papers point to their numerical importance in marine systems (e.g. Anderson and Rogerson, 1995; Butler and Rogerson, 1995; Rogerson and Gwaltney, 2000; Rogerson and Laybourn-Parry 1992), suggesting that they play an important, as yet undefined, ecological role. Given that most eukaryotic organisms in salt environments are protists, the ecological importance of grazing protozoa in hypersaline environments is increased in these simplified ecosystems. Even if the diversity of eukaryotic microorganisms able to grow in high salinities is relatively restricted, eukaryotic microbes do contribute significantly to the biota, and hence function, of hypersaline environments (Oren, 2002).

This review considers protozoa from hypersaline waters in the range 9% and above (adopting the definition by Oren, 2002). We also acknowledge the definition of alpha-hypersalinity as elevated seawater in the range of 6-7 to 10% (Por, 1980). It is contended that protozoa thriving at and above these salt levels are worthy of comment because of their remarkable adaptability and/or tolerance to such conditions. The most extreme natural environments showing elevated salinities are inland lakes such as the Dead Sea and the Great Salt Lake. Historically, these water bodies have received the most attention from protozoologists and zoologists (e.g. Elazari-Volcani, 1943, 1944; Javor, 1989; Jones, 1944; Pack, 1919; Post, 1977; Stephens, 1990; Volcani, 1944; Vorhies, 1917). Lakes such as Mono Lake (Jellison, 1996) and the Salton Sea in California (Hurlbert et al., 2001), although not as extreme (or well known), are under threat because of rapidly rising salinity levels. They are now the focus of much study since decreasing diversity is producing linear food chains in these environments. Protozoa have also been described from salterns, commercial ponds maintained by salt companies, which exhibit gradients of salinity. The average salinity is approximately 15% and higher and many ciliates, zooflagellates and amoebae have been reported attesting to their relative success in such environments (Park et al., 2003; Javor, 1989).

2. Biotic Classification Based on Salt Tolerance

In the 1920s, when it was first recognized that microorganisms could grow in habitats containing high levels of salt, several studies on halotolerance helped identify some of the features of organisms living in these environments. Microorganisms were defined either as halotolerant or moderately halophilic based on the salinity levels they could tolerate (Kushner, 1993). Although there is no clear distinction between these two terms, this thinking advanced the study of defining organisms as either halotolerant or halophilic. Inhabitants of normal seawater (3.2% salt) that can withstand elevated salinities up to 7 or 10% are generally considered halotolerant (Oren, 1999). In hypersaline environments, described by Javor (1989) as habitats with salinities above 10% (and up to 15%), some halotolerant species still survive as intermediate to halophilic species. According to Oren (1999) the 10-20% salinity range represents a highly specialized halophilic environment

to which only a few halotolerant species have adapted. Extremely halophilic organisms (halophiles) are found at concentrations from 15% salt to saturation (over 32%; Javor, 1989) and may be inactivated or killed by lower salt concentrations. In 2000, Oren maintained that in extreme hypersaline habitats over 30% primary producers and protozoa were absent. The only microbial life was dense bacterial populations living on organic matter. On the other hand, less extreme hypersaline environments, saline lakes that fluctuate seasonally from marine to hypersaline, support a wide variety of specialized halotolerant or moderately halophilic eukaryotic microbes (Por, 1972).

3. Protozoa from High Salt Environments

Protozoa are commonly found in hypersaline lakes and solar salterns as shown in the comprehensive listings given in Tables 1-3. Prior to the study by Post et al. (1983) there were only a few published accounts of protozoa from high salinity habitats worldwide. In fact, before the 1970s most of the information on protozoa was based on limited information on ciliates from San Francisco salt ponds and the Great Salt Lake (Carpelan, 1957; Flowers and Evans, 1966), flagellates from Australian salt ponds, the Great Salt Lake, the Dead Sea, and Antarctic (Baas-Becking and Kaplan, 1956; Elazari-Volcani, 1943; Flowers and Evans, 1966; Goldman et al., 1967; Pack, 1919; Ruinen, 1938; Vorhies, 1917) and amoebae from the Great Salt Lake (Flowers and Evans, 1966; Jones, 1944; Kirkpatrick, 1934; Pack, 1919; Vorhies, 1917), the Dead Sea (Elazari-Volcani, 1943), Western Australia (Hamburger, 1905) and marine environments (Mast and Hopkins, 1941). One report was published on heliozoans from the Antarctic (Goldman et al., 1967).

Prior to 1970 only 10 species of amoebae (or amoeboflagellates) were noted from hypersaline environments. This contrasts with the 35 or so species (morphospecies) recorded in the last 35 years by various authors (Davis, 1978; Golubic, 1980; Post, 1977; Post et al., 1983; Read et al., 1983; Rogerson and Hauer, 2002; Hauer, unpublished). Similarly, only 20 species of ciliates were reported from hypersaline waters before 1978, while today at least 130 species are documented (Al-Rasheid et al., 2001; Ayadi et al., 2002; Davis, 1978; Javor, 1989; Post et al., 1983; Wilbert, 1995; Wilbert and Kahan, 1981; Small, personal communication). The flagellated protozoa show a similar increase from around 8 species prior to 1970 to approximately 35 species today (Ayadi et al., 2002; Post, 1977; Post et al., 1983; Simpson and Patterson, 1996). It is clear that there has been much study over the last 25-30 years concerning heterotrophic protozoa in hypersaline environments. It can also be concluded from the data (Tables 1-3) that the ciliates are the dominant group in these extreme environments although this might be misleading and a function of their conspicuous nature (i.e. they are large and active). The data are also unreliable regarding these protozoa because several studies were incomplete and based on observational accounts of samples casually examined from Eilat salt ponds and other hypersaline water bodies (Al-Rasheid et al., 2001; Imhoff et al., 1979; Larsen, 1980; Nissenbaum, 1975; Rodriguez-Valera et al., 1985; Winkler, 1977).

TABLE 1. Naked amoebae (and amoeboflagellates) recorded from hypersaline environments worldwide. + hypersaline but salinity not measured; * no location given

SPECIES	SALINITY	LOCATION	REFERENCE
Amoeba limax	23.0%	Great Salt Lake Utah	Vorhies, 1917; Pack, 1919; Post, 1977
Unidentified amoeba	20.0-26.0%	"	Kirkpatrick, 1934
Unidentified amoebae	"	"	Flowers and Evans, 1966
Tetramitus sp.	"	"	"
Unidentified spp.	33.0%	"	Post, 1977
Tetramitus-like organism	Up to sat.	"	"
Unidentified spp.	8.5-12.0%	"	Hauer, unpublished
Amoeba flowersii	>20.0%	"	Jones, 1944
Unidentified sp.	"	"	"
Heteramoeba sp.	12.5-21.0%	Hutt Lagoon West Australia	Post et al., 1983
Nagleria spp.	Up to sat.	"	"
Unidentified spp.	15.0-22.0%	"	"
Tetramitus salina	22.3%	"	"
T. cosmopolitus	12.0%	"	"
Unidentified spp.	15.0%	Dead Sea, Israel	Elazari-Volcani, 1943
Unidentified sp.	16.0-18.0%	Hypersaline ponds, Salton Sea, California	Rogerson and Hauer, 2002
Vexillifera aurea	"	"	"
Parvamoeba rugata	"	"	"
Unidentified spp. thecate-like	"	"	"
Cochliopodium sp.	"	"	"
Unidentified genus	"	"	"
Vannella mira	"	"	"
Vannella/Platyamoeba	"	"	"
V. septentrionalis	"	"	"
Platyamoeba sp.	"	"	"
Paratetramitus sp.	"	"	"
Unidentified spp.	21.0-31.0%	Salt ponds, Eilat, Israel	Hauer and Rogerson, unpublished
Paratetramitus sp.	16.0-21.0%	"	"
Amoeba salina	+	West Australia	Hamburger, 1905
Paratetramitus jugosus	>20.0%	Laguna Figueroa, Mexico	Read et al., 1983
Vannella ebro	Up to 9.0%	Ebro delta, Spain	Smirnov, 2001
Unidentified amoeba	3.0-27.0%	Southern Spain	Garcia and Neill, 1993
Vahlkampfia limax	5.0-21.3%	Salt ponds, Long Island, Bahamas	Davis, 1978
Unidentified spp.	9.0%	Mono Lake	Davidson, personal communication
Tetramitus sp.	13.0-27.0%	Salt Ponds, Yallahs Jamaica	Golubic, 1980
Amoeba mira	0.1% to sat.	*Marine	Mast and Hopkins, 1941
Tetramitus salinus	20.0%	*Marine	Page, 1983
T. cosmopolitus	3.0% to sat.	Australia	Ruinen, 1938

TABLE 2. Ciliates recorded from hypersaline environments worldwide.

SPECIES	SALINITY	LOCATION	REFERENCE
Trachelocerca conifer	16.5-22.3%	West Australia, Hutt Lagoon	Post et al., 1983
Metacystis truncata	16.5-19.9%	"	"
Ciliophrya (*Procodon*) *utahensis*	27.8%	"	"
Rhopalophrya salina	17-33.2%	"	"
Nassula sp.	16.5%	"	"
Podophrya sp.	16.5-23.2%	"	"
Trematosoma bocqueti	19.2%	"	"
Uronema marinum	16.5%	"	"
Condylostoma sp.	22.0-31.1%	"	"
Fabrea salina	16.5%	"	"
Cladotricha sp.	20.0%	"	"
Blepharisma halophila	16.5-31.0%	"	"
Palmarella (*Palmarium*) *salina*	27.8%	"	"
Euplotes sp.	Up to sat.	"	"
Euplotes sp.	15.0-20.0%	Great Salt Lake	Reddy, 1972
Podophrya sp.	20.0-26.0%	Great Salt Lake	Flowers and Evans, 1966
Ciliophrya (*Procodon*) *utahensis*	Sat.	Great Salt Lake	Pack, 1919
Ciliophrya sp.	+	Dead Sea	Volcani, 1944
Rhopalophrya salina	34.8%	Brine	Kudo, 1966
Cyclidium glaucoma	2.9-37.0%	Muskiki Lake, Saskatchewan, Canada	Kahl, 1930; Bick and Kunze, 1971; Wilbert and Kahan, 1981; Wilbert, 1995
Litonotus lamella	"	"	Wilbert, 1995
Chilodontopsis depressa	"	"	"
Pseudocohnilembus marinus	"	"	"
Euplotes sp.	"	"	"
Holosticha diademata	"	"	"
Spathidium macrostomum	2.3-9.59%	"	"
Bakuella spp.	+	Canada	"
Blepharisma sp.	"	"	"
Fabrea salina	16.0%	Salton Sea, California hypersaline ponds	Small, personal communication
Cohnilembus reesi	"	"	"
Euplotes sp.	"	"	"
Lacrymeria sp.	"	"	"
Trachelophyllum sp.	"	"	"
Cristigera sp.	"	"	"
Plagiophyla sp.	"	"	"
Unidentified spp.	30.0%	Lake Eyre, Australia	Baas-Becking and Kaplan, 1956
Euplotes sp.	10.2%	Bahamian Saltern	Davis, 1978
Litonotus sp.	"	"	"
Fabrea salina	6.0-10.0%	Exportadora de Sal saltern, Mexico	Javor, 1983, Javor, 1989, unpublished

SPECIES	SALINITY	LOCATION	REFERENCE
Uroleptus packii	+	Great Salt Lake	Pack, 1919
Holophrya simplex	+	Coroong, Australia	Wilbert, 1995
Pseudocohnlembus persalinus	"	"	"
Uronema nigricans	"	"	"
Drepanomonas revoluta	9.59%	Saskatchewan, Canada Little Manitou Lake	"
Pleuronema coronatum	"	"	"
Fabrea salina	"	"	"
Strombidium styliferum	"	"	"
Bakuella marina	"	"	"
Bakuella imbricata	"	"	"
Diophrys apendiculata	"	"	"
Euplotes balteatus	"	"	"
Gonostomum affine	"	"	"
Holosticha geleii	"	"	"
Uronychia transfuga	"	"	"
Stephanoogon apogon	8.0-18.0%	Solar Lake, Sinai	Wilbert and Kahan, 1981
Enchelyodon trepida	"	"	"
Litonotus lamella	"	"	"
Uronema nigricans	"	"	"
Uronema filificum	"	"	"
Parauronema virginianum	"	"	"
Pseudocohnilembus marinus	"	"	"
Frontonia marina	"	"	"
Condylostoma reichi	"	"	"
Condylostoma patulum	"	"	"
Halteria grardinella	"	"	"
Uronychia transfuga	"	"	"
Euplotes rariseta	"	"	"
Euplotes sp.	"	"	"
Tachysoma sp.	"	"	"
Holosticha diademata	"	"	"
Condylostoma patens	5.0-15.3%	Alviso salt ponds, San Francisco, California	Carpelan, 1957
Lionotus sp.	"	"	"
Fabrea salina	"	"	"
Didinium sp.	"	"	"
Coleps sp.	"	"	"
Trachelocera sp.	"	"	"
Unidentified tintinnids	"	"	"
Vorticella-like sp.	"	"	"
Uroleptus packii	20.0-26.0%	Great Salt Lake	Flowers and Evans, 1966
Chilophyra utahensis	"	"	"
Euplotes sp.	"	"	"
Cyclidium sp.	"	"	"
Pseudocohnilembus sp.	"	"	"
Litonotus sp.	5.0-21.3%	Salt ponds, Long Island, Bahamas	Davis, 1978
Nassula sp.	"	"	"
Metopus sp.	"	"	"
Euplotes sp.	"	"	"
Cristigera sp.	"	"	"
Cyclidium sp.	"	"	"
Chaenea sp.	5.0-10.2%	Long Island, Bahamas	Davis, 1978
Diophyrs sp.	"	"	"

SPECIES	SALINITY	LOCATION	REFERENCE
Hemophrys sp.	"	"	"
Strombidium sp.	"	"	"
Blepharisma intermedium	7.0-16.0%	Al-Hassa Oasis, Saudi Arabia	Al-Rasheid et al., 2001
Fabrea salina	"	"	"
Condylostoma reichi	"	"	"
34 species	"	"	"
Euglena sp.	20.0-30.0%	Sfax, Tunisia	Ayadi et al., 2002
Blepharisma dileptus	+	*	Kahl, 1928
Blepharisma tardum	+	*	Kahl, 1928
Ciliates	23.6%	+	Jaschof and Schwartz, 1961
Fabrea salina	3.0-27.0%	Fuente de Pierda, South Spain	Garcia and Neill, 1993
Strombidium sp.	"	"	"
Cyclidium sp.	"	"	"
Euplotes sp.	"	"	"
Trachelocerca sp.	"	"	"
Uroleptus sp.	"	"	"
Trithigmostoma sp.	"	"	"
Holophrya sp.	"	"	"
Podophrya sp.	"	"	"
Bakuella salinarum	+	Münsterland	Mihailowitsch and Wilbert, 1990
Pseudokeronopsis ignea	"	"	"
Euglena sp.	3.48-23.7%	Salt ponds, Bulgaria	Pavlova et al., 1998
Fabrea salina	"	"	"
Fabrea salina	>8.0%	Hypersaline lagoon Spain	Esteban and Finlay, 2003
Holophrya teres	"	"	"
Prorodon sp.	"	"	"
Frontonia marina	"	"	"
Condylostoma remanei	"	"	"
Uronema filificum	"	"	"
Trachelocerca sagitta	"	"	"
Calyptotricha lanuginosa	"	"	"
Anophryoides puytoraci	"	"	"
Vasicola parvula	"	"	"
Parauronema virginianum	"	"	"
Cyclidium bonnetti	"	"	"
Metacystis truncata	"	"	"
Metanophrys sp.	"	"	"
Cyclidium citrullus	"	"	"
Vaginicola crystallina	"	"	"

+ hypersaline but salinity not measured
* no location given

TABLE 3. Heterotrophic flagellates recorded from hypersaline environments worldwide.

SPECIES	SALINITY	LOCATION	REFERENCE
Monosiga spp.	17.0-23.0%	Western Australia	Post et al., 1983
Bodo caudatus	20.2%	"	"
Bodo edox	20.2%	"	"
Bodo sp. #1	Up to sat.	"	"
Bodo sp. #2	16.5%	"	"
Bodo sp. #3	20.0% to sat.	"	"
Rhynchomonas nasuta	15.0-20.0%	"	"
Phyllomitus sp.	27.8% to sat	"	"
Rhynchomonas mutabilis	18.0%	Australia	Ruinen, 1938
Pleurostomum flabellatum	Up to sat.	"	"
Palustrimonas yorkeensis	Up to sat.	"	"
Acanthoecopsis unguiculata	16.5-22.8%	Antarctica	Franzmann et al., 1987
Unidentified spp.	33.0%	Great Salt Lake	Vorhies, 1917; Pack, 1919; Post, 1977
Bodo sp.	Up to sat.	Great Salt Lake	Post, 1977
Unidentified spp.	15.0%	Dead Sea	Volcani, 1943
Unidentified sp.	4.3-12.6%	Lake Bonney, Antarctica	Goldman et al., 1967
Unidentified sp.	30.0%	Lake Eyre, Australia	Baas-Becking and Kaplan, 1956
Colpodella pugnax	26.0%	Whyalla, South Australia	Simpson and Patterson, 1996
Colpodella turpis	"	"	"
Bodo saliens	4.1-15.0%	Little lagoon, Hamelin Pond and hypersaline ponds, Western Australia	Patterson and Simpson, 1996
Rhynchomonas nasuta	"	"	"
Ancyromonas melba	6.5-15.0%	Hamelin Pond and hypersaline ponds, Western Australia	"
Rhynchoboda simius	"	"	"
Colpodella unguis	"	"	"
Bodo saltans	"	"	"
Bodo saltans	18.0-26.0%	Hypersaline ponds, South Australia	"
Colpodella pugnax	Brine	Hypersaline ponds, Western Australia	"
Palastrimonas yorkeensis	"	"	"
Pleurostomum flabellatum	"	"	"
Pleurostomum turgidum	"	"	"
Ancyromonas melba	+	Antarctic	Tong et al., 1997
Flagellated protozoa	Up to 37.4%	Wadi Natrun, Egypt	Imhoff et al., 1979

While bearing these limitations in mind, it is useful to comment on the types of protozoa highlighted by the review data in Tables 1-3. Several species of ciliates were common in hypersaline environments namely *Fabrea salina*, *Euplotes* spp., (including *E. balteatus* and *E. rariseta*), and *Ciliophrya* (*Procodon*). The ciliate *Ciliophrya* (*Procodon*) *utahensis* has been isolated from West Australia Hutt lagoon, the Great Salt Lake, and the Dead Sea (Flowers and Evans, 1966; Pack, 1919; Post et al., 1983; Volcani, 1944). The ciliate *F. salina* has a remarkable tolerance to high salinities and has

been found in Western Australia Hutt Lagoon (Post et al., 1983), hypersaline ponds around the Salton Sea in California (Small, personal communication), the Exportadora del Sal salterns in Mexico (Javor, 1983), Little Manitou Lake Canada (Wilbert, 1995), Alviso salt ponds, California (Carpelan, 1957), and Al-Hassa Oasis, Saudi Arabia (Al-Rasheid et al., 2001). Another common ciliate genus *Euplotes* sp. has been isolated from six hypersaline habitats worldwide (Davis, 1978; Flowers and Evans, 1966; Post et al., 1983; Reddy, 1972; Wilbert, 1995; Wilbert and Kahan, 1981; Small, personal communication).

Throughout extensive biotic surveys conducted at Shark Bay, Australia, Patterson and Simpson (1996) found two flagellate species (*Pleurostomum flabellatum* and *Palustrimonas yorkeensis*) that had previously been reported from hypersaline habitats by Ruinen (1938). Another flagellate found in salinities of 6-15%, *Ancyromonas melba*, had previously been isolated from saline Antarctic water by Tong et al. (1997). *Bodo saltans* is a common flagellate in hypersaline sites throughout Australia and could have been the unidentified *Bodo* sp. previously described by Post et al. (1983). *Bodo saltans* has been reported from sites ranging between 6% salt to saturation and according to Patterson and Simpson (1996) might be the same halophilic flagellate that was isolated from saturation ponds by Namyslowski (1913).

Amoeboflagellates are also common in hypersaline environments. Some protozoologists include these amoeboid protozoa that frequently have flagellate stages as heterolobosean amoebae (sensu Page, 1988). On the other hand, molecular evidence indicates that they are flagellates, even although *Vahlkampfia* is a genus identified by its inability to form flagellates (Patterson et al., 2002). According to Page (1983), freshwater *Tetramitus* are known mostly as flagellates while amoeboid forms are common in the marine environment. From the literature, this genus appears to be common in hypersaline environments. Balamuth (see Page, 1983) commented that *Tetramitus salinus* grew at 20% salinity and produced a typical amoeboid stage under appropriate culture conditions. Ruinen (1938) described *T. cosmopolitus* and *T. ovoides* in flagellate form from hypersaline waters in Australia and *Tetramitus* spp. were found in the Great Salt Lake on two occasions (Flowers and Evans, 1966; Post, 1977). *Tetramitus salina* and *T. cosmopolitus* were found in an Australian hypersaline lagoon (Post et al., 1983) and *T. cosmopolitus* was isolated from hypersaline waters in Australia by Ruinen (1938). Finally, *Tetramitus* spp. were isolated from salterns in Jamaica (Golubic, 1980).

Another amoeboflagellate, *Paratetramitus* was found in a hypersaline lagoon and demonstrated to be euryhaline (Read et al., 1983). It may be the same as the euryhaline species, *P. jugosus*, identified by Page (1976). Recently, *Paratetramitus* was isolated from hypersaline ponds (16% salt) around the periphery of the Salton Sea, California (Rogerson and Hauer, 2002) and the same genus was isolated from an Eilat salt pond, Israel, at salinities of 16-21%. Elazari-Volcani (1943) found a halophilic dimastigamoeba (another amoeboflagellate or flagellate depending upon classification scheme) in the waters of the Dead Sea (15-18% salt) and showed that this species was capable of survival over the range 6% to salinity saturation.

In view of the paucity of information on naked amoebae in general, it is not surprising that little is known about these protozoa inhabiting high or variable salinity environments. With the exception of records by the authors, most recordings in Table 1 are of unidentified amoebae or incorrectly named species (e.g. *Amoeba flowersii* is an invalid name). Rogerson and Hauer (2002) found twelve species of naked amoebae in

hypersaline ponds around the Salton Sea (16-18% salt) and showed that they could grow down to 3.2%. In a recent unpublished study by the authors, samples supplied by Dr. Oren from salt ponds in Eilat (ca. 30%) revealed three unidentified species of amoebae. These were maintained in cultures between 21% and 31% salt for approximately two months. Although densities were never reached to allow full species descriptions, some interesting observations on the strains were made. All amoebae ingested cyanobacteria, which were abundant in these surface mud samples. One of the amoebae was a highly vacuolated *Vannella*-like (fan-shaped) amoeba. The other two amoebae were both limax (tubular) with sluggish movement and a markedly wrinkled appearance. It is likely that all isolates were new to science. More recent water and sediment samples from the same salt ponds in Eilat (ca. 21% salt) yielded an unusual *Echinamoeba*-like amoeba approximately 13.0-17.5 µm in length (Fig. 1a). In salinity experiments, these amoebae grew down to 11% salt. A *Saccamoeba*-like limax amoeba (18.0-37.5 µm) with steady locomotion was isolated from the same waters (Fig. 1b). In culture experiments, this amoeba grew down to 7% salt. A smaller unidentified limax amoeba, approximately 12.5 µm in length was also found.

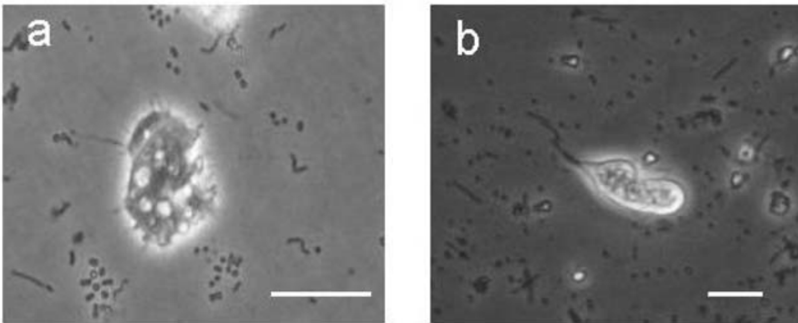

Figure 1. Unidentified amoebae from Eilat salt ponds collected from the salinity of 21%. (a) Note the abundance of cytoplasmic vacuoles. (b) Note the raised morphology that is common in amoebae from high salinity. Scale bar = 10 µm.

4. Protozoan Abundances

As pointed out earlier, many of the accounts of protozoa in hypersaline habitats are observational and there have been few attempts to quantify protozoan abundance. Generally, studies of hypersaline ponds have focused on descriptions of species with little regard for either biomass or their activity (Noel, 1984). Post (1977) did note that protozoa in the Great Salt Lake southern arm were well adapted to *in situ* high salinities. Using isolates from the Lake, he showed that numbers of both flagellates and amoebae grew up to 10^5 protozoa per ml in an aquarium filled with Great Salt Lake water. Protozoa have also been shown to reach high densities in sites with slightly elevated salinities such as the Salton Sea (up to 5.3% salt). Here, naked amoebae ranged from 14,560 to 237,120 cells l^{-1} depending upon sampling location (Rogerson and Hauer,

2002). Although similar data are not available for high salt environments, protozoa are principle consumers of bacteria and other microbes, and are probably important regulators of bacteria in high salt environments as they are in freshwater and marine environments. In studies of high saline habitats in Western Australia, where average salinities were 6.7% salt, eighty seven species of flagellates were found. These voracious consumers of bacteria were similar to the flagellate communities of marine benthic habitats. The estimated abundances of $1x10^3$ cells cm^{-3} were comparable to levels in other sediments. It is likely that their importance in these elevated salinities was greater since the higher salinities may have reduced the numbers of other predators (Al-Qassab et al., 2002).

In the Antarctic Vestfold Hills, the plankton was dominated by one ciliate, *Mesodinium rubrum* (an unusual ciliate with photosynthetic capability), that reached abundances of $2.7x10^5$ l^{-1} and spanned a salinity gradient up to 6.3% salt (Laybourn-Parry et al., 2002). High abundances of marine dinoflagellate species were reported from several of the lakes ranging from brackish to hypersaline (salinities of up to 9.8%). In hypersaline coastal lagoon waters of Mar Menor Spain, small heterotrophic flagellates were estimated around 10^4 l^{-1} (Pérez-Ruzafa et al., 2002) and in the hypersaline Laguna Madre of Texas, microzooplankton abundances of ciliates, tintinnids, copepod nauplii, and rotifers was negatively correlated with salinity. At salinities below 6% abundances ranged from 20-100 organisms ml^{-1}, and dropped to below 20 organisms ml^{-1} above 7.5% (Buskey et al., 1998). During the same study, specific growth rates for the heterotrophic dinoflagellate *Oxyrrhis marina*, a primary grazer on brown tide algae, increased at each of the three salinities tested up to 9% salt.

Primary productivity studies of salterns have sometimes considered the micrograzers. In one study, the biovolume of ciliates at 5% salinity was 12 μm^3 l^{-1} but dropped to zero at 25% (Pedrós-Alió et al., 2000). This study suggests a high grazing potential from ciliates at least at lower salinities. Very high numbers of heterotrophic nanoflagellates were reported by Pedrós-Alió et al. (2000) at La Trinitat, Spain. Around 10^6 flagellates ml^{-1} were found at 15% salinity but again densities fell off to below detection at higher salinities (30%). Park et al. (2003) also reported high numbers of heterotrophic flagellates from a solar saltern on the west coast of Korea. Here, flagellates ranged from 7 to $28x10^8$ nanoflagellates per liter, and they were credited with being important grazers of bacteria in high salinity waters where they previously have not been reported.

5. Biodiversity of Protozoa in High Salinity Environments

Lagoons, inland lakes, salt ponds and enclosed seas all provide habitats for a select few organisms that can tolerate high salt. As illustrated in Fig. 2, the number of species of protozoa decreases as salinity of the habitat increases. At 3.2% (marine waters) the numbers of species of ciliates, flagellates and amoebae typically number 194, 141 and 74, respectively (data from a taxonomic review of North Atlantic protozoa, Rogerson and Goodkov, 2001). In habitats between 10 and 20% salt, numbers of species of each protozoan group are between 3 and 16 species, while at the highest salinities (ca. 30%), typically only 1 or 2 species are present. It is interesting to note that amoebae, ciliates and flagellates are all equally represented in this database; no one group dominates at any

particular salinity level. However, since the figure is based on scant data, and often relies on the ability of protozoa to be cultivated in the laboratory before they can be recorded, further studies are necessary before hard conclusions can be drawn. For now, an increase in salinity results in a loss of diversity of heterotrophic protozoa but the few surviving protozoan species particularly at the highest salinities show impressive resilience to these extreme conditions. Their presence in these environments suggests that they are important consumers and recyclers of nutrients when higher consumers are absent. This however is speculative since Por (1980) described hypersaline waters in excess of 140 g l^{-1} as simple and imbalanced producer grazer ecosystems (where grazers exerted limited control) and in the range 200-300 g l^{-1} systems were restricted to producers.

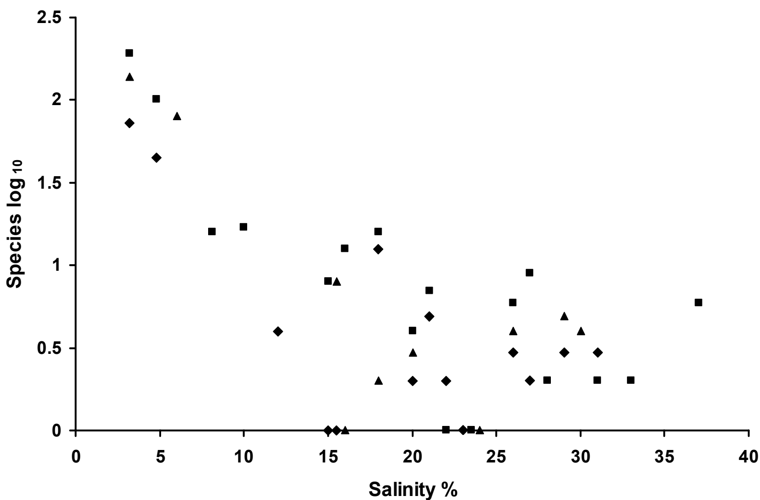

Figure 2. Number of species ($\log_{10}$) of amoebae (♦), ciliates (■), and flagellates (▲) reported in a range of salinity habitats. Only studies that were considered by the authors to be comprehensive in nature were included (amoebae: Davis, 1978; Elazari-Volcani, 1943; Flowers and Evans, 1966; Garcia and Neill, 1993; Golubic, 1980; Jones, 1944; Kirkpatrick, 1934; Pack, 1919; Post, 1977; Post et al., 1983; Rogerson and Hauer, 2002; Rogerson and Goodkov, 2001; Vorhies, 1917; Hauer and Rogerson, unpublished. Ciliates: Al-Rasheid et al., 2001; Ayadi et al., 2002; Baas- Becking, 1956; Carpelan, 1957; Costello et al., 2001; Davis, 1978; Esteban and Finlay, 2003; Flowers and Evans, 1966; Pack, 1919; Post et al., 1983; Wilbert, 1995; Wilbert and Kahan, 1981; Volcani, 1944; E. Small, personal communication. Heterotrophic flagellates: Costello et al., 2001; Elazari-Volcani, 1943; Flowers and Evans, 1966; Patterson and Simpson, 1996; Post et al., 1983; Ruinen, 1938; Simpson and Patterson, 1996).

6. Solar Salterns and Protozoa

In solar salterns worldwide there are significant differences in the types of microbes that inhabit them depending on nutrients, water retention, and effects of climatic conditions

(Oren, 2002). Solar salterns offer an experimental system with a strong gradient in biodiversity for studying primary and secondary producers. At one end of the gradient the water is evaporated seawater up to 9% and contains a wide diversity of phytoplankton, zooplankton and heterotrophic microbes. Middle salinity ponds are from 3-7 times the salinity of seawater and protozoa have been found in these salterns at 15-20% salt (Guixa-Boixareu et al., 1996; Javor, 1989; Rodriguez-Valera et al., 1981). Here, the major groups of grazers are microzooplankton, but little is reported about the biodiversity of these heterotrophs in these systems. At the other extreme (24-32% salt), the microbial community changes significantly, and at higher salinities mesozooplankton are almost absent, and phytoplankton are often reduced to a single organism, *Dunaliella*. Experiments on enrichment culture studies of protozoan isolated in salt ponds at upper salinities have provided information concerning the possible microbial activity in these environments. As seen in Tables 1, 2 and 3, approximately a third of all types of protozoa studied in hypersaline environments were found in salterns. While most of the studies of organisms in salterns are descriptive, the salinity range maintained within solar salterns controls biodiversity and offers an environment to test the future relationships between biodiversity and production within a marine system of low biomass and high productivity (Javor, 1989; Oren, 1999; Ventosa, 2004).

7. Adaptations to High Salinity Environments

Much remains to be discovered about protozoan adaptations to the salinity stress. In extreme environments, mixotrophy is often observed in hypersaline environments (Al-Qassab et al., 2002; Garcia and Neill, 1993; Laybourn-Parry, 2002; Laybourn-Parry et al., 2002) and other unusual nutritional strategies may hold the key to the adaptations of some protozoa. For example, the flagellate *Colpodella*, found in 24-26% salt in a hypersaline pond in South Australia, has been classified as phylogenetically significant because it is heterotrophic but more closely related to the apicomplexan parasites than any other known group of organisms (Simpson and Patterson, 1996). This may give this protozoan an unusual nutritional edge. In fluctuating salt environments, some protozoa alternate between flagellate, amoeboid and resilient cyst stages as is common in *Paratetramitus* sp. and *Tetramitus* sp. (Flowers and Evans, 1966; Golubic, 1980; Post, 1977; Post et al., 1983; Read et al., 1983; Rogerson and Hauer, 2002; Ruinen, 1938). Some protozoa might form particularly resistant cysts in response to salinity fluctuation. Ciliates and amoebae were recovered from the salt crust in the Hutt Lagoon in Australia and were believed to be from resilient cysts (Post et al., 1983).

8. Morphological Changes in Protozoa Growing under High Salt Conditions

Morphological changes in the protozoa in response to salt may be a consequence of adaptation or a direct result of facing gradients of salinity. Changes have been demonstrated in the morphology of amoebae over a range of salinity challenges (Rogerson and Hauer, 2002). Generally, amoebae are active and flatter at lower salinities and markedly sluggish, and raised or domed at high salinities. This is well exemplified in the case of a recently described amoeba, *Platyamoeba pseudovannellida* that grew over

the range 0 to 13.8% salt (Hauer et al., 2001). At the lowest salinities it was active and fan-shaped but at the highest salinities it was morphologically very different being rugose and rounded. Although the mechanisms responsible for these changes are interesting (and as yet unknown) the immediate importance is in identification. This is particularly true in the case of naked amoebae that are identified to genus on the basis of cell morphology. Another morphological feature common in amoebae at high salinity is the appearance of numerous cytoplasmic vacuoles, as is illustrated in the unidentified amoeba in Fig. 1a (21% salt). Changes in shape have been reported in the much-studied autotrophic protist, *Dunaliella.* Here, cells immediately respond to changes in salinity by shrinking and swelling as reported in Oren (2002).

Other changes reported for protozoa include ciliary reversal in the ciliated protozoan, *Fabrea salina* (Dryl et al., 1982) and the abnormal test formation in foraminiferans from hypersaline lagoons in Brazil (Debenay et al., 2001). There have been cautionary notes by Pack (1919), Gaievskaia (1925), and Reddy (1972) who all warn that morphology can be greatly altered by changes in salinity thus new taxa should be reported only after careful study.

9. Osmoregulation Adaptations

The chemical and physical boundaries associated with extreme salt environments and the physiological adaptations of the organisms inhabiting these waters are largely unknown (Fenchel, 1987). However, it is recognized that the major problem facing halophiles is control of their osmotic pressure. Without adaptations to high salinities, they would lose water to the surrounding environment. Not surprisingly, given its importance in hypersaline environments, adaptations in the alga *Dunaliella salina* have been well studied. This protist synthesizes high concentrations of intracellular glycerol to balance the external osmotic pressure and thereby control the cell's water balance (Avron and Ben-Amotz, 1979; Borowitzka et al., 1990; Roberts, 1998).

Far less is known about adaptations in protozoa although the control of water relations has been reviewed by Smith (1978) and Brown (1990). Protozoa regulate their electrolyte composition and osmotic pressure by three mechanisms: active ion transport across the cell membrane; regulation of the concentration of low-molecular weight solutes, mainly amino acids, in the cytoplasm; and by active excretion of sodium and uptake of water. This latter process probably takes place in the spongiome, a system of endoplasmic membranes, and the water is then in most cases collected in a contractile vacuole before being expelled from the cell. The physiology of osmoregulation and contractile vacuoles in protozoa was reviewed by Patterson (1980). Eukaryotic microbes have evolved a variety of biochemical adaptations that allow them to cope with osmotic and ionic stress. The ciliate *Paramecium calkinsi*, the amoeba *Acanthamoeba castellanii* and the flagellate *Ochromonas malhamensis* all adjust their intracellular amino acids in response to salinity challenges (Aaronson, 1980; Cronkite et al., 1991; Drainville and Cagnon, 1973). *Ochromonas* also increases carbohydrates (e.g. fluoridoside) in response to increasing external osmotic pressure (Kauss, 1977).

10. Final Comments

Water bodies with elevated salinity represent an enormously overlooked habitat since, according to Mianping et al. (1998) the total volume of saline water in the world is equivalent to the combined volume of inland freshwater lakes and rivers. For the purposes of this paper, we have generally focused on heterotrophic protozoa (ciliates, flagellates, and naked amoebae) inhabiting hypersaline waters above 9% salt, although where relevant, we include some species from 6-7%. This review has clearly shown that these protozoa can be found in all water bodies regardless of salinity although at extreme salinity (i.e. saturation) they are rare and often represented by only one species.

Prior to the 1970s, only a few species of protozoa were reported from hypersaline waters (around 38 in total) but with the increased interest over the last 35 years, this number has grown to around 200 species, as indicated by the data in Tables 1-3. Even so, there is not enough information to comment authoritatively on ubiquity of species in different salt habitats worldwide or to have confidence in abundances of protozoa reported from salt stressed habitats. It is likely that some protozoa are cosmopolitan such as the conspicuous ciliate *Fabrea salina* that has been reported from many salt habitats worldwide. However, since many biodiversity and enumeration studies of protozoa rely on enrichment cultivation methods, future work should focus on improvements in culture methods. Amoebae, for example, can be kept in culture at elevated salinity but they rarely become abundant. This suggests the media and conditions being used are less than ideal and that other cryptic forms might be overlooked. The bias in the available literature towards ciliated protozoa attests to this since these protozoa are easy to observe in fresh samples and are the most frequently reported. To truly understand the role of protozoa in hypersaline habitats requires more work, particularly on the less conspicuous flagellates and amoebae. For now we can conclude that total numbers of species of protozoa in marine waters (3.2% salt) are around 409 species and that this falls to around 10 species at between 10 and 20% salt and down to 1 or 2 species at the highest salinities (30%).

Other than information on types of protozoa and scant information on biodiversity and abundances, virtually nothing is known about the physiological adaptations exhibited by protozoa to life in extreme saline habitats. This review falls short in this regard which merely emphasizes that future research is urgently needed. There are suggestions that some protozoa have adopted unusual nutritional strategies in stressed environments, have alternate life stages, morphological adaptations, and cytoplasmic osmoprotectants, but most information is almost anecdotal rather than firm science. Several earlier studies have realized the importance of solar salterns as experimental systems. In the absence of effective laboratory cultivation methods, salterns with their steep salinity gradients, offer ideal systems for future studies on protozoa.

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HETEROTROPHIC FLAGELLATES IN HYPERSALINE WATERS

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1. Introduction

Protozoa have been observed for a long time to occur in hypersaline (> 300 psu [practical salinity units]) waters (Patterson and Simpson, 1996; Park et al., 2003; Post et al., 1983; Ruinen, 1938; Volcani, 1944). Heterotrophic nanoflagellates, ciliates and amoebae are the major groups of protozoa adapted to life in hypersaline environments (Javor, 1989; Post et al., 1983). Of these groups, heterotrophic nanoflagellates are numerically dominant, as in other aquatic environments. However, studies of nanoflagellates in hypersaline environments have been as limited as in other extreme environments (Atkins et al., 2000). Recently, quantitative measurements of heterotrophic nanoflagellate abundance and grazing by heterotrophic nanoflagellates on prokaryotes in hypersaline environments have been made. This information gained from these studies, together with a taxonomic analysis of the heterotrophic nanoflagellates found in hypersaline environments, raises some interesting questions. Below, I discuss aspects of the ecology and evolution of heterotrophic nanoflagellates from hypersaline environments.

2. Heterotrophic Nanoflagellates: Abundance and Prokaryote Grazing in Hypersaline (> 300 psu) Waters

Heterotrophic nanoflagellates can be observed frequently enough to be identified in hypersaline waters (Post et al., 1983). However, quantitative measurements of the abundance of nanoflagellates in hypersaline waters were made only recently in solar salterns in Spain and Korea. Heterotrophic nanoflagellates were usually recorded as absent from hypersaline ponds in solar salterns in Spain, but abundance of ca. $3x10^4$ cells ml^{-1} was recorded at a salinity close to 300 psu (Pedrós-Alió et al., 2000b). In a Korean solar saltern, the abundance of nanoflagellates in crystallizers ranged from 7 to $28x10^3$ cells ml^{-1}, steadily decreasing from April to August (Park et al., 2003). This abundance is about one order of magnitude higher than that found in coastal waters (Park and Cho, 2002; Sanders et al., 1992). Prokaryotic abundance in crystallizers was about two orders of magnitude higher than in coastal waters, and ranged from 69 to $276x10^9$ cells l^{-1}, increasing from April to August. Abundance ratios of heterotrophic nanoflagellates to prokaryotes were close to those found in marine systems in April, but decreased ca.

16-fold in August compared to April. In crystallizers, the prokaryotic loss per day due to viral lysis was lower than 2% (unpublished data), similar to values (< 5%) observed in a Spain solar saltern (Guixa-Boixareu et al., 1996). However, prokaryote mortality due to grazing by heterotrophic nanoflagellates was on average 14 times higher than that due to viral lysis, indicating that bacterivory, not viral lysis, controlled the abundance of heterotrophic prokaryotes in crystallizers, as in non-hypersaline aquatic systems (Pedrós-Alió et al., 2000a).

To determine whether nanoflagellates in hypersaline waters are actively grazing on prokaryotes, direct microscopical observations are desirable. Fluorescently labeled prokaryotes (Sherr et al., 1987) were prepared in the laboratory, using natural prokaryotes sampled from a crystallizer or saltern pond, and the uptake of these labeled prokaryotes by nanoflagellates was measured. In the crystallizers of a Korean solar saltern, nanoflagellates grazing rates on prokaryotes ranged from 1.4 to 13.0×10^8 cells l^{-1} h^{-1} (Park et al., 2003). In contrast, nanoflagellates grazing rates on prokaryotes in solar salterns in Spain were negligible (Guixa-Boixareu et al., 1996). The nanoflagellates grazing rate in the Korean solar saltern was highest in summer, turning over natural prokaryotes in nine days, corresponding to 358% of prokaryotic production as determined by ^{3}H-thymidine incorporation. This high grazing activity was calculated to support a doubling time of 2.3 days for heterotrophic nanoflagellates, based on their estimated ingestion rate, the biomass carbon of nanoflagellates and prokaryotes, and a gross growth efficiency of 10% (Park et al., 2003). This estimate of nanoflagellate growth was slightly lower than that observed in culture of a 10 μm filtrate of hypersaline samples (doubling time 1.9 days). A culture of mixed heterotrophic nanoflagellates from a crystallizer had a doubling time of 1.1 days. Thus, it is evident that in hypersaline conditions nanoflagellates can grow rapidly. An estimate of 10% gross growth efficiency for species from hypersaline samples is lower than the values estimated for marine heterotrophic nanoflagellates (Fenchel, 1982). Although more precise determinations of the gross growth efficiency are needed, this indicates that nanoflagellates in hypersaline waters can thrive even if they have to pay a higher energetic cost.

Grazing by active nanoflagellates in high salinity waters may provide a simple explanation for the rapid ammonium turnover recently observed in a crystallizer at the Bras del Port solar saltern system, Alicante, Spain. Joint et al. (2002) reported that a considerable recycling activity of ammonium in the high salinity waters balanced the demand. In seawater, the major agents of ammonium regeneration are microzooplankton and heterotrophic nanoflagellates (Glibert, 1982; Harrison, 1978). In the Bras del Port solar saltern system, zooplankton (mainly *Artemia*) were present in hypersaline water (ca. 320 psu), but were absent at salinities greater than 350 psu (Gasol et al., 2004). Microzooplankton herbivory was also not observed in a crystallizer (370 psu) in the same solar saltern system (Joint et al., 2002). Although the previous work reported an absence of heterotrophic nanoflagellates and lack of grazing activity in a crystallizer (> 300 psu) at the Bras del Port site (Guixa-Boixareu et al., 1996), an abundance of nanoflagellates similar to that found in a Korean solar saltern was occasionally observed at around 300 psu (Pedrós-Alió et al., 2000b). In the hypersaline ponds of the solar salterns, the prokaryotic loss per day due to viral lysis was lower than 5% (Guixa-Boixareu et al., 1996), providing only a small fraction of the observed ammonium regeneration rate in the

crystallizer. Thus, bacterivory by nanoflagellates in the crystallizer could be mainly responsible for the observed ammonium regeneration. This would also be in agreement with the general belief that solar saltern ecosystems are markedly similar all over the world and that conclusions drawn from the study of specific salterns should have a general validity (Oren, 1994).

In measurements of grazing rates, natural assemblages of heterotrophic nanoflagellates have been used. Thus, it is difficult to determine whether such nanoflagellates in hypersaline samples are truly extremely halophilic based on measured grazing rates. Cultivation of the species dominant in the natural assemblages in pure culture is necessary. A culture of *Pleurostomum flabellatum* (Fig. 1) recently isolated in my laboratory from hypersaline samples showed maximum growth at 250-300 psu, and did not grow below 100 psu (unpublished data). Thus, *Pl. flabellatum* is extremely halophilic. A culture of a bicosoecid (Fig. 1) showed maximum growth rate at 125 psu and grew at >300 psu (unpublished data), indicating its extreme halotolerance.

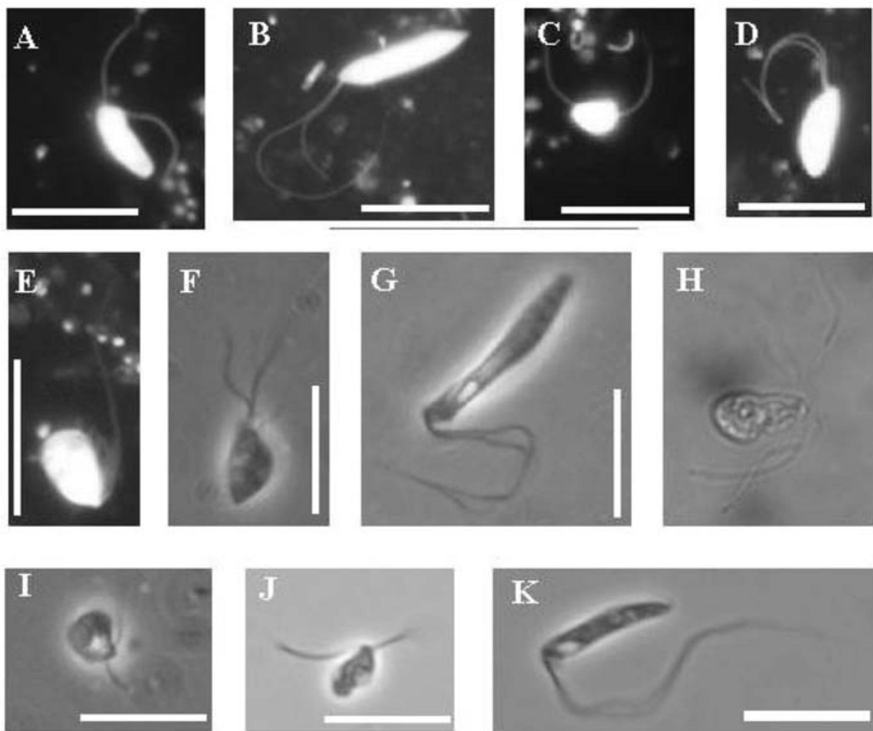

Figure 1. (A-E) Epifluorescence micrographs of fixed flagellates from hypersaline (>300 psu) samples visualized under a UV-filter set. Phase contrast micrographs of live flagellates (F-I) and cultured bicosoecid (J) and *Pleurostomum flabellatum* (K) from hypersaline samples. Bars: 10 μm.

3. Taxonomy of Heterotrophic Nanoflagellates from Hypersaline Environments

A variety of nanoflagellates can survive in hypersaline environments. Species identified in recent studies include *Bodo saltans*, *Bodo* spp., *Colpodella pugnax*, *Palustrimonas yorkeensis*, *Phyllomitus* sp., *Pleurostomum flabellatum*, *Pl. turgidum* and *Tetramitus* spp. (Patterson and Simpson, 1996; Post et al., 1983). Considering the presence of unidentified species and the limited geographic regions studied so far (Table 1), more species, including new ones, will undoubtedly be found in hypersaline waters. Molecular studies on eukaryotes in crystallizers also support this view (Casamayor et al., 2002). However, it seems that diversity of heterotrophic nanoflagellates in hypersaline waters is rather restricted. Table 1 also shows the different species reported from two hypersaline sites in Western Australia: Hutt Lagoon marine embayment and a saturated salt puddle. It is possible that geographically different areas might have different species compositions. However, some nanoflagellate species reported from the two Western Australia sites appear also to be present in a Korean solar saltern. Probably, some species may occur over a wide geographic range.

TABLE 1. Species of heterotrophic nanoflagellates observed in hypersaline (>300 psu) environments.

Species	W. Australia [a]	W. Australia [b]	Korean solar saltern [c]
Bodo saltans		+	
Bodo spp.	+		+
Colpodella pugnax		+	
Palustrimonas yorkeensis		+	
Phyllomitus spp.	+		+
Pleurostomum flabellatum		+	+
Pl. turgidum		+	
Tetramitus spp.	+		+

[a] Hutt Lagoon marine embayment, Western Australia (Post et al., 1983).
[b] W. Australia (Pattern and Simpson, 1996).
[c] Seoshin, Korea (Park et al., 2003).

Most of the protozoa in the salt crust of Hutt Lagoon, Western Australia are presumed to be present in the form of cysts and to germinate on dissolution of the salt (Post et al., 1983). Formation of cysts in hypersaline environments with subsequent propagation by physical processes to other locales and colonization of similar hypersaline environments might have occurred over geological periods. For instance, cysts of nanoflagellates in dried hypersaline environments may become mixed with ambient seawater and then carried via currents to other locales. During propagation, processes of sinking, resuspension and dispersal of cysts by currents may have repeatedly occurred. Alternatively, cysts may be propagated over long distances as wind-borne particles. During such propagation, landing on surfaces and resuspension into the air would be repeated many times. A recent observation of airborne cysts of soil nanoflagellates (Rogerson and Detwiler, 1999) suggests that aerial transport as cysts is a plausible

pathway. The propagation of the cysts of nanoflagellates from hypersaline environments via the two pathways mentioned above could explain their occurrence in similar hypersaline habitats in geographically isolated areas in the world.

In a study of heterotrophic nanoflagellates in a natural salt-saturated site in Western Australia, a total of five species were reported, including a new species (*Pl. turgidum*) (Patterson and Simpson, 1996). Interestingly, this level of diversity is similar to seven species of prokaryotes determined by the reassociation rate of DNA isolated from a Spanish salt-crystallizing pond (Torsvik et al., 2002). Apparently, stress due to high salinities could restrict numbers of successfully adapting species of both prokaryotes and nanoflagellates. In this respect, *Bodo saltans*, assuming its correct identification, is a very interesting organism because *B. saltans* would have the greatest possible range of halotolerance, occurring from freshwater to hypersaline habitats (Patterson and Simpson, 1996). Furthermore, *Bodo saliens* and *Rhyncomonas nasuta* are well known to occur in diverse environments, including garden soils, hypersaline (up to 150 psu) water, marine sediments, and deep-sea hydrothermal vents (Atkins et al., 2000). These cosmopolitan species would require a means of dispersal for a global distribution. However, cyst formation has not been reported for *B. saltans* (von der Heyden et al., 2004). Possibly, those cosmopolitan species, including *B. saltans,* may have a great genetic variability among strains from diverse environments, and thus form distinct ecotypes with different habitat preferences. For example, freshwater and marine morphotypes of the cosmopolitan bodonid *Bodo designis* may be genetically distinct (von der Heyden et al., 2004). However, it also should be noted that some heterotrophic nanoflagellates (*P. yorkeensis*, *Pl. flabellatum*, *Pl. turgidum*) may be restricted to hypersaline waters.

4. Molecular Approaches to Study Heterotrophic Nanoflagellates of Hypersaline Waters

Denaturing gradient gel electrophoresis-polymerase chain reaction-sequencing (DGGE-PCR-sequencing) of DNA extracted from natural samples has been successfully used to study the species diversity of dominant groups of both prokaryotes and eukaryotes in diverse communities. Casasmayor et al. (2002) found 11-12 eukaryotic operational taxonomic units in hypersaline ponds from the Bras del Port solar saltern system using this technique. Most of the operational taxonomic units found in hypersaline samples appeared to be absent from lower salinity ponds. However, since sequence identifications of each operational taxonomic unit were not made in that study, comparison with existing lists of heterotrophic nanoflagellates identified by light microscopy is not possible.

In my laboratory, DGGE-PCR-sequencing was also employed on DNA extracted from crystallizers. The choice of primers for PCR is important in studying nanoflagellates from hypersaline waters. For example, we did not recover large subunit (LSU) ribosomal RNA gene sequences belonging to such nanoflagellates using a set of primers employed for the amplification of dinoflagellates LSU rDNA sequences (Scholin et al., 1994); *Dunaliella*-like and *Gymnodinium*-like sequences were obtained instead (unpublished data). The ‘universal’ eukaryote primers, Euk A and B (Medlin et al., 1988) amplified sequences belonging to a bicosoecid and *Pl. flabellatum* from two pure cultures of

biflagellated heterotrophic nanoflagellates. Further development of appropriate primers would be required for the study of heterotrophic nanoflagellate diversity and phylogeny (e.g. Atkins et al., 2000). In this respect, their isolation from hypersaline waters would be interesting because some are not readily assigned to any of the recognized higher taxa within eukaryotes. Molecular phylogenetic and ultrastuctural studies on isolated strains from hypersaline waters will provide resolution of the systematic placement of well-known species with previously uncertain taxonomic affinities such as *Pl. flabellatum*. With the advent of appropriate 18S rRNA probes for nanoflagellates of hypersaline waters, the FISH-TSA (fluorescence *in situ* hybridization-tyramide signal amplification; Not et al., 2002) technique will allow us to answer questions related to community structure, species-specific grazing on prokaryotes and responses of nanoflagellates to changes in environmental factors in hypersaline environments.

5. Summary

The presence of heterotrophic nanoflagellates in hypersaline (> 300 psu) environments has been known for a long time. Limited numbers of taxonomic studies have been available for such nanoflagellates in hypersaline environments. It seems that the number of species in hypersaline waters is rather restricted. Recently, ecological studies on heterotrophic nanoflagellates have been made in a few solar salterns. In hypersaline waters, nanoflagellates actively graze on prokaryotes and can achieve rapid growth. Although limited, the available information on nanoflagellates of hypersaline environments raises many interesting questions: How can we explain the occurrence of extremely halophilic or halotolerant species in similar hypersaline habitats in geographically isolated areas in the world? Is water or aerial transport of cysts an efficient means of their dispersal? How do extremely halophilic heterotrophic nanoflagellates adapt to hypersaline environments? To what clades of the eukaryotic tree do these species belong, and what implications will the new information have on our understanding of the evolution of extremely halophilic or halotolerant flagellates?

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Section VII. Viruses

Haloviruses and their hosts
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HALOVIRUSES AND THEIR HOSTS

Recent progress in the cultivation of haloarchaea, including square haloarchaea of Walsby, and the isolation of novel haloarchaeal viruses

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1. Introduction

This year marks a significant advance into the study of the microbial ecology of salt lakes. Not only do we have the molecular tools to be able to examine and describe the diversity and dynamics of their microbial populations, but remarkable gains have been made in their cultivation, including that of the dominant, square haloarchaeon, first discovered 25 years ago by A.E. Walsby. In addition, the diversity of viruses that infest hypersaline waters, thereby significantly modulating haloarchaeal communities, is gradually being discovered and studied in their own right.

The ability to culture the dominant haloarchaea is clearly a major step forward in being able to sample the virus diversity of salt lakes, and to study the interactions between organisms representing the major players in this extreme ecosystem. We will deal with the microbial diversity first, then outline the current state of halovirus research.

2. Molecular Detection of the Dominant Haloarchaea in Solar Saltern Crystalliser Ponds

Solar salterns are useful model systems for studying microbial communities in hypersaline waters as they are carefully managed and salinity levels controlled, with a series of ponds of increasing salinity until the final crystalliser ponds where the salt concentration remains at saturation until the pond is drained and the salt harvested. The distinct pink-red colour of the crystalliser ponds is indicative of their high concentrations of microbes, the vast majority of which are extremely halophilic Archaea (family *Halobacteriaceae*). Other microorganisms that are present at lower levels in saturating (or near-saturating) brines are green algae (e.g. *Dunaliella* spp.) and Bacteria (e.g. *Salinibacter*). Extensive reviews of the microbial ecology of salt lakes and salterns have been published over the years (Rodriguez-Valera, 1991), but relatively few studies of the microbial diversity of salt lakes and crystalliser ponds have

utilized 16S rRNA gene sequence libraries, and most have been limited to the northern hemisphere (Antón et al., 1999; Casamayor et al., 2002; Ochsenreiter et al., 2002). To expand the knowledge of the microbial diversity of such ponds, particularly those in the southern hemisphere, we studied a solar saltern crystalliser pond on the outskirts of Geelong, a city on the southern coast of Australian, near Melbourne, Australia (38º 09.841' S, 144º 25.274' E) (Burns et al., 2004a). Cells from water samples were collected by centrifugation, total DNA extracted, and prokaryotic 16S rRNA genes amplified by PCR using consensus primers. Fifty-seven clones were sequenced, and a summary of the results is shown in Table 1 (right most column).

Overall, there was a remarkably low microbial diversity, a common feature of extreme environments. The predominant group, 49% of clones, consisted of close relatives of the *Halorubrum* genus, and these sequences were found to fall into two distinct subgroups based on sequence similarity (subgroups 1 and 2). The second most dominant group (33%) was the SHOW group (Square Haloarchaea Of Walsby). The third, and unexpected group (16% of clones) were closely related to sequences recovered from Antarctic hypersaline lakes, such as Deep Lake (e.g. Genbank accession AF142879, clone DEEP-5) (Bowman et al., 2000). These we refer to as the Antarctic Deep Lake (ADL) group. The smallest group (2%) consisted of sequences most closely related to members of the *Natronomonas* genus.

TABLE 1. Distribution of phylotypes detected in studies of saltern crystalliser ponds at different geographic locations.

Phylotype	% sequences		
Location	Fuencaliente, La Palma, Spain	Alicante, Spain	Geelong, Australia
Halorubrum	64	-	49
SHOW	0	61 [a]	33
ADL	0	-	16
Haloarcula	34	< 0.1	0
Haloferax	0	-	0
Other haloarchaea	2 ('cluster 1') [b]	14 [c]	2 [d]
Bacteria	-	18 [e]	-
Reference	Ochsenreiter et al., 2002	Antón et al., 1999	Burns et al., 2004a

[a]Equivalent to SphT phylotype. [b]Probably related to the ADL group. [c]Includes *Halorubrum*-like sequences. [d]*Natronomonas*-like sequences. [e]Mainly *Salinibacter* (Benlloch et al., 2002).

How do these results compare to northern hemisphere studies? Table 1 compares our results with those of two other studies of crystalliser ponds (Antón et al., 1999; Ochsenreiter et al., 2002). The three studies show some interesting differences. For example, SHOW group sequences varied widely between the different sample sites,

from 0 to 61%. *Halorubrum*-related sequences were frequently recovered in two of the three studies (49-64%), and the level of *Salinibacter*-related sequences was 0-18%. Although the Ochsenreiter et al. (2002) and Burns et al. (2004a) studies did not specifically look for Bacteria in the construction of their sequence libraries, they did not detect any Bacteria in cultivation studies of the same water samples. The reasons for such wide variation in the proportions of a relatively few haloarchaeal groups are yet to be determined, and many questions are raised. For example, does the presence of significant numbers of *Haloarcula* restrict the growth of SHOW group haloarchaea?

3. Cultivation Studies of Crystalliser Ponds Using Conventional Media and the Isolation of Members of the Antarctic Deep Lake (ADL) Group

We also tried various cultivation conditions to try and isolate representatives of all the major microbial groups in the Geelong crystalliser pond that were detected in the 16S rRNA gene sequence library (Burns et al., 2004a). Solid media (using washed, Difco-Bacto agar as the gelling agent) were tried first, with a range of selected substrates, generally at low concentration (Benlloch et al., 2001; Janssen et al., 2002; Oren, 1995). The salt concentration was 25% and plates were incubated in sealed plastic containers at 37°C for up to 12 weeks. Viable counts, and microbial diversity (as measured by 16S rRNA gene sequencing of randomly selected colonies) were monitored and the results summarized in Fig. 1. Viable counts increased significantly when the incubation

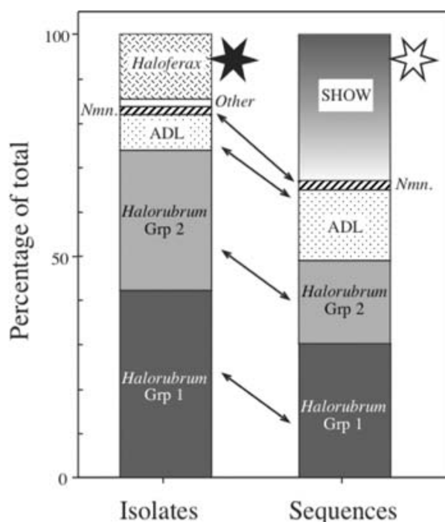

Figure 1. Comparison of isolates and 16S rRNA sequences from a study of microbial diversity in a saltern crystalliser pond (see Burns et al., 2004a for details). Abbreviations for the various groups (phylotypes) of isolates and 16S rRNA gene sequences are given in the text. Arrows show corresponding groups. Stars indicate major groups found in only one of the analyses (cultivation or sequence library). *Nmn.* = *Natronomonas*.

was extended to 12 weeks, with a significant rise occurring between 3 and 8 weeks. Diversity also increased with incubation time, so that while only 2 sequence groups were found after 3 weeks (close relatives of the genera *Haloferax* and *Halorubrum*), at 8 weeks a further three sequence groups were found (ADL group, *Natronomonas*-associated group, and CSW 2.24.4 - an isolate related to *Halogeometricum borinquense*).

There were significant differences between the microbial diversity disclosed by 16S rRNA gene sequences and by culture. *Haloferax* spp. were readily isolated but were not detected in the sequence library, whereas members of the SHOW phylotype were frequent in the sequence library but not detected on the plates. These observations are similar to previous studies. No member of the SHOW group has been cultured since their discovery 25 years ago (Walsby, 1980), and *Haloferax* spp. are commonly isolated from salt lake samples yet make up an insignificant percentage of the actual population (as deduced from 16S rRNA gene sequence libraries). The over-representation of *Haloferax* spp. probably reflects their rapid growth and high cultivability on solid media.

The ADL group isolates were generally slow growing, and the morphology of pure cultures was quite variable. Among smaller round cells, many isolates possessed characteristically long, rod shaped cells, often displaying cell wall ‘blisters’. The characteristics of this group are currently under investigation and will be formally described in the near future.

4. Cultivation of SHOW Group Organisms

Some organisms do not form colonies on agar media (Simu and Hagström, 2004). Therefore we next tried to isolate SHOW organisms using a liquid medium, MGM (0.1% yeast extract, 0.5% peptone, 23% salts), that was found to support growth of members of all the other sequence groups found in this pond (see above). In order to seed cultures with single cells, crystalliser water was serially diluted in MGM, and tubes incubated at 37°C for 3 weeks. Turbid growth was seen in all tubes down to the 10^{-7} dilution, after which the fraction of turbid tubes diminished rapidly. Of 15 turbid cultures at the 10^{-8} to 10^{-9} dilutions, all contained cells similar to *Halorubrum* species, and their 16S rRNA gene sequences closely matched *Halorubrum* sequences (>99%). Non-turbid cultures did not contain cells (by light microscopy). Since SHOW like cells were highly represented in the original water sample, it appeared that rich media, like MGM, were not suitable for their culture.

Some organisms are inhibited by peptone or yeast extract (Rappé et al., 2002), so natural water, with or without nutrient additives, was used instead of MGM for dilution cultures. The low nutrient levels precluded the likelihood that cultures would show visible turbidity, so growth was assayed by PCR, using both consensus and SHOW-specific primers (i.e. a multiplex PCR). The most successful formulation was natural saltern water, supplemented with 50 μM amino acid mix and 0.5% (wt/vol) pyruvate. After 3 weeks incubation, dilution cultures with this medium were screened by PCR and three were found to have SHOW-specific amplification products, and their sequences confirmed this. By light microscopy, all three cultures had square cells similar to those observed in the original water sample. The best growing isolate, C23,

was passaged further and its properties examined in detail (Burns et al., 2004b). By negative-stain electron microscopy cells were uniformly square (average of 2.3 µm per side) or rectangular. Rare triangular forms were seen. Cells often contained dark granules of poly-hydroxy-butyrate (also seen in phase contrast light microscopy), and gas vesicles that were long, thin cylinders with conical ends (as first noted by Walsby). Cells could form large sheets in which cell borders were difficult to discern, but when smeared across a glass slide, such sheets were seen to fall apart into individual cells. Growth rate increased with passage in culture, from a doubling time of 5.5 days to about 1 day, and final cell densities also improved to around 10^8 cells ml. Cultures were visibly turbid, slightly pink, and cell pellets (after centrifugation) were bright red in colour.

Given the ecological significance of organisms belonging to the SHOW group, the isolation of a representative of this group is a major advance in the study of salt lake microbiology. Our method of isolation is straightforward and rapid (considering the intrinsic slow growth rate of these organisms), and should be applicable to other salt lakes. We believe the method can be improved further, by optimization of the initial isolation medium, the use of laser-tweezers to inoculate single cells, and the application of robotics to increase the number of cultures that can be processed (i.e. seeded, cultured and examined for growth). Independently, H. Bolhuis and colleagues have also grown SHOW organisms, using a different strategy (Bolhuis et al., 2004; see also elsewhere in this book).

5. Future Work in the Study of Haloarchaeal Diversity and the Microbial Ecology of Salt Lakes

We are now able to grow all the major groups of haloarchaea that have been observed in conventional (neutral) saltern crystalliser ponds, and that also occur in many natural salt lakes. The full diversity of salt lakes is still being studied, and there remain many 16S rRNA sequences discovered that lie outside of the formally accepted taxa. These results indicate that there is much left to understand regarding the microbial diversity of salt lakes with saturating or near saturating salt concentration, such as:

a) examining the distribution, dynamic changes and ecological significance of known haloarchaea.

b) identifying novel haloarchaea *and* culturing representatives of these groups.

c) understanding why certain groups tend to reach significant fractions of the microbial population, while others do not.

d) characterizing the metabolic characteristics of the dominant groups.

These topics will be fruitful areas of study for many years to come, and should provide the insights necessary to understand what is happening in such waters around the world, as well as their significance in terms of biodiversity, biological productivity, and ecology. The biotechnological potential of salt lakes and haloarchaeal enzymes is likely to exceed our current, modest predictions based on examples such as bacteriorhodpsin and carotenoids.

6. Halovirus Diversity and Molecular Biology

Viruses outnumber cells in aquatic environments (including hypersaline waters) by 10-100 fold (Oren et al., 1997; Wommack and Colwell, 2000), making them by far the predominant organism. In the Dead Sea, virus-like particles were estimated to number 7 x 10^7 particles/ml (Oren et al., 1997), with unusual, spindle-shaped particles being more common than head-tail (typical bacteriophage) particles. Protozoa that graze on bacterial cells are absent in ponds above 20% salinity, and viral lysis of infected cells is believed to be responsible for the majority of (host) population turnover and nutrient cycling. Indeed, virus levels increase significantly at salinities above 15% (Pedrós-Alió et al., 2000) and viral lysis is estimated to affect 7% of the total population per day, a significant figure given the long doubling time of about 2 days for the *in-situ* prokaryotic population.

Given the dominance of viruses in fresh, saline and hypersaline waters, it is not surprising that they play key roles in the evolution and dynamic population structure of their prokaryotic hosts (e.g. Proctor and Fuhrman, 1990; Wommack and Colwell, 2000). Examples of host resistance mechanisms (e.g. restriction-modification), lysogenic conversion (e.g. virulence factors in pathogenic bacteria), and transduction spreading genes throughout a population (e.g. antibiotic resistance genes) are well known in bacteria. Genome sequences of viruses and their hosts have shown that significant fractions of host DNA can be made up of integrated phage genomes (eg. about 10% in *Bacillus subtilis*) and recombination events between viruses or between virus and host are common and widespread. Over the last ten years we have isolated several novel haloviruses directly from Australian salt lakes, and have been analyzing them at the molecular level (Dyall-Smith et al., 2003).

Of the head-and-tail haloviruses, the best studied are two pairs of related viruses: ΦH/ΦCh1, and HF1/HF2. The first pair (ΦH/ΦCh1) was described by Reiter et al. (1988) and Witte et al. (1997), respectively, and was isolated from laboratory strains of haloarchaea. They are temperate, head-tail viruses with linear dsDNA genomes of 55-59kb (Klein et al., 2002; Reiter et al., 1988; Stolt and Zillig, 1994; Witte et al., 1997). Unfortunately, ΦH and its host (*Hbt. salinarum*) suffer from extraordinarily high mutation rates caused by active insertion sequences and work on it stopped in the 1990s (W. Zillig, personal communication). The ΦH/ΦCh1 group may also include another described halovirus, Hs1 (Torsvik, 1982; Torsvik and Dundas, 1980), however a problem with all of the early halovirus isolates is that no extant samples exist in culture collections and it is believed that many have been lost, probably including Hs1.

HF1 and HF2 are lytic, have similar morphologies (68 nm head diameter, 90 nm tail length), and contain relatively large linear dsDNA genomes (76-77 kb). Recent, preliminary work on virus structural proteins indicate HF2 virions possess 2 major proteins (~40 and 28 kDa), presumably corresponding to the major head and tail proteins, and at least one larger, minor protein (B. Russ, unpublished data). The two genomes share 94% sequence identity but the differences are all clustered in the late gene region (structural, assembly and packaging genes). The first 48 kb of their 77 kb genomes are identical (except for a single base change), and we believe this unusual pattern indicates a recent recombination event, indicating that high rates of recombination occur between these haloviruses in nature (Tang et al., 2004).

Transcription in HF2 appears to be closely regulated, with early, middle and late transcripts being observed over the course of an infection cycle (Tang et al., 2002). In 2003, we made a preliminary survey of putative HF2 promoters identified in the transcription mapping studies, and were surprised to discover that the strongest promoters were a set of long, intergenic repeat sequences (seven class 1 and two class 2 repeats). These repeats were recognized at the time the HF2 genome was sequenced, and were at strategic positions relative to the transcription of open reading frames (ORFs), but their function was mysterious. When tested for promoter activity using the *bgaH* (halophilic β-galactosidase) reporter gene, the class I repeats (found between early-middle genes) gave very strong signals (T. McAlpine, H. Camakaris, and B. Russ, unpublished data), and we are now studying them in detail to determine the critical residues for activity and regulation (B. Russ, work in progress).

Viruses with a spindle or lemon-shaped morphotype have only been found in Archaea (Stedman et al., 2003), and are a dominant morphotype in hypersaline lakes (Guixa-Boixareu et al., 1996; Oren et al., 1997). The only isolated halovirus examples are His1 and His2 (Bath and Dyall-Smith, 1998, 2004; Dyall-Smith et al., 2003), which both produce clear plaques on *Har. hispanica*, do not form prophage states, and could be classified as lytic. However, it is possible that they exit through the cell wall without requiring cell lysis, with cells falling apart later (from a lack of cell maintenance). This would resemble SSV1, a lemon-shaped virus of *Sulfolobus* (a thermophilic archaeon), that can exit through the host cell wall with little or no lysis (Prangishvili et al., 2001). Although morphologically similar, SSV1 shares no sequence similarity to His1/His2 and has a different genome structure (circular dsDNA) and replication strategy (Stedman et al., 2003). Surprisingly, His1 and His2 genomes have terminal proteins, inverted terminal repeats, and code for a putative DNA polymerase with similarity to protein-primed DNA polymerases (C. Bath, unpublished data). While His1 and His2 share almost no sequence similarity to each other, the predicted protein sequences of their DNA polymerase genes show they are specifically related (C. Bath, manuscript in preparation). Although only speculative at present, it might be anticipated that the spindle-shaped virus-like particles observed in hypersaline lakes encompass a wider range of virus types than exemplified by His1/His2.

Another pair of novel haloviruses, that may represent a significant morphotype in hypersaline lakes, is PH1 and SH1. They are lytic (but may also exit the same way as His1/His2) and infect *Har. hispanica* and a natural *Halorubrum* isolate. Particles are round (~70 nm diameter), have a layered structure, and may contain an internal lipid membrane. Their genomes are linear, dsDNA of about 28-30 kb (Dyall-Smith et al., 2003); unpublished data; work in collaboration with Prof. D. Bamford). In the study of Oren et al. (1997), the second most frequent virus morphotype found in the Dead Sea, about half the concentration of lemon-shaped particles, were ‘polyhedral’ (i.e., round or icosahedral in outline). A proportion of these may represent heads that have dislodged from head-tail phages, but we suspect the majority were intact halovirus particles, similar to PH1 and SH1.

7. Future Studies of Haloviruses

The true diversity of haloviruses is yet to be assessed. At present, a handful of halovirus genomes have been sequenced, but the ease and power of DNA sequencing will allow much more comprehensive surveys, of both isolated and naturally occurring examples (e.g. metagenomic sequencing of salt lake DNA). It is to be hoped that this information will provide a useful framework and background for deeper studies of specific isolates, and allow the development of tools, such as microarrays, for following virus successions over time and across geographical regions.

At the level of individual viruses, we are still at the early phases of study. While they may possess novel characteristics, or show similarities to other known viruses, they are also relatively simple organisms that can reveal much about archaeal genetics, particularly the control of gene expression. Haloarchaeal genetics still suffers from comparison to *Escherichia coli* genetics, where there are many sophisticated methods and a wide range of host mutants, coupled with a rapid growth rate, that greatly assist investigators. Gene reporters have been developed, including *bgaH* (Holmes and Dyall-Smith, 2000) and GFP, but as yet there is no *in vitro* transcription system for haloarchaea, no reliable gel shift assay for studying DNA-binding proteins, no tightly-controllable and convenient haloarchaeal plasmid vector for protein over-expression, nor are there any suppressor host strains (for any Archaea). These gaps, among others, need to be addressed.

The balance between haloviruses and their hosts is also a fascinating area of study. Salt lakes are remarkable for their low biological diversity and, given that all the major groups of microorganisms present in these lakes can now be grown, the dominant viruses should also be accessible. It is possible to imagine comprehensive studies of natural salt lake ecosystems, where changes in hosts and parasites can be closely monitored over extended periods of time in order to understand the selective pressures and their evolutionary consequences.

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