Russell H. Vreeland *Editor*

Advances in Understanding the Biology of Halophilic Microorganisms

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To paraphrase a well-known axiom: behind every man there is a great woman. In my case it is two especially great women.
One is the late Dr. Carol Litchfield. Without Carol I would never have discovered salt loving microbes. Without Carol's dedication to students, her teaching and her willingness to look past one's surface layers I would never have had the chance to go to graduate school. If I had never met Carol I am not sure where I would be today but I know it would not be editing this book. Prior to her death in 2012 Carol was my friend and mentor for over 40 years, she gladly listened to my ideas (some nutty) always provided sound counsel as well as several hundred letters of reference and often reviewed or edited my writings. Carol involved me in societies and intellectual discussions, she taught me science and how to be a scientist. She was the genesis of my career.
The second great woman who has my back is my best friend, my confidant and my conscience Susan Vreeland. For nearly 30 years this patient woman has put up with me as an absent minded professor and scientist

who leaps before he thinks and never quite listens properly. She has corrected the mess when I paint the wall (and the floor), saved the plants I forgot to water, watched my bees fly (or not) when I head off to some scientific or other gathering and just as often encouraged me to do things I never thought I would like doing. Susan finds hobbies I never knew I'd enjoy then patiently allows me to discover that enjoyment. Her love gets me out of the house and introduces me to new friends (Steve and Donna Doan to name some) or activities (think US Coast Guard Auxiliary, Ruritans, beekeeping). She knew before I did that it was time to retire from teaching then she worked on the house while I finished my duties. She raised our three children (I did help but she led the way) along with uncounted furry "kids." She is still leading the way as we move into new phases of life with our fossil hunting, our little farm and many other new adventures.

This book is dedicated to these two incredible women, one gone and one present, but without both of them I would not be here today. I love them both more than I can express.

Russell Vreeland *Editor*

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Chapter 1
Approaches Toward the Study of Halophilic Microorganisms in Their Natural Environments: Who Are They and What Are They Doing?

Aharon Oren

Introduction

Hypersaline lakes with salt concentrations exceeding 250 g/l are often characterized by very dense communities of halophilic microorganisms imparting a red coloration to the brines. Such red waters can be found in the North Arm of Great Salt Lake, Utah, in crystallizer ponds of solar salterns for the production of salt from seawater, and in many extremely hypersaline alkaline lakes. At times even the magnesium chloride-rich waters of the Dead Sea have become red as a result of massive development of pigmented salt-loving microorganisms.

As conditions become more extreme with respect to salinity the microbial diversity decreases, but community densities can become very high: cell counts of over 10^7 prokaryotes/ml are very common, and even numbers above 10^8 ml have been recorded (Javor 1983, 1989; Oren 2002a). Among the organisms inhabiting such brines the most prominent are generally *Archaea* of the family *Halobacteriaceae*, pigmented pink-red by carotenoid pigments (α-bacterioruberin and derivatives) and possibly by bacteriorhodopsin and other retinal pigments as well. Other types of microorganisms may also contribute to the red color of the brines: the extremely halophilic *Salinibacter ruber* (*Bacteria*, *Bacteroidetes*) and the unicellular green alga *Dunaliella salina* which in addition to the photosynthetic pigments chlorophyll *a* and *b* may under certain conditions accumulate very large amounts of β-carotene within its chloroplast. Not all halophiles are pigmented, as shown, e.g., by the *Halomonadaceae*, a large family of moderately halophilic *Gammaproteobacteria*.

Our understanding of the variety of halophilic microorganisms inhabiting hypersaline water bodies and other high-salt environments is rapidly increasing and much information is available on the microbial diversity at high salt concentrations and on the properties of the organisms isolated from hypersaline lakes, soils,

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Dedicated to the memory of Carol D. Litchfield (1936-2012) who taught me much about halophiles.

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and other habitats. The different chapters in the present book are devoted to the taxonomy, physiology, and molecular biology of the diverse world of halophilic microorganisms.

This chapter explores the different approaches used to obtain information on the life of the communities of halophilic microorganisms in their natural environment, especially in the brines of hypersaline lakes and saltern ponds. The key questions to be asked can simply be summarized as “how many organisms are present?”, “who are they?”, “how do they make a living”, and “how do they interact with each other?” These are all basic questions, to be asked in microbial ecology studies of any environment, not only for high-salt ecosystems. However, the special properties of many groups of halophilic microorganisms, as deduced from pure culture studies, can often be exploited to learn more about the behavior of the microbial communities in their natural environment. Much of our understanding of hypersaline systems has of course also been contributed using “standard” techniques commonly used in ecological studies of “conventional” microbial ecosystems, whether or not in modified form as dictated by the special properties of the samples to be processed for analysis and the unusual features of some of the organisms present. The paragraphs below therefore provide an overview of the different approaches used in the study of hypersaline microbial ecology and the nature of the information gained while using each of these approaches, with emphasis on those approaches and methods that exploit the special nature of specific groups of halophilic microorganisms present in the environments being explored. Table 1.1 gives an overview of some of the most important approaches used in such studies, as well as examples of representative studies that have employed those techniques.

Characterization of the Microbial Communities in Hypersaline Brines—Qualitative and Quantitative Approaches

General Considerations

For the enumeration of microorganisms in hypersaline brines, microscopic techniques are used that are no different from those employed in other aquatic environments. Most known halophiles, *Archaea* as well as *Bacteria*, have rather large cells (1–2 μm and more in diameter), and their cell density can be assessed quite reliably using a Petroff-Hauser counting chamber (1/50 mm depth) and a microscope equipped with phase-contrast optics. If necessary the cells can be easily concentrated using high-speed centrifugation. This simple method has been successfully used for the routine monitoring of prokaryote cell densities in the Dead Sea and in saltern crystallizer brines with cell numbers exceeding 5×10^6 ml (Oren 1983a; Oren and Gurevich 1995; Oren et al. 1996). Cells can also be counted in the fluorescence microscope after fixation with formaldehyde and staining with DAPI (3′,6-diamidino-2-phenylindole) (Pedrós-Alió et al. 2000a). This technique

Table 1.1 Some Approaches Used in the Study of Planktonic Communities of Halophilic Microorganisms in Salt Lakes and Saltern Ponds. For Details See Text

	Experimental approach	Key references	Comments
Community size and structure studies—general techniques	Microscopic enumeration by DAPI stain	Antón et al. 1999	
	Fluorescence *in situ* hybridization for detection of specific groups of organisms	Antón et al. 1999	Commonly used fixation and staining techniques needed modification at high salt
	LIVE/DEAD stain	Leuko et al. 2004	The standard protocol was adapted for use in hypersaline brines
	Flow cytometry	Estrada et al. 2004	Seldom used as yet
	Cultivation—isolation	Burns et al. 2004	With skill and patience most types of microorganisms in hypersaline environments can be cultivated
	Genetic fingerprinting methods	Casamayor et al. 2002	
	Analysis of 16S rRNA gene libraries	Benlloch et al. 1995	
	Use of other molecular markers, e.g. the *bop* gene	Pašić et al. 2005	
	Microarray studies—“Phylochip” and “GeoChip”	Parnell et al. 2010	
	DNA melting profile studies	Øvreås et al. 2003	Yields information on the complexity of the community and on the G+C content of its dominant component(s)
	Environmental genomic and metagenomic approaches	Legault et al. 2006; Narasingarao et al. 2012	
	Remote sensing for detection of chlorophyll and other pigments	Oren and Ben Yossef 1997	Little used; enabled the monitoring of the development of a bloom of *Dunaliella* in the Dead Sea
Community size and structure studies—approaches specific for hypersaline environments	Quantification of non-coccoid *Archaea* by dissolution of their cell walls with bile acids	Oren 1989	

Table 1.1 (continued)

	Experimental approach	Key references	Comments
	Characterization of archaeal polar lipids by thin layer chromatography	Oren and Gurevich 1993	A convenient and rapid method to obtain information on the types of *Archaea* present
	Characterization of archaeal and bacterial lipids by electrospray ionization mass spectrometry or MALDI-TOF/MS	Corcelli et al. 2004; Lopalco et al. 2011	A high-resolution and quantitative method to obtain information on the lipid content of the community
	Use of fatty acids as biomarkers for specific groups of organisms	Ionescu et al. 2007	Can be used to obtain information on the types of *Bacteria* and *Eukarya* in the community
	HPLC separation and quantification of hydrophobic pigments	Oren and Rodríguez-Valera 2001	Can be used to separate archaeal bacterioruberins, and carotenoids of algae and photosynthetic and non-photosynthetic *Bacteria*
	Quantification of bacteriorhodopsin	Oren and Shilo 1981	
	Qualitative and quantitative determination of osmotic solutes in natural communities	Oren et al. 1994	
	Assays for the presence of halocins	Kis-Papo and Oren 2000	
Assessment of microbial activities—general techniques	Assessment of the potential metabolism of different carbon sources on Biolog plates	Litchfield et al. 2001	Does not function well at salinities above 140–150 g/l
	Monitoring of the effect of additions of substrates on community respiration rates	Warkentin et al. 2009	
	Measurement of incorporation rates of radiolabeled substrates (thymidine, amino acids)	Pedrós-Alió et al. 2000a	

Table 1.1 (continued)

	Experimental approach	Key references	Comments
Assessment of microbial activities—approaches specific for hypersaline environments	Use of specific inhibitors (antibiotics, bile acids) in labeled substrate incubation studies	Elevi Bardavid and Oren 2008b	Can be used to discriminate activities due to *Archaea* and to *Bacteria*
	Measurements of the availability and turnover of key compounds such as compatible solutes	Oren 1993	Relevant compounds are e.g. glycerol and glycine betaine
	Assessment of the availability and turnover of metabolites produced from compatible solutes	Oren and Gurevich 1994	Relevant compounds are, e.g., lactate, acetate, and dihydroxyacetone

also allows the detection of smaller cells. Recently a new group of halophilic *Euryarchaeota*, distantly related to the class *Halobacteria*, with cells of only about 0.6 μm in size, was discovered in saltern crystallizer ponds in Spain and California and in Lake Tyrrell, NW Victoria, Australia. These "Nanohaloarchaea" appear to be abundant in many hypersaline brines. No representatives of the group have yet been cultured, but based on their genome they probably lead a photoheterotrophic and polysaccharide-degrading life style (Ghai et al. 2011; Narasingarao et al. 2012). High-throughput enumeration of cells in hypersaline brines by flow cytometry after staining with DAPI or other fluorescent markers, a technique that has gained popularity in marine microbiology, has not yet been used in hypersaline waters to my knowledge. Phototrophic planktonic cells can be detected by flow cytometry thanks to the autofluorescence of chlorophyll and (for specific groups such as cyanobacteria) phycocyanin and phycoerythrin. This enabled the enumeration of primary producers in the plankton of saltern ponds of different salinities (Estrada et al. 2004). To what extent the procedure is effective for the enumeration of *Dunaliella salina* in crystallizer ponds is not yet clear; these cells often have little chlorophyll and their massive amounts of β-carotene will effectively absorb the excitation light (wavelength 488 nm as used in the setup employed by Estrada et al. 2004) used for detection of chlorophyll. Therefore more basic research is needed to assess the suitability of flow cytometry for phytoplankton studies in hypersaline brines, and if necessary to modify the standard protocols and optimize these for use in high-salt environments.

The determination of colony counts on agar plates or assessment of growth following dilution of the brine sample in tubes with liquid medium (the "most probable number" approach for the quantification of numbers of viable cells) generally give numbers much below those obtained by microscopic enumeration of cells in the same samples. This is by no means surprising as the "great plate count anomaly" is a general phenomenon in microbiological studies of environmental samples, and it

is by no means restricted to hypersaline environments. For the same reason it is generally not possible to draw any significant qualitative conclusions about the nature of the microorganisms dominating the community from the properties of the most frequently encountered type of cells in culture experiments.

Recent advances in our understanding of the nature of the community of prokaryotes most often found in saltern crystallizer ponds, as based on molecular, culture-independent studies (see below), have taught us that in many, if not in most salterns worldwide, the community in the salt-saturated ponds is dominated by the flat square archaeon *Haloquadratum walsbyi* (an organism that resisted attempts toward its cultivation for nearly a quarter of a century since its existence was first recognized in 1980) and the rod-shaped bacterium *Salinibacter ruber*. Both these organisms were only recently brought into culture (Antón et al. 2002; Burns et al. 2007), and the first is still rather difficult to isolate and cultivate. This does not necessarily imply that culture-dependent techniques based on the incubation of dilutions of brines on agar plates may not be useful to obtain qualitative as well as quantitative information about the community present. In some studies the numbers of colonies grown on agar plates approached the number of cells observed microscopically after staining with DAPI. Thus, brines of 300–330 g/l salt from Aran-Bidgol salt lake (Iran) yielded $2.5–4 \times 10^6$ colonies/ml, compared with $3–4 \times 10^7$ microscopic counts per ml (70–75 % *Archaea*, 25–30 % *Bacteria*, as based on fluorescence *in situ* hybridization) (Makhdoumi-Kakhki et al. 2012). A study in a saltern crystallizer pond in Australia showed that nearly all types of prokaryotes that could be identified using culture-independent, 16S rRNA gene sequence-based methods could also be cultured. Appropriate growth media should be used, preferentially media with low nutrient concentrations, but the main factor of importance is the use of long incubation times (2–3 months and longer) (Burns et al. 2004). Generally few microbiologists have the patience to wait so long, which explains why the method is not used more generally. Still, the success obtained in growing all or nearly all types of organisms present implies that the saltern crystallizer environment may well be the first ecosystem for which the “great plate count anomaly” no longer exists.

Many methods used in studies of microbial communities in freshwater and marine environments can be used for hypersaline brines, if necessary with minor modifications as dictated by the nature of the samples. Thus, use of the LIVE/DEAD® *Bac*Light™ kit to discriminate between intact, potentially viable and dead cells with a damaged membrane has been explored for hypersaline waters as well. Control experiments in which cultures of living and killed cells of different species were added to brines showed that the method can detect live and dead cells not only of *Bacteria*, but also of halophilic *Archaea* (Leuko et al. 2004). Detailed protocols have been described for use of the method in hypersaline environments (Stan-Lotter et al. 2006), but the adapted protocol has not yet been extensively used in field studies to assess the live/dead status of cells in brines of natural salt lakes and salterns.

One of the most powerful techniques to obtain qualitative as well as quantitative information on the types of microorganisms present in natural communities of *Archaea*, *Bacteria*, as well as *Eukarya*, is fluorescent *in situ* hybridization (FISH). Here fluorescently labeled probes are synthesized, designed to react with specific

nucleic acid sequences, generally targeting sequences on the small-subunit rRNA (16S for prokaryotes, 18S for eukaryotes). Based on sequence information derived from the study of pure cultures or from DNA extracted directly from the environment it is possible to synthesize probes to specifically stain almost any desired group of organisms, from general probes ("all *Bacteria*," "all *Archaea*") to probes that will react only with one genus or one species. The FISH technique was adapted for use in hypersaline brines by Antón et al. (1999), and the method has been applied to characterize the microbial communities in saltern ponds in Spain and in Peru (Antón et al. 2000; Maturrano et al. 2006). Application of FISH methodology to samples from crystallizer ponds in Spain first led to the recognition that a slightly curved rod-shaped extremely halophilic member of the *Bacteria* is consistently present in large numbers in these salt-saturated brines (Antón et al. 1999), a recognition that rapidly led to the isolation of the novel type of organism and its description as *Salinibacter ruber* (Antón et al. 2002).

Use of Specific Biomarkers in Ecological Studies in Hypersaline Environments

The specific nature of the types of halophilic microorganisms that abound in natural hypersaline lakes and in solar salterns, *Archaea*, *Bacteria*, as well as *Eukarya*, and the often very specific cellular components they contain, can be exploited in qualitative as well as in quantitative studies of hypersaline ecosystems. Some of these components can be used as general biomarkers for a broad group of organisms, while others are more specific for certain taxa. While some of these biomarkers are easily assayed, more advanced analytical facilities are required for the detection and quantification of others.

Most halophilic *Archaea* of the family *Halobacteriaceae* have a cell wall built of subunits of a high-molecular-weight acidic glycoprotein. This glycoprotein cell wall easily breaks down in the presence of low concentrations (20–50 mg/l) of bile acids such as desoxycholate or taurocholate, causing lysis of the cells. This phenomenon was first documented more than 50 years ago and already then proposed as a technique to discriminate between different types of halophiles (Dussault 1956), but it was subsequently forgotten to be rediscovered in the late 1980s (Kamekura et al. 1988). Members of the genus *Halococcus* form an exception as they contain a complex thick and highly stable polysaccharide cell wall; halophilic representatives of the *Bacteria* are also resistant to lysis by such low bile acid concentrations. Cell lysis by bile acids was therefore proposed to differentiate between non-coccoid halophilic *Archaea* and other types of microorganisms, and the approach was used to estimate the contribution of *Archaea* to the prokaryote community in saltern ponds of different salinities (Oren 1989).

Polar lipids are perfect biomarkers to monitor the community composition of halophilic microorganisms in hypersaline brines. First of all, the nature of archaeal

lipids (diphytanyl diether lipids) is fundamentally different from the lipids of *Bacteria* and *Eukarya* (glycerol with ester-linked predominantly straight-chain fatty acids). Also among the different genera of *Archaea* of the family *Halobacteriaceae* there is considerable variation with respect of the types of polar lipids present, and certain lipids can be used as biomarkers for specific genera. All known genera have the diphytanyl diether derivatives of phosphatidylglycerol and the methyl ester of phosphatidylglycerol phosphate. Phosphatidylglycerol sulfate is absent in some genera. Most non-alkaliphilic members of the family possess at least one type of glycolipid, with some having sulfated diglycosyl diether lipids (the most commonly encountered type), while others have triglycosyl and/or tetraglycosyl lipids, with or without one or more sulfate groups. Presence or absence of these polar lipids can easily be assessed by thin layer chromatography of a lipid extract on silica gel plates followed by staining with specific reagents (staining phosphate, glycolipids, etc.), while pure cultures of representatives of different genera can serve as standards. Thin layer chromatography of polar lipids in extracts of biomass collected from the hypersaline brines can yield information about what genera of *Halobacteriaceae* may be present, and also give some semi-quantitative indications in case more than one marker lipid is detected. The approach has been extensively used in the analysis of the archaeal communities present in a red bloom in the Dead Sea (Oren and Gurevich 1993) and in saltern evaporation and crystallizer ponds (Oren 1994; Litchfield et al. 2000; Litchfield and Oren 2001). Thin layer chromatographic analysis of a saltern crystallizer brine community dominated by the square gas-vaculolate archaeon later described as *Haloquadratum walsbyi* provided information on the lipid composition of this elusive organism (Oren et al. 1996), information that was confirmed later by electrospray ionization mass spectrometry (ESI-MS) after the organism had been brought into culture (Lobasso et al. 2008).

The technique of electrospray ionization mass spectrometry is technically much more demanding than simple thin layer chromatography, but it enables the analysis of complex mixtures of lipids, archaeal as well as bacterial (Lattanzio et al. 2002; Corcelli et al. 2004; Corcelli and Lobasso 2006). Figure 1.1 gives an example of the power of the method when applied to an extract of lipids obtained from the biomass sampled from an Italian saltern crystallizer pond at Margherita di Savoia. Most of the signals could be assigned to known archaeal polar lipids, showing that *Archaea* dominated the community. Other peaks can be attributed to the presence of glycerol ester lipids in the extract, including the recently characterized novel type of sulfonolipid (molecular mass 660 Da) that is characteristic of *Salinibacter ruber* (*Bacteroidetes*) (Corcelli et al. 2004). Application of MALDI-TOF/MS to lipid extracts of biomass collected from the same saltern system enabled a high-resolution analysis of the community by qualitative as well as quantitative detection of glycolipids: the sulfated diglycosyl glycolipids characteristic of the genera *Haloferax*, *Halorubrum*, and *Haloquadratum*, other glycolipids attributed to *Halobacterium* and to *Haloarcula*, as well as the specific sulfonolipids of *Salinibacter* (Lopalco et al. 2011).

Bacterial- and eukaryotic-type fatty acids can in some cases serve as biomarkers as well. Since the lipids of the extremely halophilic *Salinibacter ruber* have a high content of 15:0 iso, a fatty acid not frequently found in most other groups of

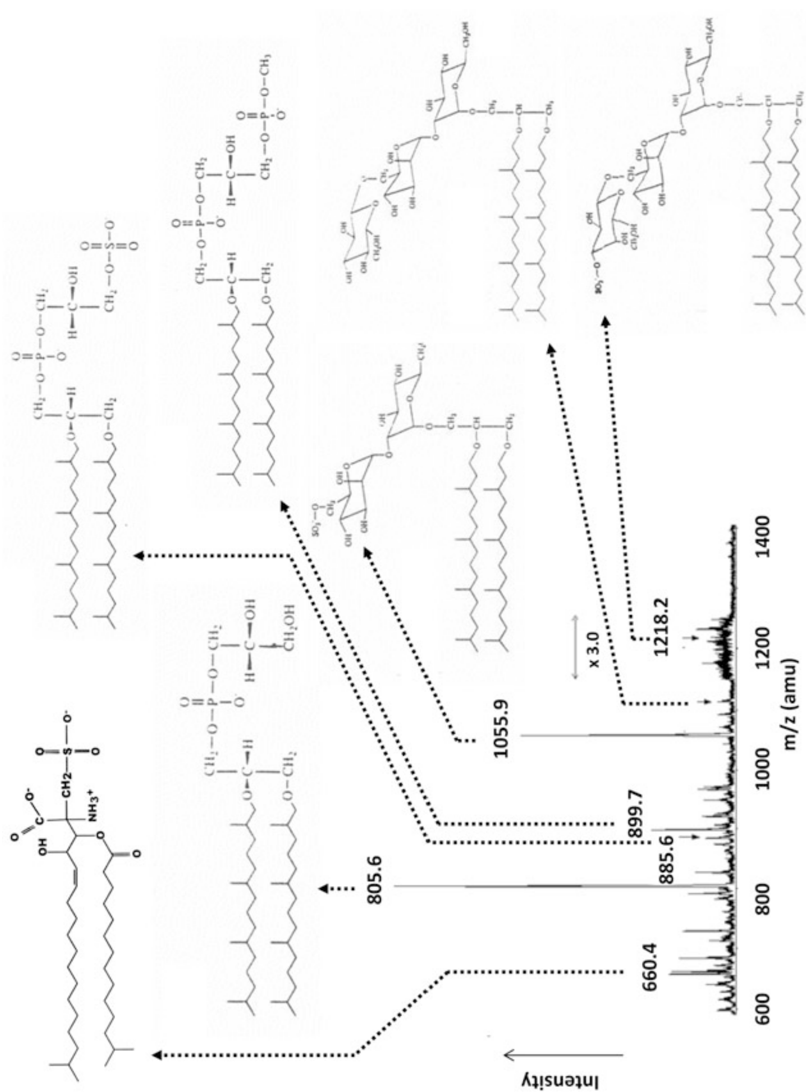

Fig. 1.1 Electrospray ionization mass spectrometry (negative ion) profile of the lipid extract obtained from the biomass of crystallizer brines of Margherita di Savoia (Italy) (reproduced from Corcelli et al. 2004; reproduced by permission of the American Society for Microbiology) (amu, atomic mass unit), and the structures assigned to some of the major peaks: the *Salinibacter* sulfonolipid ($m/z = 660.4$), the archaeal diphytanyl derivatives of phosphatidylglycerol ($m/z = 805.6$), phosphatidylglycerol sulfate ($m/z = 885.6$), the methyl ester of phosphatidylglycerol phosphate ($m/z = 899.7$), and different glycolipids: sulfated diglycosyl diether lipids attributable to genera such as *Haloquadratum*, *Haloferax* and *Halorubrum* ($m/z = 1055.9$), a triglycosyl diether lipid characteristic of the genus *Haloarcula* ($m/z = 1138$), and a sulfated triglycosyl diether lipid that may belong to *Halobacterium* ($m/z = 1218.2$)

microorganisms encountered in hypersaline environments, it should be possible to use this fatty acid as a biomarker for *Salinibacter* and related organisms (such as *Salisaeta*; Vaisman and Oren 2009). To my knowledge this option still has not yet been explored.

The use of fatty acids as biomarkers for specific types of halophilic cyanobacteria has been examined in a hypersaline gypsum crust on the bottom of a saltern evaporation pond (salinity about 200 g/l) in Eilat, Israel (Oren et al. 1995b). The presence or absence of polyunsaturated fatty acids could be correlated with the mode of life of the cyanobacteria in the different vertical layers in the crust. The unicellular *Aphanothece*-like cyanobacteria in the upper layer had a high content of di-unsaturated fatty acids, the synthesis of which requires molecular oxygen. On the other hand, polyunsaturated fatty acids were absent in the *Phormidium* layer below, and the types of mono-unsaturated fatty acids found in this layer pointed to a "bacterial," oxygen-independent fatty acid biosynthesis in this layer that at night and during a large part of the day as well is devoid of molecular oxygen and contains sulfide (Canfield et al. 2004; Oren et al. 2005, 2009; Ionescu et al. 2007).

Pigments are also very useful biomarkers in field studies of halophiles, especially at the highest salt concentrations. The diverse types of halophiles contain different types of specific pigments that, at least for the carotenoid pigments, can easily be extracted and analyzed on the basis of their absorption spectra. There is no *a-priori* need to separate the pigments by reversed phase high performance liquid chromatography before analysis. Halophilic *Archaea* of the family *Halobacteriaceae* are characteristically red because of their high content of the 50-carbon carotenoid α-bacterioruberin and its anhydro- and bis-anhydro derivatives. Only rarely are colorless halophilic *Archaea* found; the discovery of *Natrialba asiatica*, isolated from beach sand in Japan, is the exception that confirms the rule. *Salinibacter ruber*, another frequently encountered component of saltern crystallizer pond communities, is colored orange-red by salinixanthin, a C_{40}-carotenoid glycoside esterified with a fatty acid (C15:0 iso) (Lutnæs et al. 2002). A third type of red organism abundant in many hypersaline systems is *Dunaliella salina*, which under certain conditions may accumulate β-carotene to over 10–15 % of its dry weight. Quantitatively, *Dunaliella*-derived β-carotene is often the most abundant pigment in the saltern biomass, but the color of the brine is generally due to the archaeal bacterioruberin pigments. This apparent paradox is explained by the different location of the pigments within the organisms: while the archaeal carotenoids are distributed evenly over the cell membrane, the β-carotene in *Dunaliella* cells is densely concentrated in small granules located between the thylakoid membranes of the chloroplast, and therefore the pigment contributes little to the optical properties of the brine (Oren et al. 1992; Oren and Dubinsky 1994; Oren 2009). Analysis of absorption spectra of pigment extracts of biomass collected from the Dead Sea provided quantitative information on the amounts of bacterioruberin pigments at different depths and in different seasons, enabling easy monitoring of the rise and decline of archaeal blooms in the lake (Oren 1983a; Oren and Gurevich 1995). The additional possibilities for quantitative analysis provided by HPLC-based methods are illustrated in Fig. 1.2, which shows the separation and spectral analysis of the different pigments extracted from a cell pellet obtained by centrifugation of a

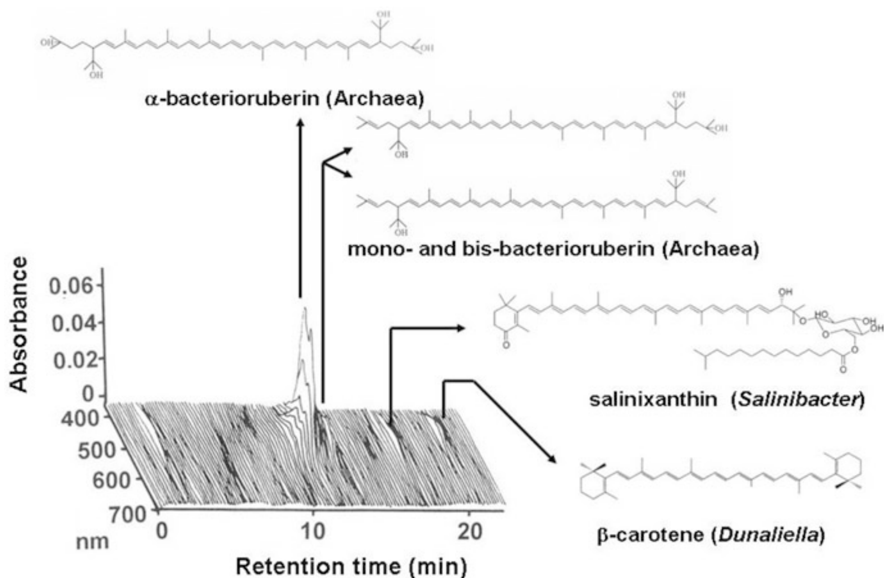

Fig. 1.2 HPLC separation and characterization of carotenoid pigments extracted from a sample of a saltern crystallizer pond in Santa Pola, Alicante, Spain. The biomass was collected by centrifugation, resulting in loss of the majority of the *Dunaliella* cells which were buoyant because of their high β-carotene content, so that levels of β-carotene were low and chlorophyll *a* and *b* were not detected in this experiment. For further details see Oren and Rodríguez-Valera 2001 and Lutnæs et al. 2002

sample from a Spanish saltern crystallizer pond. The chromatogram clearly shows the quantitative relationships between the archaeal bacterioruberins and the *Salinibacter* -derived salinixanthin that elutes later from the chromatography column (Oren and Rodríguez-Valera 2001). The amount of *Dunaliella*-derived β-carotene in the sample was small due to the way the cells were collected by centrifugation. *Dunaliella* cells with a very high β-carotene, as found in many saltern crystallizer ponds, tend to float rather than sink during centrifugation. A pigment extract of cell material collected from the same brine sample by filtration would have been dominated by algal carotenoids.

Protocols for the extraction of carotenoids by organic solvents also release chlorophyll derivatives, and their specific absorption spectra can be used to obtain quantitative information on the presence and distribution of algae and cyanobacteria in hypersaline environments such as saltern evaporation ponds (Estrada et al. 2004) and the Dead Sea (Oren and Shilo 1982; Oren et al. 1995a). To monitor the development of a *Dunaliella* bloom in the Dead Sea in the spring of 1992, remote sensing by satellites proved to be a valuable tool as well (Oren and Ben-Yosef 1997). A recent study in which techniques of emission spectroscopy and kinetic fluorometry were applied to the benthic microbial mats in saltern evaporation ponds in Eilat, Israel showed that such hypersaline systems may contain additional, yet uncharacterized photosynthetic pigment systems. Indications were obtained for the presence

of chlorosomes-containing anoxygenic phototrophs in high-salinity mats, but the nature of these organisms is still unknown (Prášil et al. 2009).

An entirely different group of pigments specific to halophilic microorganisms are the bacteriorhodopsin light-driven primary proton pumps and halorhodopsin light-driven chloride pumps present in many members of the *Halobacteriaceae*. Similar pigments were detected in *Salinibacter*, where xanthorhodopsin is the functional equivalent of bacteriorhodopsin of *Halobacterium* and related *Archaea*. Xanthorhodopsin has aroused considerable interest in the past few years as it uses the carotenoid salinixanthin as a light harvesting antenna, a phenomenon without equivalent in the halophilic *Archaea*. All these retinal pigments are small (about 25 kDa) membrane-bound proteins that contain the 20-carbon retinal as prosthetic group bound to a lysine residue. Similar light-driven retinal-containing proton pumps (proteorhodopsin) also function in marine bacterioplankton. To some extent the purple (absorption maximum at 570 nm) bacteriorhodopsin may also contribute to the red-pink color of hypersaline brines. Thus far there have only been few attempts to obtain quantitative information on the presence of retinal proteins in natural communities of halophilic microorganisms. In contrast with the methods used for extraction and analysis of carotenoids described above, there are no simple methods for the extraction and quantitative analysis of such retinal pigments. Analysis of absorption spectra of intact cells or their membranes provided some information on the occurrence of bacteriorhodopsin in the Dead Sea (Oren and Shilo 1981) and in saltern crystallizer ponds (Javor 1983); spectroscopic techniques, routinely used in studies of the presence and functioning of retinal proteins in pure cultures or in purified membrane preparations, have only seldom been applied to measurements in natural communities of halophiles (for an exception see Stoeckenius et al. 1985).

A type of biomarker extensively used in pure culture studies of halophilic and halotolerant microorganisms but almost entirely neglected in studies of the communities of such organisms in their natural environment is the presence of specific osmotic solutes. In order to withstand the osmotic pressure of the hypersaline environment each cell has to balance the osmotic pressure of its intracellular space so that no water will be lost to the environment. In most types of cells the osmotic pressure inside the cell should even be somewhat higher than that of the environment so that a turgor pressure can be maintained; however, no significant turgor pressure appears to be present in the *Halobacteriaceae*. Some prokaryotes use inorganic salts (K^+, Cl^-) for osmotic balance. This strategy of osmotic adaptation is used by the halophilic *Archaea* of the family *Halobacteriaceae*, by the anaerobic fermentative *Bacteria* of the order *Halanaerobiales*, and also by the extremely halophilic member of the *Bacteroidetes*, *Salinibacter ruber*. All other types of halophilic and halotolerant microorganisms keep their intracellular ion concentrations low, and they produce and accumulate organic solutes instead. The variety of these organic osmotic ("compatible") solutes is great; *Dunaliella* uses glycerol, halophilic cyanobacteria produce glucosylglycerol or glycine betaine, and many heterotrophic *Bacteria* synthesize ectoine, hydroxyectoine, other amino acid derivatives, and/or simple sugars. Glycine betaine is also synthesized by many anoxygenic phototrophic bacteria (see e.g., Oren 2006).

The ecological significance of the accumulation of organic osmotic solutes has been recognized long ago (Oren 1990a, 1993), even to the extent that the production and turnover of certain osmotic solutes such as dimethylsulfoniopropionate (DMSP) synthesized by marine phytoplankton for osmotic balance may have a direct influence on the global climate (Welsh 2000). Another osmotic solute of interest in studies of the community dynamics in hypersaline ecosystems is glycine betaine, produced, e.g., by halophilic cyanobacteria and other photosynthetic prokaryotes. We know different pathways for the degradation of glycine betaine under aerobic and under anaerobic conditions, some of which yield trimethylamine as one of the products (Oren 1990a). Trimethylamine is the preferred substrate of halophilic methanogenic *Archaea*, and most of the methane evolved in hypersaline anaerobic sediments is probably derived from trimethylamine (Oremland and King 1989).

Detailed protocols for the sensitive detection and quantitative determination of such osmotic solutes have been developed (Roberts 2006). Such protocols have successfully been applied to a wealth of different pure cultures of microorganisms, and this has greatly contributed to our current in-depth understanding of the diversity of osmoadaptation strategies in the microbial world. Therefore it is surprising to note that so few attempts have yet been made to apply the existing methodology also to the (undoubtedly more complex) natural hypersaline environments to characterize the osmotic solutes present, their concentrations, and their dynamics. The direct demonstration of the presence of glucosylglycerol in a cyanobacterial mat dominated by *Microcoleus* (*Coleofasciculus*) (Oren et al. 1994) and the finding of glycine betaine in a *Phormidium* or *Oscillatoria* mat in a hypersaline sulfur spring at 170 g/l salt (Oren et al. 1994) are rare examples. More extensive studies of the distribution of organic osmotic solutes in a variety of hypersaline environments will undoubtedly deepen our insight into the functioning of the ecosystems and also possibly lead to the detection of novel, yet unknown compatible solutes.

Molecular Methods

In the past decade the number of studies in which culture-independent, nucleic acid-based approaches were used to obtain information on the nature of the communities of halophilic microorganisms in salt lakes and other hypersaline environments has greatly exceeded the number of studies that employed culture-dependent methods, different biomarkers, etc., as outlined in the section above. The reason for this is obvious: the techniques for preparation of gene libraries, gene fingerprinting, sequencing, metagenomics and environmental proteomics can be universally applied, and hardly any special adaptations of the methodology are required. While many other experimental approaches that work well in freshwater and marine systems have to be adapted and carefully calibrated before they can be applied to the study of hypersaline ecosystems, as soon as DNA and/or proteins have been extracted from the community all further protocols are the standard methods now routinely used in molecular environmental microbiology.

Some modifications of the standard protocols to isolate nucleic acids from communities of halophilic microorganisms may still be necessary. Most standard methods to break the cells and release the DNA from environmental samples were developed for *Bacteria* as the main target. In most cases these standard methods will also break up the fragile glycoprotein cell wall of the non-coccoid members of the *Halobacteriaceae*, which already lyses by suspension in low-salt solutions. However, for studies targeting members of the genus *Halococcus*, which possess a complex rigid sulfated polysaccharide cell wall, the methods for DNA extraction from the community should be adapted. Leuko et al. (2008) tested different protocols, including methods based on chemical lysis, enzymatic lysis and physical disruption, to optimize the recovery of *Halococcus* DNA while causing as little damage to the DNA as possible. A protocol based on incubation for 2 h at 63 °C in buffer containing potassium ethyl xanthogenate proved to be optimal for recovery of *Halococcus* DNA from environmental samples for molecular ecological studies.

Once satisfactory DNA preparations have been obtained, all standard protocols used in molecular environmental microbiology can be applied. The examples given below do not by any means provide a complete overview of the studies performed and the conclusions obtained from them, but should be considered as a representative selection only.

In an attempt to obtain information both on the microbial diversity and on the nature of the types of organisms dominant in the microbial communities inhabiting saltern evaporation and crystallizer ponds near Alicante, Spain, Øvreås et al. (2003) measured melting profiles and reassociation kinetics of the environmental DNA. The shape of the melting profile provided clear evidence for the great abundance of the square archaeon *Haloquadratum walsbyi* in the crystallizer samples, thanks to the fact that *Haloquadratum* DNA has a far lower content of G+C (46.9 mol%) than all other known members of the *Halobacteriaceae* (commonly in the range 60–70 mol%). Based on the renaturation kinetics estimates could be made of the complexity of the microbial communities in ponds of different salinities. The apparent complexity of the metagenome of a pond with 220 g/l salt was about 7 times that of the *Escherichia coli* genome, a pond at 320 g/l salt had a more complex community (13 times that of *E. coli*), while the renaturation profile of DNA isolated from a crystallizer pond with 370 g/l salt suggested presence of a relatively simple ecosystem with 4 times the complexity of the *E. coli* genome.

A survey of the molecular approaches used thus far to characterize the microbial communities in hypersaline environments shows that by far most studies have targeted the small-subunit rRNA genes—16S for *Archaea* and *Bacteria* and (to a lesser extent) 18S for *Eukarya* (Oren 2002b). This is not surprising in view of the general popularity of the approach that enables the tentative assignment of the sequences recovered to rRNA genes of known organisms (in relatively rare cases) or yet uncultured organisms (as is generally the case) on the basis of a comparison of the environmental sequences with the sequences in the database of the Ribosomal Database Project (http://rdp.cme.msu.edu). The approach of sequencing environmental rRNA genes was first applied to a hypersaline environment in the mid-1990s by the group of Francisco Rodríguez-Valera in Alicante, Spain (Benlloch et al. 1995,

1996). These studies first led to the recognition of a thus far unknown phylotype (at the time designated the SPhT phylotype), which was later identified to belong to the abundantly present flat square gas-vacuolate archaeon on the basis of fluorescence *in situ* hybridization studies (Antón et al. 1999), now isolated and described as *Haloquadratum walsbyi* (Burns et al. 2007).

Although the exact protocols used in the different studies may differ to some extent and different primers for PCR amplification have been employed by different workers, the overall outline of the many 16S rRNA gene based studies is very similar and is not greatly different from the protocols used for similar studies in "conventional," non-hypersaline microbial ecosystems (Litchfield et al. 2006). Such studies characteristically involve a PCR amplification step of the gene(s) targeted, preparation of a clone library and/or separation of the most frequently encountered gene sequences by denaturing gradient gel electrophoresis (DGGE), and different conventional fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) and amplicon length heterogeneity to obtain information on the diversity of the sequences retrieved (Litchfield and Gillivet 2002).

The number of published studies is large, and the following list of hypersaline environments probed with these techniques provides representative examples on different continents rather than an exhaustive survey of the literature:
In Europe:

- Saltern evaporation and crystallizer ponds in Spain (Benlloch et al. 1995, 1996,2002; Casamayor et al. 2002; Ochsenreiter et al. 2002),
- The sediments of Mediterranean salterns (Mouné et al. 2002),
- Salterns in Slovenia and Kroatia (Pašić et al. 2005, 2007),
- A small pond near a slag heap of a potassium mine in Germany (Ochsenreiter et al. 2002),

In Asia:

- Sediments from a gypsum crust of a saltern, Israel (Sørensen et al. 2005),
- Aran-Bidgol salt lake, Iran (Makhdoumi-Kakhki et al. 2012),
- Salterns in India (Manikandan et al. 2009),
- Salt lakes and soda lakes in Inner Mongolia, China (Pagaling et al. 2009; Ma et al. 2004),
- Mountain lakes on the Tibetan plateau (Wu et al. 2006),
- Salterns in Taiwan (Wang et al. 2007),

In Africa:

- Saltern ponds in Tunisia (Baati et al. 2008, 2010),
- Lake Magadi and other hypersaline soda lakes in Kenya (Rees et al. 2004),
- Lake Elmenteita, Kenya (Mwrichia et al. 2010),
- East African alkaline saltern ponds (Grant et al. 1999),
- The alkaline, hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah et al. 2007),

– The deep-sea brines of Kebrit Deep and Shaban Deep in the Red Sea (Eder et al. 1999, 2001, 2002),
– The alkaline evaporation ponds at Sua pan, Botswana, used for production of sodium carbonate (Gareeb and Setati 2009),

In America:

– The alkaline hypersaline Mono Lake, California (Humayoun et al. 2003),
– Sediment of the north arm of Great Salt Lake, Utah (Youssef et al. 2012),
– The alkaline Nevada Lake, Nevada (Ochsenreiter et al. 2002),
– Sediment from Great Salt Plains, Oklahoma (Youssef et al. 2012),
– Sediment below salt heaps in a salt processing plant, Oklahoma (Youssef et al. 2012),
– The archaeal communities inhabiting the benthic microbial mats at Guerrero Negro, Baja California Sur, Mexico (Robertson et al. 2009),
– The alkaline-saline soil of the former lake Texcoco (Mexico) (Valenzuela-Encinas et al. 2008),
– Salar Guayatayoc Lake, Salinas Grandes, Argentina (Pagaling et al. 2009),
– Lake Tebenquiche (Salar de Atacama, Argentina) (Demergasso et al. 2008),
– Salterns in the Peruvian Andes (Maturrano et al. 2006).

In some studies the intergenic spacer region between the 16S and the 23S genes in the rRNA operon was used as a phylogenetic marker (Ribosomal Internal Spacer Analysis; RISA). This technique enabled the estimation of the halophilic archaeal diversity within stromatolites and microbial mats of Hamelin pool, Western Australia (Leuko et al. 2007).

A general conclusion that emerged from the above-mentioned and other studies is that the true diversity as apparent from the 16S rRNA gene sequences retrieved is much larger than that previously estimated on the basis of the characterization of the organisms cultured from the same environments. This basic conclusion is no different from that generally obtained in such molecular ecological studies of microbial ecosystems. There thus remain many novel species and genera of halophiles to be isolated and described; the rRNA sequence data may be used as a guide when looking for colonies that may harbor the phylotype of interest. Another general conclusion is that, in spite of the superficial resemblance of hypersaline systems worldwide, especially when the salt concentration approaches saturation, the composition of the microbial community in salterns and other hypersaline environments of similar salinity and similar ionic composition can be markedly different at different geographic locations. The general dominance of *Archaea* at salinities above 200–250 g/l, as observed also on the basis of polar lipid analysis (see above), is confirmed by the 16S rRNA gene-based studies, but such studies also led to the recognition of the importance of the extremely halophilic member of the *Bacteria*, *Salinibacter ruber* (Antón et al. 1999, 2000). *Salinibacter* gene sequences have since been recovered from many saltern systems and natural salt lakes of near-neutral pH approaching halite saturation. Extensive studies on the distribution and properties of *Salinbacter*

in hypersaline lakes all over the world have shown that the species is markedly homogeneous, with only very little geographic variability in 16S rRNA gene sequence and in metabolic properties (Antón et al. 2008; Rosselló-Mora et al. 2008).

In addition to the small-subunit rRNA genes, which provide phylogenetic information on the types of organisms present, a number of functional genes have been used as markers to provide information on the microbial diversity in hypersaline environments. One of the most interesting genes for such studies is the *bop* gene that encodes for the protein moiety of the bacteriorhodopsin light-driven proton pump present in some, but not all, members of the *Halobacteriaceae*. The DNA extracted from a solar saltern on the Adriatic coast yielded 10 different *bop* phylotypes (Pašić et al. 2005). In a study of the vertical distribution of different types of microorganisms at different depths and in different seasons in Mono Lake, CA (85–95 g/l total dissolved solids; pH $\sim$ 9.5), sequences of the genes *cbbL* and *cbbM*, encoding form I and form II of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) were amplified. Only form I sequences were found. It was not possible to attribute the sequences obtained to known groups of chemoautotrophic or photoautotrophic microorganisms with the exception of a single occurrence of the *cbbL* gene of the cyanobacterium *Cyanobium* (Giri et al. 2004). Microarrays were used to probe the microbial communities in Great Salt Lake, Utah, at sites of different salinities, using both a “Phylochip” microarray containing probes for 8,741 bacterial and archaeal taxa and a functional gene array (“GeoChip”) to probe for the presence of specific functions encoded by the extracted DNA (Parnell et al. 2010).

In studies of anaerobic sediments of salt lakes, functional genes specific for dissimilatory sulfate reducing bacteria and methanogenic *Archaea* have been amplified from environmental DNA and sequenced to learn about the diversity of the microorganisms involved in the terminal processes of anaerobic degradation in the sediments. For the study of sulfate reducing bacteria, the *drsAB* genes, coding for the dissimilatory sulfite reductase, are the functional genes of choice. In the sediments of the North Arm of Great Salt Lake, Utah (salt concentration 270 g/l) the major lineage of dissimilatory sulfate reducers was closely related with the genus *Desulfohalobium*; but other lineages were detected as well that clustered with the *Desulfobacteriaceae* and with known sulfate reducers belonging to the *Firmicutes* (Kjeldsen et al. 2006). Gene sequences of *dsr* retrieved from the alkaline Mono Lake suggested presence of sulfate reducers affiliated with the *Desulfovibrionales*, the *Desulfobacterales*, as well as putative species of *Desulfotomaculum* and others (Scholten et al. 2005). The *dsrA* gene was also used as a functional marker to obtain information on the nature of the sulfate reducing bacteria in the deep hypersaline anoxic L’Atalante Basin and Urania Basin, located at the depths of the Eastern Mediterranean Sea. Presence of diverse communities of sulfate reducing bacteria was indicated, most of which were affiliated with the *Deltaproteobacteria* (van der Wielen and Heijs 2007). The *dsrAB* genes were also used as probes for the study of the sulfate reducing community in a brine pool (salinity up to 140 g/l) at a depth of 650 m in the Gulf of Mexico (Joye et al. 2009). In a study in Mono Lake, the gene *apsA* coding for adenosine phosphosulfate reductase was used as an additional marker to characterize the community of dissimilatory sulfate reducers (Scholten et al. 2005).

To investigate the diversity of methanogenic *Archaea* in hypersaline environments such as Mono Lake and the above-mentioned brine pool in the Gulf of Mexico, the *mcrA* gene was selected, the gene coding for methyl coenzyme A reductase, a key enzyme in the biochemical pathway leading to methane formation (Scholten et al. 2005; Joye et al. 2009).

Approaches using the technology of metagenomics were recently introduced in the study of the microbial communities in hypersaline ecosystems. After the genome of a reference strain (not the type strain) of *Haloquadratum walsbyi* had been sequenced, a fosmid clone library was prepared from DNA isolated from the $>2\ \mu m$ prokaryotic plankton of a Spanish saltern crystallizer pond in order to assess the genetic variability of the genus within a single *Haloquadratum* population. The analysis of the clones led to the discovery of a large pool of accessory genes within the otherwise coherent species. Many transposition and phage-related genes were identified in those genomic areas where heterogeneity was most pronounced (Legault et al. 2006). Metagenomics techniques were also applied in the characterization of the microbial communities in the deep-sea hypersaline Lake Thetis, discovered in 2008 at a depth of 3,258 m in the eastern Mediterranean Sea (Ferrer et al. 2012).

Analysis of the metagenome of a complex stratified microbial mat developing at a salinity of around 90 g/l in the shallow lagoons of Guerrero Negro, Baja California, Mexico, calculating the isoelectric points of the predicted proteins on a millimeter scale, showed a markedly acidic nature of most proteins in all layers (Kunin et al. 2008). A highly acidic proteome is characteristically found in microorganisms that use potassium chloride for osmotic balance, such as the members of the *Halobacteriaceae* and *Salinibacter*. Extremely halophilic organisms are probably not present at high densities at the relatively low salinity of the microbial mats examined. Cyanobacteria, anoxygenic phototrophic bacteria, and the different types of heterotrophic microorganisms present in the mats are expected to use organic "compatible" solutes for osmotic balance. The latter strategy does not require adaptation of the intracellular proteome to the presence of salt, which generally leads to a high excess of acidic over basic amino acids, resulting in a low isoelectric point. Kunin et al. (2008) thus concluded that adaptation by enriching proteins with acidic amino acids is more widespread than previously assumed. We have reevaluated the data and conclusions by comparing the isoelectric point profiles of the Guerrero Negro microbial mats (average isoelectric point 6.8) with those of the proteins encoded by the genomes of prokaryotes adapted to different salt concentration ranges and belonging to different phylogenetic and physiological groups. Average isoelectric points below 6.8 were found not only in the proteomes of the moderately halophilic aerobic bacteria *Halomonas elongata* and *Chromohalobacter salexigens*, but even in common types of marine bacteria of the genera *Alteromonas* and *Vibrio*. We did not find clear evidence that the isoelectric point profile of the Guerrero Negro microbial mat can be considered to be the result of species-independent molecular convergence of the members of the microbial community determined by the salinity of the overlying brine (Elevi Bardavid and Oren 2012).

Approaches Toward the Study of the *In Situ* Activities of Halophilic Microbial Communities

The above-discussed culture-dependent methods, culture-independent characterization of the microbial communities based on the sequencing of phylogenetic or functional marker genes, and the use of specific cell components as biomarkers, all provide qualitative and/or quantitative information on the types of cells present in the community. However, they tell us little, if anything, about the *in situ* microbial activities in the community: how fast do the cells divide, what substrates are used and how these are processed, and how the different components of the community interact with each other.

A survey of the studies performed in hypersaline environments in the past decades shows that, compared to the extensive research devoted to the isolation and characterization of halophilic microorganisms and the culture-independent assessment of the extent of the yet-uncultured microbial diversity living at high salt concentrations, the number of studies devoted to attempts to assess the dynamic aspects of the processes performed by those microorganisms is depressingly small. A summary of these investigations is given in the paragraphs below.

To obtain information on the metabolic potential of natural microbial communities in hypersaline ecosystems, the use of the Biolog® system has been explored (Litchfield et al. 2006). This diagnostic system, originally developed for the characterization of the substrate utilization patterns of pure cultures of bacteria, has also found applications in microbial ecology, as it enables the comparison of patterns of substrate utilization by mixed microbial assemblages in different communities. The system consists of microtiter plates with wells that contain a large variety of different organic substrate as well as an indicator dye. Oxidation of the substrate leads to reduction of the indicator dye, accompanied by a color change. The Biolog® GN plates system was used to compare the heterotrophic communities in saltern ponds of an oligotrophic saltern system (Eilat, Israel) and a more eutrophic system (Newark, California) (Litchfield et al. 2001; Litchfield and Gillivet 2002). Considerable differences were found between the metabolic potentials of ponds of comparable salt concentrations. At salt concentrations up to 140–150 g/l the results are reproducible, but the system cannot be used at higher salinities as the salt interferes with reduction of the indicator dye (Litchfield and Gillivet 2002). It should be stressed that a positive reaction with a certain substrate shows that that substrate can be metabolized by the community, but it does not necessarily imply that that substrate is indeed available and is used *in situ* at a significant rate. At the highest salt concentrations the community densities may be so great that it is possible to directly measure respiration rates by monitoring the decrease in oxygen concentration in the dark. The effect of addition of different substrates can then show which compounds may stimulate the activity (Warkentin et al. 2009).

To assess the metabolic activities of the microbial communities in the waters of salt lakes and salterns, a number of studies have measured the uptake, incorporation and further metabolism of radiolabeled substrates. To quantify photosynthetic activity

and primary production, incorporation of ^{14}C-labeled bicarbonate, routinely used in marine and freshwater systems, can be used in hypersaline environments as well. The method was employed in Great Salt Lake, Utah (Stephens and Gillespie 1976), in the Dead Sea (Oren et al. 1995a), and in the saltern ponds near Alicante, Spain (Joint et al. 2002). In the last-mentioned study the photosynthetic activity was monitored along the salinity gradient in the saltern pond system. Maximum bicarbonate uptake rates were obtained in the low salinity ponds (80 g/l salt), while only very low rates were measured in the crystallizer ponds, in spite of the presence of a dense *Dunaliella* population and a high content of chlorophyll. It was concluded that the algae in the salt-saturated crystallizers were heavily stressed by the excessively high salinity, and therefore their activity was minimal. Studies of the inorganic carbon uptake in the Dead Sea in 1992 during a bloom of *Dunaliella* using ^{14}C-bicarbonate as a tracer were supplemented by stable isotope studies in which changes in the ^{13}C content of the dissolved inorganic carbon were related to biological phenomena in the lake (Oren et al. 1995a). The interesting possibility that part of the photoassimilation of CO_2 in hypersaline environments may be driven by light absorbed by bacteriorhodopsin and not by chlorophyll has been explored in an earlier microbial bloom in the Dead Sea (Oren 1983b). Incorporation of ^{14}C-labelled bicarbonate was measured in the brine-seawater interface of Lake Thetis, a deep-sea brine lake in the eastern Mediterranean. Metagenomic studies showed the presence of at least three pathways of CO_2 fixation: the reductive acetyl-CoA pathway, the reductive tricarboxylic acid cycle pathway, as well as RuBisCo (Ferrer et al. 2012; La Cono et al. 2011).

To assess rates of dissimilatory sulfate reduction in hypersaline sediments, $^{35}SO_4^{2-}$ can be used as a tracer by monitoring the rate of formation of $^{35}S^{2-}$. Measurements of sulfate reduction in the sediments of the hypersaline north arm (270 g/l salt) of Great Salt Lake, Utah gave rates that were an order of magnitude lower than those found in the south arm (115–125 g/l salt). Sediment slurries from the north arm showed optimal sulfate reduction rates when the salinity was reduced to 100–150 g/l salt (Brandt et al. 2001). The same approach was applied to obtain information on the rates of sulfate reduction in hypersaline coastal pans in South Africa (Porter et al. 2007) and in salterns in Israel (Canfield et al. 2004; Sørensen et al. 2004). Stable isotopes can also be used to assess dissimilatory sulfate processes in hypersaline environments: comparison of the stable isotope composition of the sulfide and the sulfate in the sediments and in the water of the Dead Sea in the 1970s (at a time when the lake was still meromictic and had an anaerobic, sulfide-rich hypolimnion) showed an enrichment of ^{32}S in the sulfide, and this isotopic fractionation was brought forward as evidence for the biological nature of the sulfide (Nissenbaum and Kaplan 1976).

For the assessment of heterotrophic activities in hypersaline water bodies, different radiolabeled compounds have been used. Incorporation of labeled thymidine is often used to calculate *in situ* growth rates of bacterioplankton, but with the exception of two studies in salterns in Israel (Oren 1990b) and in Spain (Gasol et al. 2004) it has only rarely been used in hypersaline environments. [^{3}H]Leucine and ^{14}C-labeled mixtures of amino acids have further been used in studies in which heterotrophic activities in saltern ponds of different salinities were compared (Pedrós-Alió et al. 2000a; Oren 1990c, 1992). Other labeled organic compounds tested in such studies

are glycerol and acetate (Oren 1992, 1993, 1995a). Although the concentrations of radioactive tracer compounds added in all these studies were low, the question should always be asked what were the *in situ* concentrations of the same compounds, and to what extent did the addition of the labeled substances significantly increase the concentrations of the compounds and thereby increased the rates at which they were taken up and metabolized. It is therefore well possible that at least in part of the studies the rates measured are potential rates rather than true *in situ* process rates.

Glycerol is a compound not usually employed in studies of heterotrophic activities in "conventional" environments, but its inclusion in the study of hypersaline ecosystems has a special reason. *Dunaliella*, the main or sole primary producer in salt lakes and salterns with over 200 g/l salt, produces glycerol as its osmotic solute, and the compound may accumulate within its cells to concentrations exceeding 5–6 M. Therefore glycerol can be expected to become available to the heterotrophic community of halophiles as one of its major sources of carbon and energy. Glycerol is also known as a substrate that stimulates growth of many members of the *Halobacteriaceae* (Elevi Bardavid et al. 2008). High uptake rates and short turnover times of glycerol (in the order of hours) were measured in the saltern ponds of Eilat, Israel (Oren 1993, 1995b).

Whether the dominant types of heterotrophic prokaryotes commonly found in saltern crystallizer ponds—the archaeon *Haloquadratum walsbyi* and the bacterium *Salinibacter ruber*—do indeed use glycerol effectively *in situ* is not yet completely clear. In a study in which brine from a Spanish crystallizer pond was incubated with different radiolabeled substrates (glycerol, acetate, amino acids), the uptake of the compounds by the different components of the community was monitored using a combination of fluorescence *in situ* hybridization to detect cells of known phylogeny and microautoradiography to assess the labeling of each cell ("MAR-FISH"). Both *Haloquadratum* and *Salinibacter* readily took up acetate and amino acids, but the cells did not take up glycerol under the conditions of the experiment (Rosselló-Mora et al. 2003). This finding was unexpected as pure cultures of *Haloquadratum* and *Salinibacter* readily incorporated glycerol (Elevi Bardavid and Oren 2008a; Sher et al. 2004). The reason for the apparent discrepancy between the results obtained using different experimental approaches remains to be elucidated.

Studies on the metabolism of glycerol by different types of halophilic prokaryotes in pure culture and in mixed natural assemblages showed that a substantial part of the glycerol added may be converted to incomplete oxidation products, this in addition to complete oxidation to carbon dioxide and incorporation into cell material. Many members of the *Halobacteriaceae* excrete acidic products when incubated with glycerol. Acetate, pyruvate, and D-lactate were identified as the products of incomplete oxidation of glycerol by species of *Haloferax*, *Haloarcula*, and *Halorubrum*. Such incomplete oxidation of the substrate occurs not only in the presence of high glycerol concentrations: when micromolar concentrations of [^{14}C]glycerol were added to Dead Sea water at the time of an archaeal bloom or to the red crystallizer brine from a solar saltern, up to 12 % of the added label was recovered as D-lactate, acetate, and pyruvate (the latter being detected in Dead Sea brine only) (Oren and Gurevich 1994).

Another incomplete oxidation product of potential interest in the food web of saltern crystallizer ponds and other salt-saturated water bodies is dihydroxyacetone. When *Salinibacter* is fed with glycerol, part of the substrate is incorporated into cell material; part is respired to CO_2, but a significant fraction of the added carbon accumulates in the medium as an incomplete oxidation product (Sher et al. 2004), subsequently identified as dihydroxyacetone (Elevi Bardavid and Oren 2008a). As dihydroxyacetone is readily used by *Haloquadratum* (Elevi Bardavid and Oren 2008a), the compound may well connect the metabolism of the two main components of the heterotrophic community in many ecosystems in which these organisms coexist. Unfortunately no sufficiently sensitive and specific methods have yet been developed to assess the *in situ* concentrations of dihydroxyacetone, lactate, and other potential key compounds in the community metabolism of hypersaline ecosystems.

High-salt environments, even the most hypersaline ones saturated with NaCl, are inhabited both by representatives of the *Archaea* (the family *Halobacteriaceae*) and by salt-tolerant and salt-requiring *Bacteria* of which the recently characterized genus *Salinibacter* is currently the most halophilic one known (Antón et al. 2002). The question should therefore be asked how much each group contributes to the heterotrophic activity of the community. To answer this question, differences in sensitivity toward antibiotics and other inhibitors have been exploited to differentiate between the activities due to either group. As already stated in an earlier section, the non-coccoid members of the *Halobacteriacae* are lysed by low concentrations of bile acids that do not significantly affect the known species of *Bacteria* inhabiting high-salt environments. Therefore taurocholate was used in experiments in which saltern pond samples of different salinities were incubated with radiolabeled amino acids or glycerol, assuming that the residual incorporation may be due mainly to the activity of *Bacteria*. Taurocholate at a concentration of 50 mg/l had little effect on the heterotrophic activity in ponds up to 200 g/l salt, but caused nearly complete inhibition of amino acids and glycerol incorporation by the communities growing at salt concentrations above 250 g/l, suggesting that there the *Archaea* are responsible for most, if not all, of the activity (Oren 1990d). Antibiotics targeting the protein synthesis machinery were also employed in such studies: anisomycin to selectively inhibit archaeal protein synthesis, and erythromycin or chloramphenicol which are known as inhibitors of the bacterial ribosome. The conclusions were similar to those obtained using taurocholate (Oren 1990c; Gasol et al. 2004).

Following the discovery of *Salinibacter* and its recognition of as a potentially important component of the heterotrophic community in saltern crystallizer ponds (Antón et al. 1999, 2000), a reevaluation of the contribution of *Bacteria* to the heterotrophic activities in crystallizer ponds was necessary. *Salinibacter ruber*, when suspended in saltern crystallizer brine, took up labeled amino acids at two orders of magnitude lower rates than did *Haloqudratum walsbyi* (Elevi Bardavid and Oren 2008b). Chloramphenicol, used in earlier attempts to differentiate between bacterial and archaeal activity (Oren 1990c) indeed inhibited *Salinibacter*, but also caused significant inhibition of *Haloquadratum*, especially at the highest salt concentrations. Erythromycin inhibited *Salinibacter* without affecting amino acids incorporation by

Haloquadratum, and is therefore to be recommended as a differentiating agent in such studies (Elevi Bardavid and Oren 2008b).

Fungi are also potential contributors to the heterotrophic activity in hypersaline waters. In recent years, different species of fungi, especially black yeasts such as *Trimmatostroma salinum* and *Hortaea werneckii*, were consistently found in marine salterns (Gunde-Cimerman et al. 2000; Zalar et al. 2005). No information is yet available on their activities *in situ*. No indications have thus far been obtained to show that they provide a quantitatively significant contribution to the aerobic breakdown of organic compounds in the saltern ecosystem.

Protozoa, Viruses and Other Factors Controlling the Abundance of Halophilic Microorganisms

There are surprisingly few studies that have examined the factors that may cause the decline and death of halophilic microorganisms in their natural environments: protozoal grazing, lysis by viruses, or action of halocins.

The existence of flagellate, ciliate and amoeboid protozoa living in hypersaline brines, in some cases even up to salt saturation, is now well established. However, their importance in controlling the communities of unicellular algae, *Archaea* and *Bacteria* at the highest salt concentrations is probably minor. Eukaryotic predators were the most important factor controlling the abundance of heterotrophic prokaryotic plankton in Spanish saltern ponds of intermediate salinity, but they contributed little to the dynamics of the halophilic communities in the crystallizer ponds (Pedrós-Alió et al. 2000a,b; Gasol et al. 2004). Up to 6×10^3 protists were counted per liter in the anoxic brine (348 g/l salt) of the hypersaline deep-sea basin Lake Thetis in the eastern Mediterranean; numbers at the seawater—brine interface were around 1.1×10^4/l. Based on FISH studies and small subunit rRNA gene libraries, fungi were the most diverse group of protists, followed by ciliates and stramenopiles (Stock et al. 2012).

Viral lysis is probably the most important loss factor to the prokaryotic communities at the highest salinities. The first study of the ecology of halophilic viruses was an investigation of the populations of phages infecting *Halobacterium* in a transient brine pool in Jamaica (Wais and Daniels 1985). Virus-like particles were found in great abundance in electron microscopic examination of Spanish saltern brines (Guixa-Boixareu et al. 1996), Dead Sea water samples collected during the decline of an archaeal bloom (Oren et al. 1997), and in the alkaline hypersaline Mono Lake (Jiang et al. 2004). Numbers of virus-like particles typically exceeded those of the prokaryotic cells by 1–2 orders of magnitude. Studies of viral abundance in Spanish saltern ponds, using tangential flow filtration, ultracentrifugation, DNA extraction, and pulsed-field gel electrophoresis, showed the viral community to be less diverse than in the marine environment (Diez et al. 2000). The diversity increased from 40–150 g/l salt, to decrease at the higher salinities (Sandaa et al. 2003). Using similar techniques of phage concentration and analysis, at least 27 different viruses were

recognized in the waters of Mono Lake (Jiang et al. 2004). Metagenomic studies of the crystallizer brine of the Alicante, Spain saltern resulted in the elucidation of the sequence of halophilic phage EHP-1 (Santos et al. 2007).

In the past few years a number of in-depth studies of the viral communities in hypersaline brines have been published. Lake Retba, Senegal (345 g/l salt) was reported to harbor 3.4×10^8 prokaryotes/ml and 6.9×10^8 virus-like particles /ml, as assessed microscopically using 0.02 μm pore size filters and fluorescent staining with SYBR Gold. Transmission electron microscopy of viruses collected by polyethylene glycol precipitation showed a great morphological diversity of viruses, including spindle-shaped, head-and-tail, and several novel viral morphologies (Sime-Ngando et al. 2011). Different types of virus-like particles, including head-and-tail and untailed viruses, were observed in the stratified water column of alkaline, hypersaline Mono Lake, California (1.3×10^8 virus-like particles /ml in the epilimnion, $0.5–0.8 \times 10^8$ ml in the hypolimnion, with $8.3–9.3 \times 10^6$ prokaryotes/ml at all depths (Brum and Steward 2010). In the framework of a comparative metagenomic study of waters of different salinities, ponds of 60–80, 120–140, and 270–300 g/l salt of the Chula Vista, California salterns were sampled at different frequencies to elucidate the dynamics of viruses and their prey organisms (Rodriguez-Brito et al. 2010). In a study of the viral community of the crystallizer ponds of the salterns in Alicante, Spain, Garcia-Heredia et al. (2012) reconstructed viral genomes from fosmid clones. A total of 42 different viral genomes were retrieved. Analysis of these genomes, and in particular the presence of Clustered Regularly Interspaced Short Palindromic Sequences (CRISPR) spacer sequences shared by one of the available strain genomes, suggested that most viruses probably prey on *Haloquadratum*, “Nanohaloarchaea”, and *Salinibacter*. A metatranscriptomic analysis of viral expression in the Alicante crystallizer ponds, in which clones from a metaviromic library immobilized on a microarray were used as probes against total mRNA extracted from the hypersaline community, showed that the halovirus assemblage was highly active; the viral groups with the highest expression levels were those related to high GC content haloarchaea and *Salinibacter* (Santos et al. 2011). The different approaches for culture-independent studies of viruses from hypersaline environments, including methods of transmission electron microscopy, pulsed-field gel electrophoresis, and metagenomic approaches, were reviewed by Santos et al. (2012). Additional more detailed information about halophilic viruses is presented in this book in Chap. 4. Finally there is an interesting class of molecules produced by some halophilic *Archaea*: the halocins, peptide antibiotics secreted to inhibit the growth of other members of the same family. Production of such compounds has been well documented in pure cultures, and protocols for their concentration and detection have been described (Shand 2006). There has been a single study documenting attempts to detect presence of halocins in the brines of saltern crystallizer ponds (Eilat, Israel and Newark, California); if halocins were at all present in the brines, their concentration was below the limit of detection of the methods applied (Kis-Papo and Oren 2000). Whether such halocins are indeed present in significant amounts in natural communities of *Halobacteriaceae* in natural salt lakes and salterns, and whether their activity may influence the interspecies competition in these communities, remains to be determined.

Final Comments

The above survey was intended to provide an overview of the approaches used in studies of halophilic microorganisms in their natural environments. Many of the techniques in use are similar to those commonly employed in freshwater and marine ecosystems. Sometimes modifications of the standard methods are necessary to overcome problems caused by the presence of high salt concentrations. Other approaches discussed are unique to hypersaline environments, and are based on the unique properties of specific groups of high-salt-adapted microorganisms.

Most studies published on the ecology of hypersaline environments in the past decade dealt with the analysis of small subunit rRNA gene sequences recovered from environmental DNA. Here the situation is probably no different from that in other types of microbial ecosystems. Such studies indeed yield valuable understanding of the diversity within the community present, but the amount of knowledge gained remains limited as the organisms harboring the 16S/18S rRNA genes have in most cases never been isolated and their properties remain unknown. Methods of metagenomics are now also applied to high-salt environments, shedding light not only on the on the phylogenetic diversity, but also on the functional metabolic potential within the community.

Hypersaline environments pose a number of specific questions to the investigator, and overall relatively little research effort has been devoted to obtain the answers. A few studies have tried to assess the contributions of different groups of halophilic microorganisms (*Archaea*, *Bacteria*, protozoa, fungi, viruses) on the activities and the microbial communities and their dynamics at high salinities. A wide range of methods have been employed, including use of specific inhibitors to differentiate between the activities due to different groups. There also have been attempts to assess the possible role of a few low-molecular-weight substrates of special interest, such as glycerol, dihydroxyacetone, lactate, acetate, amino acids, etc. as key nutrients for different types of halophiles in their natural environment. However, our true understanding of the interactions between the community components is still limited.

Not all microbial processes known from low-salt environments function also at the highest salinities. A survey of the upper limit at which different dissimilatory processes are active and the microorganisms responsible for these processes led to a coherent model explaining why some types of metabolism can function up to salt saturation, while others cannot (Oren 1999, 2001, 2011). Little effort has yet been made to assess the fate of compounds that are easily metabolized in freshwater and marine environment but for which no degradation process is yet known at high salt concentrations.

Summarizing: there still are many gaps in our current understanding of the microbiology of hypersaline environments, in spite of the in-depth studies of so many halophiles in pure culture. To answer the many remaining questions, a combination of *in situ* observations and measurements as well as molecular techniques such as metagenomic and metaproteomic approaches will be needed. Such multidisciplinary approaches will surely lead to new insights in the functioning of hypersaline ecosystems in the years to come.

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Chapter 2
Media and Conditions for the Growth of Halophilic and Halotolerant Bacteria and Archaea

Mark A. Schneegurt

Introduction

An awareness of haloarchaea has existed since ancient times, with published descriptions of "red waters" associated with salt mining, the "red heat" of salted hides, and the "reddening" of salted fish (Bass-Becking 1931; Kurlansky 2002). For a society without refrigeration, the economic impact of codfish deterioration garnered particular attention, with Farlow (1878) oft cited as the first to publish on what were presumably haloarchaea. The early growth media of Eddington (1887) and Le Dantec (1891) reflected natural high-protein substrates, using beef peptone, gelatins, and fish broths, solidified with agar, flour, or bread paste. While some early studies used pieces of fish soaked in various brines (Høye 1908; Klebahn 1919; Harrison and Kennedy 1922), many included ground cod or a cod broth, or media based on beef bouillon or beef gelatin (Beckwith 1911; Bitting 1911; Becker 1912; Kellerman 1915; Clayton and Gibbs 1927; Velu 1929). Milk was introduced as a preferred organic constituent by Bitting (1911) and Kellerman (1915), but was popularized by Lockhead (1934). Rice flour, wheat flour or whole rice grains often were used as gelling agents (Clayton and Gibbs 1927; Robertson 1931; Boury 1934; Gibbons 1937). Silica gel was suggested to reduce organic content of solidified media (Hanks and Weintraub 1936; Moore 1940, 1941). It was recognized that alkaline culture conditions were useful for growing certain halophilic microbes (Stather and Liebscher 1929) and that halophilic obligate anaerobes could be grown on a cooked meat medium (Baumgartner 1937). The seminal paper of Harrison and Kennedy (1922) focused on the difficulties of growing the organisms responsible for red discolorations on salted fish, trying many media recipes including those based on cider, milk, broths, sugars, and potatoes. While the red organisms proved difficult to isolate, as an aside, the paper discusses a broad diversity of non-red halophilic organisms that were more easily isolated on these media.

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The influential work of Lockhead (1934) provided three recipes, a medium with skim milk and salt, a medium with codfish broth, peptone, glycerin, and salt, and a medium with beef extract, yeast extract, peptone, starch, and salt. These were widely used and included in the studies of Gibbons (1937), and of Hess (1942), who also used a medium based on Irish moss extract (carrageenan), seawater, and solid gypsum. Weber (1949) advanced the Lockhead media, and this became the basis of recipes reflected in more contemporary media (Dussault and LaChance 1952; Katznelson and Lockhead 1952). Defined media recipes often trace their history to Petter (1931) whose medium included asparagine and glycine, and again to Weber (1949). These early media led to the common media types used today, developed by Sehgal and Gibbons (1960), Rodriguez-Valera et al. (1980), Tindall et al. (1980), and Vreeland et al. (1980). Initial work on Dead Sea mud by Lortet (1892) was greatly expanded through the seminal work of Volcani (1944). His wide variety of hypersaline media was designed for aerobes, fermenters, denitrifiers, methanogens, and others. These media were based on Dead Sea waters supplemented with organic sources, ranging from cellulose to paraffin to kerosene. Oren (1983a, 1983b) continued the evolution of media using Dead Sea waters.

Early literature on organisms from salted foods and solar salt interjects a running debate on the nature of adaptation to hypersaline environments. Smith (1938) reviewed the arguments, which center on whether halophilism is an evolutionary consequence or simply the adaptation of a single generation. Rubentschik (1929) and Golikowa (1930) are credited with the first distinction between halophiles and halotolerant organisms. The former require high salinities for growth, while the latter can grow in both low and high salinities. Horowitz-Wlassowa (1931) equated halophilicity with halotolerance, introducing the term “halobe” for obligate halophiles, while Hof (1935) defined halophiles as organisms that can grow at 3 M salt. Flannery (1956) defined obligate halophiles as those requiring 2 % or more salt, while facultative halophiles grow best at greater than 2 % salinity, but also grow with less salt.

An important review by Larsen (1962) outlined a scheme that has relevance today. Nonhalophiles are those microorganisms that grow best below 2 % salt. Slight, moderate, and extreme halophiles are those that grow best in media containing 2 to 5 %, 5 to 20 %, and 20 to 30 %, respectively. Kushner (1968) then distinguished between obligate moderate halophiles and obligate extreme halophiles, which require 0.5 to 3.5 M and 3.0 M to saturated salinities, respectively. Kushner later (1978) added a definition for borderline extreme halophiles that grow best at 0.5 to 2.5 M salinity. These definitions were codified in the last edition of this review volume, but Kushner (1993) expressed concern about the use of “grows best” in the definitions of Larsen, as this could be misleading, suggesting broad optima even for extreme halophiles. Thus, halophiles require a minimum salinity for growth. Halotolerant organisms then are nonhalophiles that can grow at high salinities. Facultative halophiles require high salt only under certain environmental conditions.

For this review, halophile will be used to describe any organism that requires salinities higher than typical seawater for growth. Organisms that do not require high salt, but can grow at salinities above that of seawater will be considered halotolerant. It

can be argued that many marine organisms are slight halophiles under Kushner's scheme. The current review does not include a discussion of media for marine organisms that do not exhibit a greater degree of halotolerance or halophilicity. A wide variety of artificial seawater preparations are available (Zobell 1946; Provasoli et al. 1957). The media discussed here typically contain no less than 5 % salinity. This review also focuses only on media for halophilic and halotolerant bacteria and archaea. Saline media for eukaryotic algae (McLachlan 1960; Ben-Amotz and Avron 1983), fungi (Pitt and Hocking 1985; Gunde-Cimerman et al. 2009), or protists (Post et al. 1983; Esteban and Finlay 2003) are not discussed.

Growth Media

General Comments

The preparation and use of hypersaline media presents challenges unique to high salinities, as well as, some of the same concerns inherent with any microbial culture system. Choosing appropriate media and growth conditions is important and published media are typically associated with a particular microbial genus or species. As with other microbial discovery research, when working with environmental samples harboring communities of novel microbial populations, the media and growth conditions chosen will enrich for certain populations and not others. As a general rule, halotolerant and moderately halophilic bacteria are found at lower salinities in the environment and are cultured at room temperature with perhaps 10 % salinity. Extreme halophiles are predominantly archaea and are cultured at warmer temperatures (37 °C) with salinities of 20 % or more. The segregation of isolates into these classes using this enrichment scheme is not thorough, but the trends support this strategy. Specialized media and conditions are used to enrich for microbes from specific biogeochemical guilds, anaerobes, and alkaliphiles.

Hypersaline media can be divided into complex media that include organic components for which exact chemical formulae are not known and defined media where all components can be described by chemical formulae. There is a wide range of organic ingredients used for hypersaline media, the most popular of which are yeast extract, peptone, tryptone, and casamino acids (v.i.). The predominant salt is nearly always NaCl. Additional salts are often constituted like seawater, since the bulk of hypersaline research has been done in marine solar salterns or other thalassohaline environments. Extreme halophile media often have elevated levels of magnesium, particularly for Dead Sea isolates. The source of water used for hypersaline media preparation varies, with some media based on natural waters from the sea or hypersaline lakes (Volcani 1944; Madeley et al. 1967; Oren 1983a,b; Paterek and Smith 1985; Franzmann et al. 1987; Yu and Kawamura 1987; Wais 1988; Bertrand et al. 1990). Growth media can be prepared with tap water to provide trace minerals or with distilled water to avoid potential toxins. If phosphate is included in a medium with

substantial magnesium or calcium levels, it is commonly prepared as component solutions that are mixed after autoclaving to avoid precipitates. Other additives, mainly vitamins, are typically filter-sterilized and added to cooled media after autoclaving. One must take care when preparing media with very high salt concentrations to adjust the amount of water used to dissolve the salts such that the initial solution is not saturated and such that the final volume is not exceeded during preparation. Standard precautions used for the preparation of anaerobic media also apply to hypersaline media.

Microbial growth in media of high salinity is often slow, so it is not unusual to maintain cultures for weeks rather than days. Evaporation from liquid cultures, especially shake-flasks at elevated temperatures, can be problematic and lead to changes in salinity with time and even salt precipitation. Agar plates already present a relatively dry environment, so the addition of high salt exacerbates potential limitations. It is prudent to wrap plates in plastic paraffin film to retain moisture. It is advisable to store plates in a moist chamber, as first suggested by Le Dantec (1891). This can be as simple as sealed plastic bags (Post 1977; Rodriguez-Valera et al. 1985) or a plastic tub with a secure but unsealed cover, in which an open beaker of water or brine is kept (Caton et al. 2004). Plates will remain hydrated longer and are less likely to begin crystallization.

Another general consideration when working with hypersaline cultures is that the appearance and growth habit of microbial isolates can change depending on salinity. For *Halobacterium* and some halococci, red pigmentation is increased at higher salinities (Kushner 1993). In contrast, *Haloferax* may be more highly pigmented at lower salinities and colorless at high salinities (Rodriguez-Valera et al. 1980; Kushwaha et al. 1982). Colonies that are less highly colored, appearing cream or yellow, can exhibit more subtle changes in color at different salinities. Colonies may become smaller or mucoidy with increasing salinity. Cells may be smaller at higher salinities, often falling in the submicron range, making staining protocols more difficult. In addition, classic staining and biochemical tests have to be modified for higher salinities and may not be as consistent. For instance, carbon substrate utilization analysis using the Biolog system can give unreliable results in hypersaline solutions. Responses also may change with nutritional needs at different salinities (Litzner et al. 2006) and with changes in active transport systems (Kushner and Kamekura 1988). Variations in envelope characteristics and lipid composition are seen at higher salt concentrations, with increases in negatively charged phospholipids (Vreeland 1987; Kushner and Kamekura 1988).

Media Composition

Modern hypersaline media can trace their roots to a handful of influential media recipes, readily modified to meet specific needs. The recipes for several hypersaline media directed at haloarchaea are given in Table 2.1, while a group of common media for moderate halophiles and halotolerant bacteria is given in Table 2.2. Complex media from the Gibbons laboratory (Brown and Gibbons 1955; Abram and Gibbons

Table 2.1 Compositions of common extreme halophile media

Component	Medium composition (g L^{-1})					
	A	B	C	D	E	F
NaCl	250	125	234	250	220	200
KCl	2		6	2	5	4
K_2SO_4		5				
KNO_3					1	
K_2HPO_4						0.5
$(NH_4)_2SO_4$						1
$MgSO_4{\cdot}7H_2O$	20		29		10	20
$MgCl_2{\cdot}6H_2O$		50	19.5	20		
$CaCl_2{\cdot}6H_2O$		0.12	1.1	0.2	0.2	
NaBr			0.8			
$NaHCO_3$			0.2			
$FeCl_2$	0.023				*	
Na-citrate	3				3	
Casamino acids	7.5					
Yeast extract	10	5	5	5	1	
Tryptone/peptone		5		5	5	
Glycerol						2.5
Pyruvate						2.5

A, Sehgal and Gibbons 1960; B, Mullakhanbhai and Larsen 1975; C, Rodriguez-Valera et al. 1980; D, Tomlinson and Hochstein 1972; E, Caton et al. 2004; F, Vreeland et al. 2002. (Adapted from Kushner 1993).
*Includes trace minerals with iron.

Table 2.2 Compositions of common moderate halophile media

Component	Medium composition (g L^{-1})				
	A	B	C	D	E
NaCl	80	98	29–174	29–174	80
KCl		2		0.02	
$MgSO_4{\cdot}7H_2O$	20	1	0.1	2	20
$CaCl_2{\cdot}6H_2O$		0.36			
NaBr		0.23			
$NaHCO_3$		0.06			
$FeCl_3{\cdot}6H_2O$		0.001			
Na-citrate	3				3
Casamino acids	7.5				7.5
Yeast extract	1	10			1
Tryptone/peptone	5	5			3
$(NH_4)_2SO_4$			2		
$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$	0.05				0.005
K_2HPO_4	0.5		3.12		7.5
KH_2PO_4			0.28	0.01	
NH_4Cl			2		
Glucose		1	10	10	
Glutamate				2	
Trace minerals		yes			

A, ATCC 1097; B, Caton et al. 2004; C, Forsyth and Kushner 1970; D, Kamekura et al. 1985; Vreeland et al. 1980.

Table 2.3 Hypersaline ATCC Media Suggested for Common *Bacteria* and *Archaea*

Target	ATCC medium
Bacteria	
Bacillus	1659, 1660
Chromohalobacter	87, 1097
Halobacillus	925[a]
Halomonas	87, 1097, 1582, 1689, 1725, 1740, 2049, 2084, 2096, 2097, 2168
Marinococcus	87, 800
Salinibacter	2402
Archaea	
Haloarcula	1218, 1230
Halobacterium	213[b], 1218, 1270
Haloferax	974, 1270
Halorubrum	1218, 1394, 1682, 2168, 2402
Halosimplex	2235
Selective	
Alkaline[c]	1392, 1590, 2049, 2096, 2097
Anaerobic	1275, 1279, 1302, 1453

[a]Also, *Pseudomonas halosaccharolytica.*
[b]Also, *Halogeometricum and Haloterrigena.*
[c]Includes *Natronomonas, Natrialba,* and *Natronococcus.*

1960; Sehgal and Gibbons 1960) are the bases for a number of both moderately saline and extremely saline media. For instance, the *Halomonas* medium of Vreeland et al. (1980) is based on Abram and Gibbons (1960). In time, as new isolates were obtained, some haloarchaeal media were more successful after the addition of carbohydrates (Tomlinson and Hochstein 1972a,1972b, 1976). An important class of hypersaline media uses a mixture of salts that resembles the composition of concentrated seawater (Rodriguez-Valera et al. 1980). Defined media for the extreme halophile, *Halosimplex*, has broad application (Vreeland et al. 2002). The medium of Payne et al. (1960), originally designed for isolates from Lake Magadi, is the basis of many hypersaline alkaline media (Tindall et al. 1980). More than 30 hypersaline media recipes are suggested by ATCC. ATCC media for common halotolerant and halophilic microbes are given in Table 2.3. A summary of the prevalence of specific ingredients in ATCC media is given in Table 2.4.

Salinity

The selection of media for halophilic and halotolerant aerobic heterotrophs often is based on salinity. Media for halotolerant bacteria typically contain lower salinities than media specific for halophilic archaea. A survey of media recipes suggested by ATCC finds that nearly half have salinities between 5 % and 10 % (Table 2.4). Media with salinities above 20 % are suggested for halophilic archaea. Related *Halomonas* media have 8 % salinity (Table 2.2; Vreeland et al. 1980; ATCC 1097). A group of media used for enrichments of moderately halophilic and halotolerant bacteria

Table 2.4 Composition of hypersaline ATCC Media. Number of media recipes containing each component are given

Major salts		Minor salts		Complex additions	
NaCl		*Iron*		*Organic C*	
5–10 %	13	As $(NH_4)_2(SO_4)_2$	4	Yeast extract	20
11–19 %	7	as Cl_2	4	Peptone/Tryptone	14
≥20 %	8	as SO_4	2	Casamino acids	8
Magnesium		*Calcium*		Citrate	8
as citrate	1	as Cl_2	11	Glucose	5
as SO_4	14	as SO_4	1	Glutamate	2
as Cl_2	11	*Trace*		Glycerol	2
as NO_3	1	HCO_3	6	Soluble starch	1
>50 g/L Mg salt	2	Br	4	Succinate	1
Sulfate		B	4	Pyruvate	1
as Mg	20	Cu	4	pH buffer	1
as K	2	Mn	4	Vitamins	4
Phosphate[a]		Mo	4	Complex anaerobe[b]	3
as Na	1	Zn	4		
as K	8	Co	3		
Nitrogen		Ni	2		
as NH_4Cl	4				
as NH_4SO_4	3				
as NO_3	2				
Potassium					
as Cl	17				
as PO_4	7				
as SO_4	2				
as NO_3	1				

[a]≥As $NaHPO_4$ and K_2HPO_4.
[b]ATCC Medium 1279, 1302, and 1453.

(*Bacillus, Halobacillus, Halomonas, Salibacillus*, *Salinibacter*) has approximately 10 % salinity (Rodriguez-Valera et al. 1980; Quesada et al. 1983; Caton et al. 2004). While the popular halophilic archaeal medium of Mullakhanbhai and Larsen (1975) has a salinity of 12.5 %, other popular halophilic media have 23 % or greater salinities (Table 2.1). These media support the growth of a wide range of haloarchaea including *Haloarcula, Halobacterium, Halococcus*, *Haloferax,* and *Halorubrum*.

For some media, NaCl is supplemented with other salts in an effort to mimic the composition of concentrated seawater. These media are best suited for organisms from solar salterns fed with seawater or other thalassohaline waters and soils. The most popular of these media are based on Rodriguez-Valera et al. (1980) that derives its salt mixture from earlier work by Subov (1931). It includes 2 % KCl and lesser concentrations of $CaCl_2$, NaBr, and $NaHCO_3$.

It is not uncommon for media to be developed using natural saline waters or salt mixtures obtained from the natural sources where microbial specimens were collected (Eimhjellen 1965; Madeley et al. 1967; Mathrani and Boone 1985; Yu and Kawamura 1987; Franzmann et al. 1988; Wais 1988). Even the popular *Halomonas*

medium of Vreeland et al. (1980) originally included solar salt from a study site in the Netherlands Antilles. A wide range of media developed by Volcani (1944) is based on waters from the Dead Sea diluted to different salinities and then extended by Oren (1983a,b). Filtered seawater or salt plains brine were effective bases for f/2 medium (Guillard and Ryther 1962) in growing halotolerant cyanobacteria and algae (Garcia-Pichel et al. 1996; Henley et al. 2002; Kirkwood and Henley 2006), as was Hamelin pool water as a basis for BG-11 medium (Goh et al. 2010). Wais (1988) was successful in isolating haloarchaea using natural brines from thalassohaline lagoons and suggested that conventional media may be less effective for enrichment cultures.

The early work of Schoop (1935) replaced NaCl in growth media with KCl, KNO_3, Na_2CO_3, and $NaNO_3$. None of these replacements were suitable for obligate halophiles, but facultative halophiles grew with these substitutions. Based on the medium of Sehgal and Gibbons (1960; supplemented with $FeCl_2$), studies on the extreme halophile *Halobacterium cutirubrum* have examined the effects of replacing NaCl with other salts by observing cell morphology and leakage (Abram and Gibbons 1961; Boring et al. 1963). At salinities below 1.5 M, significant cell lysis occurred, while at salinities below 3.5 M NaCl, cell morphology was altered. In media containing 0.1 M NaCl, cells remained intact with 1–2 M concentrations of $CaCl_2$, $MgCl_2$, Na-acetate, or $Na_2S_2O_3$. Growth can be supported in media where NaCl is partially replaced by other solutes, such as KCl for haloarchaea (Brown and Gibbons 1955; Gibbons 1969; Kushner 1985) or sucrose for halotolerant bacteria (MacLeod 1965; Adams et al. 1987). However, Na^+ seems to be a broad requirement of haloarchaea and halotolerant bacteria. Generally for the halophilic archaea, NaCl is needed at a concentration of at least 1.5 M, in addition to other salts (Mohr and Larsen 1963).

Suitable substitutes for *Halobacterium volcanii* were shown to be NaBr, $NaNO_3$, $MgCl_2$, and KCl (in that order), but not Na_2SO_4 (Mullakhanbhai and Larsen 1975). The haloarchaea seem to require Cl^- as well as Na^+ ions for growth (Brown and Gibbons 1955). *Halomonas elongata* was found to grow well with NaCl replaced by NaBr or $NaNO_3$, but not NaI or Na_2SO_4, while *Deleya halophila* could use NaBr, Na_2SO_4, and $Na_2S_2O_3$, but not other sodium salts (Vreeland and Martin 1980; Quesada et al. 1987). *Vibrio costicola* was able to grow on NaBr, $NaMO_4$, $NaPO_4$, and Na_2SO_4, with weak growth on LiCl, KCl, and $MgCl_2$ (Flannery et al. 1952). In a study of 168 halophilic bacterial isolates, 75 strains required NaCl, while 21 strains grew well on media with 1–4 M KCl (Onishi et al. 1980). A wide variety of salts at 1 M concentration supported growth of *Micrococcus varians* including KBr, KCl, KNO_3, RbBr, RbCl, and a number of Na salts (Kamekura and Onishi 1982). In this case, media made with KI or NaI showed growth after a long lag period, and strains capable of growth with high concentrations CsCl and LiCl were isolated. As discussed below, some microorganisms from oligohaline environments may be osmotolerant or osmophilic, but naturally depend on salts other than NaCl.

Magnesium

Requirements for Mg salts are broad among halotolerant and halophilic microorganisms. While Mg^{++} is a common component of microbiological media, haloarchaea are generally considered to require higher concentrations of Mg^{++} for growth. However, not all do and the growth of some halophilic and halotolerant microbes is inhibited by higher Mg^{++} concentrations (Soliman and Trüper 1982; Juez 1988). *Halobacterium cutirubrum* required at least 0.1 M Mg^{++} for growth, helping cells maintain normal morphology at lower (2.5 M) salinities (Boring et al. 1963). While slow growth was observed at Mg^{++} concentrations of 0.01–0.025 M, maximum growth occurred in the range of 0.1–0.5 M Mg^{++} for *Halobacterium halobium, Pseudomonas cutirubra, P. salinaria*, and *Sarcina littoralis* (Brown and Gibbons 1955). Magnesium salts of chloride, nitrate, and sulfate were equally effective.

A special case, are microbes isolated from the Dead Sea. These waters have a $MgCl_2$ concentration of 1.1–1.5 M, in addition to 1.7 M NaCl. The early work of Volcani (1944) used Dead Sea water for a variety of media. Isolates from the Dead Sea are typically grown in media containing 0.6–1.2 M Mg^{++}, with *Halobacterium sodomense* shown to require high Mg^{++} concentrations (Oren 1983b). Calcium could partially satisfy this requirement.

The common haloarchaea media of Table 2.1 include 2–5 % Mg salts, as chlorides or sulfates. *Halomonas* media also contain 2 % Mg salts, while other common halotolerant bacteria media contain lower amounts (Table 2.2). A survey of media from ATCC (Table 2.4) shows that the chloride and sulfate salts are most popular, with only one medium using the nitrate salt. Only 2 of the 28 hypersaline media contained more than 5 % Mg salts.

Potassium

KCl is typically added up to 2 %, to media that mimic concentrated seawater (Rodriguez-Valera et al. 1980; Caton et al. 2004; Caton et al. 2009), and is likely a component of media based on natural brines. *Halobacterium halobium, Pseudomonas salinaria, P. cutirubra*, and *Sarcina littoralis* failed to grow in media that did not contain K^+ (Brown and Gibbons 1955). Maximum growth was seen at 1–3 mM, but was not inhibited at 3 M KCl. The K^+ requirement could not be filled by NH_4^+, Cs^+, or Li^+, but higher concentrations of Rb^+ supported growth, as did the addition of ash from yeast extract. *Halobacterium* did not grow below 12.5 mg L^{-1} K^+ and grew best at 1 % K^+ (Gochnauer and Kushner 1969). A survey of ATCC media (Table 2.4) shows that most use the chloride salt, while others use a phosphate salt, likely to provide pH control. Media containing nitrate or sulfate salts also have been suggested. Common haloarchaea media contain between 0.2 and 0.5 % K salts (Table 2.1). The potential for K salts to partially or completely replace Na salts in media for halophilic and halotolerant microbes is discussed above.

Sulfur, Phosphate, and Nitrogen

Complex media typically rely on organic materials as sources of sulfur and nitrogen. In defined media, these can be supplied as inorganic chemicals or as amino acids. Nitrogen can be provided as NH_4Cl, $NaNO_3$, or $(NH_4)_2SO_4$ (Table 2.4). The amino acids asparagine, cysteine, glutamate, glutamine, and histidine have been used (Flannery and Kennedy 1962; Onishi et al. 1965; Forsyth and Kushner 1970; Grey and Fitt 1976; Yu and Kawamura 1987; Kauri et al. 1990). Sulfate often appears as a counterion for Ca, Fe, Mg, N, and K salts, while phosphate is a counterion in K and Na salts. Aerobic, chemolithoautotrophic, sulfur-oxidizing *Thiohalobacter* uses thiocyanate as an electron donor (Sorokin et al. 2010).

Minor and Trace Salts

Iron is often added to both defined and complex hypersaline media as chloride, citrate, or sulfate salts, or as a double salt with ammonium sulfate. Typically it is not provided in the chelated forms common for plant media. The influential medium of Sehgal and Gibbons (1960) is often supplemented with $FeCl_2$ (Boring et al. 1963; Kushner and Bayley 1963). Iron has been shown to be essential for growth of halophilic and halotolerant microbes at concentrations similar to those used for other bacteria and archaea (Brown and Gibbons 1955).

Calcium salts are included in mixtures that mimic seawater salts and in defined media, mainly $CaCl_2$. It can be found at 0.4 M in Dead Sea waters. No specific studies address requirements for calcium or the trace minerals. Seawater mimics include NaBr and $NaHCO_3$ in addition to $CaCl_2$ (Rodriguez-Valera et al. 1980; Caton et al. 2004; Caton et al. 2009), at low concentrations (<0.5 %). Trace mineral mixtures can include B, Co, Cu, Mn, Mo, Ni, or Zn, but generally no trace minerals are added to hypersaline media. With such high salinities, it can be expected that trace mineral requirements will be filled by contaminants in the laboratory chemicals. Trace minerals are likely present in media made from natural brines, tap water, or solar salts.

Organic Components

Work with halophiles began with organisms found on salted fish and meats, so early media recipes focused on protein and amino acid sources of C, including ground fish, peptone, and skim milk (Le Dantec 1891; Beckwith 1911; Kellerman 1915; Klebahn 1919; Stuart et al. 1933; Lockhead 1934; Stuart 1940). For the extreme halophiles, it was thought that carbohydrates were not good C sources. This paradigm changed with the isolation of *Halobacterium saccharovorum* that grows on a range of simple carbohydrates (Tomlinson and Hochstein 1972a,1972b, 1976). In addition, *Haloarcula*

marismortui, *Haloarcula trapanicum, Haloarcula vallismortis*, and *Halobacterium sodomense* grow well on a variety of carbohydrates (Juez 1988; Oren 1994). Some carbohydrates can support the growth of *Haloferax mediterranie, Haloferax volcanii*, and *Natronobacterium pharaonis*. Essentially no growth is obtained with carbohydrates for *Halobacterium cutirubrum, Halobacterium salinarum*, and *Halococcus morrhuae*. This last species is considered to be equivalent to the earliest haloarchaea studied, formerly designated as *Sarcina litoralis* and *Micrococcus morrhuae* (Juez 1988). Halotolerant bacteria typically have wider metabolic abilities than haloarchaea, thus, many carbon sources are suitable (Ventosa et al. 1982; Quesada et al. 1983; Litzner et al. 2006).

Yeast extract is the most popular carbon source in complex media for halophilic and halotolerant bacteria and archaea (Dundas et al. 1963; Tomlinson and Hochstein 1972a,b; Post 1977; Rodriguez-Valera et al. 1980; Ventosa et al. 1982; Caton et al. 2004). It was a component of several Gibbons' media (Abram and Gibbons 1960; Sehgal and Gibbons 1960) that form the bases of modern media recipes. Yeast extract is found in 20 hypersaline ATCC media (Table 2.4) and derivatives of popular moderate halophile media (Vreeland et al. 1980; Quesada et al. 1983, 1985). Taken together, the second most broadly used carbon sources are peptones, trypticases, and tryptones, widely known from the influential work of Weber (Hof 1935; Weber 1949; Dussault and LaChance 1952; Shiio et al. 1956; Mullakhanbhai and Larsen 1975; Ishida and Fujii 1970). Casamino acids and citrate, which were included in the medium of Sehgal and Gibbons, appear in media recipes today (Post 1977; Vreeland et al. 1980). In some cases, compounds, such as carbohydrates or glycerol are the main carbon sources, but yeast extract or another amino acid source is added in low quantities (Forsyth and Kushner 1970; Ducharme et al. 1972; Tomlinson and Hochstein 1972a,b; Mullakhanbhai and Larsen 1975). These complex ingredients also can supply some mineral nutrients and complex growth factors such as vitamins.

Glucose is the most common simple carbohydrate added to complex hypersaline media (Forsyth and Kushner 1970; Rodriguez-Valera et al. 1980; Quesada et al. 1983; Oren 1986; Caton et al. 2004). Aliphatic and aromatic hydrocarbons also can serve as carbon sources (Bertrand et al. 1990; Kulichevskaya et al. 1992; Nicholson and Fathepure 2004, 2006; Al-Mailem et al. 2010). Glycerol can be used for halotolerant bacteria (Chan and Leung 1979; Vreeland and Martin 1980). Succinate and glycerol have been used for *Haloferax* (Mevarech and Werczberger 1985; Kauri et al. 1990) and acetate for *Halobacterium* (Boring et al. 1963). Mixtures of acetate, glycerol or pyruvate are used in defined media for *Halosimplex* (Vreeland et al. 2002). Media for microbes isolated from foods can contain related foodstuffs as components (Clayton and Gibbs 1927; Kono and Taniguchi 1960; Ōmata et al. 1961). A medium designed using only household materials and foodstuffs is suitable for inexpensive secondary science classroom laboratory activities (Schneegurt et al. 2004).

It should be pointed out that there have been issues in the past with certain brands of peptone (Kamekura et al. 1988). Apparently there was contamination with bile salts (glycocholic acid and taurocholic acid) in some batches, at

concentrations high enough to lyse sensitive haloarchaea such as *Haloarcula, Halobacterium, Haloferax*, and *Natronobacterium*, while not affecting *Halococcus* and *Natronococcus*. The damaging effects of bile salts had been suggested earlier (Dussault 1956). It seems that carbohydrates offer some protection from bile salt contaminants (Oren 1990). Oleates and stearates at 0.5 % or detergents also appear to lyse haloarchaea (Bertullo 1960–1961; Abram and Gibbons 1961).

Defined hypersaline media generally include amino acids and several amino acids appear to be required by certain microbial species (Petter 1931; Dundas et al. 1963; Onishi et al. 1965; Ducharme et al. 1972; Grey and Fitt 1976; Kamekura at al.1985; Plakunov and Lobyreva 1985; Lobyreva et al. 1987). Amino acids are often added to meet these needs or as a N source, while C is supplied as carbohydrates or glycerol (Katznelson and White 1950; Onishi et al. 1965; Grey and Fitt 1976).

Other Components

Vitamins and other growth factors have been tested for their ability to stimulate growth in hypersaline media. These may be supplied with the addition of yeast extract (Kauri et al. 1990). Some vitamins are useful in *Halobacterium* and *Haloferax* medium, including biotin, thiamine, folate, and B_{12} (Onishi et al. 1965; Gochnauer and Kushner 1969; Franzmann et al. 1988; Kauri et al. 1990), and some vitamins appear in halotolerant bacteria media (Flannery 1955; Chan and Leung 1979). Nucleic acid bases did not have much effect on growth (Katznelson and Lockhead 1952) and are rare media components (Onishi et al. 1965).

Antibiotics have been used for the purpose of selecting for particular organisms in enrichment or maintenance cultures. Penicillin is most popular, but ampicillin and streptomycin have been used (Torreblanca et al. 1986; Montero et al. 1988; Wais 1988; Kulichevskaya et al. 1992). A combination of penicillin G, erythromycin, and cycloheximide were used to select for archaea at different pHs from subzero hypersaline methane seeps (Niederberger et al. 2010). Generally pH buffers are not included in neutrophile media, but have appeared in several (Tomlinson and Hochstein 1972a,b; Tomlinson et al. 1986; Tardy-Jacquenod 1998). The pH of alkaline media is generally set by the addition of carbonates (Brown 1963; Tindall et al. 1980; Kobayashi et al. 1992; Kanai et al. 1995).

Specialty Media

Most work with halophilic and halotolerant microbes has been done on strict aerobes and at neutral pH. However, a body of literature deals with various anaerobes, including fermenters and methanogens, and with organisms found in alkaline environments rich in natron. These groups require special media and growth conditions.

A detailed rendering of all of the published niche media is outside the scope of this review, but a few are discussed here.

Enrichment and maintenance media for *Clostridium, Halanaerobium*, and *Halobacteroides* from the Dead Sea have been presented by Oren (1983a, 1984, 1986). These are rich complex media that may include amino acid sources, glucose, glutamate, lactate, pyruvate, salts, and vitamins. These are set at a relatively low pH (6–6.5) and include components often found in media for anaerobes such as ascorbate, cysteine, resazurin, and thioglycolate. Oren also has studied the fermentative and respiratory abilities of *Halobacterium* and *Haloferax* using DMSO, TMAO, and fumarate (Oren and Trüper 1990; Oren 1991). A medium specific for a halophilic nitrate respirer, *Halobacterium dentrificans*, is rich in organic compounds and includes a pH buffer and KNO_3 (Tomlinson et al. 1986).

Methanogens from hypersaline environments have been studied using complex media that typically include trimethylamine (Mathrani and Boone 1985; Paterek and Smith 1985; Zhilina 1997; Yu and Kawamura 1987; La Cono 2011). These can be organically rich media with peptone and yeast extract, and often include amino acids and vitamins. Additives to lower the reduction potential of the medium, such as cysteine and sulfides are included. Sulfate reducers are known at higher salinities and have been isolated on anoxic media (Tardy-Jacquenod 1998). The medium for *Desulfotomaculum* is moderately hypersaline at 4 % NaCl, is buffered with MOPS, and includes Na-lactate and yeast extract.

Alkaline media often mimic common hypersaline media, but the pH is set from 8 to 10 with the addition of Na_2CO_3 (Payne 1960; Tindall et al. 1980; Tindall et al. 1984; Kobayashi et al. 1992; Kanai et al. 1995; Zhilina et al. 1997; Pikuta et al. 2003). These media are specific for isolates from soda lakes such as Lake Magadi in Kenya and the Wadi An Natrun in Egypt. Many of these alkaliphiles, including strains of *Natronobacterium pharaonis* and *Natronococcus occultus*, can be inhibited by Mg^{++} concentrations higher than 0.01 M (Soliman and Trüper 1982; Juez 1988), as their parent body of water is very low in soluble Mg^{++} and Ca^{++}. Anaerobic alkaline media are known, including media for isolates from Mono Lake (Guffanti et al. 1986; Blum et al. 1998; Mesbah et al. 2007). Halophilic acidophiles appear to be rare, although a growth medium set at pH 4.5 has been reported for a haloarchaean (Minegishi et al. 2008).

Photosynthetic organisms are found in hypersaline environments with the eukaryote *Dunaliella* receiving the most attention (Ben-Amotz and Avron 1983; Rodriquez-Valera et al. 1985; Oren 2000; Henley et al. 2002; Kirkwood and Henley 2006). Media for halotolerant cyanobacteria are typically either made from seawater or artificial seawater, such as Provasoli's enriched seawater (Yopp et al. 1978; Tindall et al. 1978; Starr and Zeikus 1987; Garcia-Pichel et al. 1998). Common media such as f/2 or Chu11 are found supplemented with salts for halotolerant phototrophs (Dor and Hornoff 1985; Garcia-Pichel et al. 1996). It has been suggested that maintaining elevated temperatures (45 °C) suppresses the growth of *Dunaliella*, allowing cyanobacteria to bloom (Dor and Hornoff 1985).

Environmental Conditions

Generally haloarchaea grow best above room temperature. Most laboratories use 37 °C (Abram and Gibbons 1960; Boring et al. 1963; Dundas et al. 1963; Ducharme et al. 1972; Matheson et al. 1976; Tomlinson and Hochstein 1976; Tindall et al. 1984; Montero et al. 1988; Yu and Kawamura 1987; Kamekura and Dyall-Smith 1995), while others work somewhat lower (35 °C) or higher (40 °C) (Kushner and Bayley 1963; Mullakhanbhai and Larsen 1975; Oren 1983b, 1986; Torreblanca et al. 1986), and optimal growth temperatures of 50 °C have been reported (Gibbons 1969; Hochstein 1988; Cayol et al. 1994, 2000; Mesbah et al. 2007). Halophilic and halotolerant bacteria are often grown at room temperature or at a slightly elevated temperature (30 °C) (Forsyth and Kushner 1970; Vreeland et al. 1980; Oren 1983a, 1986; Caton et al. 2004). Psychrotrophic and psychrophilic organisms are maintained at 5–10 °C, but can grow at or below –5 °C at high salinities (Madeley et al. 1967; Franzmann et al. 1987, 1988; Niederberger et al. 2010).

The salt response of halophilic and halotolerant microbes can be affected by growth temperature and optimal growth temperature can be affected by salinity (Ishida 1970; Mullakhanbhai and Larsen 1975; Novitsky and Kushner 1975, 1976; Vreeland and Martin 1980; Quesada et al. 1987). *Halomonas elongata* exhibited different optimal salinities and permissible ranges with changing temperatures (Vreeland and Martin 1980). Growth at higher salinities (4 M) was enhanced at 30 or 40 °C relative to 20 °C. *Planococcus halophila* required 0.5 M salt for growth when cultured above 25 °C (Novitsky and Kushner 1975). A broader study of 48 strains of *Brevibacterium* and *Flavibacterium* and from solar salt found that increasing growth temperatures from 27 to 35 °C increased minimum salinity requirements, but salinity did not affect optimal growth temperature (Ishida 1970).

Most halophilic and halotolerant microbes isolated to date are neutrophiles, growing best in media with pHs from 6.8 to 7.5 (Brown and Gibbons 1955; Abram and Gibbons 1960; Flannery and Kennedy 1962; Tomlinson and Hochstein 1972a,b; Mullakhanbhai and Larsen 1975; Vreeland et al. 1980; Mevarech and Werczberger 1985; Vreeland et al. 2002; Caton et al. 2004), although some media use pHs outside of this range (Boring et al. 1963; Kushner and Bayley 1963; Forsyth and Kushner 1970; Ishida and Fujii 1970). Anaerobes can be maintained over a similar pH range of 6–7 (Oren 1983a, 1986; Yu and Kawamura 1987). As discussed above, alkaliphilic organisms are grown at pHs of 8–10 using carbonates (or natron) to maintain the pH (Tindall et al. 1980; Kobayashi et al. 1992; Soliman and Trüper 1982; Kanai et al. 1995).

Most of the halotolerant and halophilic microbes studied to date are aerobes, although anaerobes are known (Oren 1983a, 1986; Mathrani and Boone 1985; Tomlinson et al. 1986; Zhilina 1997; Yu and Kawamura 1987). A challenge for aerobic organisms in hypersaline systems is that oxygen solubility decreases with increasing salinity, dropping by half at 10 % salinity and by over 80 % at 30 % salinity. Therefore, more vigorous aeration should be considered when culturing at higher salinities.

Illumination conditions are typically not specified in published reports on halotolerant isolates and the presumption is that microbial cultures should be maintained in the dark. Haloarchaea have been shown to contain photosensory pigments that control gene expression and responses, light-driven ion pumps, and bacteriorhodopsins that can support phototrophic growth (Sharma et al. 2007). Light has been used to drive anaerobic growth of *Halobacterium* at more than 3×10^5 lx, near saturation for bacteriorhodopsins (Hartmann et al. 1980; Oesterhelt and Krippahl 1983). There have been reports that light can damage haloarchaeal cells as noted in colorless mutants and can inhibit respiration by reducing available ADP levels (Dundas and Larsen 1962; Oesterhelt and Krippahl 1973). Algal and cyanobacterial cultures are maintained under moderate illumination of approximately 3,000 lx (Yopp et al. 1978; Dor and Hornoff 1985; Garcia-Pichel et al. 1998; Kirkwood and Henley 2006).

Other Salinophiles

Not all environments with high salinity are rich in NaCl. Osmotolerant microbes are typically obtained at high NaCl concentrations (halotolerant), although some are known from environments rich in sugars or other salts (Ingram 1957; Grant 2004). After the precipitation of halite in solar saltern crystallizer ponds, the remaining bitterns are typically dominated by $MgCl_2$ and KCl (Ratton 1877). It is oft cited that bitterns are “apparently devoid of life” since Javor (1982, 1983, 1984) had difficulty cultivating microbes from Guerrero Negro salterns using complex media (Sehgal and Gibbons 1960; Oesterhelt and Stoeckenius 1974). However, others report on microbes isolated from similar bitterns (Rodriguez-Valera et al. 1985; Butinar et al. 2005; Cantrell et al. 2006; Hallsworth et al. 2007). Typical hypersaline growth media were used and these were not specifically aligned with the composition of bitterns. The hypersaline anoxic Discovery Basin in the Mediterranean Sea is naturally 5 M $MgCl_2$ with low NaCl (van der Wielen et al. 2005). While *Bacillus* were isolated from this environment, the growth medium was enriched seawater that did not reflect the composition or anoxic nature of the basin (Sass et al. 2008).

Basque Lake in the Kamloops region of BC and Hot Lake near Oroville WA are athalassohaline epsomite lakes that contain precipitating concentrations of $MgSO_4$ (Epsom salt) and virtually no NaCl or other chlorides (Handy 1916; Anderson 1958; Hammer 1986; Nesbitt 2004). Organisms growing at high $MgSO_4$ concentrations might be called salinotolerant or osmotolerant, but these terms seem too broad. It is suggested that these organisms be characterized as “epsotolerant” or “epsophilic,” either growing at or requiring high concentrations of $MgSO_4$, respectively (Crisler et al. 2012). While growth of halotolerant and halophilic organisms at high $MgSO_4$ concentrations has been reported (Markovitz 1961; Markovitz and Sylvan 1962; Boring et al. 1963; Crisler et al. 2012), no epsophilic organisms are known. One report from Basque Lake used media with 2 M $MgSO_4$; however, the enrichment cultures were not fully described (Foster et al. 2010). Epsotolerant bacteria were isolated from efflorescences on degraded stone surfaces using media with $MgSO_4$

concentrations as high as 25 % (Laiz et al. 2000; Mandrioli and Saiz-Jimenez 2002). An initial study (Crisler et al. 2010) of waters and sediments from Basque and Hot Lakes demonstrated good microbial growth on organically rich medium containing 2 M $MgSO_4$, with dozens of microbial isolates capable of growth in 10 % NaCl or 10 % $MgSO_4$ media, including algae and cyanobacteria. The isolates generally appear to be broadly epsotolerant and dominated by bacteria.

Concluding Remarks

Growth media for halophilic and halotolerant bacteria and archaea trace their composition to the high-protein fish and hides where these organisms first attracted attention. Media recipes have bifurcated into those directed at extreme halophiles and those directed at moderate halophiles and halotolerant microbes. As more isolates have been obtained from varied environments, more types of media have been modified to hypersaline variants. Development of new media and growth conditions in the future will likely be driven by the isolation of novel organisms from unique hypersaline environments.

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Chapter 3
Taxonomy of Halophilic Archaea and Bacteria

Antonio Ventosa, M. Carmen Márquez, Cristina Sánchez-Porro, and Rafael R. de la Haba

Introduction

Microorganisms that inhabit hypersaline habitats are designated as halophiles and they are extremophilic organisms that must cope not only with the high ionic composition but also with other environmental factors such as alkaline pH values, low oxygen availability, high or low temperatures, presence of heavy metals and/or other toxic compounds, etc. They are normal inhabitants of natural saline environments such as saline lakes and soils, marine and inland salterns and several other hypersaline habitats. Besides, they are found in a variety of food products and several other saline derived goods (Oren 2002; Ventosa 2006).

The response of microorganisms to salt has been studied by many authors and several classifications have been proposed. The most widely accepted classification was proposed by Kushner and Kamekura (1988) and is based on the optimal growth of microorganisms with respect to the concentration of NaCl. On the basis of this classification, halophilic microorganisms are divided in the following categories: **extreme halophiles**, able to grow optimally in media with 15–30 % (2.5–5.2 M) NaCl, **moderate halophiles**, growing optimally in media with 3–15 % (0.5–2.5 M) NaCl, and **slight halophiles**, represented by most marine microorganisms, which are able to grow optimally between 1 and 3 % (0.2–0.5 M) NaCl. In contrast, **non-**

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halophilic microorganisms are those organisms that grow optimally in media with less than 1 % (0.2 M) NaCl; however, some of them are able to tolerate high NaCl concentrations and they are defined as halotolerant or extremely tolerant (Kushner and Kamekura 1988).

Classically, two physiological groups of halophilic microorganisms have been differentiated in the hypersaline environments: the extremely aerobic halophilic Archaea (haloarchaea) and the moderately halophilic Bacteria. However, studies carried out during the last two decades using different approaches from a variety of hypersaline environments have permitted the isolation and characterization of a large number of species and in some cases genera and other higher taxa, reflecting a wide physiological and metabolic diversity. Besides, as we will point out later the halophilic Archaea are represented not only by the haloarchaea and on the other hand, not all species of haloarchaea can be considered as extremely halophilic since some of them are able to grow optimally at lower NaCl concentrations than those described for extreme halophiles and thus, they should be considered as moderately halophilic microorganisms. Moreover, the presence of haloarchaea in environments with low salinities has been reported (Purdy et al. 2004) and the general assumption that they were confined to hypersaline habitats should be revised. On the other hand, the halophilic Bacteria are currently represented by a large number of species included on different phylogenetic branches, reflecting their broad metabolic activities; although classically they have been considered as slight or moderately halophilic, several bacterial species have been described as extremely halophilic. This fact would reflect their ecological ability of adaptation to a wide range of different habitats that in most cases are not stable and might have changing saline conditions. A special attention should be devoted to the extremely halophilic bacterial species *Salinibacter ruber*, which has been found to be very abundant in many hypersaline environments, in which it constitutes the largest bacterial community together with the haloarchaeon *Haloquadratum walsbyi*.

On the next sections we will review the taxonomy of halophilic Archaea and Bacteria as well as the features and criteria which are used for their taxonomic characterization. We will emphasize those aspects that could be of interest for scientists for the correct characterization of these microorganisms.

Taxonomy of Halophilic Archaea

The halophilic Archaea are included in the phylum *Euryarchaeota*. They are represented by the extremely halophilic aerobic Archaea, also designated as haloarchaea, currently included within the class *Halobacteria* (Grant et al. 2001). Besides haloarchaea some halophilic methanogenic Archaea have also been isolated and described from hypersaline environments.

Methanogenic Archaea are strictly anaerobic microorganisms that obtain energy by formation of methane. They can grow by dismutating methyl compounds (methanol, methyl amines, or methyl sulfides). They have been found in many hypersaline environments but few halophilic species have been characterized in detail. Taxo-nomically the halophilic methanogens are included in the orders

Table 3.1 Archaeal genera which include validly published halophilic species names within the classes *Halobacteria* and *Methanomicrobia* (except otherwise indicated taken from de la Haba et al. 2011). In brackets are indicated the number of halophilic species within each genus

Taxon	Genera
Phylum *Euryarcheota*	
Class *Halobacteria*	
Genera	*Halobacterium* (4), *Haladaptatus* (3), *Halalkalicoccus* (2), *Halarchaeum*[a] (1), *Haloarcula* (7), *Halobaculum* (1), *Halobiforma* (3), *Halococcus* (7), *Haloferax* (11), *Halogeometricum* (1), *Halogranum*[b] (1), *Halomicrobium* (2), *Halonotius*[c] (1), *Halopelagius*[d] (1), *Halopiger* (2), *Haloplanus* (1), *Haloquadratum* (1), *Halorhabdus* (2), *Halorubrum* (24), *Halorussus*[e] (1), *Halosarcina* (1), *Halosimplex* (1), *Halostagnicola* (1), *Haloterrigena* (8), *Halovivax* (2), *Natrialba* (6), *Natrinema* (6), *Natronoarchaeum*[f] (1), *Natronobacterium* (1), *Natronococcus* (3), *Natronolimnobius* (2), *Natronomonas* (2), *Natronorubrum* (4), *Salarchaeum*[g] (1)
Class *Methanomicrobia*	
Genera	*Methanohalobium* (1), *Methanocalculus* (1), *Methanohalophilus* (3), *Methanosalsum* (1)

[a]Minegishi et al. (2010); [b]Cui et al. (2010c); [c]Burns et al. (2010); [d]Cui et al. (2010b); [e]Cui et al. (2010a); [f]Shimane et al. (2010); [g]Shimane et al. (2011).

Methanosarcinales and *Methanomicrobiales.* Only four genera include halophilic species (Table 3.1).

The haloarchaea are currently included within a single order and family, named *Halobacteriales* (Grant et al. 2001) and *Halobacteriaceae* (Gibbons 1974), respectively. At the time of writing (March 2011), the haloarchaea includes 115 species which are classified into 34 genera (Table 3.1). The complete descriptions of the features of these taxa can be found in the original articles, as well as in the second edition of *Bergey's Manual of Systematic Bacteriology* (Grant et al. 2001). The characterization of haloarchaeal species is currently based on a combination of phylogenetic analysis obtained by comparison of their 16S rRNA gene sequences, phenotypic and genotypic features as well as polar lipid analysis. It is noteworthy that the ICSP-Subcommittee on the taxonomy of the *Halobacteriaceae* published in 1997 the recommended Minimal Standards for describing new taxa within this family, a document that may be very useful to researchers for the correct taxonomic characterization of new species and genera of haloarchaea (Oren et al. 1997).

Haloarchaea are considered as the prokaryotes best adapted to high salt concentrations and in fact they cannot grow in freshwater media where most of them are lysed. They can grow easily aerobically in media with 20–25 % NaCl. They produce red to pink-pigmented colonies (except some species of the genus *Natrialba*) due to the presence of C_{50}-carotenoids designated bacterioruberins and thus, they are partially responsible of the typical pink pigmentations of the saline lakes and most highly salt concentrated ponds of salterns where they predominate. Some species are not only halophilic, but also are able to grow optimally at alkaline pH values and thus, are defined as haloalkaliphilic. Haloarchaea have typical archaeal characteristics such as the presence of ether-linked lipids that can be easily detected by thin-

layer chromatography (TLC) and they are used as a key feature for the differentiation of taxa, especially at the genus level. All haloarchaea contain phytanyl ether analogues of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Many species also have phosphatidylglycerol sulfate and one or more glycolipids and sulfated glycolipids. Haloarchaea have diphytanyl ($C_{20}C_{20}$) glycerol ether core lipids and some species may have additional phytanyl-sesterterpanyl ($C_{20}C_{25}$) glycerol core lipids as well as disesterterpanyl ($C_{25}C_{25}$) glycerol core lipids (Grant et al. 2001). Thus, as pointed out previously polar lipid profiles are used for the chemotaxonomic differentiation of many haloarchaeal genera. However, currently some genera of the *Halobacteriaceae*, such as the genus *Haloterrigena*, include species with different polar lipid profiles and their taxonomic position remains to be assessed (Oren et al. 2009).

Taxonomy of Halophilic Bacteria

In contrast to the halophilic Archaea, the species of Bacteria which have been reported as moderately or extremely halophilic are included in many different phylogenetic branches (phyla). We should also consider that besides the species that have been characterized following the currently accepted standards and whose names have been validly published, there are a large number of halophilic bacteria that have been isolated and studied from other points of view, such as by their production of enzymes or other compounds of interest, which have not been properly characterized. When the *Approved Lists of Bacterial Names* was published (Skerman et al. 1980) the number of species that were recognized as moderately halophilic was reduced: *Micrococcus halobius* (currently *Nesterenkonia halobia*), *Planococcus halophilus* (lately reclassified as *Marinococcus halophilus*), *Vibrio costicola* (currently *Salinivibrio costicola*), *Flavobacterium halmephilum* (currently *Halomonas halmophila*), *Paracoccus halodenitrificans* (currently *Halomonas halodenitrificans*) and *Spirochaeta halophila*. Besides, most of them had been isolated from salted foods (*V. costicola* and *P. halodenitrificans*), unrefined salt (*M. halobius*) or as a laboratory contaminant (*P. halophilus*) and only two species, *F. halmephilum* and *S. halophila*, had been isolated from natural environments (Dead Sea and Solar Lake, respectively). For more complete information on the early taxonomic studies on halophilic bacteria several reviews can be consulted (Ventosa 1988, 1989; Vreeland 1993). Currently the number of species names that have been validly published as moderately and extremely halophilic bacteria is very large and most of them have been isolated from hypersaline habitats (e.g., saline lakes, salterns, saline soils) as well as from salt and salted products (salted foods, salted hides, etc). Halophilic Bacteria constitute a very heterogeneous group of microorganisms with different biochemical activities, ranging from Gram-positive to Gram-negative rods, cocci, spiral cells, etc., aerobic to strictly anaerobic and phototrophic to heterotrophic (Oren 2002; Ventosa 2006; de la Haba et al. 2011). Phylogenetically they are included in at least eight phyla: *Actinobacteria, Bacteroidetes, Cyanobacteria,*

Table 3.2 Bacterial genera which include validly published halophilic species names within the Bacteria (except otherwise indicated taken from de la Haba et al. 2011). In brackets are indicated the number of halophilic species within each genus

Taxon	Genera
Phylum *Actinobacteria*	
Class *Actinobacteria*	
Genera	*Actinopolyspora* (3), *Amycolatopsis* (1), *Georgenia* (1), *Corynebacterium* (1), *Haloactinobacterium* (1), *Haloactinopolyspora*[a] (1), *Haloechinothrix*[b] (1), *Haloglycomyces* (1), *Nesterenkonia* (6), *Nocardiopsis* (12), *Haloactinospora* (1), *Streptomonospora (3), Isoptericola* (1), *Prauserella* (7), *Saccharomonospora* (3), *Saccharopolyspora* (2)
Phylum *Bacteroidetes*	
Class *Bacteroidia*	
Genus	*Anaerophaga* (1)
Class *Flavobacteria*	
Genera	*Gramella* (1), *Psychroflexus* (1)
Class *Sphingobacteria*	
Genera	*Salinibacter* (1), *Salisaeta* (1)
Phylum *Cyanobacteria*	
Genera	*Rubidibacter* (1), *Prochlorococcus* (1), *Halospirulina* (1)
Phylum *Firmicutes*	
Class *Bacilli*	
Genera	*Alkalibacillus* (5), *Aquisalibacillus* (1), *Bacillus* (10), *Filobacillus* (1), *Gracilibacillus* (4), *Halalkalibacillus* (1), *Halolactibacillus* (1), *Halobacillus* (14), *Jeotgalibacillus* (1), *Lentibacillus* (10), *Oceanobacillus* (3), *Ornithinibacillus* (1), *Paraliobacillus* (1), *Piscibacillus* (1), *Pontibacillus* (1), *Salimicrobium* (4), *Salinibacillus* (2), *Salirhabdus* (1), *Salsuginibacillus* (1), *Sediminibacillus* (2), *Salinicoccus* (11), *Tenuibacillus* (1), *Thalassobacillus* (2), *Virgibacillus* (13)
Class *Clostridia*	
Genera	*Acetohalobium* (1), *Halanaerobacter* (3), *Halanaerobium* (9), *Halobacteroides* (2), *Halocella* (1), *Halonatronum* (1), *Halothermothrix* (1), *Natranaerobius*[c] (2), *Natrionella* (1), *Natronovirga*[d] (1), *Orenia* (3), *Selenihalanaerobacter* (1), *Sporohalobacter* (2)
Phylum *Proteobacteria*	
Class *Alphaproteobacteria*	
Genera	*Antarctobacter* (1), *Citreimonas* (1), *Dichotomicrobium* (1), *Fodinicurvata* (2), *Hwanghaeicola*[e] (1), *Hyphomonas* (2), *Jannaschia* (1), *Maribaculum* (1), *Maribius* (2), *Marispirillum* (1), *Marivita* (2), *Maricaulis* (2), *Marispirillum*[f] (1), *Methylarcula* (2), *Oceanibulbus* (1), *Oceanicola* (1), *Palleronia* (1), *Paracoccus* (3), *Ponticoccus* (1), *Rhodobium* (2), *Rhodothalassium* (1), *Rhodovibrio* (2), *Rhodovulum* (1), *Roseicitreum*[g] (1), *Roseinatronobacter* (1), *Roseisalinus* (1), *Roseospira* (1), *Roseovarius* (2), *Salinihabitans* (1), *Salipiger* (1), *Sediminimonas* (1), *Shimia* (1), *Sulfitobacter* (2), *Tropicibacter* (1), *Woodsholea* (1), *Yangia* (1)

Table 3.2 (continued)

Taxon	Genera
Class *Gammaproteobacteria*	
Genera	*Aidingimonas* (1), *Alcanivorax* (4), *Alkalilimnicola* (2), *Alteromonas* (3), *Aestuariibacter* (1), *Aquisalimonas* (1), *Arhodomonas* (1), *Carnimonas* (1), *Chromohalobacter* (8), *Cobetia* (1), *Ectothiorhodospira* (3), *Ectothiorhodosinus* (1), *Glaciecola* (3), *Gilvimarinus* (1), *Haliea* (1), *Halochromatium* (2), *Halomonas* (58), *Halorhodospira* (3), *Halospina* (1), *Halothiobacillus* (1), *Idiomarina* (7), *Kushneria* (4), *Marichromatium* (2), *Marinobacter* (17), *Marinobacterium* (1), *Melitea* (1), *Methylohalomonas* (1), *Microbulbifer* (1), *Modicisalibacter* (1), *Nitrincola* (1), *Oleispira* (1), *Pseudidiomarina* (2), *Pseudoalteromonas* (3), *Psychromonas* (2), *Pseudomonas* (1), *Saccharospirillum* (2), *Salicola* (2), *Salinicola* (3), *Salinisphaera* (1), *Salinivibrio* (3), *Thioalkalibacter* (1), *Thioalkalivibrio* (2), *Thiohalobacter*[h] (1), *Thiohalorhabdus* (1), *Thiohalocapsa* (1), *Thiohalomonas* (2), *Thiohalophilus* (1), *Thiohalospira* (2), *Thiomicrospira* (1)
Class *Deltaproteobacteria*	
Genera	*Desulfocella* (1), *Desulfohalobium* (2), *Desulfonatronospira* (2), *Desulfosalsimonas*[i] (1), *Desulfovermiculus* (1), *Desulfovibrio* (7), *Desulfurivibrio* (1)
Class *Epsiloproteobacteria*	
Genera	*Arcobacter* (1), *Sulfurimonas* (1), *Sulfurovum* (1)
Phylum *Spirochaetes*	
Class *Spirochaetes*	
Genus	*Spirochaeta* (4)
Phylum *Tenericutes*	
Class *Mollicutes*	
Genus	*Haloplasma* (1)
Phylum *Thermotogae*	
Class *Thermotogae*	
Genus	*Petrotoga* (1)

[a]Tang et al. (2011); [b]Tang et al. (2010); [c]Mesbah et al. (2007); [d]Mesbah and Wiegel (2009); [e]Kim et al (2010); [f]Lai et al. (2009); [g]Yu et al. (2011); [h]Sorokin et al. (2010); [i]Kjeldsen et al. (2010).

Firmicutes, Proteobacteria, Spirochaetes, Tenericutes and *Thermotogae*. Table 3.2 shows the genera which include moderately or extremely halophilic species whose names have been validly published by the inclusion in the *Approved Lists of Bacterial Names* (Skerman et al. 1980) or published on the *International Journal of Systematic and Evolutionary Microbiology* or on the *Validation Lists* that are published in this journal, which is the official organ of the International Committee on Systematics of Prokaryotes (ICSP). We should emphasize that in some cases the genera include only halophilic species but in some others halophilic and non-halophilic or halotolerant representatives are included in the same genus, which might reflect their common phylogenetic origin and that the halophilic feature is not circumscribed to a very specific group of bacteria. Most species and genera are grouped in the *Proteobacteria* (*Gamma*- and *Alphaproteobacteria*), *Firmicutes* and

Actinobacteria. Several genera include a large number of halophilic species and are especially interesting since they are used as models for the study of the mechanisms of haloadaptation and other basic studies, as well as for the exploration of their utilization in biotechnological processes. Some examples are the members of the family *Halomonadaceae* (especially *Halomonas* and *Chromohalobacter*), the genus *Halobacillus* and other related endospore-forming aerobic bacteria, such as several species of *Virgibacillus*, *Lentibacillus* or *Bacillus*. For more detailed information about the taxonomy of the halophilic bacteria we recommend readers to consult a recent review on this topic (de la Haba et al. 2011) as well as the most recent editions of the *Bergey's Manual of Systematic Bacteriology* and *The Prokaryotes*.

Methodology for the Characterization of Halophiles

Traditionally, classification of microorganisms was based on numerical taxonomy, which consists on the analysis of a large set of phenotypic (morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests) data. Lately, the elucidation of the structure of DNA molecule and the development of molecular techniques resulted in a classification system based, together with the phenotype, on genotypic features such as DNA G+C content or DNA-DNA hybridization. During the seventies, phylogenetic studies based on 16S rRNA gene sequence established the foundations of the current systematic. Nowadays, it is accepted that a suitable classification system for prokaryotes, mainly at the lowest taxonomic levels such as genera and species, must be based on a polyphasic approach, which combine as many data and techniques as possible, fundamentally phenotypic (including chemotaxonomic), genotypic and phylogenetic characteristics (Vandamme et al. 1996; Goodfellow et al. 1997). Characterization and description of halophilic microorganisms is not an exception, and this polyphasic approach should be followed.

In the last decades, minimal standards for describing new taxa belonging to different phylogenetic taxa that include halophilic microorganisms have been reported (Boone and Whitman 1988; Oren et al. 1997; Imhoff and Caumette 2007; Arahal et al. 2007; Logan et al. 2009; Schumann et al. 2009). In these publications, the minimal characteristics that authors should follow in order to describe a novel halophilic taxon are clearly provided. When such guidelines do not exist, descriptions should follow guidelines for closely related taxa. A list with the published minimal standards up to date is provided at http://www.bacterio.cict.fr/minimalstandards.html (Euzéby 2012).

Phylogenetic Analysis

Carl Woese, from the University of Illinois (USA), selected the 16S rRNA gene as a molecular phylogenetic marker and, using the sequencing methods existing at that time, obtained nucleotide sequence catalogues from different microorganisms and constructed a universal tree of life, achieving a global understanding of the

prokaryotic phylogeny (Woese et al. 1975; Woese and Fox 1977). Since then, 16S rRNA gene sequence analysis has become widespread because it provides a useful working hypothesis on which other elements may be compared when investigating the microbial taxonomy and evolution. In fact, currently, this approach remains the backbone of prokaryotic systematics (Ludwig and Schleifer1999; Ludwig and Klenk 2001). It is realistic to assume that the recognition of novel taxa often centres on the use of 16S rRNA gene-based techniques (Tindall et al. 2010).

Given the important role of 16S rRNA gene sequence comparison in modern microbial taxonomy, some key points should be taken into consideration. The first one is the quality and length of the sequence itself. The careless handling of 16S rRNA gene sequences has negativecon sequences for the systematic of prokaryotes (Stackebrandt and Ebers 2006). Contrary to the former nucleotide cataloguing technique, modern sequencing methods permit to obtain high-quality and large sequences. According to Stackebrandt et al. (2002) all species descriptions should include a complete or almost complete 16S rRNA gene sequence (>1,300 nt, <0.5 % ambiguity). Garrityet al. (2004) further defined high-quality 16S rRNA gene sequences as >1,400 nt, <4 % ambiguity and fewer than 10 missing positions. The quality of the sequence should be assessed to check ambiguities, primary and secondary structures, and overlay of potential cistron heterogeneities (direct PCR fragment sequencing) (Tindall et al. 2010). Comparisons of the new sequence(s) against a set of properly aligned sequences are required to guarantee the quality. It is important to remember that the quality should be checked before sequences are deposited in public databases, published in journals or sent to culture collections along with type strain deposits. Not only should the new sequences be checked, but also the reference sequences used in the study. Retrieval of reference sequences from databases must be accomplished carefully because the sequence databases are full of incorrectly labelled and poor quality sequences. Therefore, when characterizing new taxa, a taxonomist should use only the best quality data available, including resequencing if appropriate.

With respect to the sequences to be included into the phylogenetic analysis, although many of them are from environmental clones or have been obtained from unidentified isolates, these sequences may be included during the preliminary stages of the analysis. However, for the final presentation of the results, it is recommended the inclusion of only those sequences from species whose names have been validly published (Arahal et al. 2007). A detailed list of validly published prokaryotic species names can be found in http://www.bacterio.net (Euzéby 1997). Furthermore, sequencesfrom strains other than the type strain usually exhibit little divergence and can be removed from the final tree. Sequence duplicates (sequences derived from the same or an equivalent strain, but obtained in different studies, and essentially identical) are unnecessary in final trees, and only those possessing the highest quality should be included (Arahal et al. 2007).

The ribosomal RNA operons–which code for both the small-subunit (16S rRNA) and large-subunit (23S rRNA)–are among the very few redundant genes found in prokaryotes (Klappenbach et al. 2000). They are quite conserved in sequence within one genome, being the interoperonic heterogeneity most often below 0.5 % (Clayton

et al. 1995; Coenye and Vandamme 2003; Acinas et al. 2004). Therefore, it is generally assumed that all rRNA operons within a single cell are almost identical. However, with the availability of more complete genomes published, several exceptions have been reported for species of genera of extremely halophilic Archaea, such as *Haloarcula*, *Halosimplex*, *Natrinema, Haladaptatus, Haloferax, Halomicrobium, Haloquadratum or Natronoarchaeum*, which are known to harbour highly divergent rRNAoperons that differ at ~5–6.7 % of the nucleotide positions in the 16S rRNA gene and at 1–2.6 % of the nucleotide positions in the 23S rRNA gene (Boucher et al. 2004; Minegishi et al. 2011). In such cases, direct PCR sequencing is not recommended. Instead, divergent 16S rRNA gene copies should be cloned into a vector and later sequenced in order to determinate the intragenomic 16S rRNA heterogeneity.

Chromosomal DNA isolation and purification can be performed as described elsewhere (de la Haba et al. 2010). Amplification and sequencing of 16S rRNA gene can be achieved with primers showed in Table 3.3. Some of them are universal for prokaryotes, but others are specific for Bacteria or Archaea. The following conditions are recommended for PCR amplification: (i) 5 min at 95 °C; (ii) 25 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C; and (iii) a final elongation step of 10 min at 72 °C.

Once the new high-quality sequence(s) have been obtained, an initial database search based on pairwise sequence comparisons should be carried out to determinate the reference sequences to be included into the analysis. This search can be executed with the aid of BLAST tools (Altschul et al. 1990) or EzTaxon-e server (Kim et al. 2012). Multiple alignment of new and reference sequences must be performed. Special care has to be taken during this step. The use of expert-maintained seed alignments comprising only high quality sequence data is highly recommended, e.g., ARB (www.arb-home.de), RDP (http://rdp.cme.msu.edu/), SILVA (www.arb-silva.de),and LTP (www.arb-silva.de/projects/living-tree/). A limitation is that seed alignments may not be universally compatible with some alignment programs. Alternatively, high quality sequences that were not previously aligned can be obtained from public databases and aligned using robust multiple alignment programs (Tindall et al. 2010), for example CLUSTAL W (Thompson et al. 1994), CLUSTAL X (Thompson et al. 1997), CLUSTAL W2, CLUSTAL X2 (Larkin et al. 2007), MEGA (Tamura et al. 2007), T-COFFEE (Notredame et al. 2000), or MUSCLE (Edgar 2004). Manual inspection of the alignments generated with automatic aligners is recommended, taking into consideration the secondary structure and previous alignments (Arahal et al. 2007). Examples of programs that can display secondary structure for sequence editing are ARB (Ludwig et al. 2004), jPHYDIT (Jeon et al.2005) or RnaViz (De Rijk et al. 2003). The Comparative RNA website and project (http://www.rna.ccbb.utexas.edu/) maintained by the Gutell Lab. at the University of Texas at Austin is an excellent source of reference secondary RNA structures.

After those alignments have been made and evaluated, pairwise nucleotide sequence similarity values can be calculated. The following programs are recommended for similarity calculations: ARB (Ludwig et al. 2004) and jPHYDIT (Jeon et al.2005). Programs such as CLUSTAL (Larkin et al. 2007)or PHYLIP (Felsenstein 1989) are also suitable. Pairwise similarity values obtained from local alignment

Table 3.3 Oligonucleotide primers recommended for PCR amplification and sequencing of 16S rRNA gene of halophilic Archaea and Bacteria. Sequences are listed according to the IUPAC code for nucleotide ambiguities. Primer positions are given according to the *E. coli* numbering

Primer	Specificity	Sequence (5¢→3¢)	Position	Orientation	Use	Reference
D30	Archaea	ATTCCGGTTGATCCTGC	6–22	Forward	PCR, sequencing	Arahal et al. (1996)
16F27	Bacteria	AGAGTTTGATCMTGGCTCAG	8–27	Forward	PCR, sequencing	de la Haba et al. (2010)
16R343	Bacteria	ACTGCTGCCTCCCGTA	343–358	Reverse	Sequencing	de la Haba et al. (2010)
D33	Archaea	TCGCGCCTGCGCCCCGT	344–360	Reverse	Sequencing	Arahal et al. (1996)
16F530	Prokaryotes	GTGCCAGCAGCCGCGG	515–530	Forward	Sequencing	de la Haba et al. (2010)
B36	Prokaryotes	GGACTACCAGGGTATCTA	789–806	Reverse	Sequencing	Arahal et al. (1996)
D34	Archaea	GGTCTCGCTCGTTGCCTG	1096–1113	Reverse	Sequencing	Arahal et al. (1996)
16R1488	Prokaryotes	CGGTTACCTTGTTAGGACTTCACC	1488–1511	Reverse	PCR, sequencing	de la Haba et al. (2010)

programs,such as BLAST, are not adequate because they are primarily useful for database searches. Furthermore, corrected evolutionary distances (e.g., Jukes and Cantor 1969 model) should not be used for pairwise similarity calculations (Tindall et al. 2010). Overall sequence similarity values might be sufficient to assign a strain to defined taxa if comprehensive high quality reference datasets are available. A 97 % 16S rRNA gene sequence similarity is extensively documented as the threshold value below which two strains are not considered members of the same species (Martínez-Murcia and Collins 1990; Collins et al. 1991; Amann et al. 1992; Fox et al. 1992; Martínez-Murcia et al. 1992; Stackebrandt and Goebel 1994). Therefore, 16S rRNA gene sequences alone do not describe a species, but may provide the first indication that a novel species has been isolated (less than 97 % gene sequence similarity). Where 16S rRNA gene sequence similarity values are higher than 97 % (over full pairwise comparisons), other methods such as DNA-DNA hybridization or analysis of gene sequences with a greater resolution must be used. These methods must also be correlated with the characterization based on phenotypic tests. Concerning the taxonomic rank of the genus, at values ~95 % 16S rRNA gene sequence similarity (overfull pairwise comparisons),taxa should be analyzed by other features to establish whether they represent separate genera (Tindall et al. 2010).

When previous considerations have been analyzed, phylogenetic inference should be performed according to thestate of the art. Several sequences should be used as outgroups, but never use sequences from single distantly related organisms as (an) outgroup(s) to avoid the long-branch attraction phenomenon. The most commonly used treeing methods are based on distance matrix, maximum-parsimony and maximum-likelihood (Ludwig et al. 1998; Ludwig and Klenk 2001). The latter two are to be preferred; distance matrix methods should be used for raw screening only (Ludwig and Klenk 2001; Peplies et al. 2008). Topologicaldifferences are very likely to occur, since each treeing method relies on a different model of evolution. However, not all branches will be equally affected; those branches or groups of branches that remain unchanged regardless of the algorithm followed can be considered very stable (Arahal et al. 2007). Other means to evaluate the statisticalsignificance of the branching order are the application of filters and weighting masks and the use of resampling techniques (bootstrap analyses) (Felsenstein 1985; Ludwig et al. 1998; Ludwig and Klenk 2001). Such confidence tests are strongly recommended, especially when tree topologies are used to infer or support taxonomic conclusions. In the case of the bootstrap test, only values of 70 % or higher should be includedin the phylogenetic trees. More than 350 phylogeny programs are listed on the Felsenstein Lab. website at the University of Washington (http://evolution.genetics.washington.edu/phylip/software.html). Among the most recommended are ARB (Ludwig et al. 2004), PHYML (Guindon and Gascuel 2003), PAUP* (Swofford 2002), PHYLIP (Felsenstein 1989) and RAXML (Stamatakis 2006).

Despite the widespread use of 16S rRNA gene sequencing, there is growing interest in the use of other genes with a greater degree of resolution (23S rRNA gene and protein-encoding genes) to resolve issues that are not solved by 16S rRNA gene sequencing. 23S rRNA gene sequences have been deeply analyzed within the halophilic family *Halomonadaceae* and a good agreement between phylogenies based

on 16S and 23S rRNA gene sequences was found, although 23S rRNA showed a higher resolution capability (Arahal et al. 2002; de la Haba et al. 2010). However, 23S rRNA dataset is currently much smaller and the 16S rRNA gene sequence presently remains the gene sequence of choice (Tindall et al. 2010). On the other hand, sequences of conserved protein-encoding genes, typically called "housekeeping genes" can be used to carry out a Multilocus Sequence Analysis (MLSA) approach either compared as individual datasets or combined in concatenated sequences. Ribosomal RNA gene sequence can also be used along with protein-encoding gene sequences to conduct the MLSA. This approach has been proposed as an alternative to DNA-DNA hybridization for species delineation, but in order to do that, the MLSA scheme must be correlated with DNA-DNA hybridization data and intraspecific diversity must be evaluated (Stackebrandt et al. 2002). Up to date, only one single exhaustive MLSA dealing with haloarchaea has been published (Papke et al. 2011). In this study, MLSA was utilized for evolutionary and taxonomic investigation of the order *Halobacteriales*. For that purpose, five housekeeping genes (*atpB*, *EF-2*, *radA*, *rpoB* and *secY*) were tested across a hierarchical gradient using 52 halobacterial strains, representing thirty-three species (including names without standing in nomenclature) and fourteen genera. Results demonstrated MLSA differentiated individual strains, reliably grouped strains into species and species into genera, and identified potential new species and also family-like relationships. Therefore, MLSA is proved to be a rapid and informative molecular method that will likely accommodate strain analysis at any taxonomic level within the *Halobacteriales*. Additional MLSA studies on halophilic bacteria of the family *Halomonadaceae* have been recently reported. In this case, four housekeeping genes (*atpA*, *gyrB*, *rpoD* and *secA*) along with 16S and 23S rRNA genes have been analyzed on 52 type strains of validly published species names from the family *Halomonadaceae* belonging to nine different genera. Nevertheless, since only type strains have been studied the intraspecific diversity has not been elucidated. MLSA has showed that horizontal gene transfer (HGT) plays an important evolutionary role in the family *Halomonadaceae*; however, the impact of recombination events in phylogenetic analysis was minimized by concatenating the six loci, which agreed with the current taxonomic scheme of this family (de la Haba et al. 2012).

Phenotypic Characteristics

Phenotypic features include a large number of morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests. As previously stated, since halophilic microorganisms constitute a very heterogeneous physiological group it is not possible to suggest a single culture medium for the characterization of all of them. There are three preliminary tests that are crucial to perform in order to know the optimal conditions that support the growth of these organisms: salt, pH and temperature requirements. It is essential to test not only the range of each parameter but also the optimal conditions within those ranges. These growth experiments should be performed in liquid media and growth rate may be determined measuring

the increase in turbidity. For halophiles, the optimal NaCl concentration should be determined first and subsequent tests for other growth parameters should be carried out at the optimal NaCl concentration. Growth at different concentration of NaCl must be determined on liquid media to which different NaCl concentrations should be added: 0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25, and 30 %, w/v. To test growth in the absence of NaCl, minimal media are recommended, as well as cell inocula free of the medium in which they were grown. To determine the optimal NaCl concentration, in addition to the values previously recommended, further tests might be required to assess with more accuracy the physiological boundaries of the organisms being studied; for instance, if the strain grows better at 10 % (w/v) NaCl, growth at values close to 10 % should also be tested. The optimal NaCl concentration is that in which the organism first began to grow; therefore, it is essential to observe the growth of the inocula at different NaCl concentrations during the first 12–24 h of incubation. On the other hand, it must be taken into account that many organisms grow better with salt mixtures than with NaCl. In some cases specific ions different to NaCl or together with this salt are necessary to support growth and, therefore, these requirements should be studied in detail (for instance, aerobic halophilic Archaea may have specific requirement for MgCl in addition of NaCl). To examine the effect of pH on growth, the media with the optimal salt concentration and composition should be supplemented with suitable buffers to maintain stable pH values. Adjustment of pH must be done with an electrode that can operate at high salinities, especially for alkaline media, before and after autoclaving the liquid media. Further phenotypic tests should be carried out with cells grown under these optimal set of conditions.

Many phenotypic features used for the description of the phenotype are affected by cultural or test conditions. For this reason, a comparative experimental study of phenotypic characteristics in representatives of putative new species, and of type strains of recognized taxa in the authors' own laboratories rather than comparisons with data reported in the literature must be performed (Tindall et al. 2010).This is crucial when the organisms to be compared appear highly related on the basis of 16S rRNA gene sequences ($\geq$97 % sequence similarity). In the case that the novel isolates may constitute a new genus, the type species of the related genera must also be included in the phenotypic study. However, when the novel strain to describe is a halophilic microorganism, these recommendations are not always easy to follow since sometimes the novel organisms are closely related to reference strains that are non-halophiles or have a different optimal requirement of salts. In these cases, it is impossible to grow all strains under the same conditions.

Guidelines for the phenotypic description of halophilic Bacteriaand Archaea are found in numerous publications (Arahal et al. 2007; García et al. 1987; Márquez et al. 2008; Mata et al. 2002; Oren et al. 1997; Quesada et al. 1984; Ventosa et al. 1982; Vreeland 1993). Regarding the biochemical and nutritional tests, it is important to point out that the use of commercially available kits, such as API ZYM, API 50CH, API 20E, API 20NE or Biolog system, should not substitute the conventional methodologies, since they may often give different results for a particular test. Besides, these miniaturized systems are not generally designed for halophilic organisms, so it is important to carry out some modifications in order to adapt them to the requirements of these organisms. For instance, it is necessary to replace the inocula-

tion fluid with an appropriate saline solution in order to permit the cells to grow under optimal NaCl conditions.

Chemotaxonomic Characteristics

The chemotaxonomic features that must be taken into account as taxonomic markers are different depending on the physiological group to study. For Gram-positive halophilic bacteria, it is essential to describe the fatty acid, respiratory lipoquinone, and polar lipid composition, as well as the amino acid composition of the peptidoglycan of the cell wall, whereas for Gram-negative bacteria, only the analysis of fatty acids and polar lipids is highly recommended. On the other hand, the polar lipid composition is the most important chemotaxonomic trait useful as taxonomic marker in haloarchaea. We include here some general guidelines about the use of these chemotaxonomic markers.

Cellular Fatty Acids

The general type of cellular fatty acids is of significance for classification at the genus level whereas the quantitativeprofile of cellular fatty acids can be used for the differentiation of species. The comparison of fatty acid profiles necessitates the standardization of the cultivation conditions prior to fatty acid extraction, since the composition of media and the culture conditions have a strong influenceon the fatty acid patterns (Tindall et al. 2010). In the case of halophilic microorganisms, standardization of the salts content of the media is again essential.

The preferable method for analysis of whole cell fatty acid profiles is capillary gas chromatography of fatty acid methyl esters. The Sherlock Microbial Identification System (MIDI Inc) is a fully automated gas chromatographic analytical system, which identifies bacteria based on their unique fatty acid profiles. Although it provides a comprehensive database, this is certainly not complete and there are some discrepancies that need to be clarified or compounds that are currently not included in the database (Tindall et al. 2010). These determinations should be carried out growing the cultures under their optimal conditions (culture medium, pH, temperature and incubation time) and comparing the new organism with the reference strains (most closely related type strains) under the same laboratory conditions.

Polar Lipids

There is a vast diversity of polar lipids now known to be present in prokaryotes and in many cases their structures have yet to be fully elucidated and their biosynthesis is also not fully understood. One important distinction between Archaea and Bacteria is the chemical structure of lipids composing the cytoplasmic membrane. Whereas in Archaea the range of lipids known to occur currently is

restricted to phospholipids, glycolipids and phosphoglycolipids, in members of the Bacteria this range includes phospholipids, glycolipids, phosphoglycolipids, amino acid derived lipids, capnines, sphingolipids (glyco- or phosphosphingolipids) and also hopanoids. Analyses of polar lipids are performed in most laboratories by two dimensional TLC as described by for example Lechevalier et al. (1977), Komagata and Suzuki (1987), Tindall (1990), Kamekura (1993) and Xin et al. (2000). Many publications describing halophilic organisms report "unknown" phospholipid and glycolipid components which could not be identified at the time of analysis. Given the large diversity of polar lipid known to be present in prokaryotes, it has been suggested that authors document the lipids present by providing a figure of the thin layer plate stained with a reagent that will allow all lipids to be visualized (Tindall et al. 2010).

Respiratory Quinone Systems

Isoprenoid or respiratory quinones are a class of terpenoid lipids constituents of bacterial cytoplasmic membranes. The determination of the quinone composition is currently recognized as one of the most important traits in bacterial chemotaxonomy. Respiratory quinones are divided into two major structural groups, the naphthoquinones (which include menaquinones, demethylmenaquinones, monomethylmenaquinones, dimethylmenaquinones, and menathioquinones) and the benzoquinones (which include ubiquinones, rhodoquinones and plastoquinones) (Tindall et al. 2010). Separation of simple quinone mixtures may be undertaken using reverse phase thin layer chromatography, but a properly calibrated reverse phase HPLC column provides greater reproducibility and allows more accurate quantification. If quinones that cannot be identified are detected information such as UV-visible spectroscopy and accurately reporting behavior in thin layer and HPLC chromatography systems would be helpful. Full structural identification can usually be achieved by a combination of mass spectrometry and NMR (Tindall et al. 2010).

Peptidoglycan Structure

Peptidoglycan structure is an important tool for the differentiationof Gram-positive bacteria, however, no variation has been reported among members of the *Proteobacteria* and *Bacteroidetes* (Tindall et al. 2010). Analyses of the peptidoglycan structure can be performed at different levels. The elucidation of the detailed peptidoglycan structure according to Schleifer and Kandler (1972) and Schleifer (1985) is a rather demanding task thatrequires specific experience and rather few laboratories are equipped to analyze it. The simplest analysis is the determination of the characteristic diamino acid in the cross linking peptide. Analysis of the peptidoglycan type (A: cross-linkage of the two peptide side chains via amino acid 3 of one peptide subunit to amino acid 4 of the other peptide subunit; B: cross-linkage of the two peptide side chains via amino acid 2 of the one peptide subunit to amino acid 4

of the other peptide subunit), mode of cross-linkage (direct or interpeptide bridge and amino acids in the bridge) and complete amino acid compositions provides more detailed information. For description of members of novel Gram-positive bacteria, determination of the diagnostic diamino acid is essential, and determination of the peptidoglycan structure is essential for description of new genera and strongly recommended for all novel Gram positive species. A list of peptidoglycan variations can be found at http://www.dsmz.de/microorganisms/main.php?content_id=35.

Genotypic Characteristics

DNA Base Composition

The range DNA of G+C values within a genus is an important taxonomic criterion. The indication of the DNA G+C value of the type strainof the type species of a novel genus is mandatory and highly recommended for type strains of novel species in established genera (Stackebrandtet al. 2002). The determination of DNA G+C values can be carried out following different methodologies, such as HPLC, thermal denaturation (*Tm*), or buoyant density (Bd) (De Ley 1970; Mesbah et al. 1989). Values obtained by these methods may differ; therefore, determinations should be made by using the same methods for all organisms to be compared.

DNA-DNA Hybridization

DNA-DNA hybridization (DDH) has been used by bacterial taxonomists since the 1960s to determine relatedness between strains and currently it is still the most important criterion in the delineation of prokaryote species. A value equal or higher than 70 % DDH was proposed by Wayne et al. (1987) as a recommended standard for delineating species. However, this threshold should not be rigidly applied and should not be considered itself sufficient evidence for the recognition of a novel species, since some strains of a species may show a value lower than 70 % DDH with the type strain or other strains of the same species (Goris et al. 2007). Past experience has shown that DDH is recommended for the evaluation of species status when the value for 16S rRNA gene sequence similarity is equal or above 97 % (Stackebrandt and Goebel 1994).

Several principally different methods for the measurement of DDH values have been described (Brenner et al. 1969; Crosa et al. 1973; De Leyet al. 1970; Ezaki et al. 1989), and the use of DDH in bacterial taxonomy has recently been reviewed in detail (Rosselló-Mora2006). While the technique has the above-mentioned advantages, it also has several important drawbacks. Because relatively large quantities of DNA (in comparison with PCR-based techniques) of a high quality are required, the whole process of performing DDHs often becomes rather time-consuming and labour-intensive. Also, the diverse methods that are available can

yield different results, especially for lower reassociation values (Grimont et al. 1980; Huß et al. 1983). Its main disadvantage, however, is that because of the comparative nature of the technique no incremental databases can be built, in contrast to sequence information, for example (Gevers et al. 2005; Stackebrandt 2003). Due to these drawbacks, bacterial taxonomists are actively searching for alternative methods that can replace DDH experiments (Cho and Tiedje 2001; Coenye et al. 2005; Gevers et al. 2005).

In order to evaluate the stringency of the DNA-DNAhybridization, it is necessary that the experimental conditions(buffer system, ionic strength and reassociation temperature)are reported. Besides, it is also necessary that the integrities of the bacterial DNAs used in such studies always be checked before determining DNA G+C content and DNA–DNA relatedness values, as poor quality DNAs may yield misleading results.

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Chapter 4
Halophilic Viruses

Shereen Sabet

Introduction

It is now well-established that viruses are the most abundant biological entities on Earth and are estimated to harbor the second greatest biomass after prokaryotes, equivalent to the amount of carbon found in ∼75 million blue whales (the largest organism on Earth) (Suttle 2005). College textbooks commonly quote a global estimate of 10^{30}–10^{31} individual phages (Acheson 2007; Flint et al. 2009), and investigations have reported a range between 10^6 and 10^9 viruses per milliliter in samples taken from various aquatic habitats (Fuhrman 1999; Wommack and Colwell 2000; Jiang et al. 2004; Suttle 2005; Baxter et al. 2011; Sime-Ngando et al. 2011). While the scientific literature indicates that over 5,500 phages have been described (Ackermann 2007), much of the scientific community has focused attention on the relatively fewer pathogenic viruses of humans, animals, and agricultural crops. Nonetheless, phages (previously bacteriophages) have played an essential role in basic biological research, even becoming the basis for establishing the field of molecular genetics (Summers 2005).

The origin of viruses is still unknown, and there are differing views as to whether viruses preceded and possibly helped to give rise to cells, or if cells gave rise to viruses (Forterre 2006; Koonin et al. 2006; Forterre and Prangishvili 2009). It is, of course, entirely plausible that there may be multiple origins for viruses, including the possibility that viruses may have actually begun as a form of genetic communication between and among cells. With the discovery of certain bacteria having undergone an evolutionary reduction of their genome (Andersson and Kurland 1998; Wixon 2001) and the discovery of the largest viruses that contain some bacterial elements (CroV and Mimivirus (Filee and Chandler 2010)), it is also possible that certain viruses may actually have originated as cells that underwent reductive evolution, resulting in severely reduced genomes.

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The origin of viruses still poses a major mystery to biologists, and undoubtedly there will be continued research to try and solve it. Nonetheless, over the last thirty years, the importance of environmental viruses has become clear. While bacterioplankton play an essential role in the biogeochemical cycling of dissolved matter through the microbial loop in aquatic food webs (Jones et al. 1998; Kirchman 2000), phages play an equally significant role as a major cause of prokaryote mortality affecting microbial community composition and population dynamics, resulting in the restructuring of both prokaryotic and eukaryotic communities (van Hannen et al. 1999; Middelboe 2000; Middelboe et al. 2001). "Predation" by phages also influences the microbial loop by removing prokaryotes as a food source for grazers (e.g., flagellates), and infection rates within the aquatic environment have been estimated at approximately 10 %, although the evidence for viral-induced mortality ranges from insignificant rates to 50 %, and as high as 100 % in anaerobic waters (Fuhrman 1999). There is also evidence to suggest that viruses may even be contributors to the microbial loop by acting as a direct source of nutrient (mainly for heterotrophic flagellates) (Gonzalez and Suttle 1993).

Besides being a cause of host mortality, there is growing evidence that phages in aquatic environments also affect the genetic architecture of their hosts (Wommack and Colwell 2000; Weinbauer and Rassoulzadegan 2004). Metagenomics analyses of viral genomes (or metaviromes)—including those from hypersaline sites—showed similarity to microbial gene and amino acid sequences (Santos et al. 2010; Sime-Ngando et al. 2011). Through transduction, phages are involved in lateral gene transfer among hosts, including genes involved in metabolism (Jiang and Paul 1998). Furthermore, viral genomes have been shown to encode for prokaryotic proteins, such as a photosynthetic reaction center (Mann et al. 2003), that enhance host survival. Such findings implicate the virus as a positive symbiont, helping the host to metabolize more efficiently. Because of their ability to shape a host's genetic make-up, viruses are increasingly being viewed as fundamental contributors to the evolution of life on Earth (Weinbauer and Rassoulzadegan 2004; Tyson and Banfield 2008). Our view of viruses, therefore, may not be as simple as we initially thought as they may be playing a seemingly more sophisticated role within nature; and, as confusing as it may seem that they are technically not 'alive', they may even be viewed as bridging that fine line between inanimate and animate.

Although strides have been made in studying pathogenic eukaryotic and mesophilic bacterial viruses, there is still a dearth of knowledge regarding environmental and extremophilic viruses, especially those of the *Archaea*. There have been 25 viruses isolated that infect the Crenarchaeota (the hyperthermophiles) (Prangishvili and Garrett 2005) and $\sim$75 viruses that infect Euryarchaeota—a handful that infect methanogens (Stedman et al. 2010) and 70 that infect halophiles (Table 4.1). While halophilic *Bacteria* and *Archaea* have been studied for over a century, the field of halophilic viruses, or halophages, did not begin until the mid-1970s after accidental discovery of Hs-1, a halophage that infected *Halobacterium salinarum* (Torsvik and Dundas 1974). Since then, 9 halophage isolates infecting *Bacteria* and 56 infecting *Archaea* have been described in the scientific literature (Table 4.1). Surprisingly, viruses that infect halophilic eukaryotes (e.g., *Dunaliella*

Table 4.1 List of known halophages, including both isolates and completely sequenced environmental viruses

Halophage Name	Host Type	Isolating Author(s)	Morphology	Genotype
Hs-1	A	Torsvik and Dundas (1974)	T	n/k
Ja.1	A	Wais et al. (1975)	T	dsDNA
ϕH	A	Schnabel et al. (1982)	T	lin. dsDNA
Hh-1	A	Pauling (1982)	T	lin. dsDNA
Hh-3	A	Pauling (1982)	T	lin. dsDNA
S45	A	Daniels and Wais (1984)	T	dsDNA
ϕF9-11	B	Calvo et al. (1988)	T	n/k
ϕN	A	Vogelsang-Wenke and Oesterhelt (1988)	T	lin. dsDNA
S5100	A	Daniels and Wais (1990)	T	n/k
ϕPs-G3	B	Kauri et al. (1991)	T	n/k
ϕ7116	B	Uchida and Kanbe (1993)	T	n/k
ϕD-86	B	Uchida and Kanbe (1993)	T	n/k
HF1	A	Nuttall et al. (1993)	T	lin. dsDNA
HF2	A	Nuttall et al. (1993)	T	lin. dsDNA
B10	A	Torsvik (1982)	T	n/k
ϕUTAK	B	Goel et al. (1996)	T	dsDNA
ϕCh1	A	Witte et al. (1997)	T	lin. dsDNA
S50.2	A	Daniels and Wais (1998)	T	n/k
S4100	A	Daniels and Wais (1998)	T	n/k
S41	A	Daniels and Wais (1998)	T	n/k
S50.2 Vm	A	Daniels and Wais (1998)	T	n/k
S41 Vm	A	Daniels and Wais (1998)	T	n/k
His1	A	Bath et al. (1998)	F	lin. dsDNA
SH1	A	Porter et al. (2005)	S	lin. dsDNA
His2	A	Bath et al. (2006)	F	lin. dsDNA
BJ1	A	Pagaling et al. (2007)	T	lin. dsDNA
BJ2	A	Pagaling, unpublished	T	lin. dsDNA
SNJ1	A	Mei et al. (2007)	T	n/k
GNϕ1	A	Sabet, unpublished	T	n/k
GNϕ2	A	Sabet, unpublished	T	lin. dsDNA
HRPV-1	A	Pietilä et al. (2009)	P	circ ssDNA
SCTP-1	B	Kukkaro and Bamford (2009)	T	n/k
SCTP-2	B	Kukkaro and Bamford (2009)	T	n/k
HHTV-1	A	Kukkaro and Bamford (2009)	T	n/k
HCTV-1	A	Kukkaro and Bamford (2009)	T	n/k
HRTV-1	A	Kukkaro and Bamford (2009)	T	n/k
HHPV-1	A	Roine et al. (2010)	P	circ dsDNA
HRTV-2	A	Atanasova et al. (2012)	T	n/k
SCTP-3	B	Atanasova et al. (2012)	T	n/k
HRTV-3	A	Atanasova et al. (2012)	T	n/k
HRTV-4	A	Atanasova et al. (2012)	T	n/k
HRTV-5	A	Atanasova et al. (2012)	T	n/k
HRTV-6	A	Atanasova et al. (2012)	T	n/k
HRTV-7	A	Atanasova et al. (2012)	T	n/k
HSTV-1	A	Atanasova et al. (2012)	T	n/k
HHIV-2	A	Atanasova et al. (2012)	S	n/k
HJTV-1	A	Atanasova et al. (2012)	T	n/k
HVTV-1	A	Atanasova et al. (2012)	T	n/k
HJTV-2	A	Atanasova et al. (2012)	T	n/k
HCTV-2	A	Atanasova et al. (2012)	T	n/k

Table 4.1 (continued)

Halophage Name	Host Type	Isolating Author(s)	Morphology	Genotype
HCTV-5	A	Atanasova et al. (2012)	T	n/k
HVTV-2	A	Atanasova et al. (2012)	T	n/k
HHTV-2	A	Atanasova et al. (2012)	T	n/k
HRTV-8	A	Atanasova et al. (2012)	T	n/k
HRPV-2	A	Atanasova et al. (2012)	P	n/k
HGTV-1	A	Atanasova et al. (2012)	T	n/k
HATV-1	A	Atanasova et al. (2012)	T	n/k
HRPV-3	A	Atanasova et al. (2012)	P	n/k
SSIP-1	B	Atanasova et al. (2012)	S	n/k
HSTV-3	A	Atanasova et al. (2012)	T	n/k
HSTV-2	A	Atanasova et al. (2012)	T	n/k
HRTV-9	A	Atanasova et al. (2012)	T	n/k
HRTV-10	A	Atanasova et al. (2012)	T	n/k
HATV-2	A	Atanasova et al. (2012)	T	n/k
HTV-1	A	Atanasova et al. (2012)	T	n/k
HRTV-11	A	Atanasova et al. (2012)	T	n/k
HRTV-12	A	Atanasova et al. (2012)	T	n/k
HGPV-1	A	Atanasova et al. (2012)	P	n/k
EHP-1	n/k	Santos et al. (2007)	n/k	DNA
EHP-2	n/k	Santos et al. (2010)	n/k	DNA

A archaeal; *B* bacterial; *T* tailed; *F* fusellovirus (i.e., lemon-shaped or spindle-shaped); *S* spherical (i.e., icosahedral); *P* pleomorphic; *lin*. linear; *circ*. circular; *n/k* not known

sp. alga, *Halocafeteria* sp. nanoflagellate, and *Navicula diserta* diatom) have yet to be described in the scientific literature, and no viruses have yet been discovered that infect extreme hypersaline fungi (Nina Gunde-Cimerman, personal communication). The field of halophages has been a relatively slow one over the last three decades, but exciting progress has been made in just the last several years.

The first report to investigate the ecology of halophages in a salt pond was published in 1985. The pace of such studies was slow as ten years passed before the next set of ecologically-relevant reports was published that involved morphological analysis and enumeration of virus-like particles (VLP). The first section of this chapter will review the classical literature and also highlight the more recent ecological studies, which have employed the popular and relatively new culture-independent methodology of metagenomics to learn more about the halophage community at both the local and global levels. While there have been several ecological reports, halophage isolates have also been described in the scientific literature, but at a basic level—relatively few have been genetically sequenced and studied. The second section of this chapter will summarize information about those halophages that have been studied extensively at the genomic level. Whether investigating the environmental halophage community or individual isolates, scientific resources (e.g., protocols and products) with a hypersaline focus have become increasingly available and more refined over the past 30 years. These resources will be reviewed in the third section of this chapter, followed by suggestions, summarized in the final section, for where the field of halophage research ought to proceed.

Ecological Studies

The first study to address ecological questions about halophages was reported by Wais and Daniels who investigated shallow Jamaican hypersaline ponds (Wais and Daniels 1985). They utilized a culture-dependent approach that included viral enumeration under different salinities. They initially observed that rainfall led to the dilution of hypersaline ponds resulting in the drastic reduction of halophiles while simultaneously increasing the number of free halophages. Therefore, the decreased salinity in these ponds due to rain ultimately destroyed the halophile host community but caused an increase in the virioplankton community. They collected water samples after rainfall (i.e. post-rain) for use in phage enrichment cultures. They also collected water samples before rainfall (i.e. pre-rain) and made a second discovery. Pre-rain enrichments from small sample volumes resulted in fewer culturable phages and smaller-sized plaques, while pre-rain enrichments from larger sample volumes resulted in a greater number of culturable phages with larger-sized and clearer (i.e., more lytic) plaques. As a side note it has been well established within the scientific literature that the larger the plaque, the more virulent the phage is considered to be compared to a smaller-sized plaque (Schloer and Hanson 1968; Lipton 1980; Ramsingh et al. 1995; Daniels and Wais 1998); and turbid plaques represent lysogenic (less virulent) viruses, while clear plaques represent lytic (more virulent) viruses (Maloy et al. 1994). Therefore, the quality or morphology of a plaque would be just as informative about the virus as the number of plaques it produces. The pre-rain enrichment data from Wais and Daniels (1985) indicated that lower-virulence viruses were predominant *in situ* as their presence was detected in smaller sample volumes, as opposed to the higher-virulence viruses, which were detected only in the larger sample volumes. It may be argued here that level of virulence is not directly correlated to plaque size or to sample volume, and that increased sample volume only resulted in a higher *ratio* of viruses to host, or MOI (multiplicity of infection), which would naturally result in a greater number of plaques. However, in order to better understand the conclusion drawn by these investigators, it would be instructive to note that a higher MOI does not necessarily mean greater lysis or more virulence. For instance, some hypersaline host-phage systems, such as those in this author's collection, must employ quite a low MOI, for example, 0.0025 or 0.005 (i.e., 0.0025 viruses to 1 host cell, or 25 host cells to 1 virus, etc.), in order to get complete lysis, or turbidity clearance, in the infection flask (unpublished data). If a greater MOI was used (e.g., an MOI of 1 or higher), lysis or clearance of turbidity was not achieved. This is not an uncommon occurrence in the halophage field, as an MOI of 0.5 or 0.05 in other systems is sometimes necessary to achieve lysis in liquid culture (Michael Dyall-Smith, personal communication). In other words, ridiculously low MOIs may need to be used in order to infect a host *in vitro*. Therefore, it is not necessarily true that there is an automatically direct relationship between MOI and lysis—a higher MOI, presumably from a larger sample volume, does not necessarily result in greater lysis or more virulence, and the complete opposite may be the case. In addition, to these pre-rain observations of *in situ* virulence these initial halophage enrichment experiments that Wais and Daniels carried out showed that relatively low numbers of

viruses could be recovered from pre-rain samples, while higher numbers of viruses could be recovered from samples immediately after a rain but then virus numbers dropped once again in water samples collected 24 days after a rainfall, after the hypersaline pond returned to normal salinity and to halophile saturation levels. Cultured phages that were originally recovered from post-rain samples were unable to proliferate in high salinity media (i.e., 4.5 M NaCl). Collectively these findings led Wais and Daniels to hypothesize that less-virulent lytic halophages were dominant in extreme hypersaline ponds, compared to the more virulent halophages. Furthermore, the more virulent halophages tend to proliferate within their hosts at lower salinities and that halophilic hosts "escape" viral predation at significantly higher salinities. The argument here, then, is that at higher salinities, halophages are not active, they do not replicate, they do not reproduce—they exist as prophages—while at lower salinities they are lytic. This would help to explain the higher abundance of halophages at lower salinities (i.e., immediately after a rainfall) compared to at higher salinities (i.e., before a rainfall or after the pond has recovered from a rainfall). Extreme hypersalinity could then be viewed as a refuge for halophilic hosts from temperate halophages that would then be triggered into their lytic cycles at lower salinities. Such a strategy by halophages would ensure that they remained stable in their hosts at less-than-ideal extreme hypersalinity but then allowed them to proliferate before their hosts were "doomed" to die in the more optimal diluted salinities. One implication of these data is that this could be viewed as a mutually beneficial adaptation to both the halophilic host and phage, on the one hand allowing the host to thrive in extreme hypersalinity in the absence of viral predation, and on the other hand allowing the phage to reproduce optimally at a lower salinity if the host was threatened with imminent death. Another implication from the Wais and Daniels study is that the halophages may be considered a genetic reservoir for halophiles after re-establishment of the host population. The hypothesis that a higher salinity prevents viral predation of halophilic hosts was first proposed by Torsvik and Dundas when they made similar observations during their experiments with their Hs-1 halophage isolate. They reported that Hs-1 was lytic when its host, *Halobacterium salinarum*, was infected at relatively lower salinity, but that the phage became lysogenic when infection was carried out at higher salinity—a salinity that was optimal for host proliferation (Torsvik and Dundas 1980). They further noted that Hs-1 adsorption decreased with increasing salinity, meaning that it was less infectious, at higher salinity, implying that higher salinity favors less virulent phages, as viruses with higher adsorption rates are less able and less likely to infect at higher salinity, thereby favoring cell proliferation (Torsvik and Dundas 1980). Collectively, these lab results from Torsvik and Dundas confirm the environmental observations recorded by Wais and Daniels.

Guixa-Boixareu et al. (1996) employed virus-like particle (VLP) counts and viral lysis estimates while Oren et al. (1997) used transmission electron microscopy (TEM) to determine the abundance of hypersaline viruses in Spanish salterns and the Dead Sea. One of the goals of the Spanish salterns study was to determine the impact of bacterivory (predation of prokaryotes by protist grazing) versus viral infection and to investigate the main cause for prokaryotic loss along a salinity gradient. These

studies included a range of salinities between 3.7–37 %, and Guixa-Boixareu et al. showed that viral abundance was directly proportional to prokaryote abundance as VLP counts increased with prokaryotic counts as salinity increased (Guixa-Boixareu et al. 1996). The investigators determined that there was an indirect relationship between viral lysis and bacterivory across a salinity gradient. Bacterivory accounted for prokaryotic loss at lower salinities (below 18 %) where viral infection was seemingly non-existent. As salinity increased, bacterivory decreased while viral lysis increased. While viral infection was detected at salinities between ~15 and 37 %, viral lysis was not a significant factor of prokaryotic loss below 25 %. However, viral lysis was shown to account for all of the prokaryotic loss at and above 25 % salinity, whereas bacterivory was non-existent at those salinities. The total number of infected prokaryotes (TIP) was estimated and it was concluded that mortality of prokaryotes due to viral lysis was consistently less than 20 % of both biomass and production at all salinities where viral infection was detected.

Oren et al. (1997) collected water samples from the Dead Sea in October 1994 and determined VLP counts to be approximately 7.3×10^7 pfu/ml; however, that number decreased with subsequent collections in April 1995, November 1995, and January 1996 (Oren et al. 1997). Their data corroborated the findings of Guixa-Boixareu et al. (1996) that VLP counts directly correlate with prokaryote counts. Furthermore, they discovered that the VLP-to-prokaryote ratio varied between 0.9 and 9.5X depending on depth; however, it should be noted that in this study, samples prepared for the TEM were not fixed upon collection and were not processed immediately but were processed within one week of sample collection. It has been documented that VLP enumeration can be severely underestimated if fixed or unfixed water samples are not processed immediately or stored at −80 °C (Wen et al. 2004); therefore, the counts from Oren et al. (1997) could possibly have been even higher and, so, the VLP numbers determined in this study may be considered a minimum count. Morphological analysis of VLPs via TEM showed a diversity of shapes including polyhedral, tailed, star-shaped, and spindle-shaped, which was the most abundant morphology discovered from the Dead Sea samplings.

In 1996, water samples from several Spanish salterns were collected in January, April, and July in order to investigate the hypersaline virioplankton. Diez et al. (2000) showed via TEM that there were two morphologies present, icosahedral and spindle-shaped (or fusiform). Pulsed-field gel electrophoresis (PFGE) showed that there was less diversity of hypersaline viruses in these extreme hypersaline ponds than reported in marine or haloalkaliphilic aquatic environments (Diez et al. 2000), but that genome sizes ranged from 25–300 kb, similar to the viral genome sizes found in the alkaline moderately hypersaline Mono Lake (Jiang et al. 2004). PFGE and DNA hybridization showed that there were different viral assemblages in the different Spanish salt ponds studied, with greater abundance of halophages in concentrator ponds (lower salinity) versus crystallizer ponds (higher salinity). This study also showed, interestingly, that the method for collecting and concentrating viruses can be critical. Diez et al. compared the use of tangential flow filtration (TFF) to concentrate 20 l of hypersaline water samples to positive pressure filtration of 15 l of water, before ultracentrifugation. They discovered that TFF significantly reduced

the amount of viruses recovered (3.64×10^{10} VLP/ml) versus recovery through positive pressure filtration (6.73×10^{10} VLP/ml) (Diez et al. 2000). Although the authors of this study concluded that there was no qualitative loss in viral data (i.e., the same morphotypes were seen in the samples irrespective of recovery method), this discrepancy should be taken into account when deciding on a method for collecting environmental samples as some halophages may not be represented accurately, if at all.

Investigations of a full-salinity gradient across several aquatic sites in Senegal, including low-salinity coastal estuaries, man-made solar salterns, and natural hypersaline river estuaries and lakes, ranging in salinity from 1–36 %, provided some details regarding the tropical halophilic virioplankton (Bettarel et al. 2011). Firstly, both prokaryotic and viral counts increased with increasing salinity reaching 3.4×10^8 cell/ml and 6.8×10^8 viruses/ml at 35 % salinity. Secondly, there was a strong correlation between virus and prokaryotic abundance, meaning that as prokaryotic abundance increased with salinity, so did viral counts. Furthermore, studies revealed that the fraction of infected cells (FIC), which represent lytic viruses, was found to be the highest at 20–18 % between the salinities of 1 and 3 %, respectively, then dropping to almost undetectable levels at salinities between 4 and 8 %, before rising again to an FIC of approximately 10 % at salinities of 10–12 %. There were no infected cells detected at salinities from 19 to 36 %. Conversely, the fraction of lysogenic cells (FLC), representing temperate viruses, was very low (0.5–6 %) between salinities of 1 and 12 %, but then rose in salinities from 15 to 34 %, with the highest FLC calculated to be 63 % at 19 % salinity and ~30 % at 30 % salinity. These data indicate that haloviruses are lytic at the lower salinities—where Eubacteria are predominant—but that they preferred a lysogenic lifestyle at the higher salinities—where *Archaea*, especially the square-shaped *Haloquadratum*, predominate. The authors of this study interpret their data to support the point previously made that halophages are more likely to be chronic viruses and not strictly lytic viruses (Porter et al. 2007) and they point out that their data seem to support the observation initially made by Wais and Daniels that halophages are lytic at lower salinities (especially during and immediately after a rainfall dilutes the high-salinity pond), but are lysogenic at higher salinities, resulting in the halophilic hosts escaping viral lysis. They and another group (Kukkaro and Bamford 2009) echo the suggestion initially made that extreme salinity offers a benefit to both the halophilic host and the halovirus (Wais and Daniels 1985). Bettarel et al. also investigated the survival rate of free viruses collected from each site by enumerating them via fluorescence microscopy after they had been stored in the dark for 12 hours. Interestingly, their data showed that the viruses from low-salinity habitats (i.e., 1–14 %) had survival rates between 30 and 40 %, whereas the free viruses from high-salinity sites (i.e., 19–36 %) had survival rates between 60 and 98 % (Bettarel et al. 2011). The authors speculate that perhaps the halophages at the higher salinities are protected from virivorous predators (i.e., organisms such as heterotrophic nanoflagellates and ciliates that feed on viruses) that may exist at lower salinities, and/or that they have adapted to physically withstand the harsher chemical environment. Another explanation put forward by this group is that these higher-salinity-surviving viruses may possibly be exploiting

Table 4.2 Relative abundance of virus tail lengths as a function of depth in Mono Lake. Water samples were collected from 2, 16.5, and 35 m representing all three stratified layers of the lake

	Epilimnion (0–16 m)	Metalimnion (16–19 m)	Hypolimnion (19–38 m)
Viral tail lengths	More similar to each other		
Capsid size-frequency distribution		More similar to each other	
Tailed viruses	+ + +	+ +	+
Large untailed capsids (>150 nm)	+	+ + +	+ +
Podoviruses	+ + +	−	−
Myoviruses	+ + +	+ +	+
Siphoviruses	+	+ +	+ + +

the presence of such compounds as glycerol, which is produced in large quantities by the halophilic eukaryotic algal species, *Dunaliella*, somehow using it as an extra protective coating.

Recent work has shed more light on the various morphotypes that hypersaline viruses exhibit as well as *in situ* patterns of morphology. Brum and Steward (2010) undertook morphological characterization of the phage community in the moderately hypersaline Mono Lake. By using TEM, they demonstrated that virus assemblages can segregate within a stratified lake based on tail length and that the general pattern observed was that virus tail length increased in size with increasing depth (Brum and Steward 2010). Overall, the epilimnion (or oxic layer) harbored more tailed phages than any other depth. Podoviruses (untailed capsids) were exclusively found in the epilimnion while myoviruses (tailed viruses with moderate length) were found mostly in the epilimnion and their numbers decreased with increasing depth (Table 4.2). The opposite was true for siphoviruses (viruses with long flexible tails), which were shown to be most abundant in the hypolimnion, but decreased in abundance with shallower depth (Table 4.2). Similarly, the capsid size of large untailed viruses (>150 nm), which the authors reasoned were most likely eukaryotic viruses, also increased with depth. Furthermore, the majority of Mono Lake viruses had a capsid size >60 nm, larger than the typical range of 30–60 nm reported elsewhere (Wommack and Colwell 2000). Brum and Steward reported that ‘exotic’ viral morphologies—such as spindle-shaped, etc.—were not observed in Mono Lake, but only viruses with hexagonal-shaped capsids, prolate capsids, and capsids with single-axis symmetry (Brum and Steward 2010). Once again, this study showed that methodology is a crucial factor and must be considered carefully when working with environmental samples. Brum and Steward discovered that the widely-used and accepted protocol of 0.2 μm filtration to remove cellular material can introduce a bias into the dataset resulting in both reduced numbers and types of viruses recovered from the water samples (Brum and Steward 2010). TEM results from filtered and unfiltered water samples showed that filtration reduced the number of large viruses (> 150 nm), increased the relative number of tailed viruses, and decreased the number of viruses with shorter tails. Another study also showed how virus morphology is directly correlated to environmental conditions, in this case, salinity. Bettarel et al. collected water samples across a full-salinity gradient and demonstrated that certain

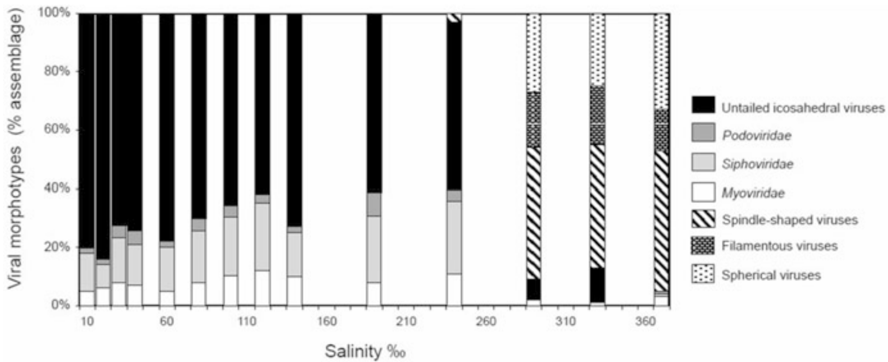

Fig. 4.1 Correlation between virus morphotype and salinity across a full-salinity gradient in Senegal. Note that salinity is presented here in per thousand (‰); hence, 10 ‰ = 1 %, etc. The salinity gradient in this study ranged from 1 to 37 %. (Figure reprinted by permission from Bettarel et al. 2011, courtesy of John Wiley and Sons Publishing)

virus types were present or absent at different salinities. More specifically, the tailless icosahedral viruses, podoviruses, myoviruses, and siphoviruses dominated between 1 and 24 % salinities and disappeared above 24 %; however, the virus shapes that dominated above 24 % salinity were the spherical, spindle-shaped, and filamentous types (Bettarel et al. 2011) (Fig. 4.1). This exciting and unprecedented finding, which parallels those from Brum and Steward discussed above, tantalizingly implies that the morphotypes of halophages, and perhaps of viruses in general, are an evolutionary adaptation to the specific—and, in this case, extreme—environment that they inhabit. More of this same work must be undertaken at various other extreme hypersaline sites in order to support this hypothesis.

A recent investigation of the hypersaline virus community in Lake Retba, Senegal, reported very exciting findings regarding the apparently widening range of halovirus morphology (Sime-Ngando et al. 2011). Via TEM, Sime-Ngando et al. observed 'typical' hypersaline virus shapes, such as classical head/tail, non-enveloped icosahedral, enveloped non-icosahedral (spherical), rod-shaped, and spindle-shaped (Fig. 4.2). They estimated that a mere 1 % of VLPs were head/tail, which is surprising given the fact that the majority of viral isolates are of this morphotype, while 13 % were rod-shaped or linear. The vast majority of Lake Retba VLPs detected by TEM were spindle-shaped (81 %), which corroborates reports by others that this morphology is predominant in hypersaline habitats (Guixa-Boixareu et al. 1996; Oren et al. 1997; Diez et al. 2000). What was astounding was the discovery that 5 % of VLPs in Lake Retba had novel or previously unidentified shapes (Fig. 4.3), including hairpin-shaped particles, bacilliform particles with an appendage at one end, chains of small globules, hook-shaped particles, tadpole-shaped particles, reed-shaped particles, filamentous particles with terminal structures, and branched filaments with spherical structures located at their ends (Sime-Ngando et al. 2011). There is no direct evidence at this time to indicate that these novel and exotic shapes represent actual viruses, but their presence intimates the possibility that halophages—like their thermophilic

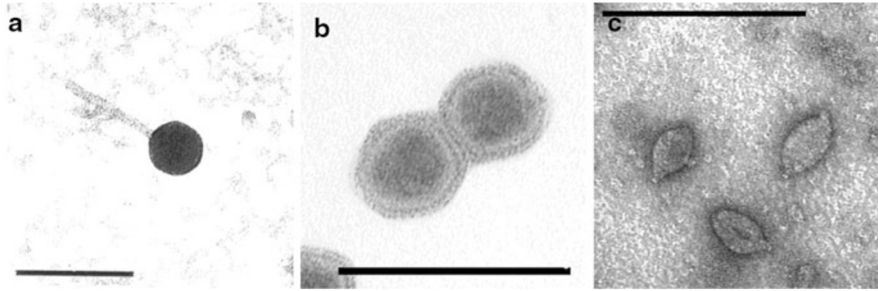

Fig. 4.2 TEM images of hypersaline viral isolates exhibiting more commonly encountered morphologies as **a**) head/tail, **b**) enveloped spherical, and **c**) fusiform (or spindle-shaped). (Figure reprinted by permission from Porter et al. 2007, courtesy of Elsevier Publishing)

counterparts (Prangishvili and Garrett 2005)—may come in a greater diversity of morphotypes than initially thought. Viral abundance in this habitat was calculated to be 6.9×10^8 per ml, which the authors claim to be the highest virus concentration of any archaea-rich environment (Sime-Ngando et al. 2011). Metagenomics analysis of the prokaryotic community showed sequence matches to some known clades, but many sequences belonged to novel clades, possibly to a basal lineage (Sime-Ngando et al. 2011). This was also seen with the metavirome (metagenomics analysis of the viral community). There were some sequence matches to known halophages including ϕH, ϕCH1, ϕBJ1, HRPV-1, His-1, as well as to environmental phages and mobile elements (e.g., halophile plasmids). Although some novel morphologies were found to be similar to their hyperthermophilic cousins, no sequence matches were made between the Lake Retba VLPs and known hyperthermophilic phages, reinforcing the observation made that although some halophages (i.e., His-1 and His-2) may resemble spindle-shaped hyperthermophilic phages, their lineage is thought to be quite distinct (Porter et al. 2007). Many Lake Retba VLP sequence matches (about 1/2 of the amino acid sequences and 1/3 of the nucleotide sequences) were made to moderate and hypersaline viral sequences found in environmental databases containing sequences from the USA and Spain, which the authors suggest shows that highly similar viruses have adapted in very distinct geographies (Sime-Ngando et al. 2011). If so, then what role does host similarity play in this? If the same or similar hosts are found at different hypersaline sites, could those hosts contribute to the adaptation of similar viruses in different locations? In regard to the issue of head/tail halophages, the authors propose that, if bacterial and archaeal head/tail viruses are closely related to each other (Krupovic et al. 2010), and if head/tail hypersaline viruses dominate bacteria-rich environments while they comprise only a small fraction of the archaea-rich environments, then perhaps head/tail phages are bacterial in origin and only recently infected *Archaea*, or, on the other hand, head/tail phages predated the divergence of *Bacteria* and *Archaea* and evolved independently (Sime-Ngando et al. 2011). Alternatively, it may be reasonable to think that *Archaea* would naturally have viruses that look similar to both *Bacteria* and *Eucarya*, since *Archaea* have characteristics of both domains. Further research in this arena is needed.

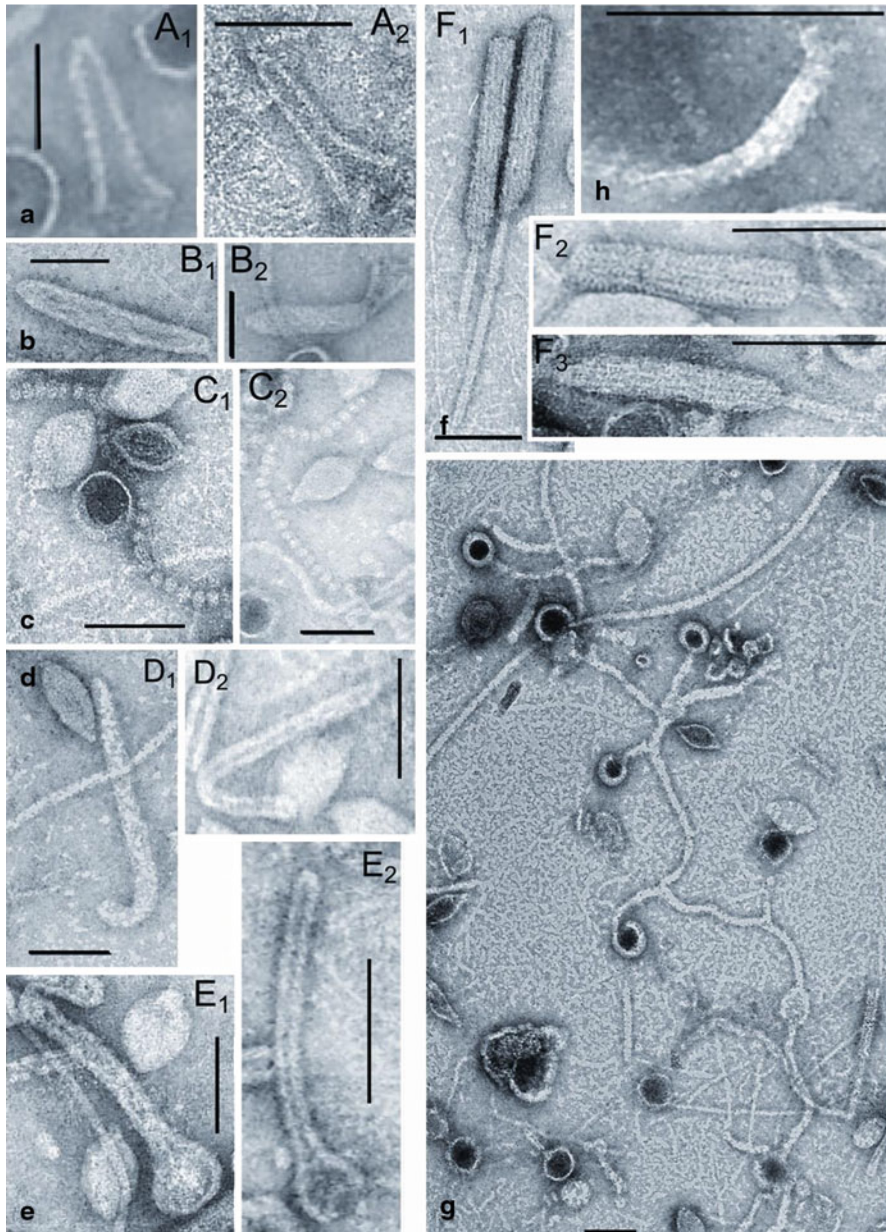

Fig. 4.3 TEM images showing uncommonly diverse and exotic morphologies of virus-like particles recovered from Lake Retba in Senegal, including **a**) hairpin-shaped, **b**) bacilliform, **c**) chains of small globules, **d**) hook-shaped, **e**) 'tadpole'-shaped, **f**) reed-shaped, **g**) branched filaments with spherical subunits, and **h**) terminal structures on filamentous particles. (Figure reprinted by permission from Sime-Ngando et al. 2011, courtesy of John Wiley and Sons Publishing)

Other recent studies have also applied metagenomics and metatranscriptomics analyses to better understand the halophage community. Angly et al. (2010) reported on their new software program called GAAS, an acronym for "genome abundance and relative size", the goal of which is to reduce error and increase the accuracy of sequence searches for metagenomics data (Angly et al. 2009). GAAS was initially used to survey the global aquatic phage population, meaning phages that reside in different aquatic ecosystems, and the data suggests that the majority of global aquatic phage genomes are small ssDNA, not double-stranded DNA. For hypersaline biomes in particular, the average phage genome length was found to be between 51 and 263 kb. Furthermore, there was no trend observed between halophage and halophile host genome sizes—i.e., halophage genome sizes were consistently smaller than host genome sizes, even as host genome sizes increased (Angly et al. 2009), which is interpreted to mean that phage genome size is not directly related to host genome size. The size of the phage genome was consistent (and consistently smaller) no matter what the host genome size was. Rodriguez-Brito et al. (2010) investigated four different aquatic environments—freshwater, low salinity, medium salinity, and high salinity—at the "coarse-grain", or species, level (i.e., known genomes were present) and at the "fine-grain" level (i.e., at the viral genotype and microbial strain level). Employing this "coarse-grain" approach, they discovered that both the microbial and viral communities were stable over a time period, ranging from between 1 day to >1 year, and that the same taxonomic groups and cellular metabolic profiles persisted over time; thus, reinforcing the concept that stable geochemistry leads to stable biology (i.e., microbial and viral taxa) and to stable metabolic potential (Angly et al. 2009). The caveat to using the "coarse-grain" approach, as the authors point out, is that it excludes unknown dominant species that are not currently in databases. By utilizing the Maxiϕ software program (for viral genotypes) and the Taxiϕ software program (for microbial strains), the "fine-grain" approach could instead be applied. This "fine-grain" analysis of all viromes (metagenomes of the virus community) and microbiomes (metagenomes of the prokaryotic community) shows that there are indeed changes in taxa within each viral and microbial community sample collected between one day, several days, or several months of each other. At the "fine-grain" level, these investigators observed a reshuffling of the dominant microbial strains and viral genotypes over time, supporting the "kill-the-winner" theory, which states that a bacterial strain that becomes dominant or overly abundant in the environment due to certain advantages (e.g., nutrient acquisition) will ultimately fall victim to virus infection and its population will be controlled by a viral epidemic allowing for less dominant, but virus-resistant strains to survive and thrive, thus maintaining microbial diversity in the ecosystem (Thingstad and Lignell 1997; Wommack and Colwell 2000). Therefore, the initial results seem to indicate that dominant microbial and viral communities apparently remain stable over time; but a closer, more refined look shows that specific strains of microbes and phages actually do fluctuate, and that these data support the hypothesis that viral predation is a main factor in shaping microbial communities (Angly et al. 2009).

In another metagenomics investigation, Santos et al. (2010) collected water from Spanish salterns, centrifuged out the cells, and concentrated the viral component

via TFF (the caveat of using this technique has already been discussed above). They created fosmid and shotgun libraries of the viral DNA—a metavirome of the Spanish salterns—and conducted sequence analysis against current databases. While studies conducted before Santos et al. (2010) have shown that most, if not almost all, environmental virus sequences have no matches in the database, in this study, there were some matches to known halophages, such as HF1, HF2, and His-1 (all Australian isolates), ϕCh1 (isolated from a Kenyan haloalkaliphile), and BJ1 (isolated from China), a peculiar similarity that overlaps data obtained in the Sime-Ngando et al. (2011), Senegalese study; and, surprisingly, there were also direct sequence matches to the San Diego metavirome discussed earlier (Rodriguez-Brito et al. 2010). However, 75–88 % of the metavirome did not match any known sequences and were considered hypothetical proteins, many of them conserved (Santos et al. 2010). One of the findings from this study involved the integrase gene. Integrase genes are used by viruses to integrate their own DNA with that of the host's. The low number of integrases discovered in this metavirome indicated that lysogeny is not a prevalent strategy used by hypersaline viruses, at least not in this Spanish saltern at the time of collection, and lends support to the view that lysis, or, more likely, chronic infection may be the preferred lifestyle of halophages as has been discussed in a previous review article (Porter et al. 2007). At this point, a definition of terminology is necessary for a more accurate understanding. Just to clarify, chronic infection from the virus' point of view is *not* the same as lysogeny. Chronic infection results in viral progeny continuously being produced from the host, but the host is not killed in the process (at least not in the short-term). On the other hand, lysogeny refers to an inactive virus, or one that has integrated its genome into the host's DNA and does not replicate at all, but is then triggered by a stimulus to become lytic. Viruses that come out of the lysogenic state are lytic and will kill the host in releasing progeny virus. Here, the virus' perspective (not the host's) is being described based on the available metavirome data in this particular study. Unfortunately, when speaking from the host's point of view, "chronic infection " means something different than when discussing the different virus lifestyles, which are (a) lytic, (b) lysogenic, and (c) chronic. "Chronic infection " from the host's point of view is actually lysogeny from the virus' point of view; but the chronic lifestyle of the virus is not lysogeny—those are two distinctly different virus lifestyles. Further analysis revealed that single nucleotide polymorphisms (SNPs) exist along this metavirome. The majority of SNPs were neutral, suggesting that genetic variants of the same species of halophages were present in this community. The relatively high mutation rate reflected in the sequences of this metavirome indicated high intra-species diversity, which means that, in this ecosystem, similar hosts are apparently infected by closely-related viruses (Santos et al. 2010). One of the most interesting findings of this study is that the halophage sequences acquired from the Spanish salterns metavirome analysis were distinguishable based upon their respective GC content. In one group, the sequences clustered together with a low GC content (∼45 %), while in a second group, the sequences clustered together with a high GC content (∼60 %). The low-GC phage cluster also included genomic sequences from *Haloquadratum walsbyi*, a halophilic host with a relatively low GC content; while the high-GC phage cluster included

genomic sequences from other halophilic hosts that are known to have a relatively high GC content, such as *Salinibacter ruber*, *Halobacterium salinarum*, *Haloarcula marismortui*, and *Natronomonas pharaonis*. This finding sheds more light on the relationship between viruses and their hosts and nicely correlates the tendency of halophages to share similar GC content with their halophilic hosts, which would be expected if those same viruses are dependent upon their respective host's replication machinery. This may seem like a confusing statement, as GC content should be an irrelevant factor in regards to the DNA replication machinery, especially in regards to the polymerase. As it turns out, however, one cannot undertake PCR on some halophilic host genomes with regular PCR reagents. Due to the high GC content, an enhancer must be added to the mastermix to get any amplification. Some manufacturers either add the enhancer to the all-in-one mastermix, or they include different buffers in the kit—one of them is called GC buffer and it is used instead of the standard polymerase buffer. So, while the polymerase itself may not be an issue—although there are 8 different polymerase families across the domains of life (5 eukaryotic (Cotterill and Kearsey 2009) and 3 bacterial)—GC content can apparently affect replication, at least in the lab. It is understood within the halophage field that phages infect hosts with similar GC content, as is observed the majority of the time, and it is always surprising (as is presented in the second part of this chapter) when a virus infects a host with a different GC content. A follow-up report by the same group describes the use of microarray technology to assess the environmental viral mRNA 'population' under normal and under stress conditions (Santos et al. 2011). Initial fluorescent *in situ* hybridization (FISH) analysis indicated that 77 % of the hypersaline water sample that the group collected was archaeal, while 17.6 % was bacterial, confirming previous data that *Archaea* dominate extreme hypersaline habitats. Total mRNA from the sample, including from hosts that were actively infected at the time of sampling, was then used to create cDNAs for hybridization onto the microarray against the metavirome that was created from their previous study (see above). Though not absolute, there was the general finding that the longest contigs gave the highest fluorescent signal on the microarray, which was interpreted as coming from viruses with the highest gene expression, or the greatest activity. In this study, the investigators were able to discriminate their metavirome sequences, or halophilic viral sequences (HVS), based on GC content into five distinct subgroups called HVS-1, HVS-2, HVS-3, HVS-4, and HVS-5. HVS-1 and HVS-2 were viruses that shared high GC content as is the case with *Salinibacter ruber*; while HVS-3, HVS-4, and HVS-5 were viruses in the second group that had relatively lower GC content as is the case with *Haloquadratum walsbyi*. They discovered that there was significant gene expression (>9 times the background fluorescence) among these different groups of viruses. They found that 65 % of the contigs in HVS-1, 69 % of contigs in HVS-2, 67 % of contigs in HVS-3, 48 % of contigs in HVS-4, and 72 % of contigs in HVS-5 showed elevated gene expression, reflecting the amount of actual viral activity within each of these different virus groups in the natural water sample (Santos et al. 2011). More specifically, they interpreted the data to mean that, *in situ* under normal conditions, the viruses in HVS-4 were the least active (with only a 48 % gene overexpression) compared to the viruses in HVS-5, which seemed to be

the most active out of all of the virus groups (with a 72 % gene overexpression). They then repeated the same analysis after they had treated the water sample with either UV radiation or dilution, and they compared those results to the untreated natural water sample to see if there were any differences in the viral gene expression under stressful conditions. After UV exposure, gene overexpression was noticeable in the HVS-1 group (15 % of contigs) and HVS-4 group (12 % of contigs) relative to the untreated, natural water sample, compared to HVS-2 (4.6 % of contigs), HVS-3 (3.3 % of contigs), and HVS-5 (2.7 % of contigs); and similar results were found when the water sample was diluted (Santos et al. 2011). The authors of this study concluded that, while there was high viral activity overall at the time of sampling, (a) viral gene expression was different for different groups of viruses and that (b) stressful environmental conditions can lead certain viruses to become even more active than others. Additionally, they noticed that the genetic variants they had identified previously through SNPs (see above) were also differentially expressed under the stress conditions. Up to 67 % of the polymorphic contigs that were overexpressed had non-synonymous sequence changes compared to the consensus sequence, and the authors suggest that these viral variants be referred to as "ecoviriotypes ", as they could represent groups of very closely related viral genomes (Santos et al. 2011).

A novel experiment carried out by Bettarel et al. (2010) aimed at investigating cross-biome infections. This group used lacustrine, marine, and extreme hypersaline water samples to conduct culture-independent cross inoculations. First, they filtered the water samples through 3 μm and then through 0.2 μm membranes. The 3 μm-filtered water samples were used as host sources, while the 0.2 μm-filtered water samples were used as virus sources. Inoculation of lacustrine and marine hosts with their "native" virus sources resulted in viral production, which was measured as prokaryotic heterotrophic production (PHP) post-inoculation, although no viral production was detected when the hypersaline host was inoculated with its own hypersaline virus source. Though this negative result may at first seem counterintuitive, there are different reasons why a host from a hypersaline source may not be immediately infected by a virus from the same water source. This author has had first-hand experience of this as have other groups (for example, see Atanasova et al. 2012 below). Such an observation is actually not unusual or strange in the least—just one observation of the relatively few that have been published about aquatic viruses or halophages. This author's first-hand experience indicates that the cell state as well as the salinity can play important factors in the lysis of hosts, neither of which was investigated in the Bettarel et al. study. Also, remember that this paper used viral production as a measure. It is possible that the virus(es) in this particular case could have been temperate, thereby producing lysogens. There are, therefore, plausible explanations for this published observation. Another observation reported in this study was that cross inoculation—inoculating the different host sources with 'non-native' virus sources—did not result in any viral production in any of the samples. Since viruses are known to be host-specific, this result may seem predictable. However, halophages have been shown to be able to infect across different *genera*; thus, some halophages have broad host ranges while others have strict or narrow host ranges (Nuttall and Dyall-Smith 1993; Atanasova et al. 2012). Because of such previous discoveries, a

cross-inoculation, or cross-infectivity, study is actually a good idea and should be considered as a regular part of anyone's phage investigations. One caveat to keep in mind regarding this study is the fact that filtration has been shown to reduce or possibly eliminate both numbers and different types of viruses (see above for discussion of Brum and Steward study). A second caveat is that the 0.2 μm-filtered water samples, representing the viral sources, were resuspended using freshwater, which may have negatively affected at least the extreme hypersaline sample by possibly degrading the viruses therein (Vogelsang-Wenke and Oesterhelt 1988; Nuttall and Dyall-Smith 1993). It is possible that the aforementioned caveats may have played a significant role in the outcome of this study; alternatively, this study shows that perhaps some viruses are very much sequestered in the environment, having evolved to exclusively infect hosts that are geographically local and physically within reach. The results of this study may support the metagenomics observations that halophage sequences are mostly unique and rarely, if ever, match known sequences in databases, reflecting unique virioplankton communities in different geographic locations. More work will need to be undertaken to clarify the lineage of hypersaline viruses within the same site, and among the different biomes. On the other hand, in another study that focused exclusively on extreme hypersaline sources, cross-infectivity experiments showed that viruses isolated from one geographic location could infect hosts isolated from a completely different geographical site, and in some cases may not infect hosts cultured from the same exact site or source. Atanasova et al. used a culture-dependent approach to isolate 61 halophilic archaea, 24 halophilic bacteria, and 49 halophages from nine geographically distinct sites in Italy, Israel, Slovenia, Spain, and Thailand (Atanasova et al. 2012). Their host isolates included such genera as *Halorubrum*, *Haloarcula*, *Natronomonas*, *Halorhabdus*, *Haloferax*, *Halomonas*, *Salicola*, *Chromohalobacter*, and *Rhodovibrio*. They then conducted cross-infectivity studies of the viral isolates against the cultured hosts from these sites as well as against strains from a culture collection. An important observation from this study is that none of the authors' 49 halovirus isolates infected both a bacterial and an archaeal host, confirming the fact that all known halophages to date infect hosts from either domain, but not both. In the case of the Dead Sea sample, five of the six host isolates from this site were *Chromohalobacter*, the sixth was a *Halorhabdus* species, and none of the viral isolates came from this site. In addition, none of the viral isolates from the other sites showed lytic activity against any of these hosts, meaning that the investigators could not achieve visible infection using any of the 49 halophages against any host isolate from the Dead Sea. However, there are other instances where the opposite was true. For example, there were several different genera of hosts and viruses isolated from Samut Sakhon, Thailand. Some of the Samut Sakhon viral isolates were able to lytically infect some of the hosts from the same site, but they were also able to infect several other hosts of the same genera isolated from two sites in Israel, two sites in Italy, one site in Spain, and some culture collection strains from the Middle East, Japan, Australia, and the USA. Similar observations were repeatedly made, for example, a single halophage that was isolated from Guardias Viejas, Spain, was not able to lytically infect any of the eight, diverse host isolates from the same site. Yet, that same halophage showed lytic activity against a host isolated from a different

Italian site as well as against three hosts from the Thai site. The data from this study indicate that halophages (and, by extension, other viruses) are able to infect hosts spread throughout the world, whether or not they infect host isolates from the same site. This implies that halophages are not restricted to a local environment and, instead, have a global potential for infection. Of course, there are some caveats to bear in mind regarding these results. First, the measure of infectivity used in this study is based on the lytic lifecycle, which is a standard technique used in virus research. However, the scientific literature indicates that halophages may naturally act more chronically and not strictly as lytic viruses (Torsvik and Dundas 1980; Daniels and Wais 1990; Porter et al. 2007). Therefore, it is very possible that some of the negative infection results in this study may actually be false negative results if viruses were indeed infecting the hosts, but not lytically. Second, in light of the fact that not all hosts are cultivable, it is also possible that the viruses isolated from a particular site in this study did not infect the host isolates from the same site only because those were not the preferred hosts, and that the preferred hosts were not cultured. Furthermore, most of the halophage isolates in this study were characterized as head-tail viruses, and we now know from environmental (uncultured) samples that other morphotypes dominate the extreme hypersaline environments (see discussion above). Therefore, the cultured hosts that were not infected by any viral isolate in this study may actually be infected by viruses of other morphotypes that were not successfully isolated. Nonetheless, this study is the most comprehensive ecological halophage research to date and has provided a more global view of the infective potential of extreme hypersaline viruses, which can then be used to compare with future studies of other sites from other parts of the world.

Highlights of In-depth Halophage Isolate Studies

While there has been a relatively virtual explosion of environmental reports on halophages in just the past few years, the number of in-depth analyses of halophage isolates has not kept up at the same pace (Fig. 4.4 and Table 4.3). Most articles describing halophage isolates have reported on basic characteristics of the virus, but then generally did not follow up with detailed genomic or host interaction experiments. A review of the literature shows that almost all of the cultured halophages are of the tailed morphology, belonging either to the *Myoviridae* or the *Siphoviridae* family. However, other morphotypes have also been isolated, namely the spherical (i.e., tailless capsid), lemon-/spindle-shaped, and pleomorphic (Table 4.1). Genomes of a few halophage isolates have been fully sequenced and analyzed and in-depth characterizations of some viruses have been undertaken. Only one halophage isolate, HRPV-1, has been described as having a circular, single stranded DNA (ssDNA) genome, while all the other isolates that have been genomically studied have been described as having a linear double-stranded DNA (dsDNA) genome, except for HHPV-1, which has a circular dsDNA genome (see Table 4.1 for author references).

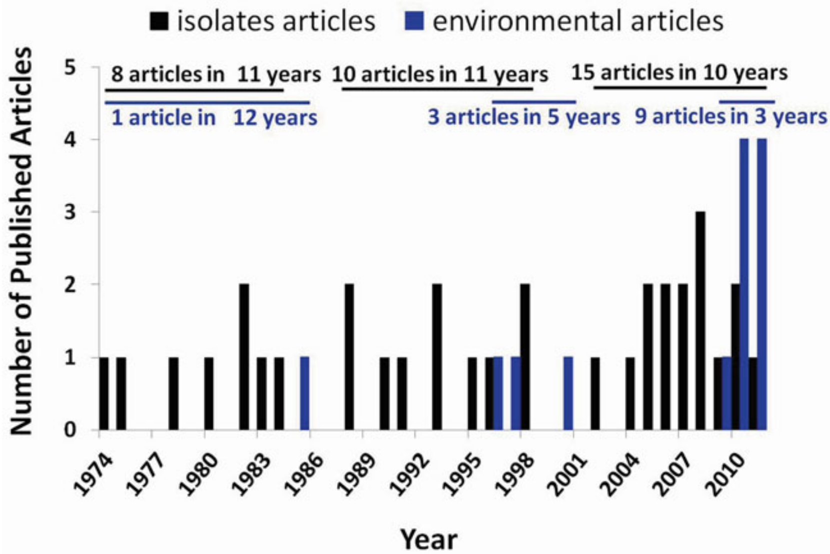

Fig. 4.4 Graph showing the publication record over a 38-year period of articles reporting on specific halophage isolates versus articles about the hypersaline community of virioplankton (i.e., environmental papers)

Table 4.3 Statistical analysis of the current halophage publication record over a 38-year period. Note that the time period that covers articles specifically about virus isolates (38-year span) differs from the publication time period for environmental articles (27-year span)

	Articles about individual halophage isolates			Articles about environmental hypersaline virioplankton		
	1974–1984 (11 year)	1988–1998 (11 year)	2002–2011 (10 year)	1974–1985 (12 year)	1996–2000 (5 year)	2009–2011 (3 year)
Average publication rate (articles/year)	0.73	0.91	1.5	0.08	0.6	3

The first extreme hypersaline virus to be analyzed at the molecular level was ϕH. Its genome was discovered to be linear dsDNA with partial circular permutation and terminal redundancy (Schnabel et al. 1982a). The ϕH genome hybridized very minimally to the host chromosome, but did hybridize strongly to a plasmid within the host leading to the conclusion that ϕH did not integrate into the host's genome, but did share sequence homology with the host's plasmid (Schnabel et al. 1982a). The authors determined that the GC content of its genome is 65 % and, like some other halophages, ϕH's physical integrity is very much dependent upon high salt concentration. Furthermore, a phage-resistant host containing ϕH DNA confirmed that this virus is temperate and exists in a covalently-closed circular state as a prophage

(Schnabel et al. 1982a). Using restriction enzyme maps, investigations of ϕH showed that the genome is 59 kbp and led to the discovery of several different variants (ϕH1–ϕH8) with highly variable genome structures and occurring at different frequencies in lysates (Schnabel et al. 1982b). These ϕH variants differed from each other through either insertions, a deletion, or an inversion that occurred at a high rate, and these variants could be classified into two groups: (i) ϕH1 – ϕH4 and ϕH6 all originated as variants from the original phage stock; and (ii) ϕH5, ϕH7, and ϕH8 originated from plaque purification of ϕH2 (Schnabel et al. 1982b). In addition, the 1.8 kb sequence within ϕH that showed homology to the host's plasmid seems likely to be a transposable element that the authors suggest was either introduced into the host's plasmid by the phage, or vice versa (Schnabel et al. 1982b). The authors further demonstrated that all phage-resistant host cells contained ϕH DNA episomally, and that, as a result, their resistance was most likely acquired through immunity against superinfection (Schnabel and Zillig 1984). Several detailed reports about ϕH's transcription process, including control over its lysogenic and lytic cycles, have been published (Gropp et al. 1989, 1992; Stolt and Zillig 1993, 1994; Stolt et al. 1994) making this halophage one of the most, if not the most, genetically-studied halovirus to date.

While the first genetic studies of a halophage were conducted in 1982, the next set of in-depth genetic investigations was not done until 1993 on the HF1 and HF2 viruses. Both viruses are tailed capsids of the *Myoviridae* family (Nuttall and Dyall-Smith 1993) and the genome sizes were determined to be ∼76 kbp for HF1 and ∼77 kbp for HF2 (Nuttall and Dyall-Smith 1995; Tang et al. 2004). Initially, both of these viruses were thought to be closely related based on morphology and protein profile via SDS-PAGE; however, they do not share the same host range. HF1 had a broader host range, being able to infect different genera (i.e., *Haloferax*, *Halobacterium*, and *Haloarcula*), while HF2 had a narrower host range, infecting only *Halobacterium sacchoravorum* and isolate Ch2—both of which were reclassified later as *Halorubrum* species (Nuttall and Dyall-Smith 1993). Both viruses were susceptible to low salinity and either required magnesium at low NaCl concentration, or, if magnesium was absent, required high NaCl concentrations in excess of 2 M. Despite the fact that these two viruses have different host ranges, they both share a 55.8 % GC content as well as 94.4 % identity across their genome. More specifically, the viruses demonstrate 99.99 % genomic identity (and 100 % protein similarity) over the first two thirds of the genome, but there is an abrupt drop in homology over the rest of the genome. These data suggest that these two viruses shared a recent and large recombination event (Tang et al. 2004). Being only the second halophage to be genetically studied up to that point, HF2 comparison with ϕH showed that halophages were similar to more well-studied mesophilic bacteriophages in their expression of lytic/lysogenic control elements, direct terminal repeats, and their replication through concatameric intermediates (Nuttall and Dyall-Smith 1995). As initially shown for HF2 (Tang et al. 2002), these viruses reflect extensive mosaicism in their genomes indicating that the genomes arose from a wide diversity of organisms, including some of cellular origin (mesophilic and halophilic prokaryotes) and of non-halophilic phages.

The first, and thus far only, virus to be isolated from a haloalkaliphile is ϕCh1. Its linear dsDNA genome was initially reported to be 55 kbp (Witte et al. 1997) and later revised to 58.5 kbp (Baranyi et al. 2000), has 62 % GC content (Witte et al. 1997), is circularly permuted, and is terminally redundant (Klein et al. 2002). Surprisingly, ϕCh1 packages both DNA and RNA molecules, with the RNA (80–700 bp) shown to be of host ribosomal origin (Witte et al. 1997). A protein profile of this virus using isoelectric focusing (IEF) showed that all of the proteins were acidic, ranging from pI 3.3 to pI 5.2, and a physicochemical analysis showed that the virus requires at least 2 M salt concentration to maintain physical integrity. ϕCh1 is a temperate virus that chromosomally integrates with the host genome resulting in a stable transfection, and, interestingly, a fraction of ϕCh1 DNA is adenine-methylated at Dam sites while another fraction is not (Witte et al. 1997). While there was no evidence of DNA modification in the ϕH genome, ϕH does encode for a cytosine methyltransferase (Schnabel et al. 1982b; Stolt et al. 1994), showing some similarity to the apparent modification systems of ϕCh1 and another halophage, ϕN (Vogelsang-Wenke and Oesterhelt 1988). Further investigations of the archaeal ϕCh1 led to the identity of its methyltransferase gene and subsequent protein product named M.ϕCh1-I. The authors showed that ϕCh1 DNA methylation occurred at a much higher efficiency in lysogenic strains compared to lytic infection and, surprisingly, M.ϕCh1 showed strong *in vivo* activity within the non-halophilic bacterium *Escherichia coli* (Baranyi et al. 2000). In *E. coli*, however, the M.ϕCh1 showed a somewhat different activity—it functioned similarly to the *E. coli* Dam methylase enzyme and was involved in the repair of genetic mutations after the cells had been treated with mutagenic agents (Baranyi et al. 2000). Comparative studies of ϕCh1 revealed that its genome organization showed a structure that is conserved across domains of head-tail viruses, illustrating homology between halophilic archaeal and mesophilic bacterial phages and leading the authors to speculate that either there is a common ancestor for both archaeal and bacterial phages before the two domains split, or that there has been massive lateral gene transfer between these two virus groups (Klein et al. 2002). The first third of the ϕCh1 genome (toward the 5’ end) is comprised of genes coding for structural proteins; the central section contains genes involved in replication and regulation of gene expression; and the last third (towards the 3’ end) includes genes mainly of unknown function. The majority of ϕCh1 genes showed highest homology to ϕH proteins and some were homologous to eukaryotic DNA replication and repair proteins (Klein et al. 2002). The capsid structure of ϕCh1 was also studied and a 34 kDa protein, protein E, was found to be 80 % homologous to Hp32, the major capsid protein of ϕH (Klein et al. 2000). The use of 2-D gel electrophoresis and anti-protein E antibodies revealed that protein E is posttranslationally processed, and the authors performed in-depth analyses to delineate the expression of protein during the infection cycle (Klein et al. 2000). Their studies indicate that protein E associates with the membrane and contains a leucine zipper, a motif that is also found in other phage structural proteins. The authors hypothesized that the protein E leucine zipper may contribute to the multimerization of the structural proteins during virion assembly and maturation (Klein et al. 2000). In another study, the investigators proposed an explanation for the discrepancy observed between the predicted size (14.4 kDa) and

apparent size (80 kDa) of ϕCh1's protein A, presumably another major capsid protein. They suggest that the larger form of protein A is possibly due to cross-linking of the protein, as has been shown for a bacteriophage, and that covalent cross-linking may stabilize the virus structure under harsh environmental conditions (Klein et al. 2002). Further studies by this group identified a repressor-operator system in the lysogenic region of the ϕCh1 genome (Iro et al. 2007) and revealed the differential expression of putative tail fiber proteins, gp34 and gp36, by inversion of the gene sequences possibly resulting in the ability of ϕCh1 to switch host range (Rossler et al. 2004; Klein et al. 2012). The report describing a domain within the gp34/36 proteins as the viral ligand to a host galactose binding site is very exciting, as this is the first report ever to identify halophage viral ligands (Klein et al. 2012).

The first halophage isolates to be described as spindle-shaped were His-1 and His-2, which was a breakthrough discovery, as the only viral isolates to have been described as spindle-, or lemon-shaped, were the hyperthermophilic phages (Prangishvili et al. 2006). While TEM micrographs of environmental samples show that spindle-shaped halophages are common (see previous section), His-1 and His-2 still remain the only halophage isolates of this morphology. However, despite their similar morphology to the hyperthermophilic phages, His-1 and His-2 are distantly related to these *Fuselloviridae* and instead make up a new family called *Salterproviridae* (Bath et al. 2006). Although isolated from geographically distinct sites, both His-1 and His-2 share similar morphology, host range, and genome structure. Both of these viruses plaque on lawns of their host, *Haloarcula hispanica*, but in liquid culture, these viruses proliferate constantly without creating lysates most of the time. In addition, virus DNA was not detected either episomally or integrated within the host chromosome, which led the investigators to conclude that both of these viruses are persistent and chronically infect their host—they are not strictly lytic and they are not lysogenic (Bath and Dyall-Smith 1998; Bath et al. 2006). Despite their initial similarities, both His-1 and His-2 have different physicochemical characteristics. They each have different tolerances to pH, temperature, salt concentration, and chloroform; however, they do share some genetic characteristics. The His-1 genome is ∼15 kbp while the His-2 genome is ∼16 kbp, and, astonishingly, the GC content for both genomes was reported to be 39–40 %, much lower than the host's GC content of 62.7 %. Genetic studies also revealed that the genome of each of these viruses is linear dsDNA containing inverted terminal repeats and bound by terminal proteins (Bath and Dyall-Smith 1998; Bath et al. 2006). Both viruses code for their own DNA polymerase that shows homology to polymerases involved in protein-priming replication—the first to be discovered in the entire domain of *Archaea*—meaning that neither RNA nor DNA intermediates but instead proteins are used to prime DNA replication (Bath et al. 2006). Later studies with His-2 confirmed that it indeed utilizes its terminal proteins in protein-priming for genome replication (Porter and Dyall-Smith 2008). Other milestones reached in halophage research were the *in vitro* transposon mutagenesis experiments carried out with His-2 that helped to identify non-essential regions of its genome, as well as the transfection experiments—only the second to be conducted since ϕH (Porter and Dyall-Smith 2008). The transfection experiments showed that His-2 could transfect six other host

strains other than *Haloarcula hispanica*, including several *Haloferax*, *Halorubrum*, *Haloterrigena turkmenica*, and *Natrialba asiatica* species (Porter and Dyall-Smith 2008).

The most comprehensively-studied halophage to date is arguably SH1, a spherical virus with a layered shell and a linear ~30 kbp dsDNA genome (Porter et al. 2005). SH1 is able to plaque on a strain that is closely related to *Halorubrum sodomense* as well as on *Haloarcula hispanica*, but what is truly interesting about the *Haloarcula hispanica* host is that there are two varieties—wild type, that was found to be much more susceptible to SH1 infection, and a variant named var.1 *Har. hispanica*, that is more resistant to infection. The var.1 host showed morphological differences compared to the wt host, namely the thicker wall that it expressed and its clustering characteristic (Porter et al. 2005). Investigators observed via TEM that viral adsorption was associated with the individual thin walled wt cells but never with the thicker-walled clusters of the var.1 cells. SH1 adsorbed much more easily and produced an average burst size of 230 pfu/cell in the wt strain; whereas adsorption to the var.1 host was much slower and the average burst size was only 30–40 pfu/cell (Porter et al. 2005). SH1 underwent an extensive investigation of its capsid structure with the use of amino acid sequencing (i.e., Edman degradation), tandem mass spectrometry, SDS-PAGE, native PAGE, and MALDI-TOF mass spectrometry (Bamford et al. 2005). These protocols revealed that SH1 has 15 capsid proteins and that most of the proteins are acidic with a predicted pI of <5, although there were also several basic proteins. SH1 shares 3 of its lipids with its *Har. hispanica* host, but the relative amounts of those lipids are different between the virus and host, indicating that SH1 selectively incorporates host lipids into its envelope (Bamford et al. 2005). Another study surprisingly showed that the SH1 structure was very similar to other mesophilic and hyperthermophilic phages—PRD1, a gram-negative phage; Bam35, a gram-positive phage; PBCV-1, an algal virus; and STIV, a hyperthermophilic phage—leading the authors to wonder if there was a shared ancestry among these very diverse viruses, especially since the combined masses of SH1's VP4 and VP7 capsid proteins equaled the masses of PRD1 and STIV coat proteins (Kivela et al. 2006). Selective dissociation of the SH1 particle resulted in the characterization of two groups of viral proteins—soluble capsid proteins and proteins associated with a lipid, DNA-containing core (Kivela et al. 2006). Based on their data, the investigators proposed that the SH1 structure is composed of a lipid membrane encapsulating the viral DNA genome, which is then surrounded by an outer proteinaceous capsid that contains spikes. In-depth comparative structural studies employing cryo-EM and image reconstruction were conducted and showed that a possible lineage among viruses could be established using Major Capsid Proteins (MCPs) (Jaalinoja et al. 2008). The authors showed that evolutionary information about viruses can be gotten based on physical structure and that SH1 is considered to be a "molecular fossil" as it utilizes single β barrels to form its complex structure instead of double β barrels as used by PRD1, STIV, and Bam35. No significant homology was found for any of SH1's proteins, except for ORF 17 (a putative ATPase) and for a protein that was homologous to a PRD1 viral protein (Bamford et al. 2005). A detailed study of SH1's

infection cycle revealed that during the early and middle parts of the infection, transcripts were of a certain, regular length, but during late infection, transcripts became longer (i.e., they were "extended") and seven of those transcripts existed in both regular and extended lengths (Porter et al. 2008). Since most of the extended transcripts were produced at 10X higher amounts than the regular length transcripts, the authors of this study hypothesized that these extended transcripts may possibly form dsRNA and could play a regulatory (i.e., inhibitory) role in blocking translation. Another interesting observation about SH1 is that, while, typically, viral capsid proteins are not produced during early infection, the capsid proteins of SH1 were transcribed early (i.e., 1 hour post infection) and increased through the infection cycle, while other structural proteins and the packaging ATPase were transcribed late in the infection (i.e., 5–6 hours post infection) (Porter et al. 2008). Genomic studies of SH1 showed that this phage relies on protein-primed replication based on the presence of terminal proteins. SH1, along with His-2 and a few other halophages, was used to successfully transfect other hosts, namely *Haloferax* and *Natrialba* (Porter and Dyall-Smith 2008).

The shortest halophage and archaeal genome to be discovered yet is HRPV-1, a *Halorubrum* pleomorphic virus with a ~7 kb circular ssDNA genome (Pietila et al. 2009). It has a restricted host range to *Halorubrum sp.* PV6, and, once again, this virus acted lytically on solid medium, but carried out chronic infection in liquid medium, just as with His-1 and His-2 (Pietila et al. 2009). The presence of a lipid envelope explained the virus' pleomorphic shape, and further analyses demonstrated that the lipid ratio between HRPV-1 and its host are almost the same, indicating that the virus non-selectively incorporates the host's lipids into its envelope (Pietila et al. 2009). However, the authors were not sure how HRPV-1 buds through the host's S layer as budding viruses were not detected during TEM analysis. An in-depth structural investigation was conducted by careful and controlled dissociation of the virion particles followed by biochemical analyses, which ultimately yielded the unusual result that there were no nucleoproteins associated with the genome of this enveloped virus (Pietila et al. 2009). Comparative studies showed that HRPV-1 had both nucleic and amino acid homology to the His-2 halophage (Pietila et al. 2009) as well as to the pHK2 temperate phage, which was initially thought to be a *Haloferax* plasmid (Roine et al. 2010).

HHPV-1 is another pleomorphic halophage, with a circular dsDNA ~8 kbp genome, ~56 % GC content, and a restricted host range infecting *Haloarcula hispanica*, that was also discovered to have genetic and protein homology to both pHK2 and to HRPV-1. Detailed sequence analyses showed that there was relatively high homology in the structural proteins among HHPV-1, pHK2, HRPV-1, and the pro-virus element of *Haloferax volcanii*, but less homology in the replication proteins (Roine et al. 2010). HHPV-1 seems to be more closely related to pHK2 and *Haloferax volcanii* pro-virus than to HRPV-1, but even the high genetic homology between HHPV-1 and HRPV-1 was surprising, considering that HHPV-1 is double-stranded while HRPV-1 is single-stranded. This led the authors of this study to suggest that perhaps genomic sequencing may not be a useful tool for virus classification purposes after all since HHPV-1 and HRPV-1 would have been classified as close relatives

despite the fact that they: (a) have different genome architecture, (b) share a different host range, and (c) do not share high homology in the replication proteins. Instead, the authors propose that capsid structure homology be used to classify viruses based on their observation that, despite the different host range of all four viruses and pro-virus elements investigated in this study, the most conserved regions among all the viral sequences were the ORFs thought to be associated with capsid proteins: e.g., 55 % identity between VP3 of HHPV-1 and HRPV-1 and 47 % identity between HHPV-1 ORF7 and HRPV-1 ORF8 versus only 9 % identity between HHPV-1 ORF1 and HRPV-1 ORF1, which is believed to be the gene for a replication protein (Roine et al. 2010). The authors propose using conserved structural proteins/ORFs/genes, which could be considered "core proteins", to classify virus relatedness based on virion physical architecture. They speculate that these core proteins could be inherited vertically, whereas other genes such as those for replication are swapped or shared horizontally.

Methods and Instrumentation

Just as with any discipline, virology in general and halophage research in particular have their own protocols and tools of the trade. There are a number of protocol resources available for those who are newcomers to the field as well as for experienced researchers alike. Protocols for more general, or basic, virus investigations can be found in the two volume set titled *Bacteriophages: Methods and Protocols* (Clokie and Kropinski 2009), which covers classical lab isolation and characterization techniques as well as applied and genomic methods. Another extensive resource is the free digital book titled *Manual of Aquatic Viral Ecology* (Wilhelm et al. 2010) published by the American Society of Limnology and Oceanography. This work reviews methods and techniques for investigating marine viruses ranging from transmission electron microscopy to nucleic acid isolation to determining rates of viral production and lysis. This book also has a chapter dedicated to methods for studying Archaeal viruses, including halophages. An indispensible resource for halophage researchers in particular is the *The Halohandbook v.7.1* (2009), another free digital resource edited by Michael Dyall-Smith. This resource instructs the halophage researcher on how to culture hosts and phages, extract nucleic acid, carry out microscopy, and prepare long-term storage of cells. Another relevant and useful resource for specifically studying hypersaline viruses is Chapter 28 in the *Extremophiles* title within the *Methods in Microbiology* series (Porter and Dyall-Smith 2006), which discusses how to purify halophages via density gradient ultracentrifugation and how to carry out initial physical characterizations of a viral isolate.

While the reader can find thorough explanations of specific protocols in the aforementioned resources, this section will be more concerned about some of the common instrumentation and techniques used in halophage research. When working with liquid samples, filtration is usually the primary method to separate the virus population from cells, whether using environmental samples or lab cultures. While there are

caveats to using filtration (see comments in earlier text above), several different types of filtration methods are employed in halophage research, including vacuum filtration; tangential flow filtration (TFF), also known as cross-flow filtration; positive pressure filtration; syringe filtration; and ultrafiltration. Vacuum filtration, familiar to a broad range of scientists, employs a flat, single filter membrane in conjunction with vacuum, or negative pressure, to separate molecules of a larger size from molecules of a smaller size (Figs. 4.5a and b). The larger-sized molecules are inhibited from travelling through the smaller-sized pores in the filter membrane and stay trapped on top of the filter membrane, while the smaller-sized molecules easily travel through the membrane pores into a vessel. In vacuum filtration, the substances in a liquid are pulled through the membrane. Positive pressure filtration, on the other hand, uses either a pressurized canister of inert gas or a peristaltic pump to instead push the substances in a liquid through a filter membrane (Fig. 4.5c). Positive pressure filtration involves the use of a steel sample container that is available in various sizes as well as a stainless steel filter holder to allow relatively high levels of pressure to be used on the filter membrane in the case of a high-solute sample, which can be very common when using environmental hypersaline water samples. While the setup for positive pressure filtration is not inexpensive, it can be quite the time-saver when working with more difficult, large-volume samples. Syringe filters utilize the same principle as positive pressure filtration, but on a much smaller scale (Fig. 4.6a). Syringe filters are available in a variety of different pore sizes and can be attached to syringes of different volumes to be customized to a researcher's immediate needs. When working with smaller volumes, syringe filters are very practical because they can be purchased as sterile units and are relatively inexpensive due to their disposable nature. However, once again, due to the high-solute nature of hypersaline environmental samples, syringe filters may be useful only up to a certain salinity and perhaps are most useful when working in the lab. Another type of device that functions essentially the same as a syringe filter is a capsule filter, or capsule cartridge (Fig. 4.6b). Instead of a single filter membrane, the capsule contains multiple layers of the same membrane, thereby resulting in a much greater surface area. An intermediate sample volume (e.g., up to a few liters) can thus be filtered in a relatively short period of time using positive pressure filtration with the capsule cartridge.

All of the filtration methods discussed thus far are also referred to as "dead-end" filtration because filtration occurs in one direction through the membrane resulting in the build-up, or entrapment, of larger particles on the top surface of the filter membrane. Furthermore, this "dead-end" filtration is used simply to separate different-sized particles from each other (e.g., viruses from cells); however, there are ways to both filter and concentrate a sample simultaneously. Centrifugal filter units are disposable ultrafiltration devices, which are employed in conjunction with centrifugation to allow very small-sized particles—such as salts and liquid—to filter through the membrane thereby concentrating the larger-sized particles—such as viruses and proteins—in the remaining amount of liquid that is left in the unit (Fig. 4.6c). Usually, the operator determines when to halt the centrifugation allowing the researcher to choose the final sample volume. Otherwise, the centrifugal filter unit is allowed to spin until the sample reaches a "dead volume", which is usually

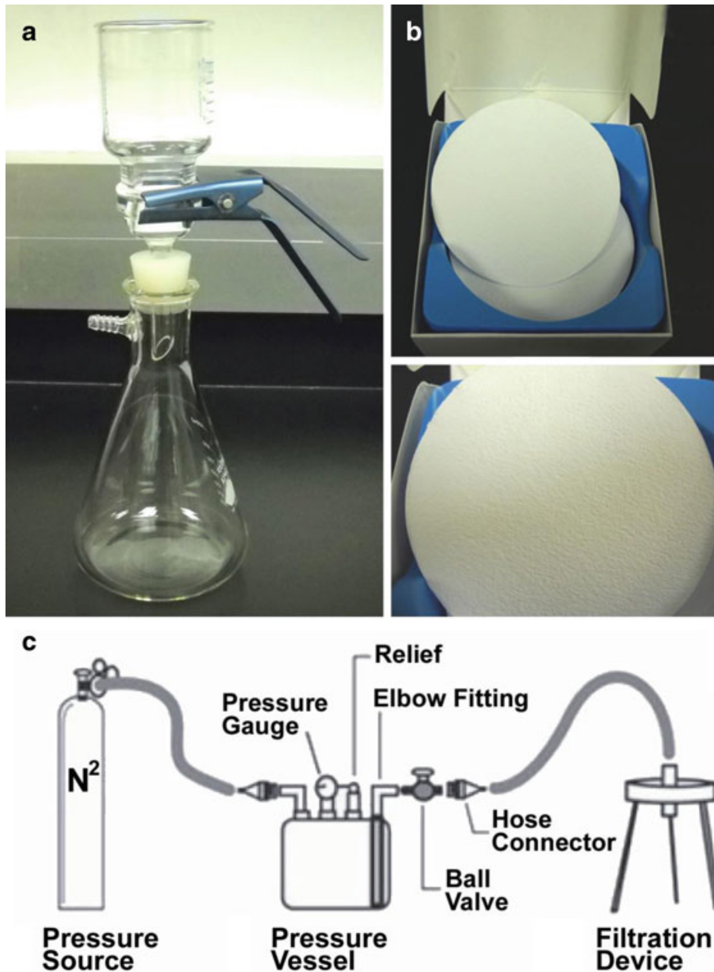

Fig. 4.5 **a**) Photograph showing the commonly-used vacuum filtration setup, also referred to as "dead-end" filtration or negative pressure filtration. **b**) Photograph of a typical flat, filter membrane used in vacuum filtration. **c**) Schematic diagram of a positive pressure filtration system showing an inert gas as the positive pressure source, the pressure vessel containing the water sample to be filtered, and the filtration device that is made up of a stainless steel filter holder containing a flat, filter membrane. The pressure vessel can range in size from approximately 5–20 l and the stainless steel filter holder, or filtration device, is available with a diameter of either 90, 142, or 293 mm. Photographs courtesy of Shereen Sabet. Diagram shown here is modified from an original diagram provided courtesy of Advantec MFS, Inc

less than 1 ml but never to dryness. This form of ultrafiltration does not result in the build-up of solutes on the surface of the membrane as in dead filtration, thereby resulting in greater efficiency by preventing the clogging of the membrane. These centrifugal filter units are used to filter-concentrate samples that are in the kilodalton range (e.g., 10,000–100,000 Da), at the macromolecular and protein level, whereas

Fig. 4.6 Examples of disposable filtration devices that are available in various sizes and used for relatively smaller sample volumes, ranging from less than 1 ml up to 70 ml, such as **a**) syringe filters, **b**) capsule cartridges (both photographs provided courtesy of Advantec MFS, Inc.), and **c**) centrifigual filtration units (photograph provided courtesy of Sartorius Laboratory Products & Services)

filter membranes used in "dead-end filtration" utilize much larger pore sizes (e.g., 0.22 μm and 0.45 μm, etc.). Ultrafiltration can be an ideal way to filter-concentrate viruses in a sample after cells have been initially removed, but their drawback is their limited volume capacity. The volume sizes available for these disposable centrifugal filter units range from 0.5 ml to 70 ml. Tangential flow filtration is another way to filter-concentrate liquid samples using positive pressure, but on a much larger scale (e.g., $\geq$5 l) (Fig. 4.7). Here, a cartridge, in the shape of a rectangular brick, is composed of multiple layers of a membrane, once again increasing the surface area to allow filtration of larger volumes. However, this time, instead of the substances being either pushed or pulled through the membrane (via positive or vacuum pressure, respectively), the sample is being pushed *across* the surface of the filter cartridge unidirectionally resulting in the smaller-sized particles traveling through the pores of the filter cartridge, while the rest of the sample is directed back to the source vessel. This, too, prevents larger particles from collecting on and clogging up the surface of the membrane. While TFF is a reusable system, investing in a complete setup, which includes a peristaltic pump, tubing, and at least one filter cartridge, can be relatively

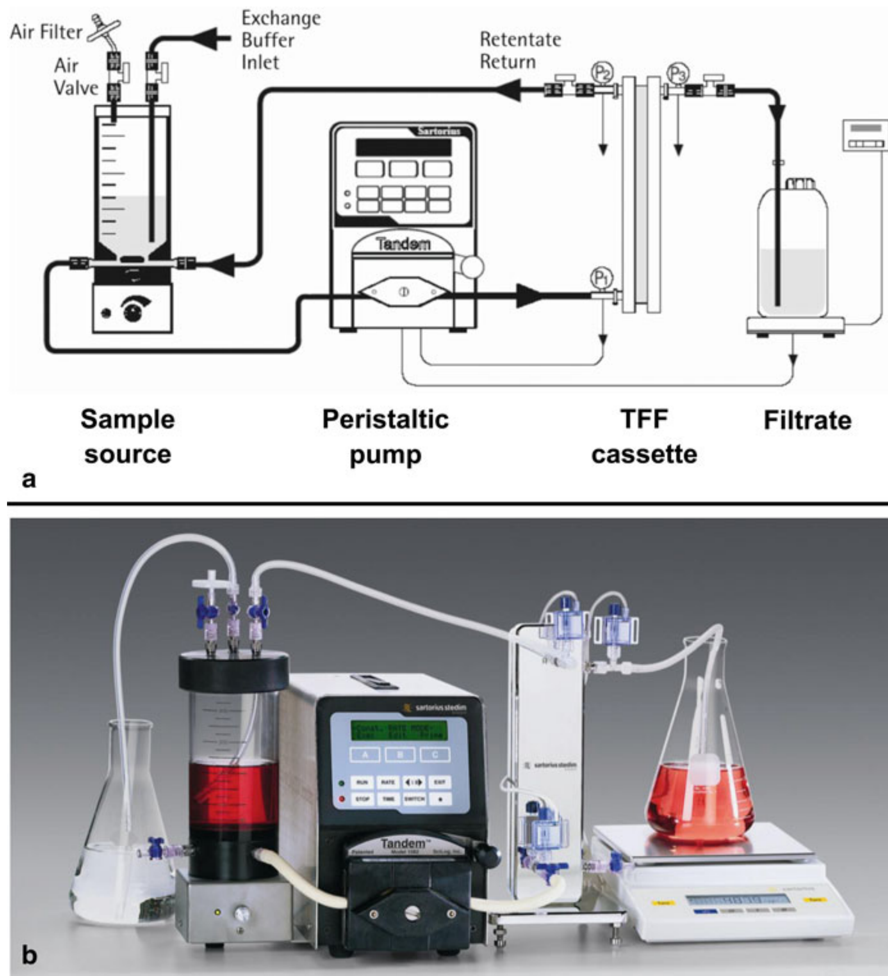

Fig. 4.7 Images showing the tangential flow filtration (TFF), or cross-flow filtration, system. **a**) Diagram illustration with the sample source volume on the far left side of the diagram and the filtrate on the very far right side of the diagram. The thin, vertical filter cassette is shown between the peristaltic pump and the filtrate. **b**) A photograph of the complete TFF system. Both images provided courtesy of Sartorius Stedim Biotech

expensive. Operation and maintenance are also more time-consuming and more involved than the disposable alternative of ultrafiltration. Ultimately, sample volume dictates which instrumentation and method the halophage researcher must employ.

Another way to concentrate viruses without filtration is by precipitating the viruses in a relatively large liquid volume via addition of polyethylene glycol (PEG). Typically, PEG is added at 10 % (w/v); however, some protocols may dictate a different concentration, especially when using an environmental sample. The PEG-virus mixture is usually incubated on a stir plate at 4 °C overnight before being centrifuged

at relatively low speeds (e.g., 10,000–16,000 rpm) to collect the viral pellet. PEG is available in different formula weights (e.g., PEG 6000, PEG 8000, etc.) and can be used to aggregate and precipitate viruses in both environmental water samples as well as in lab cultures. Specific protocols should be researched and followed according to the investigator's needs and circumstances.

PEG precipitation is a relatively inexpensive, easy, and convenient way to concentrate viruses in a solution, but it should be noted that PEG precipitation will also simultaneously precipitate any small-sized molecules, including host proteins, debris, etc. So, while filtration may have the advantage of separating viruses from all or the majority of non-viral material, filtration also involves cost, time, and may very well bias viral representation in environmental water samples (see earlier discussion above). On the other hand, PEG precipitation may be a gentler way of concentrating viruses, but further purification—in the form of density gradient ultracentrifugation—will be required to acquire a pure sample of the virus.

Other considerations the halophage researcher should keep in mind include such issues as viral DNA purification and large-scale growth and harvest of halophage isolates. Conventionally, purification of a viral isolate is accomplished via cesium chloride (CsCl) density gradient ultracentrifugation. The specific buoyancy of the viral isolate is used to separate it from other molecules, such as cellular proteins and ribosomes, which have their own individual buoyancy in the same CsCl gradient. Once a viral isolate is CsCl-purified, it is then used to extract nucleic acid to be used in downstream protocols including genome sequencing. However, with the advent of 454 sequencing, it is no longer absolutely necessary to first purify halophage isolates. One can instead successfully extract DNA with a viral DNA purification kit (e.g., Invitrogen) using viral lysate as the initial source. The DNA sample is clean enough for 454 sequencing. Nonetheless, CsCl density gradient purification is still the gold standard and should be employed for sensitive downstream applications and investigations of virus proteins and physical structure.

The halophage researcher should be aware that infection of a host may be cell state and/or salinity dependent. In addition, just because lysis is not observed when a cell strain is inoculated with its virus under certain conditions does not necessarily mean that the virus does not infect that particular cell. While an optical density at 600 nm (OD_{600}) of $\sim$0.5 for cells is typically used for an infection, one may need to experiment with different cell densities by using a cell line at different ODs, such as a low OD of $\sim$0.2, a mid OD of $\sim$0.5, or a high OD of $\sim$0.8. Compounded with this is the optimal salinity for infection of a halophage-host system. While not always the case, lytic infections have been successfully carried out using a salinity that is lower than the optimal growth salinity of the host ((Dyall-Smith 2009) and personal observations). It is believed that using a lower salinity than the host's optimal growth salinity stresses the host and makes it more vulnerable to infection. While this may seem like a daunting task, the successful halophage researcher will conduct thorough experimentation to determine the parameters for successful lytic infection in a specific host-phage system by considering different salinities, different cell states (i.e., using cells at different stages of their growth cycle), as well as the different phage-to-cell ratio, known as the multiplicity of infection (MOI).

Future Directions

The field of halophage study is nearly forty years old, and although strides have been made, especially in the last few years, this field of research is still very much understudied. While the general role of viruses in nature is better understood, especially in the aquatic environment, there is still much basic data missing from our view about extreme hypersaline viruses. In regards to the exciting discovery of novel, unclassified halophage morphologies, it would be instructive to find halophilic hosts that are visibly infected with viruses exhibiting these exotic, previously unknown shapes. In other words, it would be just as informative to view the environmental cellular samples via thin section TEM to help determine whether or not the uncultured, exotically-shaped VLPs are truly viral. In addition, since all halophage isolates to date are DNA viruses (mostly double-stranded linear with only one single-stranded circular DNA virus isolated), a search should be undertaken to detect, if not isolate, RNA halophages. What percentage of halophages is RNA versus DNA? Do RNA hypersaline viruses even exist? It would be difficult to reasonably argue against their existence with our currently limited knowledge about the halophage community.

Another very exciting find was reported by Santos et al. in 2007 who used fosmid libraries to describe the first ever complete halophage genome sequence from an uncultured halophage, named EHP-1 for environmental halophage 1. They were able to characterize this virus genomically, providing information about DNA size, G+C content, and protein predictions of putative ORFs. However, neither the morphology nor the host for EHP-1 is yet known, which greatly limits our knowledge about this halophage. In 2010, the same group reported the genome sequence of a second uncultured halophage, EHP-2 (Santos et al. 2010). Since the majority of the scientific literature on halophage isolates has been very basic and incomplete, and with ecological studies dominating the recent scientific literature, investigators should now conduct more cellular work and focus more attention on understanding the infection process and host-virus dynamics in detail and on in-depth physical, as well as genetic, characterizations of halophage isolates. One of the initial questions asked about halophages was how they were able to physically withstand and function within an extremely hypersaline habitat. This question drives to the heart of biochemistry and begs for further analysis of halophage capsid proteins and enzymes to understand their capabilities and limits within a chemically concentrated environment. Just as components of halophilic *Bacteria* and *Archaea* have contributed to industry, halophage investigators could also focus their attention to see if these viruses can be of benefit in medical or commercial use. Such knowledge can only be acquired through experimentation of isolates.

Field sites have now been established all over the world and halophages have been discovered and/or isolated from places such as Europe, the Middle East, Africa, Asia, Australia, and North America. There have been no reports of halophages from South America, so further attention is warranted for this region. Most of the field sites have been aquatic in nature, such as man-made solar salterns or natural salt lakes. The halophage researcher may also wish to consider other places that harbor an extreme

hypersaline environment, such as desert salt pans, and to undertake evaporation studies to understand if or how halophages can withstand desiccation, along with their hosts.

By combining classical methods with cutting edge techniques and creative protocols, the picture of halophages is becoming increasingly clearer. Ultimately, halophage investigators will be able to snap the halophage piece into the overall virology puzzle to provide a better understanding of the place of extreme hypersaline viruses within nature, and to address a number of diverse issues, such as, among other things, how viruses have contributed to evolutionary processes and to the history of life on Earth. Questions about the limits of proteins in an extreme habitat, and possibly whether hypersaline viruses can be promising candidates in the search for life on Mars remain to be addressed. Now is certainly a very exciting and promising time in halophage research.

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Chapter 5
Microorganisms in Evaporites: Review of Modern Geomicrobiology

Tim K. Lowenstein

Introduction

The "geomicrobiology" of evaporites—microorganisms and associated biomaterials preserved in saline minerals—has seen great progress over the past decade. There are many new reports of culturing archaea and bacteria (Stan-Lotter et al. 1999, 2002; Vreeland et al. 2000, 2007; Mormile et al. 2003; Gruber et al. 2004; Schubert et al. 2009b, 2010a; Gramain et al. 2011), sequencing prokaryote DNA (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011), and identifying organic compounds such as beta carotene and cellulose (Griffith et al. 2008; Schubert et al. 2010b; Lowenstein et al. 2011) from ancient samples of halite (NaCl) and gypsum ($CaSO_4 \cdot 2H_2O$). Tiny droplets of brine trapped within evaporite minerals, called fluid or brine inclusions, seem to be an important, but not exclusive, haven for microbes and biomaterials in buried evaporites. Given the expanded interest in microbial life in evaporites, and the potential implications regarding the search for life in the solar system, it seemed worthwhile to summarize the most important findings in the geomicrobiology of evaporites. The last such summary of advances in the geomicrobiology of ancient evaporites was by Vreeland and Powers (1999), so the focus here is on the last 10 years.

Five important aspects for geomicrobiologists studying ancient evaporites form the core of this review.

1. The timing of formation of the samples studied, whether "syndepositional" and formed at the time of deposition, or soon after deposition, by processes controlled by the contemporary surface environment, or "burial" and formed by later processes that existed in the subsurface burial environment (Hardie et al. 1985).

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The syndepositional versus burial origin of an evaporite deposit in its present state should be known before any studies of biological materials are undertaken because without definitive information on the timing of formation of the samples under consideration, little can be conclusively said about the age of any microorganisms and other biomaterials discovered.

2. Most evaporite deposits formed from the evaporation of ancient seawater. Analysis of the chemical composition of fluid inclusions in ancient marine halites over the past 10 years has shown that there have been secular changes in the major ion chemistry of seawater during the Phanerozoic Eon, the past 542 million years (Lowenstein et al. 2001; Horita et al. 2002). These changes occurred slowly over periods of millions of years and most notably involved the ions Ca^{2+}, Mg^{2+}, and $SO_4{}^{2-}$, which in turn, impacted the development and evolution of $CaCO_3$ shell building organisms (Stanley and Hardie 1998). It is not known how such variations in the major ion chemistry of seawater influenced halophilic microorganisms living in concentrated marine brines.
3. Microthermometric techniques used on primary fluid inclusions in halite can document the water temperatures at which the halite originally crystallized (Roberts and Spencer 1995; Lowenstein et al. 1998, 1999; Benison and Goldstein 1999; Satterfield et al. 2005a, b). Such information is a quantitative record of the surface water temperatures at which microorganisms were trapped in fluid inclusions, and has potential significance for paleoenvironmental interpretations and for designing cultivation experiments.
4. Geomicrobiological studies of ancient evaporites have seen important advances using *in situ* light microscopy (Benison et al. 2008; Panieri et al. 2008; Schubert et al. 2009a, b, 2010b), *in situ* Raman spectroscopy (Fendrihan et al. 2009), scanning electron microscopy, and transmission electron microscopy (Griffith et al. 2008). Such studies, in particular *in situ* microscopy, help establish authenticity of microbial materials trapped in evaporite minerals and fluid inclusions.
5. Culturing studies have become more sophisticated, using new methods, improved surface sterilization techniques, and reproduction of laboratory results. Beginning in 2001, ancient DNA from halite and gypsum has been extracted, purified, amplified, and sequenced (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011).

Sedimentology and Microscopy of Evaporites and Fluid Inclusions: Syndepositional (Primary) Versus Burial (Secondary) Origin and Interpretation of Paleoenvironments

Evaporites are salt deposits that form from the evaporation of water at the Earth's surface in marine and inland lake settings with arid climates and no drainage out of the basin. Modern environments of evaporite deposition include coastal lagoons,

such as Santa Pola, Spain; inland saline lakes such as the Dead Sea, Israel and Jordan; and desiccated saline pans such as Death Valley, California. There is a spectrum of environments, from permanent density-stratified deep lakes (i.e., the Dead Sea), to shallower perennial lakes (i.e., Great Salt Lake), to ephemeral lakes (i.e., Death Valley), that may form standing bodies of water for years to days. Some evaporite environments, almost always dry, contain thick surface salt crusts and shallow groundwaters normally less than one meter below the surface.

Samples of halite used to study microorganisms and ancient DNA have so far come from borehole cores and from underground mine outcrops. Gypsum is much less soluble than halite and therefore samples for geomicrobiological studies have come from surface outcrops (Panieri et al. 2008, 2010). The ages of these halites and gypsums vary from Pleistocene, tens of thousands of years old, to Silurian, greater than 400 million years in age. The depths from which core and mine samples were obtained range from meters to hundreds of meters. For all these samples, before beginning microbiological studies, it is important to distinguish the minerals, textures, structures, and fluid inclusions of sedimentary syndepositional origin from those formed from burial alteration processes. These features are easily observed in large (5×7.5 cm) thin sections, which may be prepared without heating or dissolving samples, preferably using a diamond wire saw (Lowenstein and Brennan 2001).

Discussion of the syndepositional versus burial origin will be limited to halite and gypsum because they are the most common evaporite minerals and the only ones that have been used for geomicrobiological studies to date. Syndepositional evaporites that formed at or soon after the time of deposition, should be the focus for geomicrobiological studies because they are expected to be the richest source of living prokaryotes and associated microorganisms and biomaterials. Such syndepositional evaporites, now buried, contain biomass that was originally trapped at or near the Earth's surface. Fortunately, the syndepositional versus burial origin of evaporites, and specific surface environments of deposition can be evaluated and interpreted through sedimentologic and microscopic studies. How are syndepositional features recognized? Detailed information on the analysis of syndepositional sedimentary features in gypsum and halite is described in Hardie et al. (1985), Smoot and Lowenstein (1991), and Lowenstein and Brennan (2001). Diagnostic sedimentary structures common in gypsum and halite include layering on the millimeter to meter scale defined by textural and mineralogical variations (Fig. 5.1). Repetitious interlayering of clay or carbonate mud with gypsum or halite is common. Evaporite layers may form cross lamination and cross stratification structures which are the grains making up ripples and dunes formed by movement of grains by water currents, waves, and air (Fig. 5.2). Detrital framework textures, the settle out layers of halite crystal hoppers, cubes, and rafts, and gypsum plates, that all precipitated at the air-water interface, are widely recognized in modern and ancient evaporites (Figs. 5.3 and 5.4). Such detrital accumulations of halite and gypsum form well sorted layers of loosely packed crystals, which can later be reworked into ripples.

Fig. 5.1 Slab sample of halite from the F-salt, Silurian Salina Salt, Michigan Basin (408.5–411 million years old), showing halite beds (light) and dark millimeter-thick laminae of anhydrite (*arrows*). Such layering is common in evaporites with well-preserved syndepositional features. Scale at bottom is in centimeters. (Modified from Satterfield et al. (2005b))

Crystalline framework crusts of halite and gypsum, formed at the brine bottom by in place growth into the water column, are common in modern and ancient evaporites. These crusts are made of vertically oriented, upward widening and elongated crystals that grew competitively off a common substrate at the brine bottom (Fig. 5.4 and 5.7). Such frameworks are of great significance because the crystals in these layers are typically large, centimeters in size, with relatively abundant and large fluid inclusions. These samples have therefore been the focus of recent geomicrobiological studies because it is relatively easy to visualize fluid inclusions and microorganisms in the crystalline frameworks using *in situ* microscopy. The large crystals also simplify procedures for surface sterilization and for drilling and extracting brine from individual fluid inclusions (Mormile et al. 2003; Vreeland et al. 2007; Schubert et al. 2009a, b, 2010a, b; Panieri et al. 2008, 2010).

Fig. 5.2 Ripple marks preserved in modern halite crust, Dabusun Lake, Qaidam Basin, China; Swiss army knife for scale. The ripple marks record reworking of halite crystals by waves along a lake shoreline. *Arrow* points to ripple crest and shows direction of wave approach. Inset shows rippled bedding surface in anhydrite-polyhalite rock from the Permian Salado Formation, New Mexico, (~250 million years old) with pencil for scale

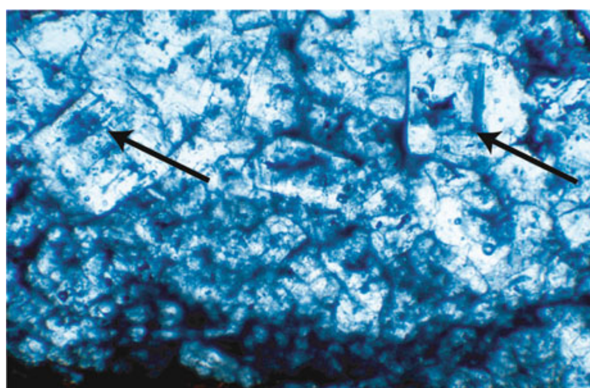

Fig. 5.3 Thin section photograph of detrital halite cubes (viewed perpendicular to layering), which precipitated at the air-water interface and settled to the brine bottom. Dark patches and bands in the cores of halite cubes are arrays of fluid inclusions (*arrows*). Dark material between halite crystals is polyhalite. Sample from Permian Salado Formation, New Mexico. Horizontal field of view is 7 mm

Layered gypsum and halite deposits may have syndepositional dissolution textures from contact with undersaturated waters. These features may be preserved as rounded dissolution cavities, truncated crystal surfaces, and vertical dissolution pipes (Figs. 5.5 and 5.6). They are important because they indicate contact with undersaturated waters, which is most likely to occur in a shallow lake, lagoon, or salt pan setting, and not in a deep brine pool. Deep saline lakes and marine saline basins are stratified and contain dense brine bodies that separate dilute undersaturated

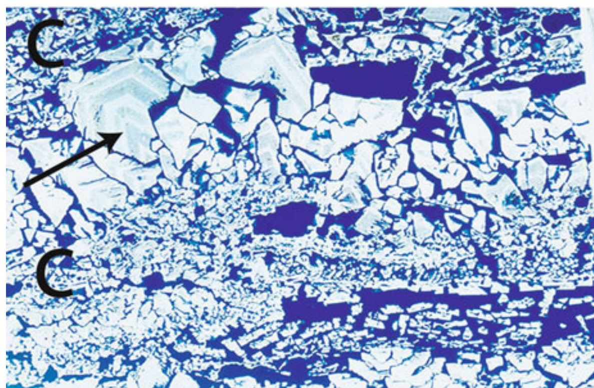

Fig. 5.4 Thin section photograph of modern halite crust from Salina Omotepec, Baja California, Mexico (viewed perpendicular to layering). Note layer of vertically-oriented, fluid inclusion banded "chevrons" in upper half (*arrow*). Smaller crystals below and above are layers made of sunken detrital rafts and cubes of halite (C). Open pore spaces are dark blue. Horizontal field of view is 5.5 cm. (Modified from Lowenstein and Hardie (1985))

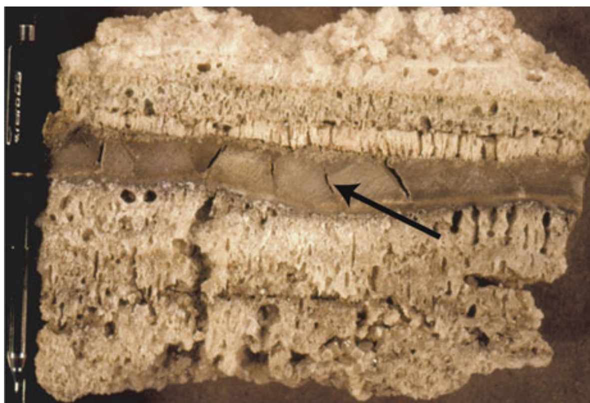

Fig. 5.5 Slabbed hand sample of modern saline pan halite from Saline Valley, California. Note the large number of vertical voids formed by dissolution of the halite crust when the saline pan is flooded. Dark mud layer in middle (*arrow*) was deposited during a flood. Sample is 10 cm thick. (Modified from Casas and Lowenstein (1989))

Fig. 5.6 Hand sample of modern halite crust from Salina Omotepec, Baja California, Mexico, from just below the surface. Large dissolution cavity (*arrow*) is lined with halite cement crystals that have grown in the cavity. Coin is 20 mm in diameter. (Modified from Lowenstein and Hardie (1985))

inflow waters from the evaporites accumulated at the brine bottom. Therefore the preservation potential of evaporite deposits formed in deep perennial settings is greater than in shallow and ephemeral systems. Dissolution features in gypsum and halite are diagnostic of very shallow water and ephemeral environments of deposition and contrast with the "pristine" unaltered deposits that commonly form in deeper water, density stratified settings. Syndepositional dissolution features found in shallow water and ephemeral deposits preserve important paleoenvironmental information.

Gypsum and halite may form "diagenetically" in the subsurface by crystallization from saline groundwaters as displacive crystals, commonly millimeter- to centimeter-sized single crystals or as nodular aggregates composed of sub-millimeter sized crystals. They may also form mineral cements that occur as cavity fillings or crystal overgrowths (Fig. 5.6). Such diagenetic cements and displacive crystals are difficult to interpret in terms of their timing of formation because they can form in either syndepositional or burial environments (Hardie et al. 1985). For example, the large halite cement crystal studied by Vreeland et al. (2000) from which a Permian bacterium was cultured, is difficult to interpret, in terms of the timing of its formation, from its texture alone. It took study of fluid inclusions from these halite cements to prove that they formed syndepositionally, early in the diagenetic history, from evaporated Permian seawater (Satterfield et al. 2005a).

Finally, because of the ease with which evaporites may be altered, one should always be on the lookout for burial diagenetic alteration features that deform, disrupt or destroy the original sedimentary features. If evaporites have sutured interpenetrating crystalline textures, recrystallized polygonal mosaics, and deformation features (folds, faults, etc.), described more fully in Hardie et al. (1985), they have been modified during burial. Such samples should not be used in geomicrobiological studies.

Fluid Inclusions in Halite and Gypsum

Fluid inclusions are cavities within crystals filled with fluid, normally water. They may also contain other liquid (i.e., hydrocarbon), vapor (i.e., CO_2 and H_2S), and a variety of solids including minerals, organic material and of course, microbes (Schubert et al. 2009a). Fluid inclusions trapped during crystal growth are called primary inclusions. Crystal imperfections and irregularities that form during crystal growth may be enclosed by the growing crystal to become fluid inclusions. It is important to note that primary fluid inclusions can form during crystal growth in either surface or burial environments. Therefore, sedimentologic and microscopic examination, outlined above, should first be conducted to determine the syndepositional versus burial origin of the deposit under consideration. Details on fluid inclusion microscopy are found in Roedder (1984), Goldstein and Reynolds (1994), Lowenstein and Brennan (2001), Schubert et al. (2009a, 2010b), and Lowenstein et al. (2011).

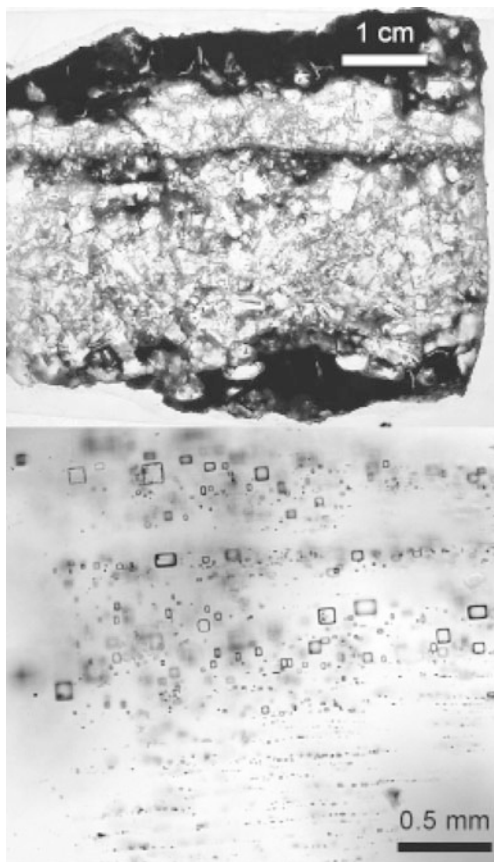

Fig. 5.7 Photomicrograph of halite in thin section from the Death Valley core, depth of 14.1 m (age of 25,000 years). *Top:* Interlayered halite (crusts of vertically oriented halite crystals grown on the bottom of an ancient lake) and dark mud (viewed perpendicular to layering). *Bottom:* Close-up of primary fluid inclusions in halite crystal, showing bands rich and poor in fluid inclusions. This sample yielded a positive culture in the genus *Natronomonas*. (Modified from Schubert et al. (2010a))

Secondary fluid inclusions form later, by healing of fluid-filled microfractures (Roedder 1984; Goldstein and Reynolds 1994) and are to be avoided in most geomicrobiological studies. First, the ages of the fluids trapped in secondary fluid inclusions are not known with certainty except that they are younger than the host mineral. In addition, secondary fluid inclusions may be related to fluids associated with burial and deformation processes, not the concern of most geomicrobiological studies if the primary aim is the isolation and study of surface microbial communities. But secondary fluid inclusions may be of interest in studies seeking to understand the activities and identification of subsurface microbes.

Fluid inclusions in halite are quite common and have been studied by geologists for decades (Roedder 1984; Hardie et al. 1985; Lowenstein and Hardie 1985; Lowenstein and Spencer 1990; Goldstein and Reynolds 1994; Roberts and Spencer 1995; Kovalevych et al. 1998; Benison and Goldstein 1999; Lowenstein and Brennan 2001; Schubert et al. 2009a, b, 2010b; Lowenstein et al. 2011). Primary fluid inclusions, composed of halite saturated brine, occur in halite crusts in which crystals grew at the bottom of the brine body as "chevrons" and vertically oriented crystals (Lowenstein and Hardie 1985) (Figs. 5.4, 5.7 and 5.8). They also occur in halite

Fig. 5.8 Photograph of Cretaceous (112–121 million years old) chevron halite crystal with well defined primary fluid inclusion bands (dark, *arrow*) that formed parallel to crystal growth faces. Crystals like this yielded live halophilic *Archaea* (Vreeland et al. 2007). Crystal is approximately 5 mm in size

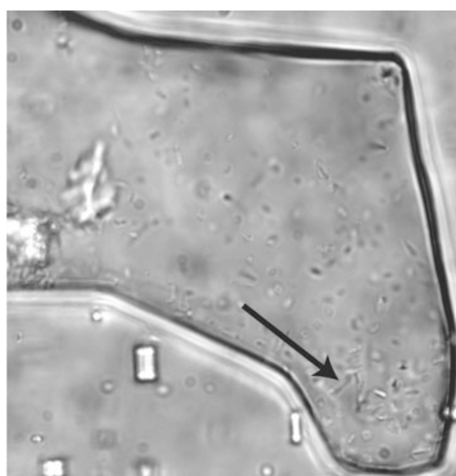

Fig. 5.9 Photomicrograph of a large, irregularly-shaped fluid inclusion in halite crystallized in Saline Valley, California, in March, 2004. Note the large number of prokaryote cells (rod and coccoid shapes, *arrow*) within the brine inclusion. Width of inclusion is ~100 µm

crystal plates, rafts and cubes that grew at the air water interface and sank down to the brine bottom to form "cumulate" crystal layers (5.3 and 5.4). Fluid inclusions in halite can be quite abundant, with as many as 10^{10} cm^{-3} (Roedder 1984). Fluid inclusions commonly occur in zones parallel to crystal growth faces (Benison and Goldstein 1999; Lowenstein and Brennan 2001) (Figs. 5.4, 5.7 and 5.8). Such fluid inclusion zonation results from variations in the rate of crystal growth, which in turn, controls the amount of ambient fluid trapped. Faster growing crystals trap more fluid inclusions, resulting in inclusion rich zones, whereas halite crystals that grow slowly have fewer fluid inclusions.

Primary fluid inclusions in halite are aqueous, negative cubes, rectangular prisms, and irregular shapes, including tubes, from <1 µm to several millimeters in size (Figs. 5.7 and 5.9). Fluid inclusions in halite are normally single phase brines because that is the medium in which they grew, but they may also contain solids and vapor.

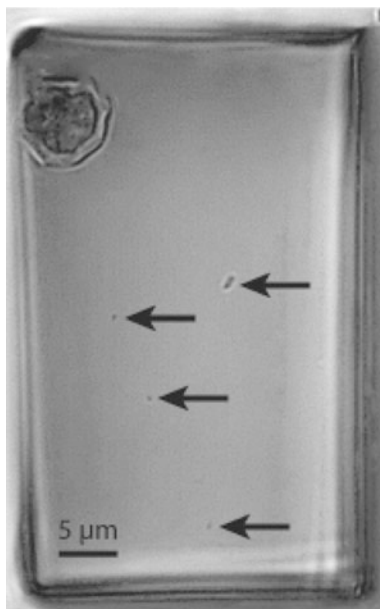

Fig. 5.10 Photomicrograph of probable *Dunaliella* cell and prokaryote cells (*arrows*) in a fluid inclusion from the Death Valley core, depth of 8.7 m (age 12,000 years). (Modified from Schubert et al. (2010b))

Fig. 5.11 Photomicrograph of a portion of a large fluid inclusion in halite, crystallized in Saline Valley, California in 2004, with numerous, small prokaryote cells and larger spherical and ellipsoidal cells of *Dunaliella*. (Modified from Lowenstein et al. (2011))

Minerals, organic materials, and microorganisms, including prokaryotes and algae, have all been observed within fluid inclusions in halite (Schubert et al. 2009a, 2009b, 2010b; Lowenstein et al. 2011) (Figs. 5.9, 5.10, and 5.11). It has been assumed that microorganisms living in the water column are passively trapped inside fluid inclusions during halite crystallization, but experiments documenting the modes and mechanisms by which microorganisms are trapped in fluid inclusions have not yet been done.

Fig. 5.12 Photomicrograph of fluid inclusions in gypsum crystal, Middle Miocene (∼11–16 million years old), Gulf of Suez, Egypt. Solid (S) and fluid inclusions (F, with liquid water and vapor bubbles) occur in planes parallel to the growth direction of the gypsum crystal. Vapor bubbles, not present in original samples, were produced in the laboratory after freezing and melting experiments. Scale bar is 60 μm. (Modified from Attia et al. (1995))

Fluid inclusions in primary gypsum have not been studied as much as in halite but are reported by Sabouraud-Rosset (1969, 1972, 1974, 1976), Attia et al. (1995), and Petrichenko et al. (1997). Primary fluid inclusions in gypsum, as in halite, are normally single phase, aqueous, and arranged in alignment with the growth direction of the gypsum crystal (Figs. 5.12 and 5.13). Primary aqueous inclusions in gypsum are <1 μm to several millimeters in size. They are typically smaller than those found in halite and have a variety of shapes including negative crystals and triangular, pentagonal, or horn-shaped inclusions in two dimensions (Attia et al. 1995). The largest and easiest inclusions to visualize in gypsum occur along crystal growth bands in primary bottom growth crusts, such as those shown in Figs. 5.12 and 5.13. Although detailed studies are lacking, solid minerals, organic matter, and microorganisms (prokaryotes including cyanobacteria and charophytes) have been observed in fluid inclusions in ancient gypsum deposits (Attia et al. 1995; Petrichenko et al. 1997). Secondary fluid inclusions in gypsum are common; they are tabular-shaped, single phase and several tens of microns in size (Fig. 5.13c) (Attia et al. 1995).

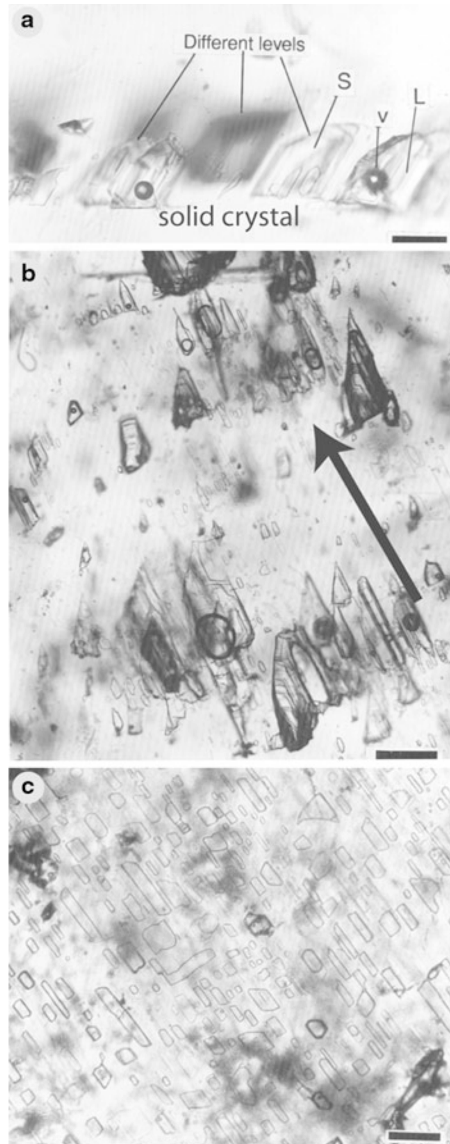

Fig. 5.13 Photomicrographs of fluid inclusions in gypsum crystals, Middle Miocene (~11–16 million years old), Gulf of Suez, Egypt. (Modified from Attia et al. (1995)). **a** Primary fluid inclusions (aqueous, single phase, S, and liquid-vapor, L-V) formed along a common surface. Scale bar is 20 μm. Vapor bubbles, not present in original samples, were produced in the laboratory after freezing and melting experiments. **b** Primary fluid inclusions aligned in rows parallel to the growth direction of the gypsum crystal (*arrow*). Scale bar is 20 μm. **c** Plane of secondary tabular aqueous inclusions along a cleavage plane. Scale bar is 30 μm

Brine Evolution and Secular Variations in the Major Ion Chemistry of Seawater

Chemical species dissolved in seawater or nonmarine waters on Earth include the major ions Na^+, Ca^{2+}, Mg^{2+}, K^+, $SO_4{}^{2-}$, Cl^-, $HCO_3{}^-$, and $CO_3{}^{2-}$, and minor to trace amounts of various other species including Li^+, Sr^{2+}, and Ba^{2+}. The

major ions in natural waters are concentrated during evaporation until the waters become supersaturated with particular minerals. The types of saline minerals found in evaporite deposits are dependent upon the chemical composition of the parent brines, which in turn, depends upon the chemistry of inflow waters, and the mechanisms by which these waters become brines. The salts formed during evaporative concentration of natural waters at the Earth's surface precipitate in order of increasing solubility. Typically, relatively insoluble calcite ($CaCO_3$) crystallizes first, followed by gypsum ($CaSO_4 \cdot 2H_2O$) and then halite (NaCl). The "bittern salts" composed of K and Mg sulfates (for example polyhalite [$K_2SO_4 \cdot 2CaSO_4 \cdot MgSO_4 \cdot 2H_2O$], kieserite [$MgSO_4 \cdot H_2O$], kainite [$KCl \cdot MgSO_4 \cdot 3H_2O$] and chlorides (sylvite [KCl], carnallite [$KCl \cdot MgCl_2 \cdot 6H_2O$]) form last, when waters become superconcentrated. These late stage bittern or potash salts are unusual because evaporative concentration of natural brines to this degree is rare. Other evaporite minerals include anhydrite ($CaSO_4$) which forms from the dehydration of gypsum, Na-sulfates (mirabilite [$Na_2SO_4 \cdot 10H_2O$], thenardite [Na_2SO_4]), Na-carbonates (trona [$NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$], nahcolite [$NaHCO_3$], shortite [$2CaCO_3 \cdot Na_2CO_3$]), and Ca-chlorides (tachyhydrite [$CaCl_2 \cdot 2MgCl_2 \cdot 12H_2O$], antarcticite [$CaCl_2 \cdot 6H_2O$]).

The guiding principle of "chemical divides" is usefully applied to the study of evaporite brines (Hardie and Eugster 1970; Eugster and Hardie 1978; Jones and Deocampo 2004). This concept greatly simplifies understanding the mechanisms by which natural waters evolve during evaporative concentration and mineral precipitation. When brines evaporate, they lose only water and all the dissolved species increase in concentration proportionally. But when minerals precipitate, they form from dissolved species in the brine and thus change the chemistry of the evolving brine. Precipitation of the early, insoluble minerals, such as calcite and gypsum, is important for determining the later brine evolution pathways. For calcite, for example, one mole of Ca^{2+} and one mole of $CO_3{}^{2-}$ are lost from the water for every mole of $CaCO_3$ formed. The equivalents (moles of charge) of Ca^{2+} versus $CO_3{}^{2-}$ + HCO_3^- in the water at calcite saturation determine whether Ca^{2+} or $CO_3{}^{2-} + HCO_3^-$ is depleted in the remaining water during precipitation of calcite. If the water has $Ca^{2+} > CO_3{}^{2-} + HCO_3{}^-$, for example, seawater, it becomes depleted in $CO_3{}^{2-}$ + $HCO_3{}^-$ and enriched in Ca^{2+}, following precipitation of alkaline earth carbonate. If $Ca^{2+} < CO_3{}^{2-} + HCO_3{}^-$, then the evolving water will become Ca-depleted and alkaline, enriched in $CO_3{}^{2-} + HCO_3{}^-$, such as Mono Lake, California and Lake Bogoria, Kenya, following carbonate mineral precipitation. In the same way, the equivalents of Ca^{2+} and $SO_4{}^{2-}$ in the evaporating water at gypsum saturation determines whether the remaining brine will be enriched or depleted in Ca^{2+} and $SO_4{}^{2-}$ after gypsum precipitates. Seawater and Great Salt Lake waters have $SO_4{}^{2-} > Ca^{2+}$, so they become sulfate-rich, Ca^{2+}-poor brines following gypsum formation, whereas the Dead Sea, with $Ca^{2+} > SO_4{}^{2-}$, becomes depleted in $SO_4{}^{2-}$ after gypsum forms.

The variety of natural waters at the Earth's surface can lead to the formation of many types of brines, but the principle of chemical divides permits easy classification into distinctive groups. Inflow waters with $Ca^{2+} < CO_3{}^{2-} + HCO_3{}^-$ precipitate alkaline earth carbonate and evolve into alkaline Na-K-HCO_3-CO_3-SO_4-Cl rich brines from which trona, halite, mirabilite and thenardite may precipitate. Such brines are

found in Mono Lake and Owens Lake California, and Lakes Magadi and Bogoria, Kenya, although sulfate is lost from some of these brines via sulfate reduction. If the inflow waters have $Ca^{2+} > CO_3^{2-} + HCO_3^-$, then Ca^{2+}-rich, carbonate-poor brines form after carbonate mineral precipitation. The resulting brines are Ca-Na-K-Mg-SO_4-Cl-rich. Then, depending on the amount of Ca^{2+} versus SO_4^{2-} in the brine at the point of gypsum precipitation, Ca-Na-K-Mg-Cl-rich brines (Dead Sea, Bristol Dry Lake, California, Qaidam Basin, China) or Na-K-Mg-SO_4-Cl-rich brines (seawater, Great Salt Lake, Death Valley) form.

Seawater, of course, is the most abundant evaporite parent water on Earth and giant marine evaporite deposits are common in the geologic record. As noted previously, it is now known from study of fluid inclusions in halite that the major ion chemistry of seawater has varied over the Phanerozoic Eon (Lowenstein et al. 2001; Horita et al. 2002), in phase with changes in sea floor spreading rates, global volcanism and global sea level. Seawater had high Mg^{2+}/Ca^{2+} and relatively high SO_4^{2-} during the Permian (299–251 Ma), Triassic (251–199.6 Ma) and much of the Cenozoic Era, from 0 to 40 million years ago. In contrast, seawater had low Mg^{2+}/Ca^{2+} ratios and relatively high Ca^{2+} and low SO_4^{2-} concentrations during the Cambrian (542–488 Ma), Silurian (444–416 Ma), Devonian (416–359 Ma), Jurassic (199.6–145.5 Ma) and Cretaceous (145.5–65.5 Ma) periods. Seawater has always had $Ca^{2+} > HCO_3^- + CO_3^{2-}$, except perhaps during the earliest history of Earth, but changes in the amount of Ca^{2+} versus SO_4^{2-} have had a major impact on brine evolution and the formation of marine evaporites. During those times when $Ca^{2+} > SO_4^{2-}$ at the point of gypsum saturation (Cambrian, Silurian, Devonian, Jurassic and Cretaceous), seawater evolved into a Ca^{2+}-rich, SO_4^{2-}-poor brine during evaporative concentration. Marine evaporites from these periods lack $MgSO_4$ salts and contain late stage K-, Mg-, and Ca-chloride salts such as sylvite, carnallite, and tachyhydrite (Lowenstein et al. 2001). When $Ca^{2+} < SO_4^{2-}$ at the point of gypsum saturation, seawater evolved into a SO_4^{2-}-rich brine, as occurred during the Permian, Triassic, and much of the Cenozoic Era. Evaporite deposits of those ages contain $MgSO_4$ salts such as polyhalite, kainite, and kieserite. Such changes in seawater chemistry, now well documented, have had a major impact on the evolution of shell building organisms (Stanley and Hardie 1998), but little is known about the impact of secular variations in seawater chemistry on halotolerant and halophilic marine microbial communities. The detailed changes in seawater chemistry of different ages can be found in Lowenstein et al. (2001, 2005), Horita et al. (2002), Brennan et al. (2004), Satterfield et al. (2005a, b), and Timofeeff et al. (2006), which may be a useful guide for media preparation when attempting to culture halophilic microorganisms from marine evaporites.

Knowledge that Permian seawater differed chemically from modern seawater, with respect to Mg^{2+} and SO_4^{2-}, for example, helped demonstrate the Permian, 250 million-year-old age of the fluid inclusions from which Vreeland et al. (2000) cultured the bacterium *Virgibacillus* sp. 2-9-3 (Satterfield et al. 2005a). In that study, fluid inclusions in halite cement crystals from the Permian Salado salts, Waste Isolation Pilot Plant in New Mexico, were chemically analyzed for Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, and Cl^-. It was found that the Permian fluid inclusions have lower

SO_4^{2-} concentrations than modern seawater, but very similar concentrations to fluid inclusions in other Permian halites (Satterfield et al. 2005a), which suggests a Permian age of the fluid inclusion waters. In addition, the Salado fluid inclusions are different in chemical composition from modern potash mine brines and mine weeps in the Salado salts, which demonstrates that the halite cement crystals that housed the bacterium did not precipitate from modern brines in the Salado salts released by fracturing and deformation associated with mining operations. Fluid inclusions have thus helped show that evaporite crystals have retained brines for periods of hundreds of millions of years.

Fluid Inclusion Microthermetry: Paleobrine Temperatures

Fluid inclusions in halite can be used to establish the temperatures of the waters in which the crystals grew. The method, called microthermometry, uses the homogenization temperature of fluid inclusions to infer ancient brine temperatures. Primary single-phase aqueous inclusions in halite at room temperature are required as the starting material. Halite crystals with these inclusions are then cooled in a laboratory freezer or on a fluid inclusion heating-freezing stage in order to nucleate a vapor bubble. The vapor bubble (water vapor at very low pressure) forms because of the volume decrease of the inclusion water that occurs during cooling, which is much greater than the volume change of the solid halite host crystal. Once vapor bubbles are nucleated in fluid inclusions, halite crystals are transferred to a heating-freezing stage mounted to a transmitted light microscope. Crystals and incorporated fluid inclusions are then slowly heated while being observed under the microscope. With warming, the volume of the water in inclusions increases and the vapor bubbles shrink. At some point, called the homogenization temperature, the vapor bubble disappears completely. The homogenization temperature, if from a primary fluid inclusion, is a record of the water temperature at which the crystal originally grew. This information, actual measurements of the water temperatures at which crystals grew and fluid inclusions were trapped, has been used for paleoclimate studies because there is a direct relationship between water temperatures, air temperatures and climate (Roberts and Spencer 1995; Lowenstein et al. 1998, 1999; Benison and Goldstein 1999; Satterfield et al. 2005a, b). Homogenization temperatures can also guide the design of conditions used for culturing ancient microorganisms trapped inside halite.

The Importance of Microscopy

Geomicrobiological studies of ancient evaporites (halite and gypsum) have seen important advances in the last 10 years using *in situ* light microscopy (Mormile et al. 2003; Fendrihan and Stan-Lotter 2004; Adamski et al. 2006; Fendrihan et al.

2006; Benison et al. 2008; Panieri et al. 2008; Schubert et al. 2009a, b, 2010a, b; Lowenstein et al. 2011). A report by Griffith et al. (2008) used transmission electron microscopy to identify cellulose fibers that were obtained from fluid inclusions and solid crystals of the Permian Salado halite of New Mexico.

In situ microscopy is particularly important because the identification of microorganisms within fluid inclusions confirms their authenticity and provides strong evidence that they are the same age as the crystals in which they are found. Microscopic studies are also important for determining the mode of preservation of microbes and understanding their populations. Such studies have recently revealed complex microbial communities in fluid inclusions in modern and ancient halite, including prokaryotes (some alive), eukaryotes (the alga *Dunaliella* and other single celled species), organic material of unknown origin, and inorganic crystals (Figs. 5.9, 5.10 and 5.11) (Schubert et al. 2010b; Lowenstein et al. 2011). Identification of such fluid inclusion ecosystems has led to hypotheses for long-term survival of halophilic *Archaea* via starvation survival and prokaryote miniaturization, as well as possible nutrient sources including glycerol (Schubert et al. 2009a, b; 2010b; Lowenstein et al. 2011).

Transmitted and epifluorescence microscopy, using a 100X oil immersion objective, and environmental scanning electron microscopy (environmental SEM), were combined to assess microbial populations in subsurface halite from Death Valley (Schubert et al. 2009a, b; 2010a, b; Lowenstein et al. 2011). *In situ* microscopy was used to document prokaryotes, eukaryotes, and associated biomolecules within fluid inclusions. Examination of nearly 7,000 fluid inclusions from Death Valley halite showed that microorganisms occur almost exclusively in halites deposited in perennial hypersaline lakes that existed 10,000–35,000 years ago, which shows that trapping and preservation of prokaryotes in fluid inclusions in halite is influenced by the surface environment in which the halite originally precipitated. Some of these halites have prokaryotes in fluid inclusions comparable in abundance to those found in modern hypersaline systems (2×10^7 microbes/ml). The same fluid inclusions contained cells of the alga *Dunaliella*, some green or orange in color, and with a cup-shaped chloroplast, which suggests preservation of intact pigments, such as chlorophyll and carotenoids (Schubert et al. 2009b; 2010b; Lowenstein et al. 2011). In contrast, prokaryotes found in Death Valley halites (>10,000 years old) appear quite different from those trapped in fluid inclusions in modern halite. Ancient prokaryotes are coccoid-shaped and miniaturized, with cell diameters <1 μm, much smaller than the rod (1–10 μm long, ~0.5–1 μm wide) and coccoid-shaped prokaryotes (typically ~1 μm diameter) typical of modern surface brines. The differences in size and shape between modern and ancient prokaryotes trapped in fluid inclusions resemble the starvation-survival forms reported for prokaryotes living in soils and in the ocean (Novitsky and Morita 1976; Morita 1982, 1997; Grant et al. 1998). It is well known that some prokaryotes living under nutrient-poor conditions adjust by reducing their size and changing shape by rounding from rod to coccoid (Kjelleberg et al. 1983). Similarly, it appears that once trapped inside fluid inclusions, prokaryotes resort to starvation-survival strategies, but the timing and triggering mechanisms are not known.

Raman spectroscopy is ideal for the study of biomolecules and other species generated through biological processes (such as CH_4 and CO_2) in fluid inclusions because it is an *in situ*, non-destructive technique capable of characterizing solid and liquid materials within cells or free in fluid inclusions (Wopenka and Pasteris 1993; Burruss 2003). Using a laser-excitation source focused through an optical microscope and into a fluid inclusion, spatial resolution on the micron scale is possible (Burruss 2003). Most covalently-bonded solids, liquids and dissolved species may be identified on the basis of Raman peak positions: peak intensities (or areas) provide information on relative concentrations of species in the analytical volume. Until recently, analysis of many organic and biological materials with Raman was limited owing to the strong fluorescence induced by some visible-wavelength laser excitation. However, in recent years the application of near-infrared and UV lasers has shown considerable promise for analyzing a wide range of biological samples (Petry et al. 2003), and there is now a large database of Raman spectra of biological molecules, including nucleic acids, amino acids, metabolites, and others such as β-carotene and chlorophyll DeGelder et al. 2007) available to interpret the Raman spectra. *In vivo* measurements of individual live cells of the alga *Dunaliella* yielded strong spectra for chlorophyll *a* and β-carotene (Heraud et al. 2007), and Fendrihan et al. (2009) identified C_{50} carotenoid compounds from single cells of halophilic *Archaea* in fluid inclusions in laboratory-grown halite. Organic compounds such as glycerol, that are soluble in water, also produce Raman spectra with characteristic peaks (Mudalige and Pemberton 2007), as do dissolved covalently bonded gases such as CO_2 and CH_4 (Burruss 2003).

Microbiological Considerations

Cultivation experiments and efforts to extract DNA have used three techniques: (1) dissolution of surface sterilized crystals, (2) grinding surface sterilized gypsum crystals to powder, and (3) microdrilling into crystals and extracting individual inclusion fluids with a syringe. The preferred technique depends upon the particular samples and minerals involved and the goal of the experiments.

Successful revival of prokaryotes trapped within ancient crystals of halite using cultivation techniques is reported in nine publications since 1999 (Stan-Lotter et al. 1999, 2002; Vreeland et al. 2000, 2007; Mormile et al. 2003; Gruber et al. 2004; Schubert et al. 2009b, 2010a; Gramain et al. 2011). Halites used for culturing ancient prokaryotes range from hand samples, obtained from underground mines and borehole cores, hundreds of grams in weight (Stan-Lotter et al. 1999, 2002), to individual crystals (Vreeland et al. 2007; Schubert et al. 2009b, 2010a; Gramain et al. 2011), to single fluid inclusions within a crystal (Vreeland et al 2000; Mormile et al. 2003). Many of these studies screened samples to target primary halite crystals with primary fluid inclusions (Mormile et al. 2003; Vreeland et al. 2007; Schubert et al. 2009b, 2010a; Gramain et al. 2011). Work was performed in clean laboratory conditions, under a laminar flow hood, using sterilized equipment. The most rigorous treatments

to decontaminate crystal surfaces involve immersion of individual halite crystals in concentrated sodium hydroxide (NaOH) and hydrochloric acid (HCl) (Rosenzweig et al. 2000; Vreeland et al. 2000, 2007; Schubert et al. 2009b, 2010a). Once crystals were surface sterilized, they were dissolved in growth media containing high salt concentrations and a carbon source such as yeast extract, casein-derived amino acids, pyruvate, or glycerol. The two studies that targeted individual fluid inclusions in halite used a microdrill to breach the inclusion cavity (Vreeland et al. 2000; Mormile et al. 2003). The inclusion brine was then removed with a micropipette and inoculated into growth medium. Contamination by younger organisms is an important concern in any study claiming to revive ancient prokaryotes and therefore reports of ancient microorganisms in halite should be viewed as controversial.

All prokaryotes cultured from ancient halite are halophilic *Archaea*, with the exception of the halotolerant bacterium *Virgibacillus* sp. 2-9-3 reported from the Permian Salado salts of New Mexico (Vreeland et al. 2000). A number of haloarchaea have been cultured from Permian-Triassic (200–300 million-year-old) halites in England, Germany, and Austria. One of these, *Halococcus salifodinae*, isolated from geographically separated areas, was interpreted by Stan-Lotter et al. (1999) as the trapped microbial remains of marine brines that once covered western Europe. The genus *Halobacterium* is the most widely cultured ancient halophilic archaea (Mormile et al. 2003; Gruber et al. 2004; Vreeland et al. 2007; Gramain et al. 2011). Schubert et al. (2009b, 2010a) cultured halophilic *Archaea* from 5 halite crystals (22,000 to 34,000 years old) out of 881 tested from the Death Valley core, showing the rarity of microbial survival in fluid inclusions. The five halophilic *Archaea* are from the genera *Haloterrigena*, *Natronomonas,* and *Halorubrum*. Supporting evidence showing that these halophilic *Archaea* were not contaminants included: (1) well-preserved primary halite and fluid inclusions (Fig. 5.7) sampled only from interior sections of the Death Valley core, (2) *in situ* microscopic confirmation that prokaryotes existed in fluid inclusions in all halite crystals that yielded growth (Fig. 5.10), (3) intra-laboratory reproducibility, in which repeated growth of related taxa of halophilic *Archaea* (*Haloterrigena*) was achieved for one interval, and, (4) inter-laboratory reproducibility, in which two halophilic *Archaea* (DV462A and *Natronomonas* sp 2-24-1) with 99.3 % similarity of DNA from the 16S rRNA gene, were cultured at separate laboratories from different halite crystals of the same cored interval (Schubert et al. 2010a). Schubert et al. (2009b, 2010b) hypothesized that glycerol and other metabolites leaked out of *Dunaliella* cells supplied heterotrophic prokaryotes trapped in fluid inclusions with the carbon and energy sources required for their prolonged survival. Support for this hypothesis comes from the fact that all five halophilic *Archaea* cultured from fluid inclusions in Death Valley halite were isolated in media containing glycerol as a carbon source.

Ancient DNA from halite and gypsum has been extracted, purified, amplified, and sequenced (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011). Halite samples from underground mines and borehole cores from the Permian-Triassic of Germany and Austria were found to contain haloarchaeal DNA similar to *Halobacterium, Halorubrum, Haloferax, and Halogeometricum* (Radax et al. 2001). Haloarchaeal and bacterial DNA fragments were

recovered by Fish et al. (2002) from primary crystals in halite deposits between 11 and 425 million years old from Poland, Thailand and the United States. These studies, like all others, amplified the 16S rRNA gene, followed by cloning and sequencing. Park et al. (2009) similarly sequenced haloarchaeal DNA related to the modern genera *Haloarcula*, *Halorubrum and Halobacterium*, from halites 23, 121 and ~419 million years old. Panieri et al. (2010) extracted and amplified the oldest known cyanobacterial DNA from gypsum crystals of the late Miocene (Messinian, 5.8–5.9 million years old) from the northern Apennines, Italy. Those samples are unusual because they contained microbial filaments trapped within the solid portions of primary gypsum crystals. Sampling for DNA in that case was accomplished by surface flaming using ethanol, followed by grinding of the gypsum into a powder, from which DNA was extracted (Panieri et al. 2010). Finally, Gramain et al. (2011) detected DNA from the genus *Halobacterium* from primary fluid inclusions in halite cements from the Pliocene (>1.8 million years old) subsurface halite of the Salar Grande, northern Chile. It should be noted that recent testing of surface sterilization protocols by Gramain et al. (2011) and Sankaranarayanan et al. (2011) has shown that many of the methods used in previous cultivation studies are not fully effective in destroying DNA attached to halite crystal surfaces. These studies both indicate the need for longer soak times, 20 min in each of bleach (6 % sodium hypochlorite), NaOH, HCl and ethanol (Gramain et al. 2011), or 15 min in each of NaOH, HCl, bleach or HCl and bleach (Sankaranarayanan et al. 2011). Whatever the method used to study ancient microorganisms and DNA, Gramain et al. (2011) and Sankaranarayanan et al. (2011) have shown that surface sterilization using a combination of concentrated HCl and bleach are required to completely remove potentially contaminating surface-bound DNA.

Conclusions

A community of microorganisms (bacteria, archaea, algae) has been found in ancient fluid inclusions in halite and gypsum. Syndepositional evaporites, that formed at or soon after the time of deposition, should be the focus for future geomicrobiological studies because they are expected to be the richest source of microorganisms and biomaterials. But within the class of syndepositional evaporites, there are only a small number of deposits formed in particular environments, such as perennial saline lakes and lagoons, that have been found to contain appreciable numbers of microorganisms (Panieri et al. 2008; Schubert et al. 2009a, b, 2010b). Other types of syndepositional evaporites, such as those formed in desiccated saline pans, contain little biomass and are thus less useful for geomicrobiological studies.

More work combining sedimentology, microscopy, geochemistry, and microbiology is needed to understand fluid inclusion ecosystems that are millions of years old. This includes more complete documentation of the suite of microorganisms that existed at the time of inclusion formation, regardless of whether they are viable. Biomaterials (DNA, chlorophyll, cellulose, carotenoids) and inorganic materials (major

elements, nutrients) associated with microorganisms in fluid inclusions also merit further study because they may hold the key for understanding the mechanisms by which prokaryotes survive for long periods inside fluid inclusions. Such knowledge is vital as studies further explore the evolution of microbial communities over geological time and the preservation of life within Earth's crust and elsewhere in the solar system where materials that potentially harbor microorganisms are millions and even billions of years old.

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Chapter 6
Searching for Microbes and DNA in Ancient Halite

Russell H. Vreeland

Introduction

This chapter discusses a variety of issues related to research on isolating microbes and DNA from ancient salts. Strictly speaking, this type of science can be accomplished on virtually any type of geological material, including Amber, shale, dinosaur fossils or ice. It is being discussed here largely because the work has been most fully developed using salt crystals and their unique properties. However one must recognize that nearly everything being discussed in this chapter relates to the overall defensibility of the ultimate results, ergo a claim that an isolate (live culture or DNA) is as old as the rock from which it has been extracted. David Hume (1711–1767) is credited with coining the phrase "A wise man, therefore, proportions his belief to the evidence." This was later brought to a more modern understanding by Marcello Truzzi as "An extraordinary claim requires extraordinary proof" (Truzzi 1978). Certainly such an admonition should be considered by any and all scientists attempting to carry out and publish this kind of research. This chapter outlines the various types of evidence that should be considered in order to make claims that any isolate, or DNA sequence is as old as the crystal (or sample) in which it was found. Adherence to these ideas will never make claims of ancient life immediately accepted without question or debate. However, experience has shown that having these various bits of information on hand will at least make the debate more likely to have a positive outcome.

Historical Perspective

Every scientist knows about the typical introduction section of journal articles. In that important part of the manuscript the authors generally trace the background of their topic. Usually it consists of a series of declarative sentences that set the stage for the research to be discussed in the following material. This introductory portion

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focuses on the data collected or the conclusions drawn by previous researchers. But before I launch into a purposeful discussion of looking for microbes in ancient salts it might be appropriate to consider a different use for those old historical papers used in introductions. Once in a while we should look at them not for the data or conclusion, rather we should use them to understand the mistakes and problems that were missed or that led to a community wide (taken here as the scientific community) rejection of the conclusions as being erroneous. Taking this historical perspective helps a scientist moving into a new field or contemplating a major new experiment to avoid those embarrassing moments when someone asks a question that the scientist had not considered or worse yet had not controlled. So before I get into the specifics of this topic it might be informative to provide some specific examples of how this worked for the isolation of microbes from truly ancient materials.

Throughout the twentieth century there were sporadic reports of microorganisms being isolated from numerous ancient sources. The first such report came from Lipman (1931, 1937) who claimed to have isolated microbes from coal. Another set of reports occurred in the early 1960s when two different groups, Reiser and Tasch (1960), Tasch (1963) and Dombrowski (1963, 1966) reported isolating microbes from ancient sodium chloride. After another 30 years elapsed several more reports of isolations surfaced, starting with Stan-Lotter et al. (1999, 2000, 2001), Norton and Grant (1988), Norton et al. (1993), Grant et al. (1998) then culminating in the work of Rosenzweig et al. (2000) and Vreeland et al. (2000). There is, of course, a great deal of research that has occurred since that date but all of it has built and improved upon these previous reports. Consequently, this portion of the chapter, pointing out various problems faced by these earliest researchers will be restricted to those up to and including that of Vreeland et al. (2000). This is not to say that the field has not developed or that successive work is somehow perfect, it is not. Simply put, the problems now being encountered are beyond the scope of this chapter or really are fodder for future developments.

Early Samples

This was likely one of the two most serious problems for all of the early research involving finding ancient microbes inside geological materials. The first real attempts in this area utilized samples of Anthracite coal. Certainly coal represents an old material. However, due to its very nature of formation and to the regions where it is mined it is difficult to impossible to document a particular sample as being primary or untouched by more recent waters and gases. For instance, virtually all coal mines have water entering the mine. Some of this water is trapped within the seams and is consequently old. However, most of the water moves through the formation rock along interfaces between the small coal seam and the surrounding rock. Generally, this water is relatively old but is still far younger than the coal itself. Further, this report provided no provenance for the sample only that it was provided by a friend. Similar problems occurred during the work of Dombrowski (1963, 1966)

and Reiser and Tasch (1960). Neither of these articles described the locations from which samples were taken or any description of the samples themselves. Predictably this was the first area that critics attacked leaving the researchers with little to defend. The later research by Norton and Grant (1988), Norton et al. (1993), and Stan-Lotter et al. (1999, 2000, 2001) did not suffer the same fate since both groups clearly identified their salt sources but both groups utilized large composite salt rocks made of many crystals with lots of interstitial spaces. Consequently, neither group could assert the age of the organisms stating only that the organisms isolated could represent "remnants" of ancient populations. This claim was however difficult to substantiate since the isolation of the specific geologic formation from which the samples arose was not fully verified by the authors. Vreeland et al. (2000) did attempt to learn from these mistakes by carefully selecting and describing the salt formation and by using only a single (interpreted as primary) salt crystal. These authors however created their own problems when geological experts recognized the crystal used as being a cement crystal, that could have formed long after burial and which therefore was not as old as believed. This error meant that it would require several more years of research to establish that this crystal was primary (Hazen and Roedder 2001; Powers et al. 2001; Satterfield et al. 2005).

Sterilization

All microbiologists realize that sterility is a probability and not an absolute. Unfortunately this was not admitted early on. Some of the earliest researchers (Lipman 1937; Tasch 1960; Reiser and Tasch 1960; Dombrowski 1963, 1966) barely acknowledged the procedures they used and assumed that things like ethanol flames were "beyond question." In fact one actually made that specific claim. When made to microbiologists such a statement was tantamount to throwing red meat to the lions. Later workers did improve on that situation by increasing the length of exposure to sterilant and to incorporating control cultures but still did not consider providing the actual sterilization probabilities. At this point it might also be informative to digress a bit and mention one of the best early studies on ancient microbes, that of Cano and Borucki (1995) who described the isolation of a live microbe from 65 million year old Amber. These authors developed an excellent sterilization process for their Amber. This involved soaking the samples in sterilant (Bleach and formaldehyde) and checking each piece for contamination by suspending it in enrichment media before sampling. However, this process became controversial when it was revealed that contaminated Amber (identified by growth in the enrichment) was then run through the sterilizing process over and over until no growth appeared. While this would seem to indicate successful sterilization the problem here was two-fold. First there was no indication of how often this occurred with the samples (a measure of sterility assurance). Second and perhaps worse the high numbers of cells developed in the enrichment check meant that a very contaminated sample was then re-sterilized. There was also a problem with proving that these actively growing cultures could not

somehow reach protected cracks and fissures in the sample and ultimately contaminate the later experiment. Basically any contaminated samples should be thrown out of the experiment. The mistake made by all of the early researchers was the same, no mention of the sterilization effectiveness (or contamination probability) for the samples used.

Physical Laboratory

When contemplating conducting research on isolating ancient microbes (or DNA) from geologically aged materials of any type, the physical laboratory needs to be considered as described below. A careful reading of the earliest papers shows that none of these researchers worried about the physical separation between the ancient sampling work and their other projects. Consequently, criticisms about contamination from modern microbes could not be refuted whenever the isolates were shown to be similar to putatively modern organisms. While one might argue that expecting vast differences between ancient microbes and modern microorganisms reflects an incorrect bias (Vreeland and Rosenzweig 2002), it is still a serious issue since there is no way to prove either position. In reality, the first researchers who considered this important separation were Cano and Borucki (1995) who went to great pains to describe their physical lab. They also were able to describe extensive cleaning and sterilization protocols. This work has actually formed the basis of most of the best evidence for the existence of ancient microbes and DNA.

To reiterate the main point of this section, when examining the historical aspects of a field, it is sometimes more helpful to closely examine how experiments were performed and the mistakes made during the early research. This can be as informative (even more informative) than the earlier data or conclusions.

Sampling Parameters

As described within the preceding historical material, early geological microbiology showed relatively little concern for the quality of rock sample being examined and often even less for the formation from which the sample arose. If one is simply looking at a bulk underground or even an individual rock sample for the presence of microorganisms or DNA this is probably okay. If however, the research being conducted is aimed at paleontological, evolutionary or long term survival questions a large number of parameters must be considered before using any particular sample. This section is meant to provide information on these parameters as they relate to site selection, laboratory configuration or set-up, basic sampling techniques, sterilization and information about how these criteria can be assembled to make such claims defensible.

Criteria for Site Selection

A number of separate criteria must be taken into account when selecting a formation for such studies (Vreeland and Powers 1998; Vreeland and Rosenzweig 1998, 2002). Many might feel that the age of a formation is one of the most important, that however is not true and may actually be of lesser importance than those that follow. Certainly age may have a high degree of interest but more important is really how those ages were determined and if different dating methods agree within experimental limits. Among the most important are the overall stability of the formation, the ability to identify primary (only formed once) individual subsamples and the degree to which the formation is isolated from recent water movements. For microbiologists this may be a difficult task in that general training does not provide the type of background knowledge necessary to make such decisions alone. Consequently, the first recommendation is to ally oneself (especially in the early going) with a geologist knowledgeable in sedimentary and especially crystalline rock systems. Each of these aspects is described in more detail below.

Age and Stability of Formations

There is no doubt that older formations and minerals are inherently more interesting to both the public and the professional. This might simply be because they offer a greater degree of the unknown. At the same time the older the formation the less is really understood about the environmental systems existing at that point in time so they may offer a more intellectually satisfying challenge. However researchers must recognize that simply jumping huge time gaps will invariably create greater controversy over contamination and disbelief. Also the older the formation the fewer undisturbed truly primary materials are available and the less likely the possibility for actually getting positive results. Therefore as one works with older and older materials one must devote increasing time and resources to the research. Consequently, for this type of science there is great value in having both types of research with some working backward in time in relatively small steps while others attempt longer leaps. Finally, one must remember that the older the formation the less secure the dating and the larger the standard error around the dates. For materials deposited near the end of an epoch or the beginning of another, this could create serious problems with interpretation.

While a full description of the techniques used to date formations is well beyond the scope of this chapter a brief account of the different methods used and how they apply may be beneficial.

One of the primary mechanisms for dating involves geological mapping of the formation itself as well as examination of the sedimentary layers above and below the target area. This is particularly important on several basic fronts. First in some cases, even the formations that look best on a gross level will contain areas where re-crystallization has occurred (Fig. 6.1). This can occur quite naturally during the repeated flooding and evaporation events that generally happen over millions of years

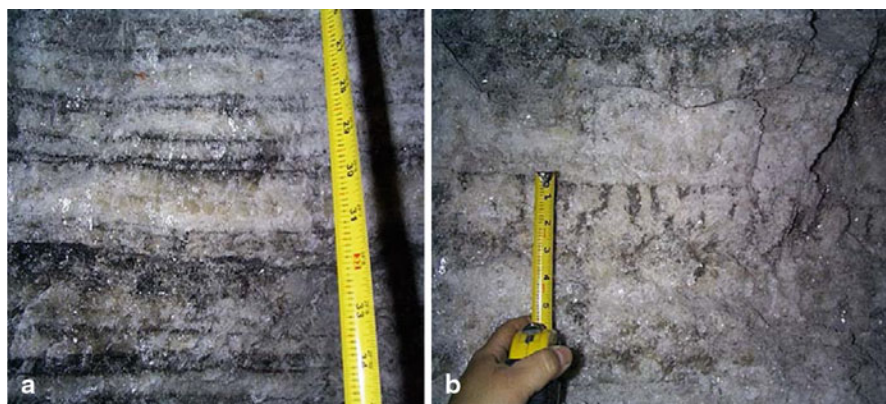

Fig. 6.1 Layering within a well preserved salt formation. Image **a** shows a series of salt layers (white) interspersed with layers of mud and sediment from periods without overlying brine. Image **b** (*right*) shows a salt layer that was overlain with fresh water which penetrated the underlying salt a short distance and in doing so carried mud into the pit. Both are examples of primary undisturbed formations. Note also the horizontal nature of the layers indicating that very little disruptive movement has occurred since this system was deposited 270 Ma

and that ultimately build the formation. In these instances non-saturated waters reach underlying layers through openings in the overlying sediment and dissolve the salt. The more water (or the lower the original salt content of the inflow) the larger this dissolution pit becomes. The salty brine then evaporates slowly forming large slow growth crystals. This is generally a natural process and does not necessarily mean that the salt rock is different in age from the surrounding primary material. A second mechanism for forming these pits and pipes (Holt and Powers 1990) is much more problematic since it occurs when water flowing through overlying rock gains entry to the buried salt formation and dissolves the salt which then crystallizes as slow growth crystals that are indistinguishable from those formed in the first situation. Examination of the surrounding layers can however, differentiate these situations since in the first instance the pit or pipe is truncated within the salt formation and does not contain any material that is contemporaneous with the overlying materials. Stated more simply the overlying layers have not penetrated into the underlying salt. Also a well documented examination of the overlying layers can determine if the layer is or ever was water bearing and might penetrate the salt. Finally, by carefully examining and counting the overlying layers it is possible to obtain a basic date for the formation based upon sedimentary rates and dating of similar layers in other parts of the Earth. Examining the underlying layers further constrains the age of the target formation (basically as a range) while at the same time providing assurance that layers have not been overturned, that geologic forces have not caused older areas to slide up and over younger ones (Bobst et al. 2001; Chernikoff 1999; Lowenstein et al. 1999).

A second dating method is relatively simple that being fossil evidence (Vreeland and Powers 1998). Modern day hypersaline environments collect the carcasses of hapless birds and other animals that die of dehydration after landing in or attempting

to drink these brines. These carcasses then become fully brined. Yet one curious aspect that many researchers note is the fact that these large underground salt areas are themselves devoid of larger fossils. That is not true of the surrounding rock or sedimentary layers. Consequently placing additional age limits on the formation occurs simply through identifying fossils (both macroscopic and microscopic) around the target formation. This can sometimes be accomplished during the geological examination described in the preceding paragraph but more often than not requires a separate dedicated examination.

The third way the age of a formation might be determined is with radiometric analyses. Unlike the first two methods the actual system used depends upon the suspected age of the formation being examined so no specific procedure will be described here. Basically, radiometric techniques can be used for any age less than a hundred years (Carbon-14, or Calcium-41) to several billions (potassium/argon). In addition each measurement system requires a different piece of expensive equipment and expertise.

Regardless, Vreeland and Powers (1998) recommended using formations whose ages have been well established with at least two of the three different methods being in agreement before selecting a particular formation for study.

Laboratory Configuration

There is certainly no exact or single configuration for laboratories that are being set-up to perform research involving studies of ancient microbes or what might be called "microbial/molecular paleontology" (Fig. 6.2). There are however some generalized criteria that any laboratory conducting this type of research should meet. Possibly the most important criterion is the availability of a "clean room" area whose primary design is to minimize the possibility of contaminating the sample with other "modern" materials. Simply put this means the design protects the sample from the workers. This is not necessarily a Biological Safety Laboratory (BSL) whose primary design protects the worker from the sample being studied. However a properly renovated BSL facility (preferably of US Biosafety Level 3 design) can suffice as long as the room is not also being actively used for work with modern microbial cultures or animals. Assuming a clean room type facility is available for the research the next important aspect is to create a unidirectional sample flow (Fig. 6.3). Basically that means all samples move in a single direction from the "dirtiest" laboratory area toward the clean room then into the analytical zones and never back. Ideally these various areas should be physically separated from each other which certainly aids in a researchers ability to defend conclusions or discoveries of very old materials which are guaranteed to be controversial (Fig. 6.3b). If physical separation of laboratory functions is not possible, one can still carry out such work very effectively by subdividing larger laboratories creating clean spaces and effective "one use areas" (Fig. 6.3a). This set-up was used quite successfully by Cano and Borucki (1995) when they isolated a living microbe from 25–40 Ma amber, earning Dr. Cano a prime position on the cover of Time magazine.

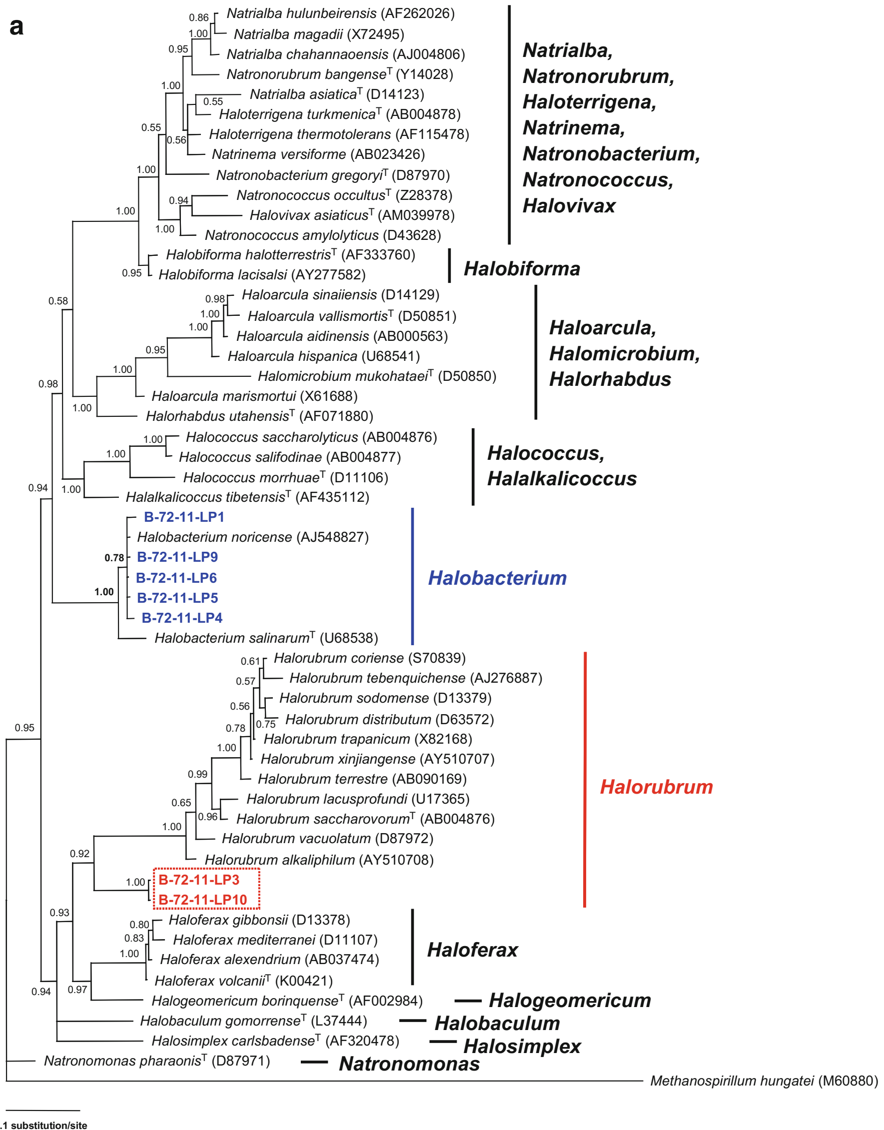

Fig. 6.2 **a** 16S rRNA gene tree showing the phylogenetic position of haloarchaea from 121 million years old (MYA) sample based on 841 unambiguously aligned sites. Seven sequences (B-72-11-LP1, 3, 4, 5, 6, 9, and 10) studied here are amplified with archaeal-specific primers. ^{T}: type strain in Haloarchaea. **b** Phylogenetic tree inferred from comparisons of haloarchaeal 16S rRNA gene sequences. Ancient sequences in the phylgenetic tree were amplified with haloarchaeal-specific primers. A total of 179 unambiguously aligned sites is retained for phylogenetic analysis. B-72-7: 8 MYA sample, B-72-11: 121 MYA sample, B-72-9: 412 MYA sample. Dotted box: the phylotypes have a previously unidentified intron in their 16S rRNA genes. Note: The bootstrap values from maximum likelihood (ML; 200 replicates) and Bayesian posterior probability (MB) are shown at the nodes and are presented in the order ML/MB

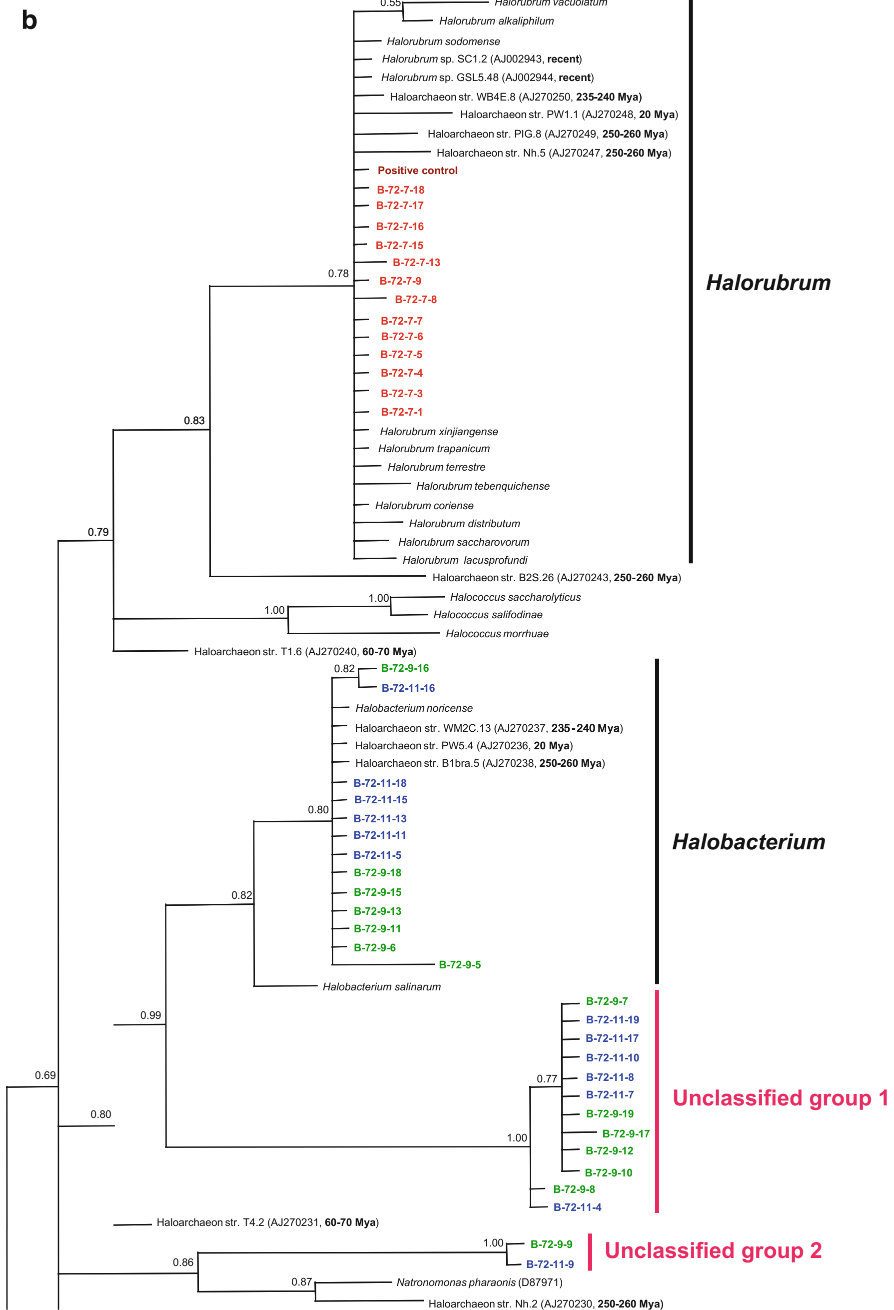

Fig. 6.2 (continued)

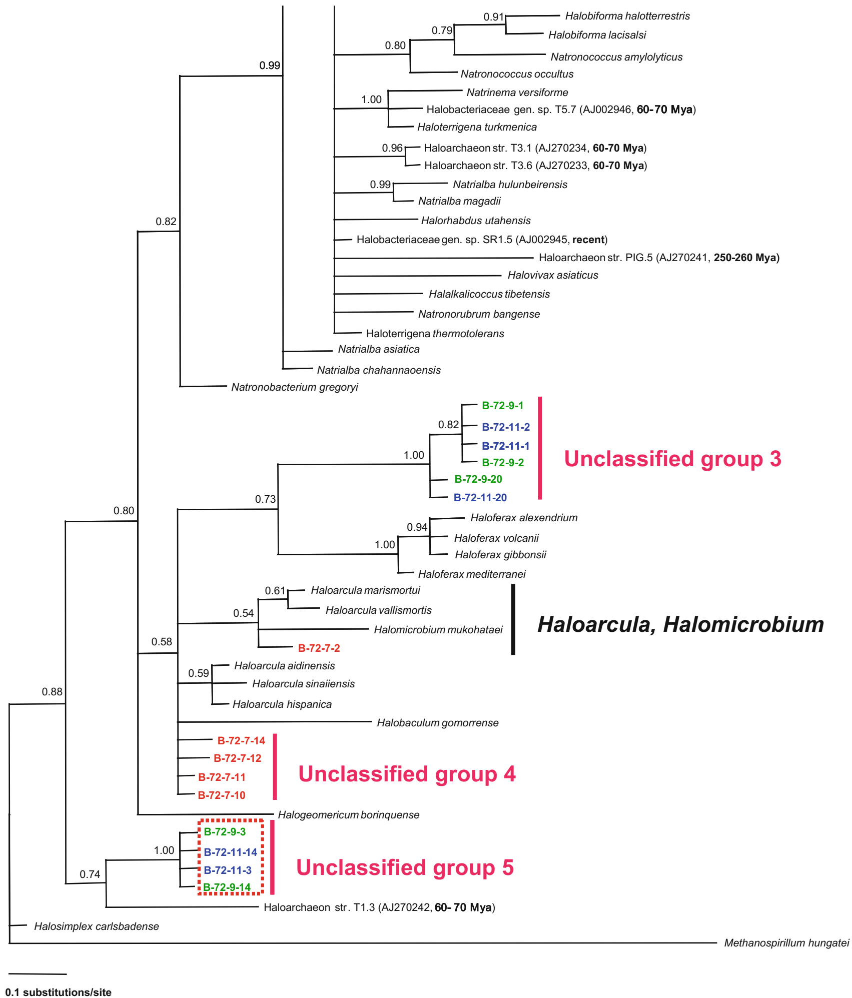

Fig. 6.2 (continued)

One other aspect of the laboratory configuration that should be mentioned is the airflow system. Generally clean rooms and BSL facilities have opposite overall goals. A clean room setting is designed to protect a critical sample from the external "dirty" world. This is certainly the case for ancient materials of all types where the amount of available biological material is very low compared to the surrounding environment. Consequently these samples need to be protected from external contamination. On the opposite side BSL's are designed to protect the external environment from the organisms or materials being studied inside the laboratory. Both types of laboratories have primary separation barriers and in the higher level systems, airlocks and gowning facilities. One major difference however relates to the direction of airflow. Most clean-rooms are constructed to have positive pressure so that air flows from the clean area toward the "dirty" areas helping to protect the samples. Generally,

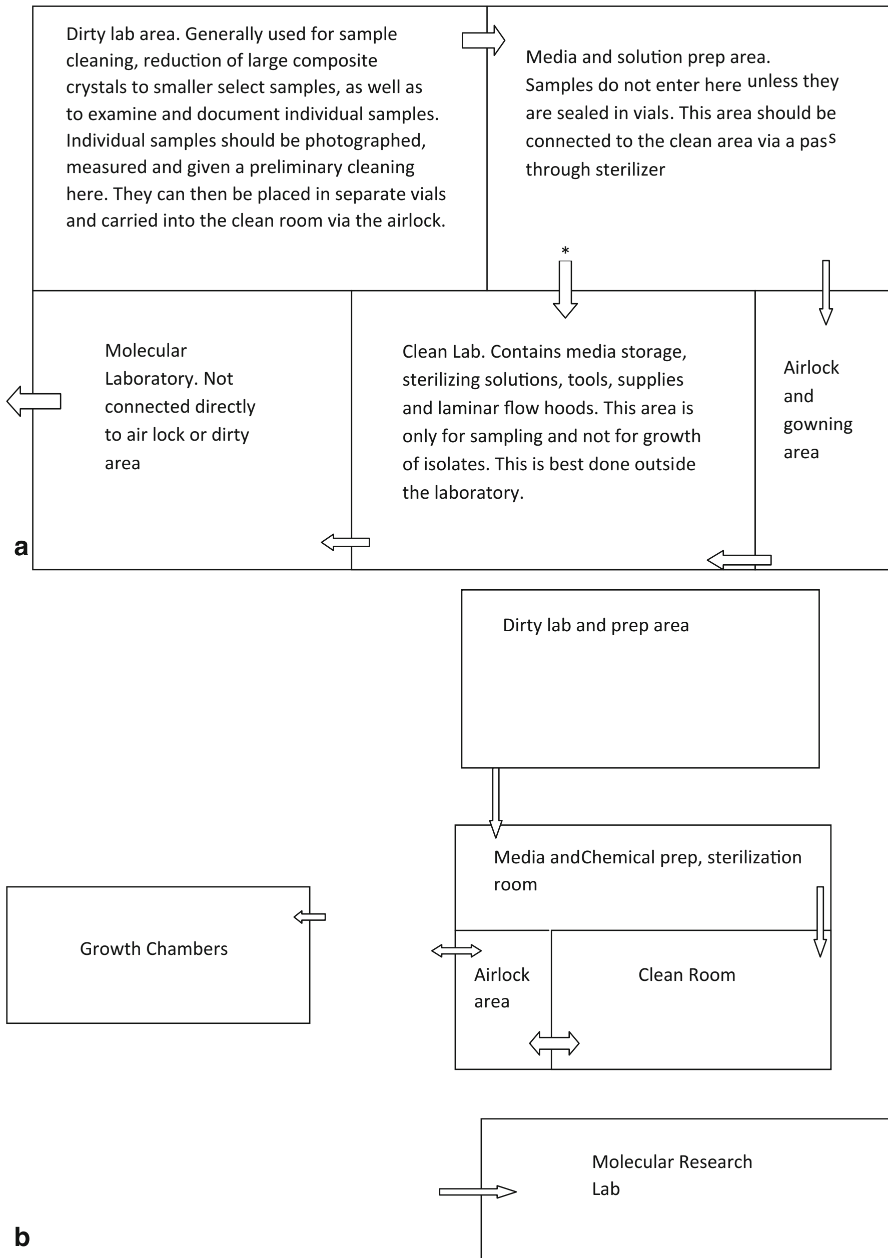

Fig. 6.3 Basic laboratory design considerations. **a** Single interconnected facility: Arrows indicate the preferred direction for the flow of samples, personnel and materials being sterilized (*). Note there is no direct connection between the various laboratory areas. Doors are not aligned and no two should be open simultaneously except in case of emergency situations. **b** Non-interconnected facility: Most situations will not be conducive to establishing a single interconnected laboratory. Under those situations an adequate area can be established as follows. The double arrows are used to indicate areas where samples must move in both directions. However the general rules of one way movement still apply

BSL facilities are the opposite with air flowing into the room (negative pressure). Part of the problem for any work with ancient samples is that one really wants a bit of both. Most biology/microbiology departments are set up with BSL laboratories rather than pure clean rooms. Consequently, the most expedient thing we have found is to utilize (HEPA) filtration on the inflow of a negative pressure system. Further if the air handling system is powerful enough one can install a second and final filter at the outflow. In the negative pressure clean room of the author this set-up resulted in very low airborne particulate levels (sometimes below 2000 total particles per cubic meter). While having a dedicated clean room is the ideal set-up for this type of research it can be very difficult to accomplish in the normally tight spaces of a typical research laboratory. Consequently the next best alternative is a laminar flow hood system. Once again under the ideal circumstances such a hood would be dedicated to the isolation of ancient materials, meaning that it would not be used for live culture transfers, DNA or RNA isolations or shared with multiple researchers. Also if at all possible this hood should be separated from the general flow of the laboratory environment. Again however such a thing may not be feasible and a single laminar flow hood may need to be used by several researchers or for several projects. At the minimum however some rules must be accepted and followed. Probably the most critical aspect would involve a willingness of all users to adhere to the cleanliness and cleaning protocols required by the project with highest burden of proof. Generally, this will be research on ancient materials but if not the ancient materials work will benefit anyway.

Finally, no research of this type should be conducted inside a clean room area in particular or a laminar flow hood without access to some sort of air monitoring equipment. There are several types on the market from one that is simply a type of particulate counter and provides data on the size distribution of the particles to others that are far more sophisticated. Without providing any specific endorsements new active air samplers are now available. These units provide a wide variety of sampling opportunities including sampling directly onto agar surfaces (impact sampling). The agar systems can be purchased premade or some corporations are willing to provide sterile dry plates which can be filled with any medium being used in the facility. Alternatively and perhaps even better in the long run is an attachment that holds a gelatin based membrane filter. This filter will retain airborne particles containing bacteria, DNA or even viruses. When placed onto growth media the filters dissolve within minutes allowing for an ultimate check on the presence of contaminants that can grow on any medium being tested. In addition the gelatin membrane will not inhibit polymerases so the air can be checked for DNA via normal PCR systems and finally the same membranes can be used in fluorescent microscopy or EM systems providing a check on any airborne viruses.

Basic Techniques

The techniques for working with ancient materials are as varied as the materials themselves. Since this particular text deals with studies related to salt loving microbes the following discussion will focus primarily on the techniques used with halite.

However, a brief generalized overview might still be informative. It does not require a seasoned professional to recognize that a large part of this research is devoted to establishing, maintaining and most importantly quantifying the techniques being used in any particular laboratory. No single technique is best and no single technique will work in all instances. At the most fundamental level the techniques that need to be considered involve first being certain one has a primary sample, second protecting that sample from external modern contamination, sterilization of the surface of the sample while leaving the internal portions intact, reaching or exposing those same internal portions then amplifying or growing whatever is inside. Obviously this final step is the goal, however due to the ever present possibility of modern contamination the focus should always be on being certain that the preliminary procedures are of sufficient quality to allow verification of the ultimate discovery.

Sample Considerations

Halite samples can be obtained in all shapes, sizes and configurations. Depending upon the overall goal of the research all of them may be used. However, the best samples will actually serve for nearly all types of research in this area, while the utility of the poorer samples should be strictly limited. For purposes of this part of the discussion we will assume that any crystals being studied have arisen from formations that meet all of the standard quality criteria mentioned above and by others (Vreeland and Powers 1998; Vreeland and Rosenzweig 1998; 2002; Vreeland et al. 2000) and will deal strictly with the sample from which one is attempting to isolate ancient biomaterials. The most important aspect of this type of sampling is to be certain that one has truly primary materials with primary being defined as something that crystallized only one time contemporaneously with the deposition of the larger formation itself (Fig. 6.4). Such criteria are difficult to meet (or even determine) if the sample is very large (circa 50–500g) (Fig. 6.4a) and is a composite rock containing many small crystals. In some cases individual slow growing cement crystals of this size may be obtained (Fig. 6.4b) but before their use the ages of such materials need to be verified by very careful geological analysis of the surrounding rock or by chemical analyses of the entrained fluids to demonstrate consistency with the rest of the formation.

One of the keys to having a defensible isolation and claim to the age of the biological material is to use only primary materials during the research. Salt crystals are an excellent target sample in this regard largely because their overall crystal structure generally reflects their apparent history. This subject is extensively and eloquently covered in Chap. 5 of this book.

In the opinion of this author samples from the rubble on mine floor should be avoided for a couple of reasons. First, there is no geological context for interpretation since it is impossible to determine where each sample originated in the main formation. This can have real significance. Vreeland et al. (2007) demonstrated that in one Cretaceous aged core sample, approximately 20 % of all crystals arising from a layer

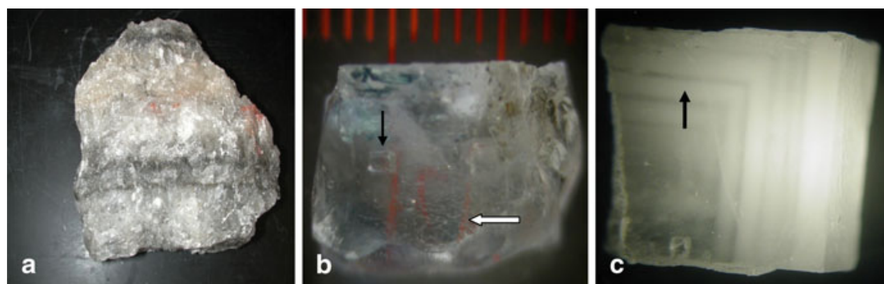

Fig. 6.4 Images of various crystals **(a)** Composite rock taken from 27 Ma formation. Note the numerous individual crystals present in this rock as well as the heterogeneity with some spots having distinct red impurities. This particular sample weighed nearly 15 kg and measured nearly 50 cm across the bottom; **(b)** smaller poor crystal from a similar rock. The large inclusion at the *left* of the image (black arrow) contains a large air bubble that could have arisen due to a crack. Further this crystal shows distinct evidence of water movement through the crystal (bottom center—white arrow). Scale is in mm. **(c)** High quality primary crystal taken from the same formation and a similar rock. The alternating cloudy/clear concentric banding patterns in the crystal are composed of high numbers of minute 100 μm fluid (brine) inclusions. The cloudy bands (arrow) represent time periods where the crystal was growing rapidly and therefore trapped a significant number of brine droplets. The clear areas were caused by slower growth during cooler periods. The 90° angles made by each band indicate the growth direction of the crystal. This crystal measured 2 cm on a side

that was 768.5m below ground surface contained live halophilic *Archaea*. Samples from what appeared to be identical salt layers less that 0.5m above and below this depth yielded no living microbes. Consequently simply picking up rubble samples could provide either an over-estimate or an underestimate of the biological content depending upon the originating layer. Second and probably even more problematic is the fact that the charges used are not shaped so the force of the blast dissipates backward into the mine face behind the blast area. When the mine face is blasted away the charges are generally placed lower on the wall. When fired, this causes the wall face to simple fall away from the rest of the workings (Myron Marcotte (salt mine manager)—personal communication). This means that the crystals lying around the mine floor have received the brunt of the blast forces and intense heating so many (if not all) of the inclusions containing biological materials have been impacted or even destroyed. Finally, these larger rocks are actually composites of many individual crystals of varying sizes (note the arrows in Fig. 6.4c).

Sterilization

In any microbiological endeavor sterility is a key aspect. However, this term is used so often that many times even microbiologists must be reminded that all sterilization processes are probability functions. There is no such thing as absolute sterility. The correct terminology is to consider probabilities of contamination or a so-called

sterility assurance level (SAL). Aside from consistently cleaning the laboratory area, sterilization of the surface of the sample is critical. The procedures utilized must be of sufficient strength and breadth to provide good assurance that external contamination has been reduced to low levels while at the same time being gentle enough to protect the fragile biomaterials inside the sample. For this reason most modern researchers have chosen to utilize chemical rather than physical sterilization approaches. One of the first techniques used for sterilizing the surfaces of ancient crystals was presented by Dombrowski (1963, 1966). In this instance the crystals were sterilized by immersion in 95 % ethanol then the ethanol was removed via flaming. This was (and still is) a somewhat standard microbiological procedure. However in the case of Dombrowski's (1963, 1966) work it suffered from numerous deficiencies. As pointed out by Vreeland and Rosenzweig (2002) one of the biggest problems is that ethanol is not really a particularly good sterilant having a phenol coefficient of only 0.4 meaning that ethanol possesses only 40 % of the sterilizing capacity of standard phenol. This can be improved slightly by using a 70 % solution of ethanol in water but then one runs the risk of dissolving some of the target crystal during sterilization. Vreeland and Rosenzweig (1998) also pointed out that the temperature of an ethanol flame is actually relatively low and of only short duration so flame sterilization is not assured. Further, NaCl crystals are fairly good heat conductors so the sudden heating of the entire surface of the sample is rapidly transmitted away from the surface and toward the center of the sample, meaning that the inside of a salt crystal actually gets slightly warmer than the surface (Vreeland and Powers 1998). Last but certainly not least, this procedure, while widely used in microbiology, has really never been quantified in terms of its overall SAL. In fact data produced in the author's laboratory and mentioned by Rosenzweig et al. (2000) has shown that the technique not only does not kill bacterial spores, there is actually a build-up of spore contamination over time on ethanol flamed utensils.

While the use of ethanol as a sterilantin the manner applied by Dombrowski (1963, 1966) and others (Reiser and Tasch 1960) cannot be recommended as a primary choice, a modification of this method was used with some success with halite (Norton and Grant 1988; Norton et al. 1993; Radax et al. 2001; Stan-Lotter et al. 1999, 2000). The principle innovations developed by these researchers involved immersing the halite samples in ethanol while at the rock facies, then sealing the tubes for transport back to the laboratory. The samples remained in the ethanol for several hours to several days prior to sampling. In addition these researchers provided some documentation of the efficacy of the technique by incorporating a test system involving laboratory grown crystals containing red rod shaped halophiles trapped within the crystal and an orange Halococcus purposefully added to the outer surfaces. At the end of the experiments data showing that the coccus had been removed from the crystals while the rod survived was very useful in providing defensibility to the isolations. This was actually an excellent choice of test microbes since the halophilic cocci are much more tenacious than are the rod shaped forms. However, the experiments were directed solely at halophilic *Archaea* and such sterilization verifications should not be used to provide assurance of sterility in the case of members of the domain *Bacteria*.

Perhaps the most rigorous sterilization protocol for use with halite is that of Rosenzweig et al. (2000). This process took advantage of the fact that pure halite which is simply NaCl is completely insoluble in concentrated NaOH and HCl. In this paper and in several ensuing articles (Park et al. 2009; Vreeland et al. 2000, 2007; Vreeland 2006) these authors have provided clear evidence of the sterility assurance level generated by the use of this technique. They were also able to show that it is effective against halophilic *Archaea*, as well as both gram negative halophilic bacteria and gram positive spore forming microbes. When used as designed the technique provides an SAL that may be better than 1.0×10^{-9}. While the effectiveness of this acid base technique is well documented its overall usage is somewhat limited as other easily obtained water bearing materials such as gypsum crystals or even other types of ancient samples (notably bone, soils, ice, etc.) could not survive under these conditions. At the same time rocks that may contain ancient materials such as amber, microcrystalline opal, or even quartz might survive this process allowing for some additional use of the procedure. There are however some cautionary points that should be considered when using the Rosenzweig et al. (2000) methods. First is the clear danger involved with using such concentrated corrosives (especially HCl). The reason acid was kept at 10M rather than straight out of the bottle (12M) was that 12M HCl fumes, releasing significant amounts of hazardous chlorine gas while 10 M fumes significantly less. Ten molar still fumes and causes problems just fewer. In addition sensitive metal equipment such as laminar flow hoods, microscopes etc need to be protected from the corrosive gases. The easiest effective way to protect these pieces is to spray all surfaces with a solution of sodium bicarbonate at the end of a work period, allow this solution to remain in contact for a short period then wipe the metal with distilled water or even disinfectant. When working in laminar flow hoods be certain the bicarbonate spray reaches the area below the primary work surface and allow time for the aerosolized bicarbonate to get into the rear airflow return of the hood. This procedure protected the author's clean room laminar flow hood for nearly 12 years despite hundreds of hours of sampling with use of the highly concentrated HCl.

Recently, several new potentially useful three part sterilants have come on the market. While these have not been tested in the type of application being described here these materials may prove to be quite useful. One of these products in particular was developed by US government laboratories following the *Bacillus anthracis* contaminated mail incidents in the United States. The best known of these disinfectants (DF-200) has been extensively tested against several pathogenic microbes including *B. anthracis and Yersinia pestis*. It has been shown in both cases to yield a 7 log reduction with only 15min of exposure (basically yielding an SAL of 1×10^{-7}) (Tadros and Tucker 2003). While this is certainly not as good as the Rosenzweig et al. (2000) technique it would have the advantage of not using highly corrosive materials. At this point the only caution with using this type of material being the fact that it has never been used with halite, or tested for its ability to destroy a large number of microbes or DNA/RNA on rough surfaces such as rocks and minerals. DF-200 is however commercially available and could quite possibly be highly useful. If it can't be used as a primary sterilant for rocks it is certainly useful for cleaning the research laboratory.

Establishing and documenting an SAL is relatively easy and can be done frequently in nearly any microbiological setting. All that is needed are several cultures of interest, each with of a known population density. Generally this would involve simply concentrating the culture to fairly high levels (i.e., 10^{8-9} viable cells per ml). Several sub-samples of these cultures are then exposed to the sterilant of being tested for a specified amount of time. As each sample time point is reached the sterilant must be neutralized (i.e., base added for acid sterilants, cultures iced for heat sterilization) and the remaining viable population determined. The population data is then displayed graphically on a logarithmic scale. The SAL is basically the time required to reduce the population levels to the desired endpoint. The easiest way to determine this is to extrapolate the graph past Y = 0 and to measure the time needed for the culture to be reduced to something like 10^{-6} (SAL = –6). In this case, the lower the exponent the more stringent the sterilization protocol becomes and the greater the overall sterility assurance. The most frequent controls used involve comparison of the new sterilization technique with some standardly accepted system (i.e., autoclave, UV light) that is similar to the "new" sterilant being tested. These procedures (including graphs, calculations and interpretations) are often illustrated in either General Microbiology or Industrial Microbiology textbooks.

What Makes a Discovery Believable?

This is probably the toughest aspect of research involving any type of ancient material especially that from very old salt rock. The key aspect that must be understood is that the burden of proof is as always in science placed squarely on the shoulders of those conducting the experiments. At the same time the author of this chapter learned that when one decides to publish a finding of anything older than a few thousand years the skeptics become very animated and numerous (Graur and Pupko 2001; Nickle et al. 2002; Hazen and Roedder 2001). So the key thing for anyone in this research area is simply defending the discovery. Much of the foregoing discussion has described the physical laboratory and other criteria that help to provide the foundational evidence needed to support a claim of antiquity. Experience and past history has shown however that there is really one aspect that should be readily available, that is a definitive SAL. Much of the earliest research and claims that have been cited in the historical section of this chapter Lipman 1931, 1937; Farrell and Turner 1932; Dombrowski 1963, 1966; Reiser and Tasch 1960; Tasch 1963) was ultimately discounted as a result of "contamination" due to the lack of a verifiable SAL. That situation began to change with the isolations of Cano and Borucki (1995) and the situation was reversed by the work of Rosenzweig et al. (2000) and Vreeland et al. (2000) who were able to provide a definitive SAL of $<1.0 \times 10^{-9}$. Thereafter, critics arguing for contamination were forced to either explain the source of the contamination or to simply say they believed the very low probability event occurred. Over the ensuing years and as more discoveries were made in different aged samples using similar SAL's those arguments have receded considerably (Table 6.1). Consequently, if there were

Table 6.1 Organisms and DNA isolated from individual crystals of varying ages

Age	Biomaterial	#Pos/total	References
22 yr	Fungi, *Bacteria*, *Archaea*, DNA	(∼100 %)	(RHV unpublished)
<0.1MYA	*Bacteria, Archaea*, DNA	60/100	(Schubert et al. 2009a, b; Vreeland et al. 2007; Mormile et al. 2003)
23MYA	*Archaea*, DNA[a]	20/100	(Vreeland et al. 2007)
121MYA	*Archaea*, DNA[a]	8/100	(Vreeland et al. 2007; Fish et al. 2002)
250MYA	*Bacteria*, archaeal DNA, Cellulose[a]	1/100	(Griffith et al. 2008; Fish et al. 2002; Vreeland et al. 2000)
419MYA	DNA[a]	<1/100	(Park et al. 2009)

[a]Heterogeneous distribution—some regions always neg. hot spots tend to yield higher positives. All represent multiple cultures

a single piece of advice that would stand out in this chapter that is it. Work in this aspect must be based on a composite of efforts that, when taken together comprise a very small SAL that is consistently present and is always being improved or verified.

Future Advances

Over the years there have been numerous isolations of organisms and DNA from many ancient rock systems. Table 6.1 presents a summary of the types of organisms found inside individual crystals and the ages of those crystals at the time of sampling. Table 6.1 demonstrates several important aspects. First the data indicate a nearly asymptotic loss of viability frequency with which individual crystals retain detectable biomaterials over time. During the early periods of deposition, the available recoveries show that nearly all crystals contain living cells from all three evolutionary domains as well as detectable DNA. Then once the materials have aged (up to ∼100,000 years) the eukaryotic representatives disappear while bacteria, *Archaea* and DNA remain. Beyond this period the available recoveries become a bit more problematic in that only archaea and archaeal DNA but no bacteria have been detected between 23 and 125MA. Then at 250MA only bacteria were isolated while only archaeal DNA was detected in materials older than 250MA (419MA being the only other salt examined to date). One aspect of these data that may help to explain this however (also noted in Table 6.1) is the fact that these biomaterials have been heterogeneously distributed within the salts and these older formations. For instance, Vreeland et al. (1998) reported on an extensive survey of the 250MA Salado accessible through the US Dept. of Energy Waste Isolation Pilot Plant. During this survey Vreeland et al. (1998) frequently found entire areas of the underground that were apparently devoid of trapped biological materials while other areas (sometimes nearby) contained viable microbial populations. This heterogeneity is likely one of the major reasons why it is often very difficult to exactly repeat reported discoveries without a major commitment to time and energy. Further as the data in this table indicate one formation could yield particular types of organisms in one region and another in a second zone. As of this writing there have simply not been enough

systematic examinations of a single formation (or two formations) with intense analyses of both the negative and the positive layers to understand why this heterogeneity may be occurring. One possible reason for such a situation is the recent observation by Schubert et al. (2009a, 2009b). In examining relatively young Death Valley salts (ca 30–100 Ka) the authors noted the presence of algal cell (*Dunaliella sp.*) remnants in every inclusion that contained living halophilic microbes. This could provide at least enough carbon and energy for microbes to repair some macromolecular damage over long periods. At the same time in the opinion of the author any long running metabolic activity would doom the trapped cells due to waste build-up within the tiny inclusions. If cell division were inhibited in some manner (possibly due to very low water activity or low oxygen levels) simple use of small amounts of energy for repair of damage might be feasible. This should certainly be a fruitful area for future research.

Recently, Park et al. (2009) reported on a molecular survey of salt crystals from several different formations and ages. This study yielded a large number of DNA fragments and a correspondingly large phylogenetic tree.This tree provided some very important insights into the potential evolution of modern halophilic *Archaea* (Fig. 6.2). There were really two most interesting aspects to these data. The first being the complete repositioning of the genus *Halobacterium* to a section of the tree that contained only sequences from 121 and 412MA salts. Most such phylogenetic trees place this genus in a separate branch generally at some mid-point within the tree but not really related to any particular modern genus. The tree of Park et al. (2009) demonstrated that this genus was clearly related more to ancient genera than to what might be considered "modern." This is particularly interesting given the original isolation of *Hbt. salinarum* from a salt cured buffalo hide which was likely cured with ancient salt, and due to the combination of *Hbt. noricense* (Stan-Lotter et al. 1999, 1963, 2001) into that same section of the tree. These results would indicate that the *Halobacterium* may actually represent an ancestral genus of the family *Halobacteriaceae*. Park et al. (2009) also amplified a set of unique gene sequences that contained a 55 base pair insert located within the V-2 region of the 16S rRNA gene. The secondary structure of this sequence indicated that it would fold into a functional rRNA. Even more interesting, artificial removal of this insert and re-folding showed that the remaining genetic sequences would exactly correspond to the 16S rRNA of the *Halorubrum* and *Haloarcula* clades. Since Park et al. (2009) were unable to find evidence of either genus in the older salts but clearly recovered them from salts younger than 23MA they concluded that these genera may have evolved only relatively recently from the loss of the insert. These data are incredibly tantalizing for a variety of reasons and as a guide to much more future research. For instance, data such as these could provide incredible information into the evolution of modern microbial lineages. It could provide better information on when important genera and even genes arose. Finally, we must be recognized that the data of Park et al. (2009) provided the first true evidence of the probable extinction of one microbe, the evolution of something new and when it occurred (Fig. 6.2).

If these studies can be performed in a more systematic manner over the next few years they may be extremely useful in shedding a clearer light on microbial

evolutionary events during past epochs. They can provide new and important insights into early environments, early biochemistry and a host of other unexpected discoveries.

Another fruitful and exciting area where this type of research can have impact comes from the discovery of intact water bearing fluid inclusions in 4.5 billion year old halite from a meteorite (Zolensky et al. 1999). This may be the most awe inspiring aspect of this type of research. It requires a thorough reexamination of issues involving microbial survival to acceleration and deceleration (Mileikowsky et al. 2000) and especially studies on the affects of long term radiation on microbes trapped in small amounts of fluid where metabolic repair systems may not be readily available. Indeed, a discussion of this aspect has already begun (Kiminek et al. 2003; Nicastro et al. 2002) but much of this currently revolves around experiments that don't mimic the actual conditions (Kiminek et al. 2003) or are purely statistical approximations based upon other research (Nicastro et al. 2002). Clearly well designed experiments need to be performed to actually quantify the survival potentials in these systems. Imagine however, the possibility that halophilic microbes (and by extension other microbes) could survive intact in meteorites that travel through space on long journeys, then these rocks split open upon impact and release their organic cargo on new worlds to begin again the process of populating a planet.

As with anything really valuable only time will tell what impact all of this will have, or even if halophilic or other bacteria will be waiting for us when humanity reaches the stars.

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Chapter 7
DNA Replication and Repair in Halophiles

Adrienne Kish and Jocelyne DiRuggiero

Introduction

Hypersaline environments are characterized by elevated temperatures, fluctuations in oxygen and nutrient concentrations, high levels of solar radiation, and periodic desiccation. Exposure of cells to these conditions can produce DNA lesions including thymidine dimers, oxidative damage to DNA bases and the phosphodiester backbone, hydrolytic depurination and deamination, and DNA strand breaks, which must be repaired to maintain genomic fidelity and cellular viability. The survival of a species also requires the accurate transfer of genetic information from parents to offspring and as such both DNA repair and replication must be highly accurate to prevent the accumulation of mutations and its deleterious effects on the cell's survival. At the same time those processes must allow for inaccuracies to generate the diversity required for Darwinian evolution.

Here we describe recent advances in our understanding of the processes and the biochemical players involved in the pathways of DNA replication, repair, and recombination in the *Archaea*. We focus on the 10 haloarchaea for which a complete genome sequence is available and that represent 10 genera: *Halobacterium* sp. NRC-1/*Halobacterium salinarum* R1 (Ng et al. 2000); *Haloarcula maris mortui* (Baliga et al. 2004b), *Natronomonas pharaonis* (Falb et al. 2005), *Haloquadratum walsbyi* (Bolhuis et al. 2006), *Halogeometricum borinquense* (Malfatti et al. 2009), *Halomicrobium mukohataei* (Tindall et al. 2009), *Halorhabdus utahensis* (Bakke et al. 2009), *Haloferax volcanii* (Hartman et al. 2010), *Haloterrigena turkmenica* (Saunders et al. 2010), and *Halorubrum lacusprofundi* (http://www.ncbi.nlm.nih.gov/genomeprj/18455). Because of evidence supporting the classification of *Halobacterium* sp.

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Table 7.1 Major Factors Involved in DNA Replication in the Three Domains of Life

Function	Archaea	Eukarya	Bacteria
Origin recognition	Orc1/Cdc6	ORC 1-6	DnaA
Helicase loader	Orc1/Cdc6	Cdc6/Cdt1	DnaC
Helicase	MCM	MCM complex	DnaB
Single strand binding	SSB/RPA	RPA	SSB
Primase	Primase	Primosome	DnaG
Polymerase	Pol B (B family)/ Pol D (D family)	Pol δ and ε (B family)	Pol III (C family)
Clamp loader	RFC	RFC	γ-Complex
Sliding clamp	PCNA	PCNA	β-Clamp
Processing of Okazaki fragments	RNase H/Fen1 DNA ligase	RNaseH/Fen1 DNA ligase	R NaseH DNA ligase

NRC-1 as a strain of the species *H. salinarum*, we will further refer in this chapter to *Halobacterium* sp. NRC-1 as *H. salinarum* (Gruber et al. 2004).

DNA Replication in Halophiles

Replication of DNA ensures the accurate and timely duplication of genetic material that is essential for a species to survive. The process of DNA replication is functionally conserved in the three domains of life and can be divided into several stages (Barry and Bell 2006): first the DNA double helix is unwound by a DNA helicase in an ATP-dependent manner at the origin of replication where it is recruited by initiator proteins. The binding of specific single-strand DNA binding proteins stabilizes the single-strand DNA, exposed by the duplex unwinding, and DNA synthesis is initiated by the generation of RNA primers by a primase. While only one primer is needed on the leading strand, multiple primers have to be synthesized on the lagging strand to accommodate the 5′ to 3′ polarity of DNA polymerases, resulting in the generation of Okazaki fragments. Processivity of the DNA polymerase is enhanced by an enzyme complex called the sliding clamp that is loaded on the DNA duplex by a clamp loader. The sliding clamp is essential in coordinating DNA synthesis on both the leading and lagging strands and it plays a role in the processing of Okazaki fragments at the end of DNA replication. The summary of the proteins that catalyze those steps (Table 7.1) shows that the archaeal and eukaryal proteins are closely related and that, in several cases, proteins non-orthologous to bacterial enzymes catalyzes analogous steps in the *Archaea*. However, the archaeal replication proteins do not represent a reduced repertoire of eukaryotic proteins but are rather a mosaic of eukaryal and bacterial systems with archaeal-specific features.

Replication Origins

Archaea and most bacteria have circular genomes whereas eukarya have linear chromosomes with a genome size several orders of magnitude larger than prokaryotic genomes. As a consequence, strategies to replicate genomes vary greatly between

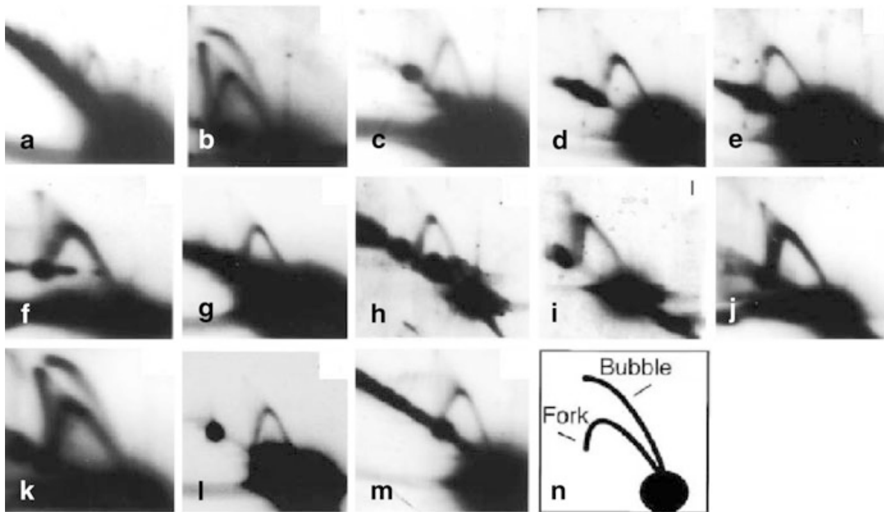

Fig. 7.1 Two-dimensional gel mapping for detection of replication origins in *S. solfataricus* at the *cdc6-1*, *cdc6-2*, and *cdc6-3* loci. DNA from asynchronous replicating cells was subjected to electrophoresis following restriction digest, transferred by Southern blot to a nylon membrane, and hybridized with specific radiolabeled probes. Panels A to M show the detection of replication intermediates by autoradiography; panel N shows a cartoon with positions of bubble and fork arcs. Panels B and K contain both bubble and fork arcs that indicate the presence of an active origin of replication, while panels C to J, L, and M, only contain fork arcs (*Y*-shaped molecule) indicative of the replication fork but no origin. (From Robinson et al. 2004 with permission)

eukaryotes and prokaryotes. *Eukarya* use multiple initiation sites along their chromosomes whereas bacteria and most archaea use a single origin of replication (Barry and Bell 2006). The first archaeal origin of replication was mapped in *Pyrococus abyssi* using pulsed field gel electrophoresis and two-dimensional (2-D) gel mapping (Myllykallio et al. 2000; Matsunaga et al. 2001). Since then, bioinformatics approaches combined with 2-D gel mapping and marker-frequency analyses have revealed archaeal genomes with more than one origin of replication. Two-D gel mapping is a particularly powerful technique to visualize whether specific loci associated with origin of replication genes (*cdc6*-like) might contain an origin of replication. In this method, DNA isolated from asynchronously replicating cells is digested with restriction enzymes and subjected to 2-D neutral-neutral agarose gel electrophoresis. The presence of a bubble-shaped replication intermediate, as opposed to just the fork-shaped replication intermediate, is indicative of the presence of an origin of replication in that specific DNA fragment (Fig. 7.1) (Robinson et al. 2004). Using this methods, the genomes of *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* were found to have three origins from which replication can be initiated simultaneously (Robinson et al. 2004). Multiple replication origins have also been mapped in *H. salinarum* and *H. volcanii* (Berquist and DasSarma 2003; Norais et al. 2007). Two origins of replication were initially proposed for the genome of *H. salinarum* (Zhang and Zhang 2003) but only one, *ori*C1 (upstream of the *orc*7 gene encoding a Cdc6/Orc1 homolog), was identified via a targeted genetic screen for automonous

replicating activity (Berquist and DasSarma 2003). In *H. volcanii,* disagreements between sequence and hybridization data make it difficult to conclude how many origins are present on the main chromosome of this organism (Norais et al. 2007). Multiple origins of replication have also been experimentally mapped in *Aeropyrum pernix* (Robinson and Bell 2007) and two putative origins were identified in *Methanocaldococcus jannaschii* by *in silico* analysis (Zhang and Zhang 2004).

The sequencing of a number of archaeal genomes has revealed the genomic organization at loci containing origin of replication genes. So far, all archaeal replication origins characterized share common features. They are several hundred base pairs in length with highly AT-rich stretches, also called duplex unwinding elements (DUEs), and multiple copies of repeat elements (Myllykallio et al. 2000; Robinson et al. 2004). These inverted repeat elements are termed origin recognition boxes (ORBs) and bind origin recognition proteins homologous to the eukaryotic proteins Orc1 and Cdc6 (Fig. 7.2a) (Robinson et al. 2004; Matsunaga et al. 2007). In *S. solfataricus*, the two origins were shown to bind different Cdc6/Orc1 homologs and the *S. solfataricus* Cdc6-1 can bind ORB elements from *P. abyssi* and *H. salinarum in vitro* (Robinson et al. 2004).

Origin Recognition Proteins

Genes for homologs of the eukaryotic initiation factor Cdc6/Orc1 are often located in close proximity to the archaeal origins of replication suggesting a functional link (Barry and Bell 2006). The archaeal Cdc6/Orc1 homologs belong to the ATPases Associated with various cellular Activities (AAA$^+$) superfamily. They are ring-shaped proteins with a highly conserved P-loop NTPase domain that contains the hallmark Walker A and B motifs required for ATP hydrolysis. The archaeal Cdc6/Orc1 homologs, in addition to a N-terminal AAA$^+$ domain, also harbor a C-terminal winged helix domain (WHD) (Iyer et al. 2004). A combination of structural and biochemical investigations in *S. solfataricus* suggest that both protein domains are key in the recognition of the replication origin and that DNA sequence and local structure are important features of the initiator binding site (Fig. 2b) (Dueber et al. 2011). Those studies used structural information from origin recognition complexes (Cdc6/Orc1) bound to DNA at the origin of replication (*ori*C DNA), and site-directed mutations of Cdc6/Orc1 protein residues, to determine the specific interactions between initiators and origin. Recent studies of the *P. furiosus* Cdc6/Orc1 showed that binding to its origin resulted in topological changes of the *ori*C DNA; it also produced the unwinding of a 12-bp long stretch of AT-rich DNA that could provide access to the replication fork for the replicative helicase (Matsunaga et al. 2010). Analysis of haloharchaeal genomes revealed 5 to 18 *cdc*6/*orc*1 gene homologs per genome whereas other archaeal genomes encode 1–4 homologs. Genetic analysis determined that only 2 Cdc6/Orc1 homologs out of 10 encoded in the genome are essential for viability in *H. salinarum* (Berquist et al. 2007); in *H. volcanii*, 3 out of 14 Cdc6/Orc1 homologs were required for viability (Norais et al. 2007).

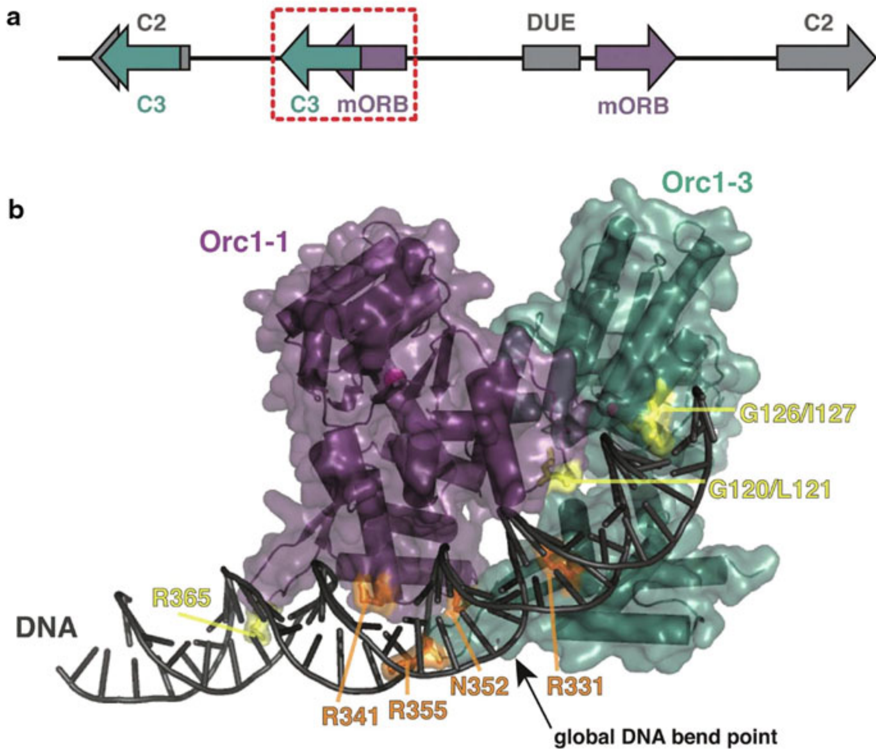

Fig. 7.2 **a** Schematic of the origin of replication *ori*C2 in *S solfataricus*; DUE, duplex unwinding element; ORB, origin recognition boxes; C2 and C3, binding sites for Orc1 proteins; dashed-red box, overlapping binding sites. Purple and teal are color-coded are binding sites for Orc1-1 and Orc1-3. **b** Schematic of Orc1-1/Orc1-3/origin DNA complex. In orange are residues with base-specific contacts with the DNA and in yellow are residues with non-specific interactions but important for origin recognition. Protein, purple and teal; DNA, gray; ADP, black sticks; magnesium ions, magenta spheres. (From Dueber et al. 2011 with permission)

Helicases

The minichromosome maintenance (MCM) protein is the replicative helicase in both the *Archaea* and the *Eukarya* and it is responsible for unwinding the DNA duplex ahead of the replication fork (Bochman and Schwacha 2009). *In vitro* assays showed that the *Methanothermobacter thermoautotrophicus* MCM complex has a processive 3′ to 5′-directed helicase activity that is ATP and Mg^{2+} dependent and can unwind up to 500 bp of double-strand DNA (Chong et al. 2000). The helicase subunit consists of a N-terminal domain needed for protein multimerization and a C-terminal domain that contains the helicase activity (Sakakibara et al. 2009). Subunit-mixing experiments, combining wild-type and mutant subunits, and protein activity assays have

successfully identified subunit composition and functional ATPase motifs within MCM complexes. Eukaryotic MCMs were found to be heterohexamers whereas most of the archaeal MCMs form homohexamers (Sakakibara et al. 2009). Most archaea encode a single MCM protein and each of the 10 haloarchaeal genomes sequenced so far only carry one *mcm* gene on the main chromosome; *H marismortui*, *H. turkmenica*, and *N. pharaonis* carry additional copies of the *mcm* gene on extra-chromosomal elements (MacNeill 2009). In the haloarchaea, the chromosome-encoded MCM proteins are phylogenetically closely related and are required for viability in *H. salinarum* and *H. volcanii* (Berquist et al. 2007; MacNeill 2009).

In *Eukarya*, the active form of MCM is found in complex with Cdc45 and the heterotretrameric GINS complex (Takara and Bell 2009). GINS are extra protein factors that enhance the MCM helicase activity and form a complex with MCM and Cdc45, called the unwindosome of CMG complex (Oyama et al. 2011). The CMG complex is essential to the template DNA unwinding reaction at the origin of replication. No Cdc45 homolog has been found in the *Archaea* but GINS proteins were found in all archaeal species (MacNeill 2010). *S. solfataricus* and *P. furiosus* encode two proteins, Gins15 and Gins 23, and the haloarchaea encode a single GINS homolog that closely resembles Gins15 (Marinsek et al. 2006; Yoshimochi et al. 2008). The haloarchaea GINS proteins are all significantly larger than their archaeal and eukaryal counterparts due mostly to the presence of a large sequence insertion between the conserved N and C-terminal domains of the protein (MacNeill 2009). In eukaryotes, GINS play an essential role in replication as structural components or regulators; they have been found essential for the survival of *H. volcanii* indicating that they may play a similar role in the *Archaea* (MacNeill 2009; Takara and Bell 2009).

Single-strand DNA Binding

Single-strand DNA-binding proteins (SSBs or RPAs) have a structural role in the replication process by coating the unwound DNA and thereby stabilizing its single-strand structure and preventing its chemical degradation (Wold 1997). Their ubiquitous distribution across the three domains of life also attests to their key role in other DNA transactions including DNA recombination and repair. SSBs bind DNA via an oligonucleotide/oligosaccharide-binding fold, called the OB fold (Murzin 1993). The functional bacterial SSB is a homotretramer while the eukaryotic SSB, called replication factor A (RFA), is heteromeric (Brill and Stillman 1991; Raghunathan et al. 2000). In the *Archaea*, the RPA/SSBs harbor various configurations and while the *Crenarchaeota* have a single RPA homolog, the *Euryarchaeota* present a multitude of homologs with various OB-fold conformations (Robbins et al. 2005). For example, *H. salinarum* has five putative RPA/SSB homologs, each resembling the *P. furiosus* RPA with respect to operon structure, and with sequence homology to eukaryotic RPAs. Two additional putative RPA homologs, with similarity to the

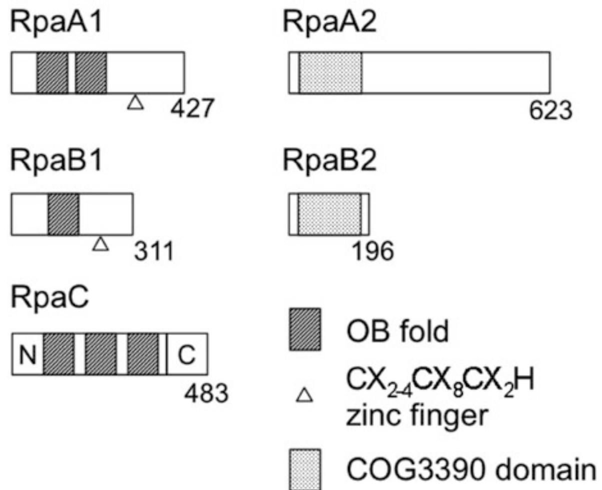

Fig. 7.3 Schematic of SSB/RPA proteins in *H. volcanii*. OB folds (dark grey boxes), COG3390 domains (light grey boxes), and the zinc finger motifs (*triangle*). Protein lengths are indicated. (Modified from Skowyra and Macneill 2011 with permission)

crenarchaeal RPA/SSBs in OB-fold structure, are each located on the two minichromosomes (Robbins et al. 2005). Five RPA/SSB homologs were also found in the model haloarchaea, *H. volcanii* (Fig. 7.3) (Skowyra and Macneill 2011). Recent study in this organism showed that two of its RPAs, RpaA1, and RpaA2, were nonessential for cell viability, whereas RpaC was essential for viability and played a key role in surviving DNA damage (Skowyra and Macneill 2011). The observation that elevated expression of *H. volcanii* RpaC enhanced cell survival when exposed to high doses of UV radiation and alkylating agents support previous observations that mutants of *H. salinarum*, evolved to higher ionizing radiation resistance, showed increased expression of three of its RPA proteins (DeVeaux et al. 2007; DiRuggiero et al. unpublished). Interactions among the various RPA homologs, and with other proteins, and the exact cellular roles of these proteins in DNA replication and repair remain to be determined.

DNA Replicative Polymerases and Sliding Clamps

Primers are extended by the action of DNA polymerases and although two families of polymerases have been found in the *Archaea*, the identity of the replicative polymerases remains unclear for this domain of life. The single-subunit family B DNA polymerases (polB) have been found in all archaea; in addition, members of the *Euryarchaeota* also encode a dimeric family D DNA polymerase (PolD) consisting of PolD1 (small subunit) and PolD2 (large subunit) (Grabowski and Kelman 2003; Barry and Bell 2006). The haloarchaea encode both PolB and PolD and several species encode additional PolB homologs (PolB2 and PolB3) (Grabowski and Kelman 2003; Barry and Bell 2006). Genetic studies in *H. salinarum* have demonstrated that PolB and PolD are essential for survival; the PolB2 enzyme was proposed to be inactive based on comparative sequence analysis and found dispensable for the survival of *H. salinarum* (Berquist et al. 2007; Rogozin et al. 2008).

Processivity of the DNA polymerase is enhanced by the tethering of the enzyme to the double-strand DNA by a structurally-conserved ring-shape protein complex, the sliding clamp (Jeruzalmi et al. 2002). In *Eukarya* and *Archaea*, the sliding clamp is called PCNA (proliferating nuclear antigen). It interacts with a number of factors involved in DNA replication and other cellular processes via a conserved motif, the PCNA-interacting protein box (PIP box), usually located at the N- or C-terminal ends of proteins (Warbrick 2000). The PCNA is a homotrimeric protein complex in eukaryotes and in the *Euryarchaeota* (Barry and Bell 2006). In contrast, up to three PCNA homologs have been found in the *Crenarchaeota* that can form both homo-trimeric and hetero-multimeric complexes (Dionne et al. 2003). Crystal structures of archaeal PCNAs has been obtained for a number of species including *A. fulgidus*, *P. furiosus*, *S. solfataricus*, and *H. volcanii* (Matsumiya et al. 2001; Chapados et al. 2004; Williams et al. 2006; Morgunova et al. 2009; Winter et al. 2009). Each haloarchaea genome sequenced so far encode a single PCNA protein and genetic studies have shown that the *pcna* gene cannot be deleted in the genome of *H. salinarum* and *H. volcanii* (Berquist et al. 2007; Meslet-Cladiere et al. 2007).

In order to load the ring-shaped sliding clamp PCNA onto double-strand DNA it must be opened and then closed; this is carried out by an enzyme complex called replication factor C (RFC) (Grabowski and Kelman 2003; Barry and Bell 2006). Clamp loaders in the three domains of life are typically heteropentamers with one large subunit and four smaller ones (Iyer et al. 2004). The haloarchaea also encode 3 RFC subunits, one large subunit (RfB) and two small subunits (RfA and RfC), with a C-terminal PIP box located at the C-terminal end of RfB, suggesting a pentameric structure (MacNeill 2009). Genetic studies with *H. volcanii* showed that the three *rfc* genes are required for viability (MacNeill 2009).

Okazaki Fragments Processing

At the end of replication, the Okazaki fragments of the lagging strand must be processed by removing the RNA primers and the newly synthesized DNA fragments must be joined. In the three domains of life, the RNA is removed by RNase H enzymes and either by DNA polymerase I in *Bacteria* or Fen 1 in *Eukarya* and *Archaea* (Barry and Bell 2006). The haloarchaeal genomes encode two types of RNase H, type I (Rnh1) and type II (Rnh2), and have a least one copy of each gene. In *H. volcanii*, RnH2 was found to be non-essential for survival (Meslet-Cladiere et al. 2007). The Fen1 enzyme was also found to be non-essential in *H. volcanii* whereas it could not be deleted from the genome of *H. salinarum* (Chen et al. 2005; Berquist et al. 2007). In the *Archaea*, Okazaki fragments are joined by an ATP-dependent ligase (LigA), as is the case in the *Eukarya* (Barry and Bell 2006). In addition, a bacterial-type NAD^+-dependent ligase (LigN) was identified in all the haloarchaea genomes, with the exception of *H. salinarum*, suggesting that the gene was acquired by lateral gene transfer from bacteria (Zhao et al. 2006). Genetic studies in *H. volcanii* showed that both ligases, LigA and LigN, can be deleted independently but not at the same time,

indicating that those enzymes share an essential cellular role (Poidevin and MacNeill 2006; Zhao et al. 2006).

DNA Repair Pathways in Halophiles

Halophiles are known to be resistant to a range of environmental stresses and the extremely halophilic archaeon *H. salinarum* has been shown to be highly resistant to desiccation (Kottemann et al. 2005), UV radiation (Kottemann et al. 2005; Whitehead et al. 2006; Shahmohammadi et al. 1997; McReady 1996; Baliga et al. 2004; Martin et al. 2000), and gamma irradiation (McCready 1996; Shahmohammadi et al. 1997; Martin et al. 2000; Baliga et al. 2004; Kottemann et al. 2005; Whitehead et al. 2006). Even after evaporation of the surrounding hypersaline medium, some halophiles are capable of surviving extended periods of desiccation trapped inside briny fluid inclusions within salt crystals. The duration of this survival ability in halophiles has been reported to range from 20 days (10 % survival) in laboratory experiments (Kottemann et al. 2005) to the retrieval of live halophiles from inside 250 million year-old environmental halite samples (Vreeland et al. 2000; Stan-Lotter et al. 2002; Gruber et al. 2004). This has set up a debate on how (and when) halophiles become incorporated into fluid inclusions inside salt crystals (Grant et al. 1998; McGenity et al. 2000; Nickle et al. 2002; Schubert et al. 2009) and how they survive long durations in this state given the potential for DNA damages during desiccation (Potts 1994). It is relevant to note that adaptations enabling survival after exposure to ionizing radiation (IR) are thought to have arisen in response to repeated cycles of desiccation that produce the same types of damage to cellular macromolecules as IR (Mattimore and Battista 1996). IR has therefore been utilized in the lab as a proxy source for desiccation, in part to reduce the time required for analyses.

Understanding the mechanisms for the maintenance of chromosomal stability under hypersaline conditions has implications not only for life in terrestrial hypersaline environments, but also for the potential for halophilic life to survive elsewhere in the solar system. Halite has been found within fluid inclusions in meteorites (Zolensky 1999) and on the surface of Mars (Rieder et al. 2004). Survival of halophiles on the Martian surface would require the ability to survive periodic desiccation as well as intense UV irradiation (Cockell et al. 2000; Patel et al. 2004; Tosca et al. 2008). Studies of halophilic microorganisms under simulated Mars conditions have shown that survival of halophiles is theoretically possible on the Martian surface, in particular if the cells are contained within brine inclusions inside salt crystals (Imshenetsky et al. 1973; Kminek et al. 2003; Fendrihan et al. 2009).

Studies of DNA damage induction and repair in halophiles have been conducted using a number of different approaches, from survival assays of wild-type cells and gene deletion mutants, targeting genes encoding DNA repair proteins, to transcriptomic surveys of stress responses after exposure to DNA-damaging agents. From these investigations, it has become clear that the ability of halophiles to survive the

extreme conditions found in their natural environment requires three combined activities: (1) protective strategies for avoiding DNA damage, (2) efficient repair of DNA damages incurred, and (3) regulated stress responses to DNA damaging agents. Thus, this section is organized along these three main themes to present the unique mechanisms for protection against DNA damage, DNA damage repair, and the regulated responses to DNA damaging agents drawn from studies using halophilic species, and to present how these studies were conducted.

DNA Damage Avoidance Strategies in Halophiles

The Role of Pigments

Due to the high levels of solar radiation found in the natural habitats of most halophiles, protection against the DNA damaging effects of radiation are of paramount importance for halophilic species. Phototaxis away from higher energy wavelengths is a common feature of all three domains of life (Jékely 2009). In haloarchaea, there are two sensory rhodopsin (SR) pigments responsible for initiating the phototactic response away from UV-blue (SRI) or green (SRII) light, and towards orange light (SRI) (Spudich and Bogomolni 1984).

Carotenoid pigments have long been known to provide protection against high levels of solar radiation (Dundas and Larsen 1962). Representative carotenoid pigments found in halophilic species include the C_{50} bacterioruberins common to haloarchaea (Kushwaha et al. 1975), the C_{40} salinixanthin identified in the halophilic bacterium *Salinibacter ruber* (Lutnaes et al. 2002), and the xanthophyll carotenoids and orange carotenoid proteins (OCP) from cyanobacteria (Kerfeld 2004; Zhu et al. 2010). While cyanobacterial OCPs and syctonemin (yellow-brown pigment) function in protection of the photosynthetic machinery against blue light and UV-A radiation, respectively, bacterioruberin in haloarchaea has been shown to have a direct role in DNA protection. Bacterioruberin provides protection against thymine degradation *in vitro* (Saito et al. 1997) and against the formation of cyclobutane pyrimidine dimers (CPD) and DNA single strand breaks (SSB) (Asgarani et al. 1999). *In vivo* studies of pigmented and colorless *H. salinarum* cultures have demonstrated the protective effects of bacterioruberin against oxidizing agents including ionizing radiation (IR), UV radiation, and hydrogen peroxide (Shahmohammadi et al. 1998; Kottemann et al. 2005).

Carotenoid pigments have been hypothesized to protect against DNA damage through the scavenging of hydroxyl radicals (Carbonneau et al. 1989). The scavenging of free radical species is also accomplished via enzymes including superoxide dismutases (for superoxide radicals), catalase and peroxidase enzymes (for hydrogen peroxide and other peroxide radicals) (Keyer et al. 1995; Cannio et al. 2000; Aguirre et al. 2005). Proteins and peptides such as thioredoxins and glutathione are also used to maintain redox homeostasis to prevent the accumulation of potentially DNA damaging free radical species.

Non-Enzymatic Shielding Mechanisms

Abiotic mechanisms have been noted for the protection of cellular macromolecules. Iron [Fe(III)] compounds have been shown to be an effective shield against UV radiation (Cockell and Knowland 1999). However, Fe(II) cofactors in many cellular proteins including some forms of superoxide dismutase have the potential to react with hydrogen peroxide increasing the production of the highly reactive hydroxyl radical through Fenton chemistry (Valko et al. 2005).

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

Haber-Weiss reaction:

$$H_2O_2 + {}^{\bullet}OH \rightarrow H_2O + O_2{}^- + H^+$$

$$H_2O_2 + O_2{}^- \rightarrow O_2 + OH^- + {}^{\bullet}OH$$

Mn(II) can be used in place of Fe(II) as a cofactor for many enzymes, including superoxide dismutase, and Mn(II) does not participate in Fenton chemistry. Many radiation resistant organisms, including *H. salinarum*, have been shown to have a higher intracellular Mn/Fe ratio than radiation sensitive organisms (Daly et al. 2004; Kish et al. 2009). Intracellular Mn complexes, amino acids and peptides have been shown to provide protection against protein carbonylation in both *Deinococcus radiodurans* (Daly et al. 2010) and *H. salinarum* (Robinson et al. 2011). Mn and other small antioxidant molecules however, were not shown to directly provide protection against DNA damage in *H. salinarum* (Robinson et al. 2011). Intracellular Mn has been hypothesized to reduce oxidative damage to intracellular proteins by the scavenging of superoxide and other peroxyl radicals, thereby preserving the functions of DNA repair proteins during periods of oxidative stress (Daly et al. 2007).

Intracellular salts, in particular halides, display protective properties for cellular macromolecules including DNA. Halophilic microorganisms achieve osmotic balance in near-saturating salt environments through the accumulation of compatible organic solutes (Roberts 2005) or by establishing an internal ionic environment equal in concentration, but not necessarily composition, to the extracellular environment (da Costa et al. 1998; Oren et al. 2002). The "salt-in" strategy is utilized by the extremely halophilic archaea of the *Halobacteriaceae* family, as well as bacteria of the order *Halanaerobiales* (Firmicutes) and *S. ruber* (Oren 2008). These halophiles concentrate K^+ in their intracellular milieu in place of Na^+ (Lutnaes et al. 2002; Oren et al. 2002; Engel and Catchpole 2005). Halide ions are transported into haloarchaeal cells via the light-driven halorhodopsin pump (Steiner et al. 1984; Kolbe et al. 2000), which was previously thought to be exclusive to haloarchaea but has now been identified—though not yet proven to be functional (Oren 2008)—in some strains of the halophilic bacterium *S. ruber* based on genomic analysis (Mongodin et al. 2005). Halides (chloride and bromide) have been shown both *in vitro* and *in*

vivo to have a direct role in the protection of nucleic acids from oxidative damage (Shahmohammadi et al. 1998; Asgarani et al. 1999; Daly et al. 2004; Kish et al. 2009).

In efforts to discover the mechanisms behind the radioresistance of *Halobacterium* cells, studies were conducted to test the hypothesis that the high intracellular Cl^- concentrations found in *Halobacterium* cells played a role in reducing intracellular oxidative stress as free radical scavengers. *In vitro* experimentation involving the γ-irradiation of DNA in aqueous solutions (Shahmohammadi et al. 1998; Asgarani et al. 1999) did in fact show fewer DNA strand breaks in the presence of high concentrations of KCl, presumably due to the scavenging of the hydroxyl radical by chloride ions. This hypothesis was confirmed *in vivo* by measuring oxidative modifications to both DNA bases and proteins in *H. salinarum* cells cultured in media containing only NaCl or a mixture of NaCl and NaBr salts, both of which are naturally occurring in hypersaline pools. DNA base oxidation was measured by GC/MS, DNA double strand breaks were visualized by pulsed field gel electrophoresis (PFGE), and protein oxidation was measured by Western blot immunoassay of protein-bound carbonyl groups (Kish et al. 2009). This experiment was limited by the fact that the presence of bromide is inhibitory to cell growth in *H. salinarum*, as has been shown for other members of the *Halobacteriaceae* (Oren and Bekhor 1999; Blaisdell and Wallace 2001; Dianov et al. 2001; Regulus et al. 2007). For this reason, it was found to be optimal to culture *H. salinarum* cells in media with a molar replacement of NaCl with NaBr of 1.7 M (1.7 M NaCl replaced with 1.7 M NaBr) rather than full replacement of NaCl with NaBr. It was demonstrated that haloarchaeal cells cultured in the presence of bromide salts incurred less oxidative damage to both DNA bases and proteins than cells cultured in chloride salts alone (Kish et al. 2009). *H. salinarum* cells also had significantly fewer oxidative DNA base modifications than *D. radiodurans* cells, which are highly radioresistant, but not halophilic, after exposure to up to 7.5 kGy of gamma radiation (Kish et al. 2009).

The protective role of intracellular salts as well as compatible solutes, with relevance for those halophiles employing the "salt-out" strategy for osmo-protection, has also been proven at temperature extremes (Santos and da Costa 2002; Garcia-Estepa et al. 2006), high pressure (Molina-Höppner et al. 2004), and during desiccation (Shirkey et al. 2003), demonstrating that these "osmolytes" play a greater role in the protection of cellular macromolecules from damages (Yancey 2005). Mycosporine-like amino acids (MAAs) found in cyanobacteria as well as eukaryotic microorganisms are effective UV-B absorbing molecules, provide protection against both osmotic stress and desiccation, and have been shown *in vitro* to have a singlet oxygen quenching activity (Oren and Gunde-Cimerman 2007; Latifi et al. 2009).

Despite the many mechanisms halophilic species possess for the prevention of damage to cellular macromolecules, DNA damage is common in the extreme environments they inhabit. Thus, a closer examination of the types of DNA repair pathways present in halophiles, both those common to mesophiles and those specific to halophiles, is required.

DNA Repair In Model Halophiles

Natural resistance to the high solar radiation (UV-A and UV-B) and periodic desiccation common to hypersaline habitats is a hallmark of halophilic organisms. UV photoproducts, nucleic acid base oxidation, and DNA strand breakage must be repaired on a continual basis to allow for the survival of halophiles in their environment. Studies of DNA damage and repair in halophiles, as with all extremophiles, begins with survival assays of halophilic cells after exposure to increasing doses of DNA-damaging agents. These assays are conducted in both exponential and stationary growth phases to determine the effects of DNA-damaging agents in both actively dividing and non-dividing cells, usually by plate counts or by measuring the optical density of liquid cultures. Survival studies typically are first conducted using DNA-damaging agents found in the natural hypersaline environment, and then progress to agents not found in the natural environment, but which produce a specific type of DNA lesion of interest. It is in this way that multi-resistant organisms (the so-call "polyextremophiles") are identified. Once survival curves are established, more detailed molecular biology experiments are conducted to determine by which pathway the DNA is repaired, which proteins are involved, and how this repair is regulated. This involves a mix of methodologies; genomics to identify putative genes encoding repair proteins, genetics to knockout or increase the expression of those genes, transcriptomics and proteomics to monitor both gene and protein expression after exposure to a DNA-damaging agent, *in vitro* biochemistry to define the activity of DNA repair proteins, and *in vivo* studies to analyze the extent of DNA damage and efficiency of DNA repair. *In vitro* biochemistry, however, is mostly limited to moderate halophiles and halophilic bacteria which use compatible solutes rather than intracellular salts to maintain osmotic balance. Haloarchaea have proteins adapted to high intracellular salt concentrations and as a consequence are largely inactive in the range of salt concentrations required for protein purification and *in vitro* biochemistry.

This section will present some of the known mechanisms used by halophiles to repair DNA damage and how they were discovered, as well as making note of those mechanisms that are currently undefined in halophilic model species. Summary descriptions of the general DNA repair pathways will be presented, and those seeking more detailed information beyond the scope of this chapter are referred to the many excellent review articles and books, such as (Friedberg et al. 2006), referenced throughout this chapter. It is also important to remember that there is a degree of overlap and interaction between the various repair systems (Swanson et al. 1999; Slupphaug et al. 2003).

Photoreactivation Repair of UV-Induced Photoproducts

UV irradiation results in the covalent dimerization of adjacent pyrimidine bases on the same DNA strand, resulting in the formation of both cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts [(6-4)PP], which produce a kink in the DNA structure. A recent study in *N. pharaonis* calculated that CPDs and (6-4)PPs accounted

for roughly 80 % of all UV induced photo-lesions (Moeller et al. 2010). These DNA lesions can be repaired via light-dependent and light-independent pathways. The role of various DNA repair proteins in the repair of UV-induced photoproducts is typically determined by quantifying the survival after UV-irradiation of a wild-type strain in comparison with a mutant strain in which the target gene encoding the repair protein is knocked out or disrupted. The study of light-independent or dark repair of UV-induced DNA lesions is more difficult than that of light-dependent repair due to the fact that all the steps, including irradiation, must be done under dark conditions.

Photoreaction (light repair) is an enzymatic process common to all three domains of life, and has been extensively studied in non-halophilic cyanobacteria (Wu et al. 1967; Tang and Asato 1978; Levine and Thiel 1987; Eker et al. 1990; Ng and Pakrasi 2001). This pathway relies upon photolyases that can bind to either CPDs or (6-4)PPs. Photolyases contain chromophores that absorb light in the blue to near-UV range and use this energy to monomerize the pyrimidine dimers before detaching from the DNA (Deisenhofer 2000). Many haloarchaeal genomes contain homologs for genes encoding two photolyases (*phr1* and *phr2*) (DasSarma et al. 2001; Capes et al. 2011). Genetic studies of the two putative photolyase genes in *H. salinarum* showed that *phr2* indeed encodes a CPD photolyase (McCready and Marcello 2003; Baliga et al. 2004), but *phr1* was not implicated in photoreactivation repair (Baliga et al. 2004). The *phr2* gene was up-regulated during light repair after UV irradiation in *Halococcus hamelinensis* (Leuko et al. 2010) but not *H. salinarum* (Baliga et al. 2004), suggesting different regulatory mechanisms between these two haloarchaea. The *H. salinarum* CPD photolyase has been shown to be highly efficient, repairing photoproducts within 30 min after UV irradiation (McCready and Marcello 2003) and underlining their adaptation to the high solar radiation environment typically inhabited by these microorganisms.

Dark Repair of UV-Induced Photoproducts (NER)

Repair of UV photoproducts in the absence of light utilizes nucleotide excision repair (NER) to excise bulky DNA lesions. The NER pathway involves recognition of the DNA lesion, bimodal incision of the DNA strand and excision of the resulting oligonucleotide, followed by repair synthesis of the DNA gap and finally DNA ligation to reseal the DNA strand (Sancar et al. 2004; Reardon and Sancar 2005; Truglio et al. 2006). The genomes of halophilic archaea encode both the bacterial type (*uvrABCD*) and the eukaryal type (*rad3a*, *rad3b*, *rad25a*, *rad25b*, *rad2*) of NER proteins (DasSarma et al. 2001; Capes et al. 2011). Excision repair of UV-induced lesions had previously been thought to be missing in haloarchaea (Hescox and Carlberg 1972; Grey and Fitt 1976; Fitt et al. 1983; Sharma et al. 1984), but a 1996 study (McCready 1996) showed evidence for a dark repair system favoring the repair of (6-4)PP over CPD. Survival assays comparing wild-type cells and mutants after targeted gene deletions of *uvrA*, *uvrB*, and *uvrC* (encoding homologs of bacterial-type NER repair proteins) revealed that these genes are required for dark repair of UV photoproducts (Crowley et al. 2006). In addition, two ligases involved

in the final step of DNA excision repair have been identified in *H. volcanii*; LigA and LigN (Zhao et al. 2006). The role of these ligases in the repair of UV photoproducts was verified by survival assays using serial dilution plating after UV-irradiation and comparing targeting gene deletion mutants with the wild-type strain. Phylogenetic analysis suggested that the presence of the NAD^+ dependant LigN is the result of lateral gene transfer from *Bacteria*, and hypothesized to be a backup for the ATP-dependant LigA; the deletion of both ligases is lethal whereas the deletion of one of these ligase genes is not.

SOS Response

In *Escherichia coli*, UV-irradiation induces the SOS response, a highly coordinated, transcriptional response regulated by the LexA repressor (Altshuler 1993; Janion 2008; Butala et al. 2009; Rastogi et al. 2010) enabling the transcription of over 40 damage-inducible genes. To our knowledge, no study has been published concerning the SOS response in a halophilic bacterium, but transcriptional analyses after UV irradiation have shown the SOS system to be absent from haloarchaea (Baliga et al. 2004; McCready et al. 2005).

Base Excision Repair of Radiation-Induced DNA Lesions

The base excision repair (BER) pathway utilizes a host of lesion-specific glycosylases to recognize and excise modified DNA bases (Friedberg et al. 2006). Many of these are specific to oxidative DNA damage, which is of particular importance to halophilic species as potential sources of oxidative damage to DNA include aerobic respiration, desiccation, and UV irradiation (Imlay 2003), all conditions common to high salt environments. The most common oxidation products are 7,8-dihydro-8-oxoguanine (8-oxoG) for purines and thymine glycol for pyrimidines, but also include 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and over 20 other types of DNA base oxidative modifications (Cadet et al. 2000; Dizdaroglu 2003; Slupphaug et al. 2003). These oxidized bases are recognized by specific glycosylases, including Fpg in *E. coli* and Ogg1 in yeast that recognize both 8-oxoG and FapyGua lesions (Cadet et al. 2000; Dizdaroglu 2003).

Glycosylases carry out the excision of the N-glycosyl bonds between DNA bases and the deoxyribose-phosphate backbone. This is followed by removal of the resulting abasic (AP) site by nicking the phosphate backbone on the 5′ side of the AP site by an AP endonuclease. Some glycosylases have an additional AP lyase activity and are able to nick instead the DNA backbone on the 3′ side of the AP site, leaving a 3′ nucleotide that must be removed by a 3′ phosphodiesterase. In either case, DNA repair synthesis adds the correct nucleotide and a DNA ligase reseals the DNA strand.

The first study of oxidative DNA base modifications in a prokaryotic species was performed using a halophile, *H. salinarum* (Kish et al. 2009), both under nominal

culturing conditions and after exposure to gamma radiation. The yield of oxidized DNA bases measured using GC/MS was found to be proportional to the IR dose from a baseline of oxidative damage found under nominal culturing conditions. Oxidized DNA bases were repaired after 2 h of recovery post-irradiation indicating that BER is highly efficient in this model halophile. The *H. salinarum* genome contains homologs of several DNA glycosylases, including *nthA1*, *nthA2*, and *nthB* (endonuclease III homologs); *ogg* (8-oxoguanine glycosylase); *mutY* (A/G specific adenine glycosylase); and *alkA* (3-methyladenine glycosylase) (DasSarma et al. 2001) which may be involved in BER in the haloarchaea.

Repair of DNA Double Strand Breaks

DNA double strand breaks (DSBs) are formed from both endogenous and exogenous sources. Aerobic organisms also produce reactive oxygen species (ROS) endogenously through the auto-oxidation of dehydrogenases involved in the respiratory electron transport chain (Imlay 2003). ROS are able to attack and modify DNA bases as well as sugar moieties, proteins, and lipids (Riley 1994). Significantly, both desiccation and ionizing radiation have been shown to produce extensive DNA DSBs (Dose et al. 1992; Mattimore and Battista 1996; Gusev et al. 2010). Oxidative damage to DNA bases and sugar moieties that occur in clusters within 2 helical turns of the DNA on opposite strands result in the formation of DSBs during attempted base excision repair (Blaisdell and Wallace 2001; Dianov et al. 2001; Sage and Harrison 2010). PFGE is the main *in vivo* method used to visualize DNA DSB formation and repair over a time-course after induction of DSBs in prokaryotic cells. The clearest interpretation of PFGE results are obtained when the chromosomal DNA is first digested with a restriction endonuclease, selecting an enzyme that results in approximately 2–10 DNA fragments so that the repair of the DSBs can clearly be observed by the re-formation over time of the pre-damage chromosomal DNA banding pattern. Without DNA digestion, DNA with extensive DSBs appears as a smear, and the precise timing of DNA repair (as demonstrated by the re-appearance of the chromosomal DNA band) is difficult to observe against this high background.

The study of DNA DSB repair in both the *Bacteria* and *Eukarya* has been conducted almost exclusively in mesophilic model systems such as *E. coli* and *S. cerevisiae.* Thus, most of the information on general DNA DSB repair pathways in two of the three domains of life has been extrapolated from mesophilic systems and hypothesized to be representative of halophilic species within these domains as well. It is only in the *Archaea* that halophilic model systems have been extensively used for studies of DNA DSB repair. This discussion will therefore concentrate on DNA DSB repair in haloarchaea, using data from both *H. salinarum* and *H. volcanii* model systems. Repair of extensive DNA DSBs produced by both desiccation and irradiation has been demonstrated within hours of damage formation in both *H. salinarum* (Kottemann et al. 2005) and *H. volcanii* (Delmas et al. 2009), showing that both model organisms possess highly efficient DNA DSB repair systems.

The pathways for DNA DSB repair can broadly be arranged into two main categories: (1) pathways based on re-ligation of the broken ends without the need for a second homologous chromosome, such as non-homologous end joining (NHEJ) and (2) pathways requiring the presence of a homologous chromosome to act as a template for repair, such as homologous recombination. The presence or absence of a second genome copy is one of the deciding factors governing DNA DSB pathway selection. In yeast, HR is confined to the S/G_2 phases due to the absence of sister-chromatids for use as templates during the rest of the cell cycle. NHEJ tends to be favored in G_0/G_1 phases of the cell cycle when only one copy of the genome is present, and has been shown to precede HR, although the HR pathway is most heavily utilized overall (reviewed in Aylon and Kupiec 2004; Sonoda et al. 2006). In contrast, both *H. salinarum* and *H. volcanii* are polyploid throughout their cell cycle. *H. salinarum* cells were found to have an average of 25 copies of the major chromosome in mid-log phase, which was reduced to 15 copies in stationary phase, while *H. volcanii* cells contained an average of 18 copies per cell in mid-log phase and 10 copies per cell in stationary phase (Breuert et al. 2006). The genome copy enumeration was verified using both quantitative PCR and what the authors describe as the "agarose block method". Briefly, this second method involves the encapsulation of cells in low-melt point agarose blocks followed by cell lysis and protein digestion, and DNA digestion with a restriction endonuclease to generate a specific 1 kb fragment near the origin of replication. The DNA was then separated by agarose gel electrophoresis along with an internal standard to determine the genomic DNA copy number by comparison of band intensities between the genomic DNA fragment and the internal standard after Southern blotting and hybridization with a single radiolabelled probe. The presence of multiple genome copies can be advantageous for the repair of extensive DNA DSBs, as it enables HR repair at any point in the cell cycle by providing a large number of homologous chromosomes to act as templates for the repair of broken DNA fragments.

Organisms in all three domains of life utilize the HR repair pathway. The general HR pathway is outlined in Fig. 7.4. There are five basic steps to homologous recombination (HR) repair of DNA DSBs: (1) DSB recognition and DNA end excision (5′-3′) to generate 3′-OH overhangs which are required for recognition by a DNA recombinase, (2) loading of DNA recombinase protein and formation of a DNA-recombinase nucleoprotein filament, (3) invasion of the homologous DNA strand by the recombinase and formation of a 4-way junction (Holliday junction) between the two DNA molecules via reciprocal exchange of DNA strands between the two molecules, (4) DNA synthesis using the homologous DNA strand as a template and the accompanying Holliday junction branch migration as DNA synthesis proceeds, and (5) Holliday junction resolution through the use of structure-specific endonucleases to restore two DNA molecules either with or without crossing-over of genetic material. Variations on this classical HR scheme include double strand break repair (DSBR), break induced repair (BIR) (Huertas 2010), synthesis dependent strand annealing (SDSA) (Paques and Haber 1999), and extended synthesis dependent strand annealing (ESDSA) (Zahradka et al. 2006), which deviate from standard HR after

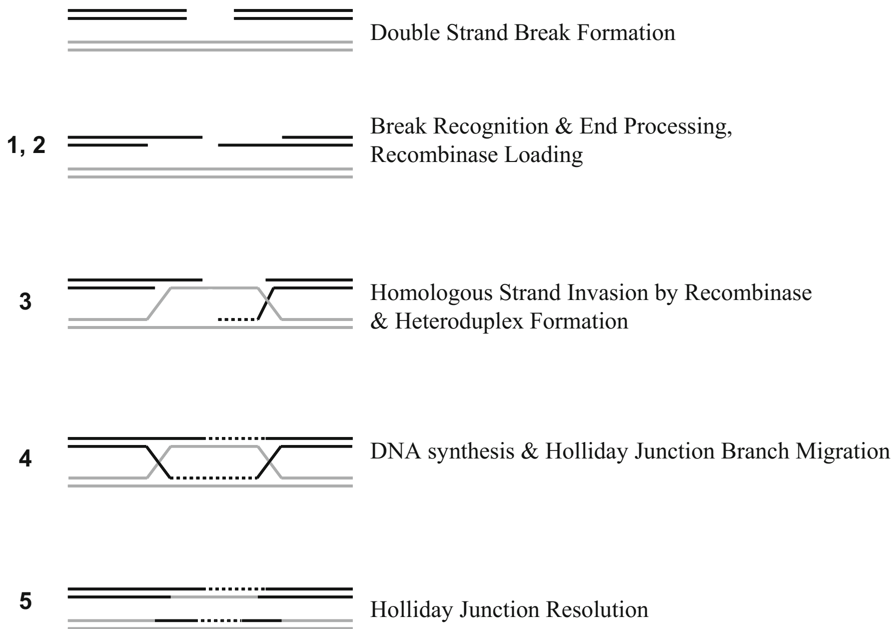

Fig. 7.4 General model of DNA DSB repair via the homologous recombination pathway showing the five major steps: (1) DSB recognition and end processing to create 3′-OH overhangs which are required for recombinase recognition, (2) recombinase loading, (3) homologous strand invasion by the recombinase forming a Holliday junction, (4) Holliday junction branch migration and DNA synthesis, and (5) Holliday junction resolution

homologous strand invasion. The bulk of the experimentation to date in haloarchaea has focused on classical HR repair, which will be presented here in greater detail.

The proteins responsible for carrying out the five basic steps of HR repair vary between the three domains, with the *Bacteria* and the *Eukarya* each utilizing a distinct set of DNA DSB repair proteins (see reviews in Kowalczykowski et al. 1994; West 2003; Krogh and Symington 2004; Wyman et al. 2004). *Archaeal* DNA repair proteins are generally homologous to those in the *Eukarya*. Due to this homology, the model of HR DSB DNA repair in the *Archaea* is based in part on comparisons with the canonical eukaryotic HR DNA repair pathway, with archaeal-specific features. Using the model of *S. cerevisiae*, DSBs in eukaryotes are first identified by the Mre11 complex, composed of the Mre11 nuclease, Rad50 ATPase, and a third partner called Xrs2 in *S. cerevisiae* or Nbs1 in humans, which is involved in DNA DSB signaling and cell cycle checkpoint activation. Rad50, an SMC-like protein, has a globular head with ATPase and DNA binding domains on either side of along coiled-coil region. Rad50 molecules can be joined together via a zinc hook structure in the middle of the coiled-coil region to form a bridge between DNA molecules. (Hopfner et al. 2002). DSB end processing to produce the required 3′ overhang is

accomplished using a number of nucleases and helicases, including Sae2 endonuclease, Sgs1(helicase)/Top3/Rmi1 complex, Dna1 (helicase/nuclease), and Exo1 (5′-3′ exonuclease) (Mimitou and Symington 2008; Niu et al. 2010). The eukaryotic recombinase is called Rad51. Recombinase loading and homologous strand exchange require a host of proteins including Rad52, Rad54, Rad55, and Rad57 (reviewed in (Symington 2002)). Evidence suggests that Rad54 acts in Holliday junction branch migration (Bugreev et al. 2006). The mechanisms of Holliday junction resolution have long remained unclear in eukaryotic systems (Heyer et al. 2003), however new studies have putatively identified a eukaryotic Holliday junction resolvase (Svendsen and Harper 2010). A full review of this intricate pathway and the numerous alternative roles of each of the players is beyond the scope of this chapter, thus readers are referred to the many excellent articles and reviews published on this topic (Symington 2002; Hopkins and Paull 2008; Li and Heyer 2008; Mimitou and Symington 2009; Rupnik et al. 2009; Williams et al. 2009; Xie et al. 2009; You et al. 2009; Cejka et al. 2010; Guo et al. 2010; Lamarche et al. 2010; Shim et al. 2010; Williams et al. 2010; Mimitou and Symington 2011).

The archaeal HR repair machinery is largely homologous to that found in the *Eukarya*. Due to the inherent limitations in biochemical investigations with haloarchaeal proteins due to the high salt concentrations required for their activity, the current model of HR in the *Archaea* results from a composite of *in vitro* protein biochemistry studies mostly coming from thermophilic archaea and *in vivo* genetic data using haloarchaea. In the post-genomics era where sequenced genomes are rapidly made available for many model organisms, identification of DNA repair genes follows a general workflow: Putative HR repair proteins are first identified with bioinformatic methods using DNA sequence similarity to eukaryotic (or bacterial) DNA repair genes or structural similarity of predicted protein structures to known DNA repair proteins. Genes or proteins with previously unknown functions may also be detected by transcriptomic or proteomic surveys as being differentially expressed after induction of DNA damage. Verification of the function of each protein can be done either *in vitro* or *in vivo*. In the case of haloarchaea, the use of *in vitro* biochemical assays using a limited set of proteins and cofactors to define the minimal set of key players essential to the DNA DSB repair is not currently possible. An array of *in vivo* methods, however, has been developed for use in haloarchaea. Survival assays comparing the survival of mutant strains lacking or overexpressing certain genes hypothesized to be key players in DNA DSB repair compared to wild-type strains are commonly used. Recombination assays are another *in vivo* method used to determine the frequency of usage of the various DSB repair pathways, or the efficacy of repair by one of the DSB repair pathways, by transforming cells with a pre-linearized plasmid bearing a selectable marker and quantifying the frequency of the observed phenotypes on selective media (Allers and Ngo 2003).

All archaeal genomes sequenced to date encode homologs of the Mre11 nuclease and Rad50 ATPase (together forming the MR complex). Together with the archaeal-specific NurA nuclease (Constantinesco et al. 2002) and the HerA bidirectional helicase (Constantinesco et al. 2004) found in thermophilic archaea, these form the

archaeal DNA DSB recognition and end processing complex (Hopkins and Paull 2008). All four proteins (Mre11, Rad50, NurA, and HerA) are encoded on the same operon in thermophilic archaea, but this operon in haloarchaea is composed of only *mre11* and *rad50*. No homologs of NurA or HerA have been identified in haloarchaea, and thus the precise mechanism of DSB end resection in haloarchaea is unclear. The archaeal Mre11 nuclease (like its eukaryotic counterpart) has a directionality (3′-5′) in opposition to that required for the production of the 3' overhang needed for recombinase loading and initiation of recombination.

Initial studies using gene deletion mutants of *mre11* and *rad50* have been conducted in both *H. volcanii* (Delmas et al. 2009) and *H. salinarum* (Kish and DiRuggiero 2008) These studies produced findings that represented some commonality between the two haloarchaeal species in terms of Mre11 and Rad50 function, but also some differences. When the survival of these *mre11* and *rad50* deletion mutants after exposure to UV-C radiation, gamma radiation, and alkylating agents was measured against their background (wild-type) strain, *H. salinarum mre11* and *rad50* deletion mutants showed survival equal to the wild-type strain but *H. volcanii* deletion mutants were found to be more resistant than the wild-type strain to these DNA damaging agents (Delmas et al. 2009). Interestingly in yeast, sensitivity to IR is one of the hallmarks of Mre11 complex mutants (Symington 2002). In the case of *H. volcanii*, the authors hypothesize that the increased survival of these mutants after UV-C irradiation was due to a failure to suppress HR in *H. volcanii* in the absence of the Mre11 complex, which may be undesirable if DNA DSB ends engage multiple homologous partners during HR repair slowing the repair process (Delmas et al. 2009). This explanation, however does not fully explain the differences between *H. volcanii* and *H. salinarum* since both are polyploid. This is not an unique situation since differences in the DNA DSB repair pathways between *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are well noted (see Krogh and Symmington 2004).

The role of the MR complex in the rate of HR repair of DNA DSBs was examined after irradiation (γ-ray in the case of *H. salinarum* and UV-C in the case of *H. volcanii*). Using PFGE to visualize DNA DSBs over a time-course after DSB induction by irradiation, it was determined that repair occurs at a reduced rate in both *H. salinarum* and *H. volcanii* strains lacking both *mre11* and *rad50* compared to their wild-type strains. In *rad50* single mutant strains of *H. salinarum* (Kish and DiRuggiero 2008), however, DNA DSB repair functioned at the same rate as in the parental strain in contrast to findings in *H. volcanii* (Delmas et al. 2009). Both studies proposed that the role of the Mre11 complex in haloarchaea might be more closely tied to regulation than repair. In eukaryotes, the Mre11 complex has been implicated in DSB recognition, cell cycle checkpoint signaling, repair pathway selection, and recruitment of repair proteins (Williams et al. 2007, 2010; Rupnik et al. 2008, 2009; Lamarche et al. 2010; Mimitou and Symington 2011). *In vivo* recombination assays suggest that in addition to HR, DSB repair may occur in *H. volcanii* by a form of accurate end-joining (Delmas et al. 2009), and that the Mre11 complex may play a

role in pathway selection, as in eukaryotic species (Williams et al. 2007, 2010; Shrivastav et al. 2008; You and Bailis 2010). Haloarchaea lack homologs of the Ku70/80 proteins that bind to DNA DSB ends to initiate canonical NHEJ, but this does not eliminate other forms of end-joining that are independent of the Ku proteins and that rely on either small (5–15 bp) regions of homology or direct repeats to guide the annealing of broken DNA ends. These alternative end-joining pathways are not currently clearly defined, (see Ciccia and Elledge 2010; Lieber 2010; Mladenov and Iliakis 2011) but appear to utilize Mre11-dependant DNA end resection prior to annealing of the DNA strands (Stracker and Petrini 2011). It is possible that other nucleases could take over DNA DSB end processing in the absence of the Mre11 nuclease for alternative end joining in haloarchaea, but this is an area for future research.

Following detection of DNA DSBs, the recombinase protein is loaded onto the DNA enabling homologous DNA strand invasion. The archaeal recombinase is a homolog of the eukaryotic Rad51. In haloarchaea, and other members of the *Euryarchaeota* (Haldenby et al. 2009), there are two paralogs; RadA and RadB. Deletion of *radA* from the genome of *H. volcanii* (Woods and Dyall-Smith 1997) revealed that RadA is responsible for DNA recombination. These findings were in agreement with *in vitro* analyses of RadA from *P. furiosus* demonstrating DNA-dependant ATPase, D-loop formation, and recombinase functions for RadA (Komori et al. 2000b). The use of powerful *in vitro* methods such as electron microscopy and gel shift assays to determine the activity and specificity of DNA repair proteins is not currently possible for the majority of haloarchaeal proteins due to the associated problems of high salt concentrations, and so the focus in haloarchaeal model systems has been on the characterization of mutant strains. *H. volcanii radA* mutants showed UV and ethylmethane sulfonate sensitivity compared to wild-type strains, similar to what is observed for bacterial *recA* and eukaryotic *rad51* mutants (Woods and Dyall-Smith 1997). The viability of *radA* mutants appears to vary between members of the *Archaea*, as attempts at deletion of *radA* from *H. salinarum* (DiRuggiero and Baliga personal communication) and the hyperthermophilic *Thermococcus kodakaraensis* (Fujikane et al. 2010) were unsuccessful. The absence of conditional promoters for archaeal model systems currently renders it impossible to determine if RadA is truly essential for some members of the *Archaea* or if other factors are inhibiting attempts to generate deletion mutants. The role of the second Rad51 homolog, RadB in the archaea, has also been investigated. Like *radA* deletion mutants *H. volcanii radB* deletion mutants were shown to be UV-sensitive (Guy et al. 2006). RadB however does not have a strong recombinase activity (Komori et al. 2000a, b). The role of RadB appears instead to be regulatory. RadB has ATP and DNA binding activities and is known to interact with both RadA and the Hjc Holliday junction resolvase *in vitro* (Komori et al. 2000a, b; Guy et al. 2006). Importantly, RadB suppresses the Holliday junction resolvase activity of Hjc in the absence of ATP *in vitro* (Komori et al. 2000a, b). A conformation change in RadB upon ATP binding may influence the regulatory activities of RadB on Hjc (Guy et al. 2006).

Hjc and Hef are both structure-specific endonucleases present in *H. volcanii*, and appear to have complementary functions in the HR repair pathway (Lestini et al. 2010). Deletion of either *hjc* or *hef* in *H. volcanii* did not affect the recombination frequency as long as the other was present. However, deletion of *hef* from a *radA* deletion mutant background resulted in a more severe growth defect than found in mutants lacking both *hjc* and *radA* (Lestini et al. 2010). The *hef* gene was also shown to be essential in the absence of the *hjc* nuclease (Lestini et al. 2010). These results indicate that while the Hef protein acts as a back up nuclease for the Hjc, Hef also has functions outside of the classical HR pathway. Indeed, *in vitro* biochemistry results from thermophilic archaea have shown that Hef is involved in the resolving of stalled DNA replication forks (Komori et al. 2004; Nishino et al. 2005), together with the Holliday junction branch migration activities of the Hel308 helicase (Woodman and Bolt 2009). Biochemical characterizations of Holliday junction helicases (Hel308, Hjm) have been conducted using proteins purified from thermophilic archaea (Fujikane et al. 2005; Li et al. 2008; Guy and Bolt 2005), and homologs of this helicase have been identified in the sequenced haloarchaeal genomes. Attempts at constructing a hel308 deletion mutant in *H. volcanii*, however, were reported to be unsuccessful (Woodman and Bolt 2009), suggesting that it may be essential.

Mismatch Repair of Replication Errors

Mismatched DNA bases arise mainly from errors in DNA replication. In bacteria such as *E. coli*, the mismatch repair (MMR) system recognizes these mismatches using MutS, which then interacts with MutL to activate the MutH endonuclease to nick the unmethylated DNA strand at a specific sequence (hemimethylated GATC). MutS and MutL then load the UvrD helicase to unwind DNA directionally from the nick site to the mismatch, followed by excision of the unwound DNA by a nuclease (RecJ or ExoVII for 3′-5′ excisions, ExoI or ExoX for 5′-3′ excisions). DNA synthesis finally replaces the excised DNA with the correctly matching DNA bases and a ligase seals the break.

Haloarchaea, unlike most archaea, encode homologs for a bacterial-type MMR system, including MutS1a, MutS1b, MutS2, MutL, and UvrD. MutL homologs appear to be missing in most *Archaea*. Also, instead of a d(GATC)-specific methylase as found in many bacteria, *H. salinarum* has been proposed to utilize a d(CTAG) sequence-specific methylase encoded by the *zim* gene (Baliga et al. 2004). Down-regulation of *zim* and up-regulation of a *recJ* homolog after exposure to UV-C radiation has been proposed to result in a transient under-methylation of DNAs that could initiate a haloarchaeal MMR system (Baliga et al. 2004). CTAG sites, however, are spaced further apart in the *H. salinarum* genome (average distance ∼2.5 kb) than GATC sites in *E. coli* (∼1 kb), casting some doubts on this model. In addition, the haloarchaeal MutS and MutL homologs were shown to be superfluous for the maintenance of a low genomic mutation rate in *H. salinarum* (Busch and DiRuggiero 2010).

Regulation of DNA Damage Repair: Transcriptional and Translational Stress Responses

Environmental challenges beyond the optimal range of growth conditions result in regulated responses by cells to counteract detrimental changes in the cellular environment. Responses can be measured at the transcriptional level, measuring differential gene expression via mRNA abundances, and at the translational level, measuring alterations in protein abundances. A systems biology approach has been used in haloarchaea to determine the overall regulatory system for stress responses in model species. This approach uses a combination of full genome, proteome, and transcriptome analyses to elucidate the overall cellular response to stress, including DNA damage, in model organisms. A number of high-throughput methods are applicable to this type of approach, including gene expression microarrays for quantifying mRNA expression, ChIP-chip for DNA-protein interactions, and quantitative proteomics via tandem mass spectrometry (ex. iTRAQ). Studies at the "local" level enable focused investigations of specific DNA repair systems that can either supplement global analyses or provide targeted information from the larger pool of halophilic species lacking a fully sequenced genome. These methods include quantitative real-time PCR and Northern blotting for gene expression, and Western blotting for protein expression.

Completed genome sequences enabled analyses of the global stress responses to DNA damaging agents in model halophiles, including the archaea *H. salinarum* (Ng et al. 2000) and *H. volcanii* (Hartman et al. 2010), and the bacterium *Chromohalobacter salixigens* (Oren et al. 2005). This has aided the study of DNA repair systems in these organisms by providing a means to putatively identify previously unannotated ORFs that may encode DNA repair proteins based on differential expression after exposure to DNA damaging conditions. In addition, systems biology studies can further elucidate the regulation of known DNA repair proteins and determine if these proteins may be active in alternative pathways. The hypotheses generated about the functions of these genes and gene products must then be verified experimentally via genetic or biochemical assays. It is important to note that transcriptional analyses are highly sensitive to the specific experimental conditions utilized including dosage, exposure time, recovery temperatures, and the growth medium composition. Care must therefore be taken when cross-comparing the results of independent studies. The most extensive library of transcriptional and translational stress responses in a halophilic organism available is for *H. salinarum*. The global stress responses of *H. salinarum* have been analyzed after UV-C irradiation (McCready et al. 2005; Baliga et al. 2004), UV-B irradiation (Boubriak et al. 2008), gamma irradiation (Whitehead et al. 2006), exposure to transition metals (Kaur et al. 2006), changes in salinity and temperature (Coker et al. 2007; Leuko et al. 2009), heat shock (Shukla 2006), changes in oxygen tension (Schmid et al. 2007), and oxidation stress (Kaur et al. 2010). Trends have emerged from these studies with respect to DNA repair in *H. salinarum*.

To reduce oxidative damages to DNA, free radical scavengers were found to be differentially regulated after exposure to IR (Whitehead et al. 2006), H_2O_2, and paraquat (PQ; produces superoxide) (Kaur et al. 2010). IR induced higher abundances for both the Sod2 superoxide dismutase protein and *trxA2* gene encoding thioredoxin (Whitehead et al. 2006). Eight dehydrogenase genes were down-regulated after exposure to IR, suggesting an attempt to minimize auto-oxidation reactions as part of aerobic respiration that would result in the production of additional ROS (Whitehead et al. 2006). Up-regulation of superoxide dismutases, a peroxidase/catalase, and carotenoid biosynthesis genes for ROS scavengers were also noted after exposure to PQ and H_2O_2 (Kaur et al. 2010).

Stalling of the cell cycle has been proposed after both UV and IR irradiation based on differential regulation of cell cycle proteins to allow time for DNA repair, along with an up-regulation of nucleotide biosynthesis genes (Baliga et al. 2004; Whitehead et al. 2006).

RadA, the archaeal DNA recombinase, has emerged as a central player in the DNA damage response under a range of conditions, although a coordinated SOS-type response appears to be absent in haloarchaea. A significant up-regulation of *radA* was noted after both high and low UV-C radiation doses (Baliga et al. 2004; McCready et al. 2005), UV-B irradiation (Boubriak et al. 2008), IR irradiation (Whitehead et al. 2006), and after exposure to the oxidizing agent paraquat (Kaur et al. 2010). Corresponding increases in RadA mRNA and protein were demonstrated over time after gamma irradiation, suggesting continual transcription and translation of RadA throughout the time course of recovery (Whitehead et al. 2006). Even in the absence of a coordinated bacterial-type SOS transcriptional regulation system, transcriptional and translational regulation of the RadA recombinase appears to be a key component to the DNA damage response in *Archaea*. This likely stems from the central role of recombinases such as RadA in multiple DNA repair pathways for DNA strand breaks or stalled replication forks (Michel 2000; Cox 2001; Haldenby et al. 2009).

RadA is not the only DNA repair protein to have shown significant differential regulation after exposure to DNA damaging conditions. Gamma irradiation produced up-regulations for *hjr* (encoding a homologous recombination Holliday junction resolvase) and *uvrD* (encoding a DNA repair helicase) (Whitehead et al. 2006), while UV-B irradiation increased mRNA abundances for a RecJ-like exonuclease, and a DNA binding protein (Boubriak et al. 2008). Transition metals (Mn, Fe, Cu, Co, Ni, Zn) altered the transcription of *uvrD*, *rhl*, *rad24a*, and *rad3b* encoding DNA helicases, with most being down-regulated. The exception was *rhl*, which was up-regulated in the presence of all metals tested except Mn (Kaur et al. 2006). The functional role of this putative helicase is not currently known.

Several glycosylases (*alkA*, *gap*, and a *mutT* homolog) thought to be involved in excision repair of oxidized DNA bases were identified after exposure to a high dose (200 J/m^2) of UV-C irradiation (Baliga et al. 2004). However at low doses (30–70 J/m^2) no induction of excision repair genes was observed (McCready et al. 2005), highlighting the differences in DNA damage induction and cellular response under different experimental conditions. An up-regulation of the *zim* d(CTAG) methylase

was also observed after exposure to high doses of UV-C radiation, suggesting the presence of an alternative type of MMR pathway (Baliga et al. 2004).

Transcriptional regulation has traditionally been more extensively studied than translational regulation, but recent studies using haloarchaea have demonstrated the importance of studying both. One study correlated mRNA and protein abundances over time after exposure to a DNA damaging agent to measure temporal separation of the transcriptional and translational responses (Whitehead et al. 2006). This study demonstrated gene-to-gene variations in terms of the lag time between changes in mRNA and protein abundances, highlighting the importance of interactions between transcriptional and translational regulatory systems in the DNA damage response. In a separate study, differential translation was compared between *H. salinarum* and *H. volcanii*. It was revealed that more than 20 % of *H. salinarum* genes and 12 % of *H. volcanii* genes were differentially translated depending on growth phase, but no overlap was found between the genes differentially translated between the two species (Lange et al. 2007). These results emphasize the need for investigations at both the transcriptional and translational levels over time, as well as the need for studies in a diverse range of model species as results cannot necessarily be generalized to related species.

Future Areas of Research for DNA Repair in Halophiles

There are several areas of DNA repair in halophiles for which there is currently inadequate information. Most significantly, there is a lack of data directly from halophilic model systems in the *Bacteria* and *Eukarya* for the repair of oxidative DNA damages and DNA DSBs and as such it is not known if the standard eukaryotic and bacterial models accurately represent the true DNA repair pathways utilized by halophilic members of these two domains of life. Alternative models to the standard *E. coli*-type HR repair of DNA DSBs are known in non-halophilic bacteria, in particular for *D. radiodurans* whose genome does not code for either RecB or RecC (Slade et al. 2009). It would be highly valuable to know if halophilic bacteria also use alternative HR pathways or if they follow the classic *E. coli* model. In the *Archaea*, there are many open questions in the mechanisms of DNA DSB repair. The exact complement of proteins that act in the initial phases of HR repair of DNA DSBs, and whether or not there are proteins that serve similar functions to NurA and HerA in the haloarchaea are questions that require further research. Cell cycle checkpoint activation after DNA DSB formation is also unclear in haloarchaea in the absence of a third partner for the Mre11/Rad50 complex corresponding to the eukaryotic Nbs1 protein, which is largely responsible for the signaling functions of the complex (Assenmacher and Hopfner 2004; Rupnik et al. 2009; Williams et al. 2010). The repair of DNA DSBs by NHEJ is currently an area of rapid discovery in all model organisms. Homologs of the Ku proteins are missing in all haloarchaea sequenced to date, suggesting that classical NHEJ in the haloarchaea either does not occur, or that it occurs using yet unidentified proteins not bearing sequence homology to the Ku proteins. A

combination of high-throughput methods quantifying gene and protein expression to identify candidate homologs that show increased expression under conditions which promote DNA DSB formation from among the unannotated open reading frames in haloarchaeal genomes, could be used together with bioinformatic methods predicting protein structure and putative function to identify candidate functional homologs of Ku70/80 in haloarchaea. The development of *in vitro* biochemistry methods applicable under near-saturating salt conditions would greatly aid research into DNA repair in halophilic species. Finally, evidence suggests that rather than a classical MMR system, an alternative pathway may be responsible for the correction of DNA mismatches resulting from erroneous DNA replication in haloarchaea, although the identity of such a pathway remains unknown.

Conclusion

The natural environment inhabited by halophiles results in continual exposure of these organisms to a range of DNA damaging agents including UV irradiation, desiccation, and oxidative stress. DNA repair and replication mechanisms described in halophilic species to date bear a great many similarities to their non-halophilic counterparts, but there is much left unknown. Knowledge derived from the biochemical characterization of individual proteins is complemented by ongoing genetic studies for which the haloarchaea, *H. salinarum* and *H. volcanii*, are good model systems. Genetic tools have been developed for both organisms and are constantly being enhanced (Allers et al. 2010). Those include efficient transformation protocols, replicative shuttle vectors along with a wide range of selectable markers, including the antibiotic novobiocin, the drug mevilonine, and multiple auxotrophic selectable markers, regulated promoter systems, and colorimetric reporter genes (Leigh et al. 2011). To date, 10 genomes of haloarchaea have been sequenced and tools for trancriptomics and proteomic analyses are available for several of them. Finally, a significant motivation for working with halophilic archaea is their ease of cultivation in the laboratory, with respect to other archaea, their resistance to contamination by non-halophilic microorganisms, and a comprehensive handbook of methods to work with halophiles developed by Mike Dyall-Smith (Dyall-Smith 2009). As a greater diversity of halophiles from all three domains of life are characterized at the molecular level, the uniquely halophilic features of protection against damaging agents, repair of DNA damages, and regulated responses to DNA damaging stress conditions will continue to emerge.

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Chapter 8
Gene Transfer Mechanisms, Population Genetics/Genomics and the Evolution of Haloarchaea

R. Thane Papke and Scott Chimileski

Introduction

Recent insights into genetics, population structure, and genomics of haloarchaea have revealed fascinating phylogenetic connections and stimulated interest in prokaryotic evolution. Because of their unique ecological settings haloarchaea are excellent model organisms for studying prokaryotic populations. They typically dominate hypersaline ponds where the salt concentration is greater than 15 %, providing a unique opportunity to study a single group of related organisms in relative ecological isolation. Isolated high salt environments can be considered microbial islands, not unlike traditional islands, which have historically yielded explosions of knowledge in basic biological concepts and paradigms. The following text synthesizes and summarizes an attempt to understand prokaryotic evolution by simultaneously focusing on the contemporary natural haloarchaeal population structure and the mechanisms of gene transfer that have, over the course of time, helped to shape it.

Part 1: The Gene Transfer Mechanisms that Shape Prokaryotic Populations

Laboratory vectors used for genetic transfer (which have become central driving forces in biological research) were invented first by Nature. Lateral gene transfer (LGT) events occur each second in the environment and likely have for billions of years. Genetic information has been cut and pasted from cell to cell long before scientists started heat shocking *Escherichia coli.* Furthermore, even laboratory strains that are chemically and genetically altered take advantage of evolved recombination machinery. The very discovery of DNA as the inherited genetic material was the

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observed because of the inherent ability for streptococci to undergo LGT through natural transformation (Avery et al. 1944). These are evolved mechanisms, driven by the advantages of recombination. The sum effect of horizontal gene flow, coupled with vertical flow by binary fission, geographic location, ecological conditions, natural selection and the passage of geologic time leads to observable phylogenetic trends.

Mating

Halobacteriales possess an interesting form of conjugation that is not pilus mediated as is found in examples from *Bacteria* like *Escherichia coli*. Moshe Mevarech's group at Tel Aviv University discovered this fascinating phenomenon in the mid 1980s and has uncovered many intriguing aspects that will be described below; however very little is known about the cellular and molecular basis of the conjugation ("mating") mechanism. It is mysterious as to why after twenty-five years so little is known, other than to note that the line of research has not been pursued. Perhaps this puzzling gap in knowledge can become an advantage for contemporary researchers when combined with modern analytical tools like microarrays at their disposal?

From the very first paper describing the morphology of *Haloferax volcanii* (originally *Halobacterium volcanii*), it was noted that cells had an unusual feature: long thin "constrictions" between cells, approximately equal in length to their flanking entities (Mullakhanbhai and Larsen 1975). Those constrictions were observed using phase contrast microscopy with 1,000$\times$ magnification, while peering at colonies on agar plates. The constrictions did not form, or at least were not reported, for cells grown in liquid medium and optimal conditions. Furthermore, these filament-like structures occurred between multiple cells, resembling beads on a string. At higher magnification (50,000$\times$) membranes were seen to be contiguous between cells, appearing funnel shaped as they extended. Electron microphotographs revealed an unbroken honeycomb-like structure of a typical archaeal cell wall, indicating multiple cells having a unified cytoplasm. Commenting on these involution forms, the authors suggested incomplete cell divisions were responsible for the observations (Mullakhanbhai and Larsen 1975).

In the decade following the initial description of *Haloferax volcanii*, an alternative explanation for the bridge-like structures was proposed. Rather than incomplete cell divisions, evidence suggested they were the opposite: cell fusion structures (Rosenshine et al. 1989). Incomplete cell divisions should only exhibit a single bridge; yet scanning electron microscopy indicated that often two or more intercellular bridges formed. Furthermore, it was observed that when the bridge-like structures were destabilized by decreasing the concentration of Mg^{2+} ions there was a significant increase in recombinants (Rosenshine et al. 1989). That observation suggested that bridges were not analogous to a pilus structure and did not act as conduits for DNA transfer between the cells, but rather initiated cell fusion analogous perhaps to a eukaryotic fertilization event. This is an important difference between pilus driven conjugation, and what is observed in the haloarchaea, which is cell fusion, and arguably analogous to an intermediate step in the evolution of sexual reproduction.

Evidence for "bidirectional," rather than unidirectional exchange of DNA (as is the case for nearly all conjugational systems, e.g., a donor and a recipient) was further demonstrated by data from exhaustive pairwise cross-matings of auxotrophs (a mutant incapable of producing key intermediary metabolic products from simple nutrients like glucose because of a nonfunctional biosynthesis pathway for an essential cellular component, like amino acids or nucleotides; the amino acid must be supplemented in media for the cells to live). When a donor/recipient relationship exists, no prototrophs (restoration of the ability to make the required amino acid(s)) would be generated from one of three possible paired mating experiments. For example, if a proline auxotroph mated with both adenine and phenylalanine auxotrophs and produced prototrophs (reverting back to the "wild type" phenotype) each time, then the proline auxotroph would be a donor and the others recipients. Donor/recipient status would be confirmed if the adenine and phenylalanine auxotrophs were mated and no prototrophs were generated. However, in pairwise matings of the three different *Haloferax volcanii* auxotrophs, protrophs were generated in all cases indicating no donor/recipient relationship: DNA was bidirectionally exchanged via a cell fusion mechanism (Mevarech and Werczberger 1985; Ortenberg et al. 1999; Rosenshine et al. 1989).

For all intents and purposes, the *Haloferax* mating mechanism is remarkably similar to sexual reproduction given its prokaryotic status: cells fuse into a single unit forming a temporary "diploid" state during which chromosomes undergo recombination (whether recombination is reciprocal or non-reciprocal is unknown) and plasmid exchange and eventual segregation into "offspring" in which "daughter" cells do not resemble each other nor their parents (Rosenshine et al. 1989). The mechanism depends upon prolonged contact between cells; attempts at mating *Haloferax* auxotrophs in a shaking culture produced no prototrophs, while cells within a pellet or on a filter are competent for mating. Critically though, unlike sexual reproduction in animals and most plants, *Haloferax* mating has been observed between species (Tchelet and Mevarech 1994), and occurs independently of reproduction via binary fission.

The observation that mating occurs between *Halferax volcanii* and *Haloferax mediterranei* indicates the mechanism for mate recognition is not species-specific, though it could be limited to genus specificity (Tchelet and Mevarech 1994). Interspecies cell fusions and recombination suggests that there may not be a lock and key type mechanism for cell fusion initiation, or if it is, then there are many locks into which a "universal" key can be inserted. Further, the observation of interspecies mating may expose a plasticity that undermines the very definition of species within single celled microorganisms. Virtually nothing is known about the molecular mechanism for mating but interestingly, a role for pilin proteins, which are responsible for binding and attaching to many different types of molecules including DNA, surfaces, and other cells, was recently ruled out (Tripepi et al. 2010). After generating a Δ*pibD* strain, Tripepi and co-workers found no difference in mating frequency compared to the wild type. That is to say, pilins are not expected to affect mating in any way, as *pibD* is a gene coding for a protein responsible for the maturation of all pilins.

Natural Competence

Natural competence, the ability to import "naked" DNA from the environment, has been reported for a large number of bacterial species (Lorenz and Wackernagel 1994), but has only been sparsely observed via serendipitous observations in *Archaea* (like finding transformants in a negative control when designing a genetic system). No in-depth analysis has ever been performed to determine a natural DNA uptake mechanism in *Archaea*, including any investigation into its regulation and the genes involved. Reasons for the lack of inquiry can only be speculative, but the observation that *Archaea* do not cause disease probably plays a significant role in this analytical deficiency. Gram-positive and Gram-negative bacteria represented mainly by pathogens have several conserved orthologous genes that are known to perform similar functions for DNA uptake in their different hosts. Additionally there also exists many features unique to one or the other of those two groups as well (Chen et al. 2005), probably due to considerable differences in cell membrane and wall construction. Though it has been 25 years since the discovery of the above described *Haloferax* mating system and 23 years since generating the first polyethylene glycol mediated chemical competence for genetic manipulation of haloarchaea (Charlebois et al. 1987), it is remarkable that natural competence was only just discovered (Chimileski et al. 2010a, b).

Two different haloarchaeal genera (*Haloferax* and *Halorubrum*) were tested for natural competence and have demonstrated the capability for metabolizing high molecular weight DNA as a sole carbon, nitrogen and phosphorus source. Furthermore, they are "picky" about the DNA that they import; the species tested metabolize DNA from other haloarchaea, but not bacterial and eukaryal DNA from *Escherichia coli*, *Micrococcus luteus* and herring sperm. Additional experiments revealed the mechanism behind the biased import was methylation-based: DNA from *E. coli* mutants lacking functional DNA methyltransferases was imported and metabolized by *Haloferax* and *Halorubrum* strains at rates equal to or greater than that observed for consuming their own DNA (Chimileski et al. 2010a, b).

The exact mechanism is not known; however, there are three key observations that suggest DNA is imported as a high molecular weight molecule and that there is a "gate keeper" system for recognizing and importing only correctly modified DNA: (i) haloarchaea methylate their own DNA (Charlebois et al. 1987; Hartman et al. 2010), yet *Haloferax volcanii* grows on DNA from divergent haloarchaeal genera. Therefore, the presence/absence of methyl groups alone does not explain discrimination of available DNA, implying closely related methylated DNA could be taken up following recognition of a specific methylation pattern. (ii) DNA is not externally hydrolyzed and imported as nucleotides. Secreted DNAses have been tested but never reported for haloarchaea (e.g., see (Ventosa et al. 2005)) and our own direct assays of *Haloferax volcanii* revealed neither a secreted DNAse homolog encoded in its genome, nor any secreted DNAse activity (Chimileski et al. 2010b). (iii) growth on nucleotides alone does not occur, or is very weak compared to growth on high molecular weight DNA.

Methylating unmethylated haloarchaeal DNA would add further evidence to the role of methylation in DNA uptake, but has not been attempted, primarily because there are many haloarchaeal methyltransferases that need to be knocked out, in single, double and multiple mutant scenarios. The use of specific methyltransferases which add methyl groups within a given recognition site may also elucidate the precise patterns enabling uptake. Undeniably the best proof that DNA is indeed being imported as high molecular weight DNA would involve demonstrating transformation. Natural transformation has been tested but not yet been verified for haloarchaea, perhaps because it may be induced by a specific unknown condition (see below). However, the points above are explained most parsimoniously through the recognition of specific DNA and importation as a high molecular weight molecule.

Analysis of genome sequence data revealed that all haloarchaea observed have chromosomal genes homologous to genes known to function in natural competence in Gram-positive and Gram-negative model organisms (e.g., *Bacillus* and *Neisseria*) (Chimileski et al. 2010a, b). Many of the putative competence genes occur in operons and also are found in the same general region of the chromosome. Aggregated, the data suggest that natural transformation is an ancient trait, or that lateral gene transfer has distributed these lateral transfer genes themselves between the domains (or some combination of both). At this point, distinguishing between the two alternatives has not been done. Bioinformatic analysis also identified many "conserved hypothetical" proteins in the putative natural competence operons suggesting that the haloarchaea though potentially sharing some of the conserved DNA uptake machinery, also have many unique genes involved in the process as expected. Of course, at this juncture, it is too premature to describe the functions of even the bacterial competence protein homologs (Chimileski et al. 2010a, b).

It is unclear at this point why haloarchaea import DNA for nutrition but have not undergone recombination in the laboratory. It is easy to point towards our lack of understanding for how haloarchaea may transform in nature. For instance, *Vibrio* species were considered incapable of natural transformation until it was eventually demonstrated that transformation only occurs in the presence of chitin (Meibom et al. 2005). Even *E. coli*, which had been described as strictly nutritionally competent (Finkel and Kolter 2001) (a subtype of natural competence in which DNA is imported but metabolized instead of recombined) has recently been demonstrated to be transformable (Tsen et al. 2002). Another possible explanation is that under nutrient poor conditions natural competence may up-regulate mismatch repair systems, which are known to prevent even identical DNA from being recombined into the chromosome (Matic et al. 1995, 1996). Despite the early inability to detect transformation, it does seem to be particularly strange that haloarchaea would be nutritionally competent, but not transformable. This statement follows from the logic that it seems unlikely cells would evolve a mechanism to discriminate available DNA if the sole purpose of the uptake system was for the metabolism of acquired DNA: why should haloarchaea reject an available and highly abundant resource? At this point there is no good reason to think that haloarchaea are not naturally transformable, and experimental successes in previously recalcitrant organisms provides hope for uncovering the proper conditions.

Part 2: Modern Haloarchaeal Species Structure: The Manifestation of Time and Gene Flow

Dip your container into a solar saltern, deep orange with trillions of haloarchaeal cells, take the sample back to the lab, isolate numerous individual colonies on plates, extract the DNA, amplify the same gene from all of the isolates and what do you have? A glimpse into the information contained within that one short stretch of a single ever-changing molecule: a snapshot of a microbial assemblage at a precise moment in time. The sequenced gene physically existed in the cells you lysed, and lives on in cells derived from the ancestral cells that inhabited this and similar such ponds throughout most of Earth's history. It probably also lives on, perhaps a bit differently, in cells across the world, within for example the halophilic microbes living in a sea bird's salty nostril (Brito-Echeverria et al. 2009). For billions of years cell after cell carried the information now on computer hard drives, passing it generation to generation. And though all the while it was replicating and changing, all we see is what it is currently [Note; progress in examining ancient DNA and cultures has been made and is ongoing, specifically in haloarchaea (e.g., see (McGenity et al. 2000)). Though, it is not without controversy (e.g., see (Griffith et al. 2008)]. Nonetheless, what it is today is a record of where it has been, and those events can be visualized in phylogenetic trees that infer/hypothesize ancestral relationships. Within phylogenetic trees, clusters of isolates can appear to have species-like characteristics. A particular population of organisms may appear to be more related to each because of vertical descent (e.g., asexual cell divisions), and also because those same organisms have a preferred DNA exchange group: frequent biased lateral gene transfer and recombination acts as a homogenizing force for the cohesiveness of clusters observed in phylogenetic trees (Gogarten et al. 2002).

Halorubrum Population Genetics Analysis: How Similar Is the Outcome of Prokaryote Gene Flow to that of Sexual Reproduction?

Despite the fact that haloarchaea have lateral gene transfer capabilities, could it be that in nature they are not used, or are infrequently used, like the largely clonal species *Staphylococcus aureus* or *Bacillus subtilus* (Chen and Dubnau 2004; Feil et al. 2003)? The answer is decidedly no, at least for *Halorubrum* species (Papke et al. 2004, 2007). Phylogenetic analysis of the same five loci (e.g., atpB, EF2, secY, bop, radA) from more than 150 *Halorubrum* strains cultivated from different habitats and geographic locations revealed three dominant clusters, phylogroups A, B, and C. Population genetic analysis testing each of the three phylogroups separately revealed that recombination with members of the same cluster was so frequent that alleles at different loci within phylogroups were randomly associated (i.e., linkage equilibrium; genes are unlinked on the chromosome). This can only be interpreted to mean that individuals within each population of the phylogroups, irrespective of

geographic location or habitat, were exchanging genes as if they were a single obligate sexually reproducing randomly mating species. This observation was certainly not the expectation of an asexually reproducing species producing cloned organisms, which would have produced linkage disequilibrium (linked genes) results upon testing. Therefore, the above parasexual mechanisms and transduction (phage mediated gene transfer) for gene transfer play an integral role in the evolution of haloarchaea.

However the observed frequent recombination measured in terms of genetic linkage yields limited information: a static view of the current population. It illustrates the outcome of recombination, but not the rate. It gives the impression that a lot of recombination has occurred, but leaves one to wonder how frequently must recombination occur to produce the appearance of sexually reproducing species? Sexually reproducing species undergoing meiosis must recombine every generation. However, there is no reason to think a recombination event in individual *Halorubrum* cells happens between every generation, and therefore the rate of recombination should by definition be less frequent than that for a sexual species. However, we shall see that such simple statements should be distrusted.

The closest estimate for the rate of recombination in *Halorubrum* strains is the relative ratio of recombination to mutation events (r:m); if the mutation rate was known, then the absolute rate could be determined. Using a binning method developed by Ed Feil (Feil et al. 2000) that identifies recombination or mutation events, it was conservatively estimated that *Halorubrum* alleles are twice as likely to be changed by a recombination than by a mutation event, and a change in any nucleotide is nine times more likely to happen by recombination than mutation (Papke et al. 2007). Though not the absolute rate, the estimate does point out that recombination plays a larger role in generating diversity than does mutation alone. Furthermore, and more importantly, the observed relative rate is sufficient to randomize the association alleles at different loci. Interestingly, and perhaps paradoxically, the r:m in *Halorubrum* is much greater than that seen in many obligate sexually reproducing species including humans, pine trees, mustard plants, and fruit flies (Lynch 2007).

What does it mean to the evolution of prokaryotic species when alleles at different loci are randomly sorted among the individuals in a population? To answer this, we again turn to what can be expected from models of sexual reproduction. For randomly mating sexually reproducing species recombination acts as a homogenizing force for maintaining the essence of the species (Coyne and Orr 2004). Indeed, a dominant concept for classifying species is by identifying who will mate with whom, an idea (biological speciation) at least several hundred years old. Because recombination is a homogenizing force, barriers to mating or fertilization must be erected before speciation can occur. Conversely, if barriers to random mating reproduction break down before speciation is complete, gene flow will homogenize the two incipient species back into a single population. The most common form of speciation is called allopatric speciation, or geographic speciation. Here, geographic barriers like mountain ranges, canyons, rivers, continental drift, and non-overlapping ranges will prevent random mating between individuals. The effect of non-random mating produced by barriers is that arbitrary independent mutations accumulate in each secluded population and induce or reinforce permanent barriers to mating (e.g.,

differential mating displays, chromosomal rearrangements inhibiting meiosis), even after removal of the original obstruction.

Is the rate of recombination in *Halorubrum* fast enough to homogenize populations, allowing each phylogenetic cluster to be defined as a unique species based on gene flow? Certainly, the fact that alleles at different loci within phylogroups are randomly associated indicates this possibility. Interestingly, computer simulation studies testing different relative rates of recombination and mutation provide additional information and insight on the required tempo needed to homogenize a population. It turns out that only a modest amount of recombination per mutation (r:m = 0.25–2.0) is required to see a homogenizing effect on populations (Falush et al. 2006; Fraser et al. 2007; Hanage et al. 2006). Given that the r:m for *Halorubrum* phylogroups is within this range and their linkage equilibrium, it seems reasonable to conjecture that a major mechanism for initiating speciation in *Halorubrum* and maybe for a majority of prokaryotes as well would be one or more barriers to recombination, like geographic isolation or an evolved feature of a parasexual LGT mechanisms, like phage immunity. However, it is unlikely to be so simple as microorganisms are known to disperse across great distances and recombine genes between species.

The Advantages and Evolution of Sex from a Prokaryotic Point of View

In the absence of recombination, an asexual reproduction event would simply make a second copy. Since all of the genes found in any asexual individual are linked, all are indefinitely committed to vertical inheritance, locked within the organism itself, and any advantageous mutation rising in an asexual population would also haul along with it the entire chromosome carrying that new allele. Thus, through the process of outcompeting other nearby relatives for the same resources, a single adaptive clone would come to dominate what was previously a genetically heterogeneous species. This process, termed periodic selection, purges the genetic diversity within asexual species and is a homogenizing force causing all individuals in the species to look similarly. Periodic selection events are also the cause of "Mullers Ratchet," a process described by Muller in his widely accepted scenario for the evolution and benefit of sexual over asexual reproduction (Crow and Kimura 1965; Muller 1932, 1964). When a single advantageous mutation sweeps through an asexual population, carrying with it the entire chromosome, any slightly deleterious mutations present will also go to fixation. Since slightly deleterious mutations are much more likely to happen than beneficial mutations, every time there is a periodic selection event, there is the simultaneous accumulation or ratcheting of mutational load. Continuous periodic selection events eventually cause the mutational load to accrue to the point that lethality results and extinction of the lineage occurs. However, bdelloid rotifers seem to be an exception to the rule as they are thought to exist for hundreds of millions of years without sexual reproduction (Judson and Normark 1996).

Sex emancipates the gene from its associated combination of genes (called the chromosome, or genome, or in essence, the organism) allowing the gene to "sample" chromosomal space: a cell, or body is arguably just a host for genes to replicate themselves with natural selection acting on the best gene or gene combination. Unlinking the fate of alleles at different loci (e.g., recombination) promotes the independent evolution of individual traits and prevents Muller's Ratchet. In a sexually reproducing population, unlike its asexual cousin, if an advantageous allele were to arise at a single locus, only that trait (or possibly nearby genes as well) would ascend to dominance in the population, not the entire chromosome. For instance, imagine every human on Earth having a pointed head, yet still different from each other in all other aspects, the only reasonable conclusion would be that selection coupled with recombination drove pointed heads to fixation. Here it is important not to confuse "dominant" traits like brown over blue eyes as having a selective advantage, but just to imagine for the sake of argument that in a heterogeneous head shape population the selective advantage is only men who have pointed heads reproduce, for what ever reason. It would not take long for pointed heads to move completely through the population. Sexual reproduction simultaneously alleviates Muller's Ratchet because slightly deleterious mutations are unlinked and not carried along with advantageous ones.

One major advantage to sexual reproduction brought on by the emancipation of genes is that the rate of evolution is increased over asexual reproduction because advantageous traits invented in different individuals are brought together more rapidly (Fig. 8.1). For example, if pointed heads and no toes were both advantageous (for any imaginable reason) and invented at the same time in different individuals, both traits could be brought together by mating the two individuals (presuming they are opposite sexes). However, if pointed heads and no toes were invented in the absence of recombination, (e.g., in an asexual, clonal haploid population) there would be no mechanism to get both traits into the same individual except mutation, therefore the two traits would be required to fight for supremacy, to the others demise (Crow and Kimura 1965; Muller 1932, 1964). For both of those traits to become fixed attributes (i.e., found in all individuals in the population) in an asexual species, the losing alleles must be reinvented via mutation in the genetic background of the winner (Fig. 8.1): for example, a no toes individual would have to be invented within a pointed head population. Therefore, the rate of evolution per generation in asexual populations is considerably slower compared to that of sexually reproducing species because the rate of fixing multiple adaptive genes is all together reduced (Crow and Kimura 1965; Muller 1932, 1964).

It turns out that *Halorubrum* phylogroups display evidence for the rise in dominance of a single allele at a single locus in a population without losing any of the genetic diversity at other loci (Papke 2009; Papke et al. 2007), implying a sexual reproduction-like evolutionary scenario despite their true asexuality. This attribute is clearly available to only those that experience a high frequency of recombination, and when gene flow is acting as a homogenizing force. The bacteriorhodopsin gene in two *Halorubrum* phylogroups effectively has zero diversity, while the other four examined loci (atpB, EF-2, radA, and secY) maintained high levels of neutral

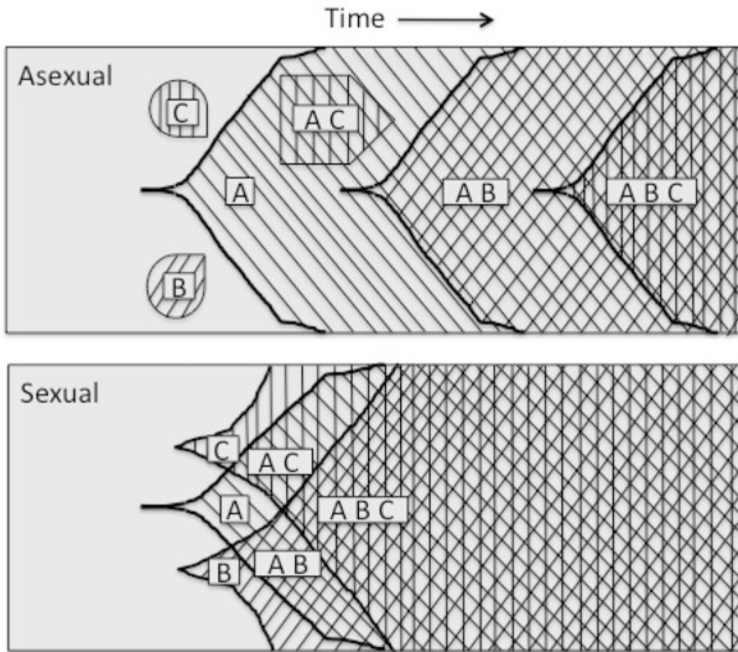

Fig. 8.1 A comparison of sexual vs. asexual populations for the fixation in large populations of multiple advantageous alleles at different loci invented simultaneously in different individuals. The vertical axis represents allele frequency in population space and the horizontal axis is time. "A", "B" and "C" represent the different advantageous alleles at different loci in the population. In the asexual model, the A allele/locus is fixed in the population first as it confers a higher selective value; B and C must go extinct because the individuals in which they were invented were outcompeted by organisms with A. The length of time to fixation depends on the advantage each allele confers to its host over the other locus' alleles. Once B or C go extinct, they can only come together with A if they are reinvented via mutation in the genetic background of A. Thus, adaptive mutations must be fixed sequentially in asexual populations. In the sexual model, the same type of direct competition between genes does not exist since recombination can put the two advantageous loci together after a single generation. The number of generations it takes for all three advantageous genes to sweep through the population is significantly quicker in the sexual model. This depiction is based on Fig. 1 by Crow and Kimura from their publication "Evolution in sexual and asexual populations" (Crow and Kimura 1965)

polymorphisms (Papke et al. 2007). This would be the equivalent of all humans having pointed heads, yet maintaining our individual uniqueness's. If it can happen once at a single locus, it is easy to imagine a pointed head, no toes scenario for *Halorubrum* phylogroups. Therefore, it is not unwarranted to expect another gene somewhere else in the *Halorubrum* pangenome (or population "chromosome") that also has no diversity due to its recently conferred selective advantage.

How is it known that the bop locus (or any other potential locus) recently underwent strong selection and rose to fixation independently of the other loci in the population? As random mutations in DNA occur, many are deleterious or neutral

and some are advantageous. If natural selection were not in play, like in pseudo genes, then any mutation, good, neutral or bad would be recorded in the genetic material at roughly the same frequency. However, if natural selection is exerting itself, the natural state of affairs, biased patterns of mutations will be observed. If natural selection removes mutations that occur at amino acid changing sites (e.g., non-synonymous changes) then there should be observed an abundance of neutral (synonymous) mutations over non-synonymous ones (the dN/dS ratio); natural selection only acts on the non-synonymous changes. This is called purifying selection, and the process prevents amino acid changes and promotes what is called gene conservation; the more important a gene is to life, the less likely a random mutation will be successfully substituted, and the more conserved it is. If there is an over abundance of non-synonymous to neutral mutations then natural selection is promoting change within the protein; this is called positive or diversifying selection and is usually thought to be responsible for adaptation.

With the above scenarios in mind, remember that there were zero nucleotide mutations for the bop locus within the entire *Halorubrum* population, not even neutral mutations. Meanwhile, the other assayed loci were replete with neutral mutations, and zero non-synonymous changes. Because of these specific observed changes, and the concept that mutations occur at a constant rate, it can be surmised that the mutation rate has not had a chance to substitute any neutral positions in the bop locus (though it did for other loci). Therefore the only logical explanation is a recent adaptive allele rose in frequency throughout the population (i.e., a selective sweep of a specific gene, but not a periodic selection event in which the entire chromosome is carried forth). Purifying selection can explain the absence of non-synonymous changes, but not neutral ones; only an insufficient amount of time to accumulate mutations can explain this. Since none of the other loci were affected by the sweep of the bop allele (all other examined loci maintained a high number of alleles in the population), a sexual model that unfetters genes from their biological hosts via recombination must be brought into play. Additionally, the claim for a recent rise in frequency is bolstered by the fact that the bop locus is the most divergent gene when comparing interphylogroup variation, indicating that bop is not highly conserved at all.

For all intents and purposes, haloarchaea are asexually reproducing organisms that experience large amounts of gene flow and appear from a population perspective to be sexually reproducing. How does this impact the way to think about the evolution of sexual reproduction? All of the above advantages and scenarios for the evolution of sexual reproduction focused on the advantages of sexual over asexually reproduction. However, it is very clear from the data on *Halorubrum* phylogroups (as well as many other prokaryotes, e.g.) that this is a false dichotomy; asexual reproducing prokaryotes via parasexual mechanisms, display all of the advantages ordinarily only claimed for sexually reproducing organisms. The seeming paradox can be understood by recognizing that recombination is intimately tied to reproduction in sexually reproducing organisms, while recombination and reproduction for asexually reproducing prokaryotes are independent events. (Perhaps a new unambiguous term describing this third possibility needs to be invented?) Therefore,

the origin and evolution of sexual reproduction can now be asked from a different vantage point; we must now ask what advantages exist for sexual reproduction over lateral gene transfer? Insight to answering this question may be gained when considering meiosis, syngamy, and fertilization as highly derived mechanisms for species-specific lateral gene transfer events.

Part 3: Surveying the Haloarchaea for LGT and Recombination

Metagenome Analysis

High rates of recombination do not appear limited to just the genus *Halorubrum*. Additional lines of evidence signify the magnitude and universal role of recombination in the evolution of haloarchaea. One important observation was made from metagenomic analysis of the crystallizer pond (saturated NaCl precipitates/crystallizes at ∼35 %) in Santa Pola, Spain, near Alicante. In this study, 23 fosmid clones (like a plasmid but with a much larger insert capability) with approximately 40 kb DNA inserts that originated from cells residing in the saturated brine environment were sequenced and compared to the sequenced strain *Haloquadratum walsbyi* DSM16790 for gene order (synteny) and recombination (Cuadros-Orellana et al. 2007). Chromosomal alignments of environmental *H. walsbyi* DNA to regions of the sequenced genome revealed some very interesting trends. Between syntenous homologous genes of extraordinary conservation (e.g. >98 % nucleotide similarity) occurred homologous genes with very low DNA similarity (e.g., 24 %). Sometimes, between conserved syntenous genes there were insertion/deletions of genes and sometimes there were non-homologous gene replacements. This level of diversity can only be explained through frequent gene gain (via LGT) and loss, and the recombination of homologous genes donated from other species. The generated diversity in membrane proteins was hypothesized to be the product of phage predation pressures; much like the human immune system is known to catalyze recombinogenetic variation in pathogenic species. Certainly, *H. walsbyi* is not the only species that exists in the hypersaline environment, nor is it the only prevalent species. Therefore, it is feasible to predict that if *Halorubrum* and *Haloquadratum* are highly recombinogenic, a high rate of gene flow is a common trait among haloarchaea.

Multilocus Sequence Analysis Across the Haloarchaea

Recent sequence and phylogenetic analysis of five different loci from 52 strains representing 14 genera have confirmed the pervasiveness of recombination throughout the haloarchaea (Papke et al. 2010). Twenty-one of the strains represented the genus *Haloarcula*, either as validly named taxa like *Haloarcula vallismortis*, or through

16S sequencing like "*Haloarcula californiae*". Phylogenetic comparison of *Haloarcula* concatenated sequence data and individual genes revealed extremely interesting patterns of seemingly random relationships. Each phylogenetic tree produced a different relationship for all the represented taxa. For instance, *Har. vallismortis*, "*Har. californiae*," *Har. quadrata*, "*Har. sinaiiensis*", and *Har. marismortui* have ambiguous, randomly associated phylogenetic relationships, representing more of a gradient than distinct clusters, suggesting that members of these groups continue to exchange genes despite their apparent divergence and varying cultivation sites (see (Papke et al. 2010) for full details).

Given the absence of clear phylogenetic clusters caused by recombination between diverged groups (different species?) an evolutionary reconstruction for how these organisms are related is difficult to produce and a definite uncertainty prevails in their taxonomy. These data indicate that traditional polyphasic approaches to taxonomy may be adequate for binning groups (e.g., classification), but the underlying process that generated the putative groups remains mysterious and often, distinct groups are not readily apparent. Species definitions impose order on classification, which is clear from the taxonomic code of prokaryotes; however, if no clear understanding of how bins of organisms came to be the way they are is inferred from their evolutionary history, what information can the terms species or common ancestor connote? Are there any true species in which common ancestry can be unequivocally identified? Philosophical meanderings aside, what is clear from phylogenetic analysis of multiple genes from closely related *Haloarcula* species is that there is a significant amount of gene flow between putative species and it clouds species identification in many cases.

What was true of *Halorubrum*, *Haloquadratum* and *Haloarcula* seems to be true for the rest of the haloarchaea. Phylogenetic analysis of the broad haloarchaeal strain diversity, including concatenated and individual genes, revealed that relationships between haloarchaeal taxa were largely incongruent (Papke et al. 2010). However, remarkably, species from the same genus were frequently but not always found together in a cluster. The observations that many haloarchaea are highly recombinogenic, that species within genera exchange genes causing fuzzy species boundaries, and yet still group together in the same genus suggests that recombination can also be thought of as a force for genus preservation.

So what is the significance of recombination as a force for genus preservation? As mentioned several times, recombination is a homogenizing force especially when it happens in a frequent and biased way. For instance, if two different *Haloarcula* species exchange genes frequently, they may or may not become the same species over time (for examples in which there is evidence for despeciation and degeneration see (Sheppard et al. 2008; Zhaxybayeva et al. 2009)), but they will certainly have identical, or nearly identical alleles and therefore are homogenized at those exchanged loci. What are the implications? Imagine that if species from the same genus share genes more frequently than with species of different genera, an expectation of genus homogenization via LGT and recombination is warranted (for example, see the evidence and arguments from (Andam et al. 2010; Gogarten et al. 2002)). That is, two species in the same genus might look more related to each other, not because

of common descent, but because of the identity of the more frequent trading partner. This statement leads to an interesting question: Can the bacterial speciation process at least in part be one of convergence via LGT rather than divergence via mutation?

An example of how convergence comes into play can be learned from the thermophilic anaerobic genus *Thermotoga*. Analysis of several *Thermotoga* genomes demonstrates that their most frequent trading partners are members of the clostridia, as indicated by the vast majority of "*Thermotoga* genes" coming from firmicutes (Gophna et al. 2005; Zhaxybayeva et al. 2009). Of course, that is one interpretation, which accepts the vast minority of genes in *Thermotoga* as being relevant for taxonomic purposes. Another interpretation being that *Thermotoga* is a member of the *Firmicutes*, but received a few distantly related genes like ribosomal protein and rRNA operons, the molecular marker of choice for taxonomic studies (operons are well-known to be frequently transferred, and are formed via the process of LGT (Lawrence 1997; Lawrence and Roth 1996)). It is more parsimonious to think fewer genes were transferred, and thus the later interpretation may be closer to the truth. However, the predominant idea is that *Thermotoga* and *Clostridia* are becoming more closely related because they have biased gene flow (Gophna et al. 2005; Zhaxybayeva et al. 2009). This is an extreme example, but it makes the point clearly. Prokaryotic organisms may appear to be related, not owing to Darwinian common descent, but because there is frequent biased gene flow and this fact must be integrated into our evolutionary paradigms.

What evidence from the haloarchaea might indicate a similar process of genus homogenization? Figure 8.2, a splits tree representation of the relationships among the haloarchaeal taxa, indicates a web-like rather than a bifurcating tree-like relationship. In the figure, it can be observed for instance in the *Halorubrum* section of the "web" (2b), that there are many more edges (threads?) woven between *Halorubrum* strains than there are connecting other genera. In fact, the main artery connecting *Halorubrum* to other haloarchaea is stout rather than web-like, indicating that recombination is occurring more frequently within members of the genus *Halorubrum* than between *Halorubrum* and other genera. The same is observed for *Haloarcula* (Fig. 8.2c). Therefore, it is reasonable to think that gene flow among members of the same genus or possibly higher taxonomic rankings (e.g., see (Andam et al. 2010)) actually acts as a homogenizing force, conserving the fundamental nature of the group.

Part 4: Concluding Remarks: A Connection Between the Contemporary Genetic Snapshot of a Haloarchaeal Population and Conserved LGT Mechanisms

Beginning about 25 years ago, analysis of haloarchaea as model organisms for understanding the genetics of the domain *Archaea* led to great insight. *Archaea* have many attributes in common with members of the *Eukarya* including among others, polyploidy, chromosomal packing by histones, shared transcriptional machinery, and cell fusion, solidifying their relationship and placement as sister groups in the greater

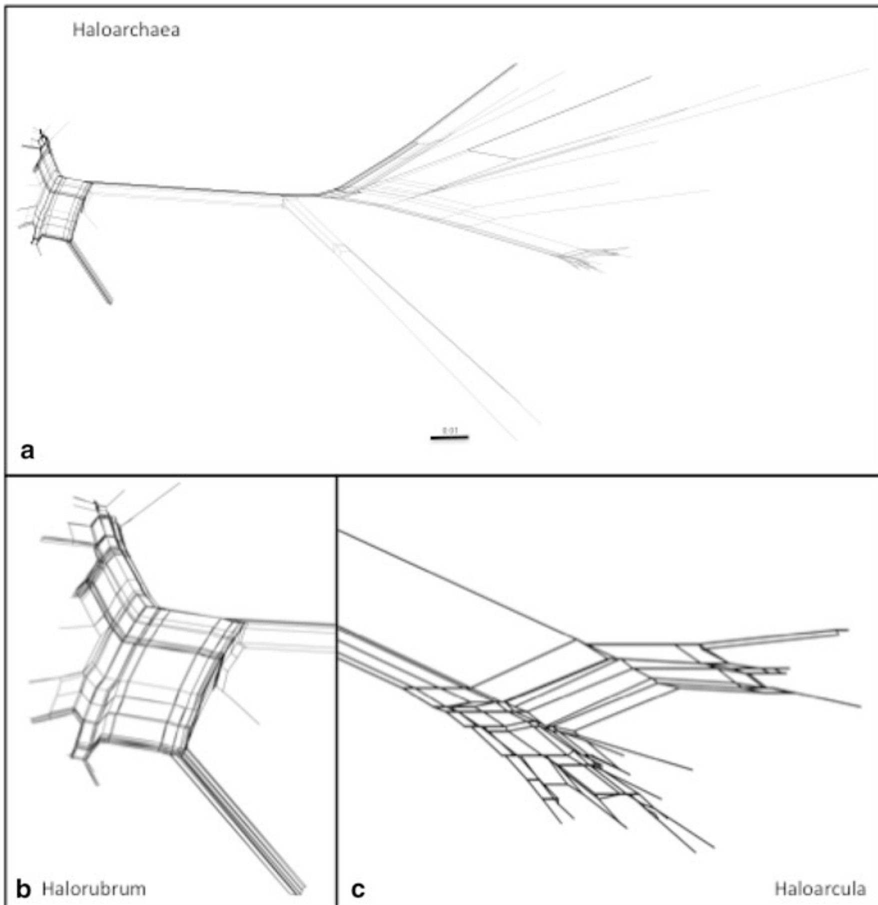

Fig. 8.2 A splits tree analysis of haloarchaeal evolution. **a** Analysis of over 150 haloarchaeal strains from (Papke et al. 2007, 2010). **b** A zoomed view of *Halorubrum* strains. **c** A zoomed view of *Haloarcula* strains. Each edge represents a possible explanation for the evolutionary relationship between any two taxa in the figure. In regions with a high concentration of edges, there are multiple evolutionary pathways to explain the relationships. Notice in panel **b** for instance that there are many more edges connecting *Halorubrum* strains than there are between *Halorubrum* and other genera. This indicates two things: (i) the relationship between members of the genus *Halorubrum* is not tree-like meaning there is a lot of recombination going on. And (ii) there is more recombination between members of the genus *Halorubrum* than between *Halorubrum* and other genera. The same can be seen in panel **c** for the genus *Haloarcula*. These data strongly indicate that different strains within *Halorubrum* and *Haloarcula* are highly recombinogenic, but they are very biased with whom they will exchange genes: *Halorubrum* strains primarily do it with other *Halorubrum* stains and the same for *Haloarcula*. Therefore, it can be concluded that recombination has a homogenizing effect on indivdual haloarchaeal genera

tree of life. However, the archaeal mating system has fallen into a bit of obscurity; during that time period, very little progress was made in determining how mating works.

Continuing in the tradition of using haloarchaea as a model system, our recent analyses using genetics, population genetics, metagenomics and bioinformatics have discovered natural competence in *Archaea*. This system may prove to be another mechanism of natural gene flow in haloarchaeal populations. Gene flow through several mechanisms may in fact be frequent enough within phylogenetically defined clusters to be indistinguishable from the outcome of mixing traits via sexual reproduction, and recombination appears to be ubiquitous and frequent among all haloarchaea. Is a new picture developing regarding the evolution of *Archaea*? Are the insights learned about haloarchaea applicable to other *Archaea*? Other model systems like *Sulfolobus*, though different in ecology and evolutionary history seem to have many of the same attributes of haloarchaea, including frequent recombination (Whitaker et al. 2005) and the formation of intercellular cytosolic bridges (Grogan 1996). By continuing to examine modern relationships between haloarchaeal groups in a systematic way, and simultaneously uncovering the parasexual mechanisms that led to the observed vast haloarchaeal diversity it will be possible to understand prokaryotic evolution with greater resolution.

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Chapter 9
Worth Your Salt: Halophiles in Education

Bonnie K. Baxter, Jaimi K. Butler and Betsy Kleba

An Invitation

Scientists are accused of being myopic-of studying one tiny corner of the natural world for decades. Perhaps microbiologists, who study the tiniest life, are most guilty of this view. The research is important, and such in-depth study provides a strong foundation on which many can build, but the impact of what you do outside the research may be equally significant. We invite halophile scientists to participate in efforts beyond the laboratory. Give a talk at your local library, speak to the newspaper, and go into schools. Work with undergraduates, design field trips for the community, and engage teachers. Our plea is discussed below, with models, rationale, and support from research in the field of science education.

Charismatic Microfauna

Halophiles are a colorful, exciting entrée into the microbial world (Fig. 9.1). These microorganisms can connect students, of all ages, to their environment. In the many years that we have studied Great Salt Lake, we have borne witness to the captivating effect of the mysterious pink water characteristic of hypersaline ecosystems (Fig. 9.2). What makes the water pink? A peek in a microscope reveals the invisible world, with unusual shapes and cell types, and the halophiles in the brine take a student deeper into reflection about the contribution of microbes in all environments. And the power to cultivate these cells brings the learning experience full circle. This perspective, from field to lab (Fig. 9.3), can make microbiology more meaningful for scientists as well as non-scientists (Jenkins et al. 2001; Dyer 2003).

Halophiles also provide an edgy "hook" to engage a young audience as they make great astrobiological models (DasSarma 2006; Baxter et al. 2007). Extremophile science is a dissection of life at its limits. This propels the work into space, as

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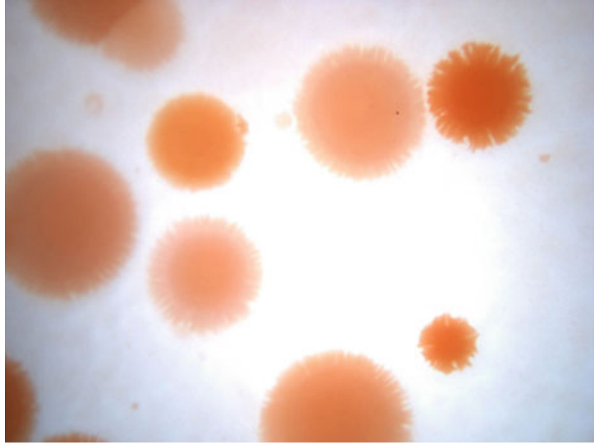

Fig. 9.1 Charasmatic microfauna. Colorful colonies of halophilic archaea grow on salt agar in petri dishes

Fig. 9.2 *Pink* water. A student samples sediment from the brine of Great Salt Lake, colored *pink* by the growth of carotenoid-containing halophilic microorganisms

extremophiles are fantastic models to fuel the very real discussion of where else life might have evolved in our universe. Halophiles are an important component; in particular in relation to Mars (Mancinelli et al. 2004) as the Rover Opportunity team discovered a salt deposit at *Meridiani Planum*. In addition, experiments sending halophiles into space demonstrated the resiliency of these cells to survive and continue vegetative growth after exposure to space conditions (Mancinelli et al. 1998).

Benefits of Outreach Efforts for the Researcher

Many granting agencies, from which halophile scientists may receive funding, require that the proposed project have a significant "broader impacts" (NSF 2006). For

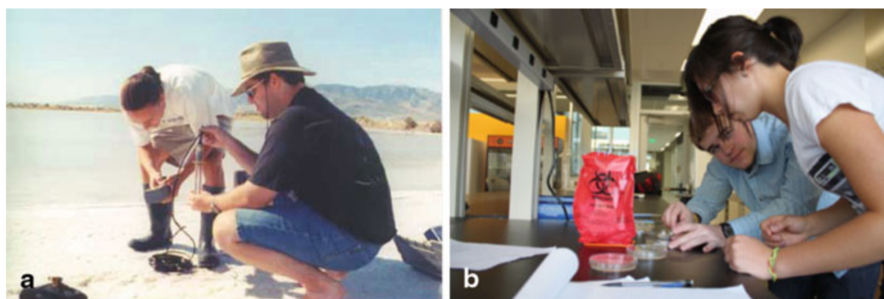

Fig. 9.3 Undergraduate students engaged in halophile research. Students work in conjunction with faculty in the department to isolate halophilc microorganisms from brine or halite crystals and genetically identify them. Panel **a** shows students sampling in the field while Panel **b** shows students in the lab. Halophile research allows the connection from field to lab

the U.S. National Science Foundation, this criterion is concerned with the promotion of teaching and learning, the participation of underrepresented groups, broad dissemination, and a consideration of the benefits to society. Scientists benefit from these types of activities as grants rich in outreach may be given priority for funding, and without such a plan, the grant would not be funded. All researchers who train graduate students will benefit ultimately when selecting from a pool of undergraduate applicants who have research experience or have varied perspectives and backgrounds. And society as a whole benefits as the public at large develops a better understanding of the work we do.

Broadening Perspectives of Life on Earth

Despite the first discussion of "three domains of life" being in 1977 (Woese and Fox 1977), many textbooks in Biology avoid discussions on archaea as a domain of life or lump bacteria and archaea in the same paragraph. Halophile scientists have a good grasp of these genetically distinct groups and can contribute to the general understanding of proper taxonomy. In hypersaline ecosystems, all three domains exist. The microbial communities are rich with archaea as well as bacteria. In addition there are hardy eukaryotes including fungi, algae and other types of protozoa. Scientists familiar with concepts in the tree of life can enrich our understanding of the natural world, and not just through their research, but also through their outreach (e.g., GSLC 2012; NOVA 2008).

Halophiles as Ideal Lab Models

For scientists who do research in the field of halophilic organisms, it makes sense to employ one's own research system in the classroom lab. There is less preparation time and energy, and it is easier to facilitate student discovery in a system with

which you are familiar. But halophiles serve as an excellent system for classroom laboratories even for the pure educator or non-halo scientist for a number of different reasons (Baxter et al. 2005; DasSarma 2012).

Laboratory Issues

Classroom settings are typically comprised of technically unskilled learners whether teaching in grade schools or at the college level, necessitating implementation of facile and tractable systems. Halophilic microbes fill this niche. High salt requirements mean that these microbes do not survive in the environment of the human body rendering halophiles non-pathogenic to people and safe for use in any classroom setting (DasSarma 2006). Moreover, most environmental contaminants cannot survive in the salty media used to propagate halophiles. So even if aseptic technique has not been mastered the risk of contamination, the bane of all culture systems, is reduced preventing time and data loss due to the presence of unwanted microbes. The instructor, however, should be mindful that students are working in a restrictive system and continually need to be reminded of proper sterile technique.

In addition to fewer problems with contamination, halophilic microbes better lend themselves to experimental set-ups in teaching labs. *Escherichia coli* and *Bacillus subtilis*, the conventional microbial model organisms, have much shorter generation times rendering many studies with these microbes impractical for classes that only meet once per week. The longer times required to cultivate mature halophile cultures is a better fit with laboratory classroom schedules that meet on a weekly or bi-weekly basis. In the event that microbial cultures at different stages of growth are required for classroom activities it is just a matter of staggering or staging starter halophilic cultures to meet ones individual needs.

Cost

A perpetual issue faced by educators is the cost of doing science in the form of equipment and consumables. But again, halophiles provide an advantageous system for science exploration in classrooms because propagation does not require specialized equipment or reagents. After an initial investment in standard glassware the primary expense will be components of the media. The recipe for typical halophile cultivation media has been reworked so that many components can be purchased at your local grocery store rather than a biological or chemical supply company (GSLC 2012), Examples include ingredients such as beef broth, brewer's yeast, plant food, sodium borate (Borax), corn syrup and sea salt.In addition, media can be prepared and sterilized using a microwave rather than a conventional and more expensive autoclave (GSLC 2012) and an inexpensive (infrared LED and phototransistor) spectrophotometer can be constructed to measure cell density, as shown in (Fig. 9.4) (TBI

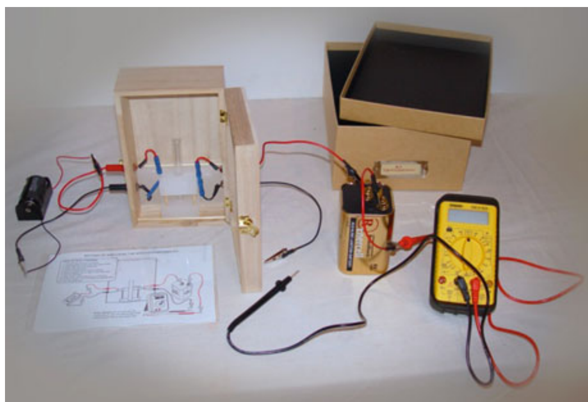

Fig. 9.4 Home-built spectrophotometer. An inexpensive instrument can replace a laboratory spectrophotometer and can be used by teachers in K-12 schools to measure cell densities and teach about growth curves (GSLC 2012)

2012; GSLC 2012). With these improvements, cost becomes a non-issue even for those operating within the confines of tight budgets.

Process Skills

A big focus in science education today is to teach not just content knowledge but the scientific process as well (e.g., Lappato 2008). And a challenge of this method is to demonstrate to students that science is a work in progress where new knowledge is continually being acquired as opposed to science as a series of well-established facts. To this end, classrooms at all grade and skill levels are striving to incorporate inquiry-based curriculums that provide opportunities for more student-driven projects and self-guided exploration. Utilization of halophilic microorganism in inquiry-based curricula is advantageous for the reasons stated above but work in this field also provides learners the opportunity to investigate a relatively understudied group of life forms and see, first-hand, where and how the boundaries of our knowledge exist and can be extended.

One case in point comes from our use of halophilic microorganisms in a genetics class taught at Westminster College (Fig. 9.3). In this course students are learning about DNA, traits and inheritance, and classification while simultaneously isolating and cultivating microbes from halite crystals. Each student is responsible for cultivating their own isolate, extracting microbial DNA, and PCR amplification of 16Sr RNA gene. PCR amplicons are sequenced and then compared to 16S sequences in public databases for identification. Typically, out of a class of about 20 students, a small number of isolates will yield 16S sequences with little identity to sequences in the database from established microbial taxa (e.g., Baxter et al. 2007). This simple series of exercises exposes students to numerous laboratory techniques, a number of genetics-related topics, and simultaneously illustrates that there continues to be whole worlds of organisms that we don't know about compelling learners to explore more!

The corollary to this classroom setting is that students who either directly or indirectly experience isolation and identification of a novel microbe walk away from the course with a tangible connection to the boundaries of scientific knowledge. Moreover, many students take their discoveries from genetics class and request further exploration of their isolates in subsequent classes (for example in a microbiology or cell biology course) or through independent research opportunities in conjunction with faculty in the department (Fig. 9.3). Altogether, this one example demonstrates that halophile research in a classroom setting provides an accessible and attractive hook for students gaining experience in science.

Acquisition and Relevance

In promoting the use of halophilic organisms in the classroom two criticisms are routinely offered for discussion. The first pertains to access to and acquisition of halophilic microbes and the second questions the broader relevance of these extremophiles in the context of the more focused content of specific scientific disciplines or courses (i.e., cell biology, medical microbiology, environmental science, etc). We are grateful to have the opportunity to formally address these important concerns and wish to alleviate whatever doubts remain about the feasibility of incorporating halophilic microorganisms into any learning environment.

First, acquisition of halophilic organisms is easily remedied. If you're fortunate enough to live near a saline body of water, a salt-mine, or a factory that releases brine effluent into the environment all you need to do is take a trip to one of these sites to collect environmental soil and/or water samples for subsequent cultivation in salty growth media (assuming permission from the appropriate authorities has been granted). If these types of environments are not readily accessible, *Halobacterium* sp. NRC-1 can be purchased inexpensively from Carolina Biological Supply Company (CBSC 2012). Thus, researchers and educators can obtain halophilic organisms regardless of geographic location.

Finally, but probably more importantly, the concern about relevance of halophiles to different students in different disciplines is easily overcome by focusing the class of interest on core themes within the discipline and allowing students to explore these core themes in the context of the safe and tractable system that halophilic microbes provide. For example, students in an environmental science course could use halophilic microbes to explore changes in community composition after environmental perturbations; cell biology classes can use halophiles to study gene expression, properties of biological membranes, and how environmental conditions influence protein function; medical microbiology classes can use halophilic microbes for units on microscopy, staining techniques, selective and differential media, and microbial growth and nutrition. To reiterate, halophiles serve as an excellent system to model basic concepts or illustrate fundamental skills in a number of different fields across many disciplines.

Support from Research on Science Education

A science education plan needs to fit the people and institutions involved. Whatever teaching endeavors halophile researchers engage in, s/he should employ elements of effective pedagogy in science. Scientists may appreciate that there is a body of evidence-based research on the topic of effective teaching, including numerous studies on engaging young students in research.

Inquiry-based Learning

Research in science education in the last two decades resulted in a variety of documents that called for change in the way we teach science (e.g., AAAS 1993; NSF 1996; NRC 2003; Lappato 2008). Each document points to the significance of inquiry-based learning and teaching as a valuable tool to gain knowledge. It is argued that inquiry-based instruction can engender an understanding of the nature of science, allows for a more rigorous understanding of scientific concepts, and engages students at a higher level of cognition.

Teaching science as discovery is distinctly different than instructing by memorization and lists of "facts." Who better to employ such real-world techniques than the active researcher? For halophile researchers who also spend time in the classroom, exciting students by allowing them an active role in inquiry results in a higher caliber of training and builds excitement about this field. We have employed such techniques in the National Science Foundation funded *Great Salt Lake Project* where outcomes of students were measured (Baxter et al. 2004). Students showed real shifts from seeing science as a product to knowing science as a process.

Undergraduate Research

Undergraduate student-faculty collaborative research is a powerful way to ignite and engage young scientists. The benefits of research in the undergraduate curriculum are far-reaching (Bauer and Bennett 2003; Elgren 2006; Lappato 2008; Fechheimer et al. 2011). Students develop sophistication in critical thinking skills and reflection. They learn to apply content knowledge in the field and laboratory. Undergraduate researchers are accepted to graduate and medical school and receive fellowships at a higher rate. One study reported that undergraduate research program participants, went to graduate school at a rate of 80 % versus 59 % for students who did not do research (Bauer and Bennett 2003). In addition, faculty mentors are enriched by these scholarship endeavors in their disciplines as they engage deeply with their students, publish their work and present at conferences. Finally, the institutions themselves clearly benefit with better student outcomes and a richer faculty.

Lappato (2008) reported on the effectiveness of undergraduate research and presented data on student and faculty outcomes. He administered surveys and other instruments to gain information on specific qualities of the experience, including contact hours with mentors, academic year research versus summer work, goal-setting, and career paths. This study measured enormous benefits for the student, the professor and the institution. Another study demonstrated marked increases in grade point averages for young students engaged in research (Fechheimer et al. 2011).

What is not measured here is the excitement generated about specific areas of study. Halophilic undergraduates can publish papers (e.g., two student authors in each study Baxter et al. 2007, 2011), present their work, and otherwise participate in science, albeit with a smaller time commitment than graduate students. Those of us in the field of halophiles should endeavor to excite younger generations about what we do, ensuring future scientists that will keep the field of salty science moving forward.

Outreach

Some academic institutions have an outreach department to coordinate school and community efforts that get faculty members beyond their campus and laboratories (e.g., TBI 2012; GSLI 2012; GSLC 2012). A call to the director can give you many ideas where you can supplement outreach efforts of others within your institutions. With infrastructure in the form of outreach staff, the scientists can contribute their expertise with minimal time and organizational responsibilities. One such example is summer camps, which may be offered to students as a recruiting tool for attracting talented and motivated university students. Other examples include participating in professional development opportunities for K-12 teachers, who are thirsty for science content and fresh ideas for classroom projects.

One novel example where undergraduate research meets outreach are the summer camps held by Great Salt Lake Institute at Westminster College, Utah (GSLI 2012). In several consecutive summers, undergraduates mentored by faculty members performed authentic research around Great Salt Lake, and then these students became mentors themselves (Fig. 9.5). Their research methods were incorporated into a science-based summer camp for high school students and paid high school teachers. The teachers, along with the undergraduate researchers, chaperone the campers and build camp curriculum, which was beta tested during the summer camp. The tested activities were subsequently published as web-based curriculum for use by a larger number of teachers, and hard copies (with equipment) in a lake "tool box" may be checked out through the local state park and natural history museum for free. (GSLI 2012; GSLC 2012).

Using this model of outreach, undergraduate researchers work in concert with teachers, high school students, community, and faculty members for the benefit of everyone. This real-world learning experience using topics that engage student

Fig. 9.5 Outreach into the community. Outreach efforts go beyond your institution and may involve members of the community, teachers, or school kids

interest can both lead to a higher level of learning and promote active engagement and interest by students (Blackboard K-12 2007; Sandoval 1995).

Conclusions

We are scientists, often funded by the public, and it is our duty to reach out to that public. Halophiles are a fantastic choice as a vehicle for broad dissemination for all the reasons discussed above especially because they allow insight into the environment using laboratory tools (Fig. 9.3). Take the time to excite non-scientists about your work or support your staff and students in such efforts. The benefits are far-reaching for the researcher and for the community. Outreach efforts could expand your own horizons and inspire an entire demographic of people otherwise unlikely to be involved in the sciences.

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Chapter 10
Halophiles in the Public Media

Russell H. Vreeland

This is simply a lagniappe chapter but few scientific books (and certainly few if any academic programs) actually deal with this rather arcane but none-the-less important aspect of a scientific career. Most of what is stated here comes from my own experience with media interviews of all types. This is really not a chapter about halophiles (although there is a section on them) this is more a chapter about communicating about halophiles to the general public. It is also being written because it is kind of fun to consider. Many traditional scientists feel that being the subject of an interview by a journalist, writer or heaven forbid television personality ranks somewhere behind having appendicitis or a root canal without anesthesia. Truthfully this is simply wrong and is, in the long term, bad for science. In this short chapter I will present some of the reasons why scientists should consent to interviews, perhaps even actively soliciting those opportunities. I will also try to provide some hints about how to handle them when they occur, what to be prepared for and a bit about how to dress. Finally I will try to provide some information on how to be sure your interview is satisfying and what you can do to be certain your information is correctly represented since you will not always be able to "proof read" the quotations before they appear.

Interviews about Halophiles

One of the first things that must be understood about working with the media for the general public is simply that few if any reporters will know anything about halophilic bacteria. So it is up to the interviewee to do a bit of educating. Since they know little about halophiles in general trying to discuss the differences between slight, moderate, extreme and halotolerant halophiles is a complete waste of time unless it is absolutely necessary. This is not a pejorative statement: as is discussed below, a journalistic education does not generally leave one much time to learn

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about scientific nuances. One thing that does need to be made clear is that there is a difference between things like halophytes (plants) and halophiles (as used to mean salt loving bacteria) halophiles can mean any organism that needs salt. Some other terms that may need to be clearly defined include *Archaea*, *Bacteria*, osmotolerance vs. halotolerance, pure culture vs. mixed culture, enrichment culture and media vs. medium. When defining each of these terms a good rule of thumb is to utilize common non-technical words (you will see this statement several times). So it is a really good idea to gloss over differences like ether vs. methyl-ester lipids, or presence/absence of peptidoglycan etc. Another thing that may need to be kept clearly separate and is obviously related to halophiles is the chemical definition of salt as opposed to strictly NaCl to the general public and most reporters there is really only one “salt” that is the kind you may want passed to you at meals. Also most reporters actually appreciate friendly comments regarding the various adjectival forms of these words as well as their respective singular and plural forms (bacterium vs. bacteria when not speaking about the Domain) and how these apply in the article being written. There is however an instance where halophiles and various types of salt crystals are especially valuable, that is as visual aids or props. A bright red culture or plate filled with red to purple colonies shows up very well in photographs. Crystals are also good as long as they are large enough and easy to show. Something about 5 cm on a side, with some detail that can be lighted works very well and are especially useful during any preliminary talks or when taping a TV spot. Other things that will help an interview about halophiles will be some environmental context. High quality pictures of salterns, or microscopic images always go over well. Sometimes good thin layer chromatography plates or easily visualized, electrophoresis gels are useful but they likely will be cut in favor of a picture of a cell swimming around. At the same time try to avoid charts or graphs that need explaining unless they are for background. Even really neat images of odd shaped halophiles do not work well and are often omitted since both reporters and the public will actually have trouble seeing them.

Why Agree to be Interviewed in the First Place?

This decision is probably the most important one you will make because once you open the office or lab door it is not only very difficult to close it again but stopping the interviews will take a long time. So why agree to this in the first place? Most scientists agree that what we really enjoy is working in the lab or in the case of lab leaders, writing our papers, grants and analyzing data. It is, after all, why we got into this field in the first place. That is fair enough but we need to face reality here. First that grant we want to write and have funded. Certainly it will be reviewed by scientific peers, it will be heavily critiqued and hopefully it will be funded. If your grant does receive funding quite literally from any source, those funds can ultimately be traced back to the non-scientific public. It really doesn’t matter simply look at Table 10.1. While this is a generalized view of the overall scientific funding sources it is fairly easy to see that all research funds come from a relatively general source.

Table 10.1 Sources of Funding for Research and Interest in General Media Publishing of Research Results

Organization	Source of funds	Interested public	Media type	Reason
Government Agency	Taxes	All Taxpayers	All media sources	Public scrutiny, politics, accountability
Foundations	Endowments, Donations	Donors, Board members	Annual reports, newspapers, magazines, TV, Radio	Need to meet goals, increase donations,
Non-Government Organizations	Donors, some endowments	Donors, Board members, Government auditing	Annual reports, Newspapers, Organizational publications, TV, Radio, local groups	Increase donor awareness, meet goals, proof of non-profit status, general PR
Corporations	Gross revenues	Stockholders and Board of Directors, General Public	Trade journals, Corporate reports, Possibly other general news media	Corporate image as "good citizens," Budget justifications, taxes and general PR

Consequently, it is always important for the funding agency to be able to justify why they are involved in supporting one research project over another, especially if the project is expensive, occurring for multiple years or is even a bit tangential to the primary mission of the organization (this is especially true of corporate sponsorship). Simply put there is no better way for any of these sources to justify funding scientific research than to be able to point toward articles in the popular non-technical media. Despite what we scientists want to believe our highly technical publications (unless they are in a very few top tier journals) really don't provide as much notoriety for our funding sources as does a single well placed newspaper article or a couple of TV minutes. I like to think about it in other terms. Given the fact that scientific funding ultimately comes from the non-scientific public I believe that we have a duty to let them know the results of the work they are paying for. We would all demand that from a mechanic or even an MD, we should demand it of ourselves. Exactly when such articles should appear in relation to the overall process will be discussed later in this chapter. For now let us look at other reasons why we should be willing to submit to interviews.

Every scientist that works for more than a few years will experience the ever present budgetary cycle wherein funding levels both increase and decrease. This is especially true with the primary funding sources of various governments. Most scientists, historians and even economists have recognized that levels of commitment to scientific studies are somewhat of a harbinger of future economic conditions within a country. Historically, high funding levels of one decade will be followed by relatively robust economic development and increased new consumer products over

the ensuing decade or even two. In other words, developments in our laboratories (especially those conducting "basic" research) require nearly 10 years in order to reach the general economy or into the household. Yet it seems that whenever a budget needs to be reduced science investment is the first thing to go. Actually that is not a perception and science funding does get cut quickly. I once asked an expert in the US Federal Budget from the non-profit Center for American Progress about this aspect. His confirmation of that perception was interesting especially when followed by the comment that science is the "low hanging fruit primarily because it doesn't have a strong public visibility, understanding and notoriety." Basically while everyone acknowledges the need for science and scientists, they are simply not made aware (ergo constant media presence) of all the things that scientists do. That is not true of medical science which constantly hits the news, but it is true of the non-medical fields like work with halophilic bacteria. So in my opinion all scientists should make an effort to let the media know about discoveries (major or not). Someone will ultimately pick up on it.

I think there is another reason why scientists should seek out and try to work with the media and that is simply to be sure that they get it right. If we become sources and resources for journalists we can all fact check valves for stories. Despite what many want to claim, journalists do want to get the story correct but they are not all trained in science and they are forced to interpret detailed technical jargon in many fields. So imagine a journalist who picks up something they find of interest and wants to verify or have a discovery explained. Due to deadline pressures or time zone differences (generally both) they may not be able to contact the author (or even worse the author is someone who won't speak to the media). If they have no scientific resources they may simply write what they think is correct and they get it wrong. But if they have a scientist they can call there is a far better chance the story will be correct.

Source or Resource?

Many scientists might think of themselves as a resource that is not being used by the media. We are willing to help them get it right, they simply don't bother to call. However, a science writer that I respect quite a bit explained it to me in a very different way. What she told me was that before one can be a resource they must first be a source. Scientifically we might not really see the difference but at the journalistic level there is a distinct difference. First of all a source is someone who brings newsworthy material to the notice of the journalist. That material must be explained simply and clearly and must be accurate. It may be early in the scientific process; but, since nearly all science writers are now free-lance the preliminary information can be used by the journalist to prepare and sell a story. Alternatively those of us in science can often see something that is not getting much press but which we recognize will either become a major breakthrough or may change the interpretations of significant issues. These are the sorts of things a valued source brings to the journalistic table.

A word of caution is appropriate here. That is that we must always respect the overall scientific process. So unless one trusts a journalist and has a track record with that individual nothing should be released until after an article is properly peer reviewed and published. One can give information in advance only so long as it is clearly (and explicitly) understood that nothing can appear in popular media until the key manuscripts are published. Also, at no time should anyone provide information about a paper or grant proposal when that individual is acting as a peer reviewer even if we know that what we have will win a Nobel Prize.

After all of that is said and done and one has become established as a good source might one then become a resource. Now the system shifts a bit and rather than needing to contact the journalist the scientist may become the one contacted. In this case you might be asked to examine a newly published paper and to provide a comment for an article. The journalist might call and ask if you think something they have found is any good or worth investigating or writing about. I personally think this is a very important event since a resource is really able to help make sure the public gets the correct message. This is probably as important as being a reviewer; some might argue it is even more important. It is also a time to be on ones finest professional behavior. Forget the fact that author of the paper has spent the last three years beating up your last paper, or that you don't like them very much, or they published in a journal that rejected you. None of that really matters and the media person calling you doesn't care as long as you give a fair and honest appraisal of the work. If however, you really can't provide a non-personal review, be honest and say so, respectfully decline to comment and preserve the relationship. When I have spoken with reporters I know they value that honesty as much and anyone, further, no reporter wants to get a negative quote then find out that you and the author have had a running feud for the last 3+ years.

Being Interviewed

So assuming you have gotten yourself into the position of being interviewed as a scientist and expert in an area what should you say? Obviously the specific answer comes from the question being asked and how you have examined the material sent to you. Recognize that if you are a resource the reporter/writer will almost certainly send you the article in question so be sure to read it and think about it carefully. However, since I have been interviewed more times than I care to count I think I can provide some useful hints.

I do have one golden rule about interviews and interview questions but first a bit of background. In 2000 following the publication of the Vreeland et al. (2000) paper in *Nature* I and my colleagues Bill Rosenzweig and Dennis Powers found ourselves swept up in a media frenzy. It went on for several weeks and I know that I personally gave over 600 different interviews during that period. They were actually scheduled for every half hour starting at 7.00 for the early shows and going sometimes to midnight for the morning shows in Europe. So you can imagine how often we were

being asked the same questions. At the very beginning all of us agreed to adopt this one golden rule—that was that no matter which interview we conducted # 1 or # 600 or how many times we were asked the same question “How did you react when you first saw the live microbe from the salt crystal” or some variation on that theme we remembered that while this was the 453rd time we gave the answer, it really was the FIRST time that reporter had asked it. Consequently we all tried to make sure that our answer carried with it the same intensity and fascination we had the first time it was asked. That wasn’t always easy but sometimes reporters would add “I’ll bet you have been asked this 200 times already” and we could respond “yes but this is the first time by you” and believe me that would change the entire tenor of the answer and the entire interview. So again the first rule of what to say is to be honest.

With the golden rule established what else should one say? First, when answering a media question avoid the scientific and subfield jargon unless it is impossible to avoid but then be sure you are ready to explain the meaning in clear terms. I once had a reporter confide to me that their newspaper editors refused to assign one person to the science beat. When I asked why they told me that every time they did make such an assignment the reporter became so interested in science they would not write about anything else! So science interviews are now often conducted by non-science majors and the professionals need to be prepared to explain concepts. These may not get into the article but the understanding always helped make the report correct. When providing the answer, try to keep it relatively short and direct (although I and most scientists fail at this leading to a great source of laughter for reporters). Don’t try to make up your own sound bites I have found that most reporters are better at finding those than I was and would often ask something like “would it be accurate (or okay) to say?” then they would re-phrase my answer into the sound bite or quote. If it was accurate (or correctly reflected my statement) I would simply say yes and let it go.

This does bring up a major, frustrating difference for both groups and one that is sometimes hard to get over. In science we are taught to carefully and accurately analyze and portray the results of experiments. Often that means parsing every nuance from the data, while at the same time trying to recognize that there is always room for a different result. However, journalists are faced with a completely different problem in that they must accurately portray a result in a column that may only be the size of an abstract or a 30 s to 2 min report. So while a scientist is often upset with the slightest inaccuracy in the report (or even being held to a definitive statement) the journalist is upset by long detailed answers that can’t possibly be given in a short column space. Also while it may not be desirable we must all recognize that newspapers, many magazines and certainly radio and TV reports are often reduced to grade school reading levels. So the accuracy that is given by large technical jargon simply does not translate. This can be a problem, without a real solution. However in my experience when these situations arose I found it easiest to first discuss this problem with the journalist (even live broadcasts often have a preliminary interview) or to simply admit that the question required a very technical answer. Often this would result in a change of the question in a way that could be answered better. With print media interviews it was also expedient to try to provide some sort of simpler analogy

which would accurately convey the result while being understandable. Once again if one goes back to the first part of this discussion the goal is to get the information out to the general public that pays the bills for the laboratory. Consequently, at least in my view, it was often better to sacrifice absolute exactitude for a clearer message, as long as that message was accurate.

Being Prepared

Being prepared for an interview may be almost impossible and is definitely not the same as preparing for a seminar, or a lecture or really anything most scientists experience. For me being prepared was about having my own background material at hand, knowing the primary focus of the interview (which I should do anyway) and by all means examining any materials sent to me by the interviewer. However, during the course of time I mentioned above I learned a few other things about being prepared. First, was to make sure I was comfortable even for a phone interview. That meant making sure I made any necessary rest room trips. It also meant having something at hand for a drink when I could get it. Second, be certain that everyone around you knows about the coming interview and has asked for any advice or supplies before it starts. Being interrupted during the interview is not only difficult but as I mentioned above sometimes you are trying to come up with an accurate or simpler analogy to express a difficult point in a more understandable way. Third, I would recommend underlining or highlighting those passages of either my report or the report I am discussing that I want to address. This sometimes meant even writing myself some brief notes about why I wanted to address those passages. Fourth, close your office door or if you don't have a door set the interview up somewhere quiet. There are several reasons for this. One is that background noise in a lab (equipment, students, hallways or other phones will distract both you and the interviewer. That is especially true for anything on air although in many of those instances there will a director present to help minimize ambient problems. A second reason is that if an interview is going over the phone, connections can be bad, alternatively, the interviewer may be someone for whom your language is secondary so you will need to concentrate on what they are saying. There is one final aspect of being prepared; that is to decide beforehand how far you are willing to go in specific instances. As a couple of personal examples, in working with a journalist for a children's magazine we used a variety of analogies and short comments that were simply meant to get the kids interested and curious, so some of the accuracy was lost. At another time during an adult radio program on science those of us involved did not permit erroneous comments about the age of the Earth to go unchallenged and responded that the caller was simply wrong. So the bottom line is set your limits and stick to them as best you can before the connections are made, it will make life much simpler.

Dressing for Success

Obviously this section is aimed more at a live (or taped) television interview or a live sit down press conference since in a radio or newspaper interview it really doesn't matter what you are wearing. Generally when one is on television the directors will ask for pastel colors, nothing really bright or flashy. Most of the time light blues and pastel yellows were the best things. At the same time people do expect to see a scientist in certain types of garb so in the laboratory wearing a lab coat is sometimes requested. On a major live broadcast a suit and tie or at least a good comfortable button down works well. One thing I did learn to avoid however was clothes cleaned with those high intensity whitening detergents. In truth the whitening agents are chemicals that actually pick up and reflect the intense lights in such a way that one seems to glow on the screen. Otherwise I found that being comfortable was again most important. Once again it is critical to remember that the goal of any science media interaction is getting a complex message out in a somewhat general manner and no matter how anyone wants to portray it someone who looks neat, well dressed and well groomed is always looked upon as having more authority.

The Interview Itself

Once a scientist agrees to do an interview there is very little that can be done to actually control what happens. The controlling party will be the director (if it is a TV moment) or the interviewer if it is a non-visual medium. There are however a few things that I found which can help. First and foremost is to ask for details about how the interview will be conducted. What will you see and hear, particularly in a live TV interview or even a radio spot. I once sat for a TV interview early in the morning. I was placed in a green room on a chair facing a camera with an earphone in one ear. I was told that everything I said would be held on a seven second delay (basically to catch swearing or improper words) I was also asked if I wanted a monitor on "So I could see the interviewer." I said yes since I felt it would be more personable. However, when I began my answer both the visual on the monitor and the speaker in my ear were relaying my answer with the seven second delay. I was completely tongue tied, they realized what occurring and killed both things but it was too late. Had I asked for more details I could have avoided the entire mess.

Another thing to understand is that the interviewer may have experience with scientists but will likely not be an expert in microbiology or particularly with halophilic microbes. Consequently they are likely to ask questions that will require long or even detailed answers. When that has happened I have always found that it is better (even in a live interview) to first comment that the answer is rather involved. Often that simple statement will cause a change in the question. Another hint is to make sure to delay a bit before answering even when the answer is in hand take a deep breath and frame the answer before you speak. Frame the response carefully, without resorting to jargon. Keep the response straightforward without any side comments

and use as few words as possible. Doing so will generally help to be sure that what is reported is correct. Visual displays that illustrate the point may also be helpful and once again will provide a mechanism to ensure accuracy. As stated before however, the goal must be clarity and understanding at the non-scientific level. This is where the real balancing act may be most difficult since it will require a considerate answer that is as accurate as possible without sacrificing understanding. In general during every interview I have had the earliest questions are the easiest to answer since they are primarily background. To me the most difficult questions came later when I was asked about things like potential implications or significance. Finally at the end of any interview (except for live broadcasts) ask for an opportunity to check the accuracy of the report. My experience is that most reporters will gladly provide it and will welcome corrections (especially grammar like bacterium [sing.] vs bacteria [plural]). At any rate if they say no and it comes out wrong scientists can console themselves with “Well I tried!”

Press Conferences

All comments aside, a press conference is an interview on anabolic steroids. Think of it as a combination professional seminar with all of the problems and vagaries of that while at the same time being an uncontrolled feeding frenzy. While they may not look like it press conferences are probably the hardest media involved interaction one can experience. Usually a press conference is set up by someone other than the scientist, possibly a granting agency or more likely the PR department of a university or corporation. The person setting it up will put out a short press release (which may or may not run past you first) which will serve as an invitation to attend. Consequently the people attending will be expected to have some interest in the topic. However, that may include anyone from the President of the company, to your college Dean, certainly your immediate supervisor local or national politicians and any number of media. Often the local media (particularly TV) will send what are called stringers. These are people in vans with camera equipment who will film the event and then send everything back to a central point from which all local and national channels will draw their video feed. This is where it can be troublesome since any mistake or miscommunication ends up magnified several fold. In one of my own Press conferences the stringer misidentified the primary crystal used for the sample with an example of one that was an example of a rejected sample. The two were clearly different but numerous stations aired the wrong one and scientists from across the country climbed all over us. Basically in a press conference you get one shot so it needs to be carefully arranged. I hope that the following couple of paragraphs will help if you have to get into one.

Basically a scientific press event will be a two stage thing. The first stage will involve the person who organized the event making a brief announcement of why folks have been invited. They will then turn to podium over to the principle scientist who is supposed to make brief comments. You should do a couple of things at this

point. First keep the comments very simple and straight forward. I recommend that you avoid showing or talking about comparisons (see the above paragraph) instead just stick with the positive material about the discovery. Everything should be as easy to see and understand as possible. Second at no point in the comments should the scientist waffle or admit that there is a chance the data conclusion is wrong. Any professional knows that no matter how hard we work there is always the chance (i.e., less than 5 % probability) of an error. In fact the worst thing one can say is to invoke something like "this experiment has a 95 % confidence interval." This will almost certainly come across as being wrong since news media too often consider being objective as always presenting an opposing view. If you want an example of that we need look no further than the interminable debate over global climate change. Nearly 1,000 scientific articles have been published on the topic, all of them say it is happening and humans are the cause. The problem is that all of these papers (and especially the models) early on admitted an uncertainty in either the timing, or in the severity of the increases. Professionally that is what must happen, it is a good thing. But that uncertainty was also given prominence in the early going and now despite the near unanimous scientific opinion 50 % of all news reports (even today) mention the uncertainty and present someone who says nothing is happening. So in the press conference situation stick to the certainties and stand on them, take the positive position "No there was no contamination we checked," or "No these data are solid and the conclusion is valid." Basically leave the uncertainty in the main paper. If the media want to read it (they will) or if they ask about it then you can frame the answer in the positive. As I stated keep the presentation short 5–10 min, keep it positive and make it easy to see. That will get you to the second part of the media event.

During the second half the floor will open for questions. Sometimes the moderator will restrict the first questions to reporters, often not because the reporters may want to hear answers to questions raised by other professionals in the room. This is where things can get ugly so this is where the principle needs to be at the top of his/her game. I have found that once the floor is opened the questions can come from all directions and at all levels from the relatively easy request for general information (like what is a halophile anyway) to very heavy attack type (such as "How can you possibly be certain you eliminated contamination?") and even to follow-up questions from reporters and anyone else. Most of the scientific press conferences I have been around are more sedate than those shout fests for politicians but the goal is always to answer as many questions as possible. So the only advice I can give here is first to keep the answers as short and direct as possible. Second, don't allow one person to ask repeated questions as this only upsets the others at the event (unless no one else has a question). Third, stick close to the data in the paper and don't allow yourself to get off into conjecture or hypotheses. Fourth, in giving the conference one should not be afraid to answer with "I don't know the answer to that" or "That is something that needs further research" those answers will help keep you out of hot water. Fifth, I tried to always give credit: Telling someone "that is a great question" makes the questioner and the audience feel good and makes them feel that they have followed your presentation. In fact if that is done early it actually lessens the number and

difficulty of subsequent questions, it actually shortens the entire event. Finally it is a good idea to spend some time with the event organizer to set up a subtle signal (very subtle almost natural) so that individual knows when to takes responsibility for ending the event. Once they stand up and state "One more question please" things wind down quickly and the principle doesn't look like they are trying to escape which minimizes the shouted questions.

Wrap Up

As I stated at the beginning of this short chapter dealing with the media is not something most scientists wish to do. I have heard some tell me that it is the last thing they would do. However, done properly it can be a very satisfying thing that helps science on a wider framework than the one-off publication. It is increasingly necessary in our information world to help the public understand how science is done, what the issues happen to be and how the data that we produce is useful. Dealing with the media also helps scientists be seen as more approachable, more down to Earth and more understandable. In the end having a wider range of scientists featured in the media will invariably stimulate the interests of more young people and could easily be the springboard to their wanting a career studying halophiles. The truth is a single media event held in one country could actually help develop the next generations of scientists in many others and that should be the goal of all of us. Try it you might find that having a root canal is worse.

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In the original version of chapter 3, two chapter authors' first name and last name were tagged incorrectly. The book has been updated with the correct author names.

The name of the authors should read as follows,
"de la Haba, Rafael R", where "de la Haba" is last name and "Rafael R." is first name.
"Márquez, M. Carmen", where "Márquez" is last name and "M. Carmen" is first name.

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