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Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications

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The phylogenetic diversity of microorganisms living at high salt concentrations is surprising. Halophiles are found in each of the three domains: Archaea, Bacteria, and Eucarya. The metabolic diversity of halophiles is great as well: they include oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens. The diversity of metabolic types encountered decreases with salinity. The upper salinity limit at which each dissimilatory process takes place is correlated with the amount of energy generated and the energetic cost of osmotic adaptation. Our understanding of the biodiversity in salt-saturated environments has increased greatly in recent years. Using a combination of culture techniques, molecular biological methods, and chemotaxonomic studies, we have obtained information on the nature of the halophilic Archaea as well as the halophilic Bacteria that inhabit saltern crystallizer ponds. Several halophilic microorganisms are being exploited in biotechnology. In some cases, such as the production of ectoine, the product is directly related to the halophilic behavior of the producing microorganism. In other cases, such as the extraction of β-carotene from *Dunaliella* or the potential use of *Haloferax* species for the production of poly-β-hydroxyalkanoate or extracellular polysaccharides, similar products can be obtained from non-halophiles, but halophilic microorganisms may present advantages over the use of non-halophilic counterparts.
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Introduction

Life exists over the whole range of salt concentrations encountered in natural habitats: from freshwater environments to hypersaline lakes such as the Dead Sea, saltern crystallizer ponds, and other places saturated with respect to sodium chloride. The diversity in the properties of saline and hypersaline habitats on Earth is reflected in the great diversity within the microbial communities adapted to life under the prevailing conditions.

Many hypersaline environments originated by evaporation of seawater (so-called thalassohaline environments). Their salt composition is similar to that of seawater: sodium and chloride are the dominating ions, and the pH is near neutral to slightly alkaline. When evaporation proceeds, some changes occur in the ionic composition due to the precipitation of gypsum ($CaSO_4 \cdot 2H_2O$) and other minerals after their solubility has been exceeded. NaCl-saturated thalassohaline brines, such as found in saltern crystallizer ponds, often display a bright red coloration due to the large numbers of pigmented microorganisms they harbor. Athalassohaline hypersaline environments, in which the ionic composition differs greatly from that of seawater, are likewise populated by microorganisms. A prime example is the Dead Sea, a lake in which the concentration of divalent cations (presently about 1.9 M Mg^{2+} and 0.4 M Ca^{2+}) exceeds that of monovalent cations (1.6 M Na^+ and 0.14 M K^+), and in which the pH is relatively low (around 6.0). Even such a hostile environment periodically supports dense microbial blooms [14]. Microbial life has adapted to environments that combine high salt concentrations with extremely high pH values. Alkaline soda lakes in Africa, India, China, and elsewhere with pH values of 11 and higher and salt concentrations exceeding 300 g/l are teeming with life. Isolation of the anaerobe *Halothermothrix orenii* from a Tunisian salt lake shows that microorganisms may even simultaneously withstand high salt concentrations (up to 200 g/l) and high temperatures (up to 68°C) [7].

Most microbiologists do not realize the true extent of the diversity of halophilic and halotolerant microorganisms in nature. This diversity is expressed both at the phylogenetic level — halophiles are found in all three domains of life, and at the physiological level — most modes of energy generation known in non-halophiles are also used by halophilic counterparts. This diversity thus supplies a largely untapped source of organisms for future biotechnological uses. The era of genomics has not passed by the halophiles either: the complete genome sequence of the first halophile (the Archaeon *Halobacterium salinarum*) has recently been published [12]. Undoubtedly, more halophiles will be sequenced in the future, thereby providing a rich source of genomic information — information that will provide deeper insights in the ways microorganisms cope with the presence of high salt concentrations, and that may also open the way to the development of novel applications.

The sections below highlight a few aspects of the microbial diversity at high salt concentrations. They discuss phylogenetic diversity of halophiles, present an overview of the ways the cells have adapted to life under high osmotic pressure, and examine the diversity displayed by halophiles in their dissimilatory metabolism. A short survey of their presently exploited and potential

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biotechnological uses is given. Finally, some recent data are presented, showing that we are only beginning to realize the true extent of the microbial diversity in hypersaline environments in nature.

Phylogenetic diversity of halophiles

Halophilic and highly halotolerant microorganisms can be found in each of the three domains of life: Archaea, Bacteria, and Eucarya. This is illustrated in Figure 1, which shows those branches in the phylogenetic tree of life in which organisms able to grow at salt concentrations above 100 g/l have been identified. It should be remembered that there is there is a continuum of lower, optimal, and maximal salt concentrations for growth found in the microbial world, and that, therefore, all classifications of microorganisms according to their requirement for and tolerance toward salt are to a large extent arbitrary.

The aerobic halophilic Archaea of the order Halobacteriales, family Halobacteriaceae, are the halophiles par excellence. They are the main component of the microbial biomass of such environments as the Dead Sea, hypersaline soda lakes such as Lake Magadi, Kenya, and saltern crystallizer ponds. Most or all of the red colorations of such lakes are due to the C-50 carotenoid pigments (α-bacterioruberin and derivatives), found in large concentrations in the membranes of most members of the family [15]. Also, the methanogenic branch of the Euryarchaeota contains halophilic representatives, and methanogenesis can occur up to salt concentrations approaching NaCl saturation (see below). No halophiles have yet been identified within the Crenarchaeota kingdom.

Within the domain Eucarya, halophiles are scarce. In fact, the only Eucaryal microorganism of importance, but one almost

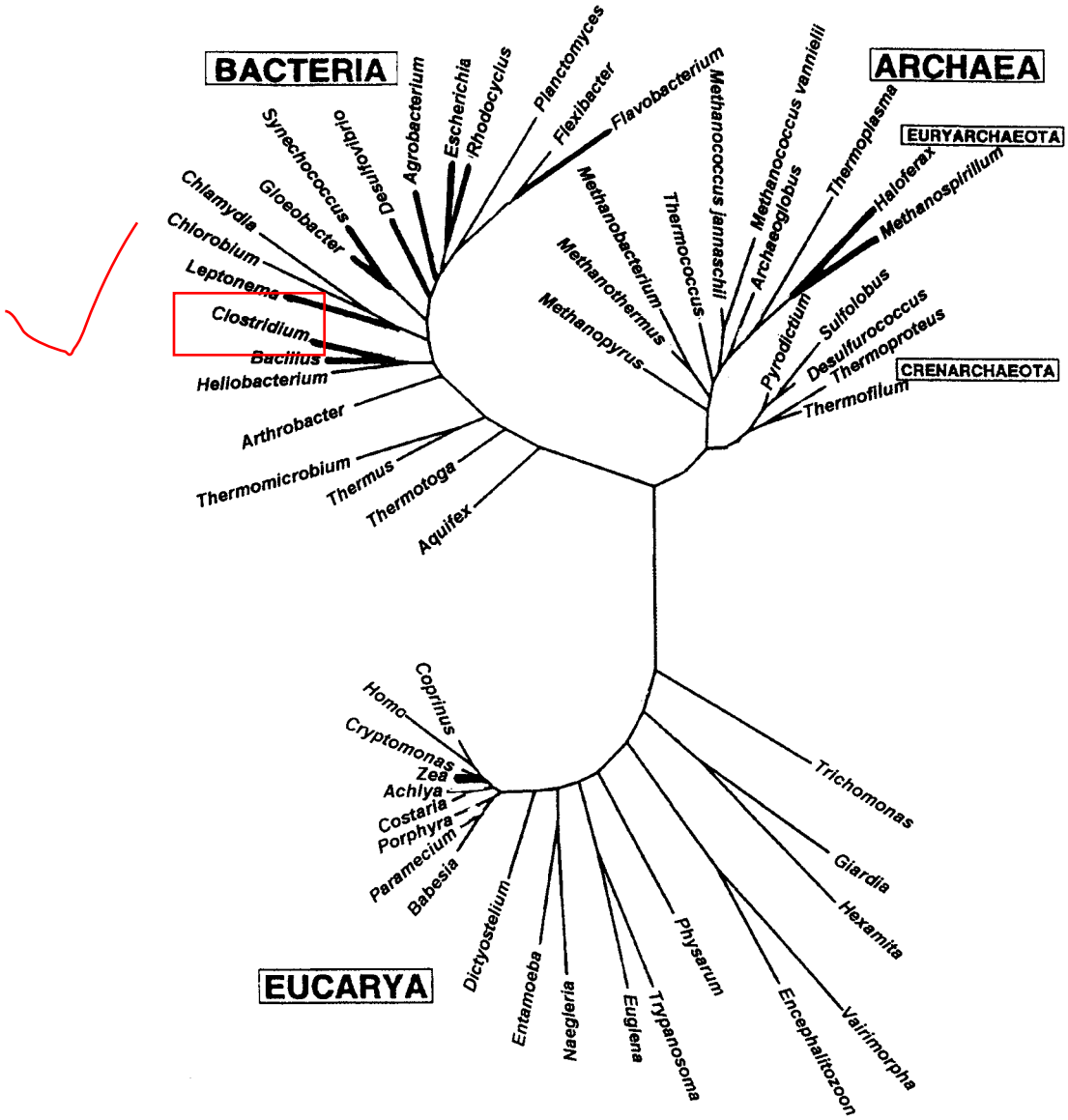

Figure 1 The universal phylogenetic tree of life, based on small subunit rRNA gene sequences. Those branches in which halophilic and halotolerant microorganisms have been found (defined here as those microorganisms that grow well at salt concentrations exceeding 100 g/l) are shown in bold lines.

ubiquitously present in high-salt environments, is the green alga *Dunaliella*. This alga is the main or only primary producer in the Dead Sea and in many other hypersaline lakes and ponds. Different species of *Dunaliella* exist, and some synthesize large amounts of β-carotene under suitable conditions, a property exploited in biotechnological operations (see below). *Dunaliella* is halotolerant rather than truly halophilic: most strains can grow over a broad range of salt concentrations, and relatively low concentrations (in the order of 1 M for the most salt-requiring types) can support growth. There is also a macroorganism that plays a role in many high-salt environments: the brine shrimp *Artemia*.

The domain Bacteria contains many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic subgroups. Many of these have been reviewed by Ventosa *et al.* [27]. The different branches of the Proteobacteria contain halophilic representatives, often having close relatives that are non-halophilic. Halophiles are further found among the cyanobacteria [19], the *Flavobacterium–Cytophaga* branch, the spirochetes, and the actinomycetes. Within the lineages of the Gram-positive Bacteria (Firmicutes), halophiles are found both within the aerobic branches (*Bacillus* and related organisms) and within the anaerobic branches. There is even an order, the Halanaerobiales, consisting of two families (the Halanaerobiaceae and the Halobacteroidaceae) that consists solely of halophilic anaerobic microorganisms [20,23].

In general, it may be stated that most halophiles within the domain Bacteria are moderate rather than extreme halophiles. However, there are a few types that resemble the archaeal halophiles of the family Halobacteriaceae in their salt requirement and tolerance. Notable examples are several photosynthetic purple bacteria of the genus *Halorhodospira* (γ-Proteobacteria), the actinomycete *Actinopolyspora halophila*, and the recently discovered "*Candidatus* Salinibacter" [1,3], an organism about which more information is presented below.

Osmotic adaptation of halophilic microorganisms

Considerable diversity also exists in the mechanisms halophilic and halotolerant microorganisms use to withstand the large osmotic pressure exerted by their highly saline surrounding medium. As biological membranes are permeable to water, all microorganisms have to keep their cytoplasm at least isoosmotic with their environment; when a turgor pressure is to be maintained, the cytoplasm should even be slightly hyperosmotic.

In all cases examined, sodium ions are excluded from the cytoplasm as much as possible. While it is still not clear why sodium ions are so harmful to the functioning of the cell, all halophilic microorganisms contain potent transport mechanisms, generally based on Na^+/H^+ antiporters, to expel sodium ions from the interior of the cell [16].

Different strategies are used by different groups of microorganisms to achieve a high osmotic pressure in the cytoplasm while keeping the Na^+ concentration low. One strategy involves accumulation of K^+ and Cl^- ions to maintain osmotic balance. This mechanism is used by a limited number of halophiles. The aerobic halophilic Archaea of the order Halobacteriales accumulate KCl at concentrations at least as high as the NaCl concentration in their surrounding medium. Within the domain Bacteria, this "salt-in" strategy has thus far been shown in one group only, the order Halanaerobiales, which consists of fermentative or homoacetogenic anaerobes. Bioenergetic calculations show that this mode of osmotic adaptation costs relatively little energy. However, the presence of high concentrations of KCl in the cytoplasm requires far-reaching adaptations of all proteins to enable all intracellular enzymatic systems to be active at high salt. More information on this issue can be found in several review articles [16,18].

The second strategy of osmotic adaptation is to exclude salts from the cytoplasm as much as possible, and to accumulate organic solutes to provide osmotic balance. A variety of compounds is used for the purpose, ranging from glycerol and other sugar alcohols, amino acids, and derivatives such as glycine betaine and ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) and its 5-hydroxy derivative, to simple sugars such as sucrose and trehalose [8]. This strategy is widely used in all three domains of life. The alga *Dunaliella* may contain molar intracellular concentrations of glycerol. The most widely used osmotic solutes within the domain Bacteria appear to be ectoine (synthesized by a great variety of organisms) and glycine betaine (synthesized almost exclusively by photosynthetic prokaryotes, but accumulated from the medium by many heterotrophic bacteria). Organic osmotic solutes have also been reported from the domain Archaea: halophilic methanogens use such solutes, and an organic osmolyte, 2-sulfotrehalose, has been detected (together with high KCl concentrations) in *Natronococcus occultus*, *Natronobacterium gregoryi*, *Natrialba magadii*, and *Natronomonas pharaonis*, all members of the Halobacteriales. Additional information on the distribution of organic osmotic solutes can be found in review articles [8,18]. The use of organic osmotic solutes requires much less far-reaching adaptations of the intracellular enzymatic machinery than does the accumulation of KCl. However, the production of such solutes is energetically expensive [17].

Metabolic diversity of halophilic microorganisms

The metabolic diversity of halophiles found in nature is as great as their phylogenetic diversity. Most microbial processes that occur at low salt concentrations can be found up to considerably high salinities, often up to NaCl saturation. Table 1 lists a variety of common dissimilatory processes known in the bacterial world, together with the upper salt concentration limit at which these processes have been documented in cultured halophilic microorganisms.

However, some dissimilatory processes known to occur in low-salinity environments have not yet been found at high salt concentrations. Examples of processes that do not appear to occur at salt concentrations above 100–150 g/l are autotrophic nitrification (both oxidation of ammonia to nitrite and oxidation of nitrite to nitrate), methanogenesis based on reduction of CO_2 with H_2 as electron donor, methanogenesis from acetate, and oxidation of acetate by sulfate-reducing bacteria. There appears to be a well-defined upper salt concentration limit for these types of dissimilatory metabolism.

An attempt has been made to explain this observation on the basis of the bioenergetic constraints related to the energetic cost of adapting the intracellular environment to the outside salinity (see above) [16]. The following processes appear to be feasible

Table 1 The upper salt concentration limit of different energy-generating processes performed by microorganisms in culture

Process	Most halotolerant representative	Approximate upper salt limit in culture
Photosynthesis		
Oxygenic photosynthesis	*D. salina*	NaCl saturation
Anoxygenic photosynthesis	*Halorhodospira halophila*	NaCl saturation
Respiration processes		
Aerobic respiration	*Hbt. salinarum*	NaCl saturation
Denitrification	*Haloarcula marismortui*	250–300 g/l
Sulfate reduction — complete oxidizers	*Desulfobacter halotolerans*	130 g/l
Sulfate reduction — incomplete oxidizers	*Desulfovibrio retbaense*	240 g/l
Fermentation		
Fermentation of L-arginine	*Hbt. salinarum*	NaCl saturation
Fermentation of carbohydrates	*Halanaerobium* species	250–300 g/l and higher
Methanogenesis and homoacetogenic metabolism		
Methane formation from H_2+CO_2	*Methanocalculus halotolerans*	120 g/l
Methane formation from acetate	*Methanosarcina acetivorans*	60 g/l
Methane formation from methylated amines or methanol	*Methanohalobium evestigatum*	250 g/l
Formation of acetate from H_2+CO_2	*A. arabaticum*	250 g/l
Aerobic chemoautotrophs and methanotrophs		
Autotrophic ammonia oxidation	*Nitrosococcus halophilus*	94 g/l
Autotrophic sulfide oxidation	*Halothiobacillus halophilus*	240 g/l
Aerobic methane oxidation	*Methylobacter modestohalophilus*	90 g/l

For additional information, see Refs. [16,18].

up to the highest salt concentrations: (1) photosynthetic processes, which are generally not energy-limited; (2) dissimilatory processes in which large amounts of energy are generated, so that the synthesis of organic osmotic compounds or the accumulation of KCl does not exhaust the energy available for growth; and (3) any process performed by such organisms that use salt (KCl) to balance the salinity of the medium, even if the amount of energy generated in the course of the dissimilatory process is small [16,18]. Processes that yield low amounts of energy are incompatible with the energetic expense of the synthesis of organic-compatible solutes.

Table 1 shows a number of examples. Processes such as nitrification, dissimilatory sulfate reduction with the oxidation of acetate, and cleavage of acetate with the formation of methane yield relatively small amounts of energy. As Proteobacteria as well as methanogenic Archaea use organic solutes to provide osmotic balance, it seems likely that the cost of adaptation to hypersaline conditions may be too high for such microorganisms. While low energy yields are also characteristic of fermentation processes, fermentative Bacteria are known to thrive at salt concentrations of 250 g/l and higher. The explanation may be found in the way such fermentative halophiles cope with the high salinity: the known anaerobic fermentative halophilic Bacteria are all members of the order Halanaerobiales. Those representatives of this group examined thus far use KCl as intracellular solute, and formation or accumulation of organic solutes has never been documented. The ability of *Hbt. salinarum* to grow fermentatively on L-arginine with the formation of one ATP per arginine can likewise only be explained by the use of the energetically favorable "salt-in" mode of osmotic adaptation. Another interesting example is the case of the hydrogenotrophic methanogens and the homoacetogens. While reduction of CO_2 with H_2 as electron donor does not appear to function at salt concentrations above 120 g/l, reduction of CO_2 with H_2 to form acetate can proceed at concentrations as high as 250 g/l, as the case of *Acetohalobium arabaticum* proves. The amount of energy generated in the acetogenic reaction is, however, slightly lower than that of the methanogenic reaction (ΔG^{0_1} values of -135.9 and -104.6 kJ, respectively, for the eight-electron reductions). Differences in the mode of osmotic adaptation used may provide the explanation. Methanogens produce organic osmotic solutes, probably at a considerable energetic cost. Although the intracellular environment of *Acetohalobium* has not yet been investigated, its phylogenetic position as a member of the Halanaerobiales makes it highly probable that it uses inorganic ions for osmotic stabilization. A full account of the ideas summarized above was given by Oren [16].

The upper salinity values for the different physiological processes shown in Table 1 represent the present state of knowledge. It cannot be excluded that new types of microorganisms may be discovered which extend the salinity limits beyond the boundaries indicated. The bioenergetics of such organisms will then deserve a thorough examination.

Biotechnology of halophilic microorganisms

Halophilic microorganisms have found several biotechnological applications. The most important of these are listed in Table 2.

Some applications of halophilic microorganisms are centuries-old, and existed long before microbiological aspects of the processes were understood. The red coloration developing in saltern crystallizer ponds worldwide, caused by halophilic Archaea of the order Halobacteriales, β-carotene-rich strains of *Dunaliella*, and possibly even red halophilic Bacteria (see below), contributes toward the absorption of light energy by the brines, thereby increasing evaporation. Benthic cyanobacterial mats may have a similar function in saltern ponds of intermediate

Table 2 Some presently exploited and potential biotechnological uses of halophilic microorganisms

Product	Producing organism	Uses and present status of technology
β-Carotene	*Dunaliella* species	Produced as antioxidant and food coloring agent
Different carotenoid pigments	Different members of the Halobacteriaceae, *Dunaliella*	Light absorption, increasing evaporation in saltern crystallizer ponds
Ectoine and hydroxyectoine	*Halomonas elongata*, *Marinococcus* M52	Produced as enzyme stabilizer ("molecular chaperone"), moisturizer in cosmetics; industrial scale production has recently started
Poly-β-hydroxyalkanoate	*Halof. mediterranei*	The organism has a high potential for PHA production; not yet industrially exploited
Salt-tolerant enzymes	Different halophilic Bacteria and Archaea	Not yet industrially exploited
Soy sauce, fish sauce	Different halophilic and halotolerant microorganisms, Bacteria as well as Archaea	Halophilic bacteria are involved in the production; no pure cultures are used; the microbiological aspects are poorly controlled
Bacteriorhodopsin	*Halob. salinarum*	Potential uses include use as holographic storage material, computer memories and processing units, photoelectric converters, and others; all these uses are yet in the experimental stage
Extracellular polysaccharides	*Halof. mediterranei*	The organisms have high potential for the production of valuable polysaccharides to be used, e.g., in the recovery of oil from oil wells; to our knowledge, not yet industrially exploited
Halophile cell biomass for cosmetics	*Dunaliella* species	*Dunaliella* is being used as an additive in cosmetic antiwrinkle preparations

For details, see Refs. [9,11,13,24,26].

salinity [19]. The production of certain traditional fermented foods in the Far East, such as fish sauce and soy sauce, involves the activity of a variety of halophilic and/or highly halotolerant microorganisms. The microbiology of some of these processes is not well understood even today.

In recent years, the number of biotechnological uses of halophilic microorganisms has increased, and additional applications are under development. The uses of halophiles in biotechnology can be divided into a number of categories. First, the halotolerance of many enzymes derived from halophilic microorganisms can be exploited wherever enzymatic transformations are required to function at low water activities, such as in the presence of high salt concentrations. Second, some organic osmotic stabilizers produced by halophiles have found interesting applications. Third, some halophilic microorganisms may produce valuable compounds that can also be found in non-halophiles, often without any direct connection with their halophilic properties, but halophiles may present distinct advantages for the development of biotechnological production processes.

Several halophilic enzymes have been tested for potential biotechnological applications, including amylases, nucleases, and proteases [10]. Salt-resistant, and even salt-dependent, cytoplasmic enzymes can be found inside those groups of microorganisms that maintain high intracellular ionic concentrations, such as the Archaea of the order Halobacteriales. In addition, all exoenzymes excreted by halophiles have to be active in the presence of the high salinities found in their medium, even when the organisms that produce them may maintain low intracellular ionic concentrations. Many halophilic enzymes are also considerably thermophilic.

Some of the organic solutes accumulated in high concentrations by many halophilic microorganisms as osmotic stabilizers are being exploited for different purposes. While attempts to grow *Dunaliella* for the commercial production of glycerol have not resulted in the development of an economically feasible process, ectoine and hydroxyectoine are now being produced commercially. These molecules have a strong *in vitro* stabilizing action on many otherwise labile enzymes, thereby increasing shelf-life and activity of enzyme preparations. This function is the basis for the commercial production of ectoine from *Halomonas elongata* and hydroxyectoine from *Marinococcus* M52. Nucleic acids are stabilized by the ectoines as well. Moreover, addition of ectoine to cosmetics is being tested in an attempt to increase the moisturizing activity of skin creams. Preparations of *Dunaliella* cells have also been found to be valuable additives to antiwrinkle creams [11].

There are a number of products that can be obtained from non-halophilic organisms as well, but halophiles may have distinct advantages when developing biological production processes. β-Carotene is a prime example. This pigment is in high demand as an antioxidant and food coloring agent. While β-carotene is present in many algae and higher plants and can also be synthesized chemically, its biological production from red strains of *Dunaliella* is highly successful. Certain *Dunaliella* strains may contain up to 10% or more of β-carotene in their dry weight, including a large percentage of the valuable 9-*cis* isomer. *Dunaliella* is now grown in different places in the world for β-carotene production [4].

It has also been suggested that β-hydroxyalkanoate as a source of biologically degradable plastic material can be obtained in a high yield from the halophilic Archaeon *Haloferax mediterranei*. The organism produces a copolymer of β-hydroxybutyrate and β-hydroxyvalerate that can be used for the production of thermoplastics ("biological polyesters") with excellent properties such as high strength and low melting point, similar to polypropylene. A poly-β-hydroxyalkanoate-based thermoplastics ("Biopol") is presently produced by ICI, using the non-halophile *Ralstonia eutropha*, and is used for the production of bottles for cosmetics. *Hfx. mediterranei* can accumulate as much β-hydroxyalkanoate as *Ralstonia*, and it can use starch as a cheap carbon and energy source. When grown on starch, the polymer produced was found to contain a minimum of 9% β-hydroxyvalerate, yielding a product of excellent consistency. Lysis of the cells in the absence of salt provides a simple process for recovery of the polymer. Growth is rapid, and because of the high salt concentration of the medium, there is little danger of contamination.

Hfx. mediterranei also produces copious amounts of anionic exopolysaccharides. Such compounds can be used as gelling agents, stabilizers, and thickeners. The sulfated acidic heteropolysaccharide of *Haloferax* species has a high viscosity at low concentrations, its rheological properties are excellent, and it is resistant to extremes of pH and temperature. It has been suggested that this polymer may be used to enhance oil recovery from low-productivity oil wells. Its stability to extremes of salt concentration, temperature, and pressure encountered in such environments may be advantageous [24]. In addition, the membrane lipids of the Archaeon may act as surfactants, improving the oil-carrying properties of the water. However, no large-scale production of the *Hfx. mediterranei* exopolysaccharide has been initiated as yet.

A special case is the retinal protein bacteriorhodopsin. This unique molecule, discovered in the early 1970s, is produced by *Hbt. salinarum* and by a few other representatives of the Halobacteriaceae. It is located in the cell membrane, where it serves as a light-driven proton pump. Upon excitation by light of a suitable wavelength (the absorption maximum of the purple ground state [B state] is 570 nm), the molecule undergoes a complex photocycle; one of the intermediates is the yellow M state, in which the molecule absorbs light of 400–450 nm. In contrast to virtually all other proteins of *Halobacterium*, it is not a truly "halophilic protein," as it is stable and active in the absence of salts as well. Bacteriorhodopsin is easy to immobilize on glass plates or to embed in polymers, and it maintains its photochemical properties over long periods. A large number of possible uses have been suggested for bacteriorhodopsin. Some of these are based on the conversion of light energy into chemical energy. Others are exploiting the photocycle of the retinal moiety, which undergoes transformation from the all-*trans*- to the 13-*cis* conformation upon excitation. Bacteriorhodopsin can therefore be used for holographic image storage [13]. The holographic interference patterns are registered as purple or yellow areas. Two alternative recording systems have been proposed. One mechanism is based on the photoreactivity of bacteriorhodopsin in the ground state after illumination with 500–600 nm (transition B→M). Alternatively, the M→B transition can be recorded with light of 400–450 nm, using a film previously converted to the M state by illumination in spectral range of the B state. Such M-type holograms offer certain advantages, especially when a mutant bacteriorhodopsin is used with a prolonged lifetime of the M-state. As the transitions are reversible, a bacteriorhodopsin holographic matrix can be used repeatedly. Another possible future use of the molecule may be the construction of "bioelectronic" elements of computer memories and information processing units. Bacteriorhodopsin then would serve as an optical switching element, again based on the B→M and M→B transitions, analogous to the conducting/non-conducting stages in a semiconductor. A high density of information storage is possible, and all the information can be processed simultaneously (parallel processing) [13].

The above list of possible biotechnological uses of halophilic microorganisms is by no means exhaustive. For a more complete account, specialized review articles should be consulted [9,24,26]. The tremendous diversity of halophilic microorganisms found in nature is still far from being fully exploited. The application of genomics will undoubtedly contribute additional information that may lead to novel applications. *Hbt. salinarum* has now been fully sequenced [12], the sequencing of *Hfx. volcanii* is soon to be completed, and we may expect more useful genomic information on additional halophiles in the future.

Microbial diversity at the highest salt concentrations — the case of saltern crystallizer ponds

We have recently obtained new insights into the microbial diversity present at the highest salt concentrations in saltern crystallizer ponds. In these ponds, NaCl precipitates as halite crystals, which are then collected, purified, and marketed. The brines in saltern crystallizer ponds are generally colored intensely red due to the presence of dense communities of pigmented microorganisms.

Until recently, the prevailing view was that the dominant microorganisms in the crystallizer ponds are carotenoid-rich *Dunaliella* cells and one or more species of halophilic Archaea, thought to belong to such well-known genera as *Halobacterium*, *Haloferax*, *Haloarcula*, and *Halorubrum*, representatives of which have been isolated in the past from such ponds. However, as is commonly observed in most ecosystems, the number of colony-forming bacteria recovered from the ponds is generally several orders of magnitude lower than the number of microscopically recognizable bacteria. Therefore, those organisms isolated with the highest frequency are not necessarily those that are present in the highest numbers.

Clues as to the true identity of the types of prokaryotes dominant in saltern crystallizers came from a combination of microscopic studies, chemotaxonomic work, and molecular biological approaches. Square, gas-vesicles-containing microbes are often seen to dominate the community. Such square cells were first recognized by Walsby [28] in a brine pool in Sinai, Egypt, and they have since been reported from many hypersaline environments [17]. They have never been cultured. Characterization of the polar lipids present in a saltern crystallizer pond in Eilat, Israel, dominated by such square cells, showed the presence of a single glycolipid, chromatographically identical with the sulfated diglycosyl diether lipid S-DGD-1. The diether derivative of phosphatidyl glycerosulfate was also present [22]. These observations suggest that this as yet uncultured organism is not a member of the genera *Halobacterium*, *Haloferax*, or *Haloarcula*.

Further information came from the application of molecular biological methods. Sequencing of 16S rDNA genes recovered from saltern crystallizer ponds in Alicante, Spain, consistently showed one phylotype to be dominant, a phylotype not closely related with any of the thus far recognized genera within the Halobacteriaceae. Its closest, but still very distant, relative is *Haloferax* [5,6]. The crystallizer ponds in Eilat yielded the same phylotype [25]. The link between this phylotype and the yet uncultured square bacteria was recently established when it was shown that fluorescent 16S rRNA probes, designed to specifically detect ribosomes of this new phylotype, interacted with microorganisms with the characteristic square morphology [2].

PCR amplification of bacterial 16S rRNA genes from saltern crystallizer ponds from different sites in Spain yielded a number of closely related phylotypes, clustering near the

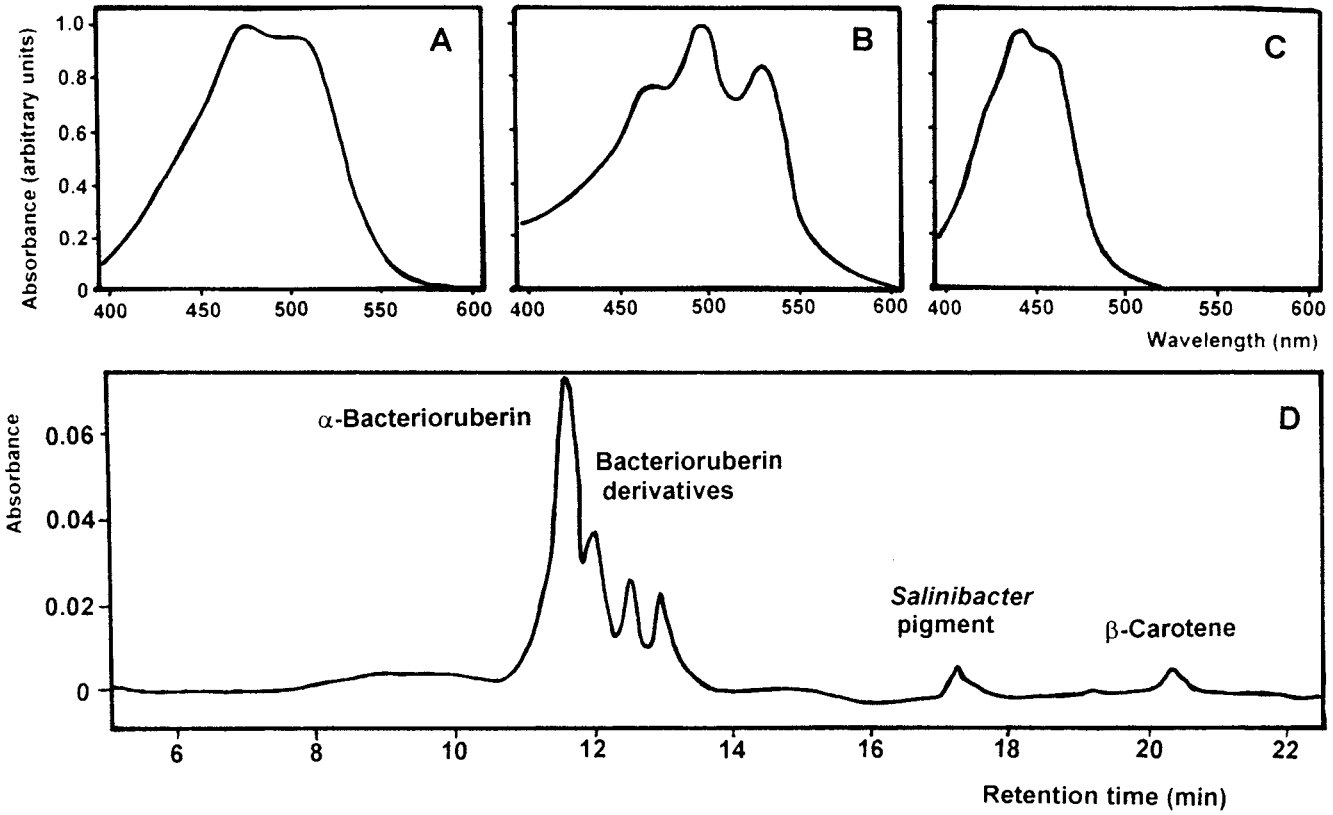

Figure 2 Absorption spectra of carotenoid and other solvent-extractable pigments from "*Candidatus* Salinibacter" isolated from the Santa Pola salterns, Alicante (A), *Hbt. salinarum* (B), and *Dunaliella salina* (C). Panel D shows the HPLC separation of pigments extracted from the biomass collected in March 2000 from a crystallizer pond of the Santa Pola salterns, and the identification of the pigment fractions. Cell pellets were extracted in 1 ml methanol–acetone 1:1 (vol/vol) for 4 h. Pigment extracts were injected through a 100-μl sample loop into a LiChrospher 100 RP-18 column (5 μm, 250×4 mm; Merck) and were eluted at a rate of 1 ml/min by a gradient of acetone and water (from 70–30% to 85–15% in 10 min, from 85% to 100% acetone in the next 5 min, followed by 6 min 100% acetone) using a Merck-Hitachi (Darmstadt, Germany) HPLC setup, including pump L-6200A and UV–visible detector L-4200. The elution of pigments was monitored at 490 nm.

Cytophaga–Flavobacterium–Bacteroides group. The closest relative was *Rhodothermus*, a genus of thermophilic marine bacteria isolated from marine hot springs. Probing environmental samples with fluorescent probes designed to specifically interact with the 16S RNA of this new type of halophilic Bacteria showed that the organisms are rod-shaped, and represent between 5% and 25% of the total number of prokaryotes in saltern crystallizer ponds near Alicante and on Mallorca. The new type of halophilic Bacteria was described on the basis of these environmental studies as "*Candidatus* Salinibacter" [3].

We recently succeeded in isolating Bacteria from saltern crystallizer ponds in Spain with 16S rDNA sequences nearly similar to the environmental sequences of "*Candidatus* Salinibacter" [1]. These isolates are rod-shaped, heterotrophic, motile microorganisms that grow optimally at NaCl concentrations as high as 200–250 g/l, and do not grow below 120–15 g/l salt. Their salt requirement and tolerance is thus similar to that of the halophilic Archaea of the order Halobacteriales. Similar to most halophilic Archaea, they are colored brightly red. HPLC analysis showed that a single pigment was present, with an absorption maximum at 478 nm and a shoulder at 510 nm (Figure 2A). The absorption spectrum thus differs from the archaeal bacterioruberin carotenoids (absorption maxima at 496 and 530 nm, with a shoulder at 470 nm; Figure 2B) and that of the β-carotenoid-rich *Dunaliella* (Figure 2C). We have detected significant amounts of this red pigment in extracts of biomass collected from the Alicante saltern crystallizers (Figure 2D) [21]. Thus, the red coloration of saltern crystallizer brines may be due not only to halophilic Archaea and β-carotenoid-rich *Dunaliella* cells as assumed thus far, but a bacterial pigment may contribute as well. Only traces of this pigment, if at all, were found in the Newark, CA, salterns [21], and it was not detected in samples collected from crystallizer ponds of the salterns of Eilat, Israel.

Epilogue

The information presented above shows that hypersaline environments may harbor surprisingly diverse microbial communities. Only now are we starting to obtain a reliable picture of this diversity, which is much greater than what could be inferred on the basis of the study of those halophilic microorganisms that have thus far been brought into culture.

Halophiles appear to be highly diverse in their metabolic potentials and in the ways they cope with the osmotic stress caused by the high salt concentrations in their medium. Some of the unique properties of halophiles have already found their way toward biotechnological applications. It may be expected that

more biotechnological uses of halophiles will be found in the future. The search for halophiles with useful applications will in its turn deepen our understanding of the functioning of hypersaline ecosystems.

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