

Unusual micro-organisms from unusual habitats: hypersaline environments

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INTRODUCTION

Thomas D. Brock defined extreme environments, considering that there are environments with high species diversity and others with low species diversity. Those environments with low species diversity, in which whole taxonomic groups are missing, are called 'extreme' (Brock, 1979). It is not easy to find a definition that is completely acceptable for all environments that are considered as extreme, but we observe that in some habitats environmental conditions such as pH, temperature, pressure, nutrients or saline concentrations are extremely high or low and that only limited numbers of species (that may grow at high cell densities) are well adapted to those conditions.

Hypersaline environments are typical extreme habitats, in which the high salt concentration is not the only environmental factor that may limit their biodiversity; they have low oxygen concentrations, depending on the geographical area, high or low temperatures, and are sometimes very alkaline. Other factors that may influence their biodiversity are the pressure, low nutrient availability, solar radiation or the presence of heavy metals and other toxic compounds (Rodríguez-Valera, 1988). With a few exceptions, most inhabitants of these environments are micro-organisms that are called 'halophiles'. However, different groups can be distinguished on the basis of their physiological responses to salt. Several classifications have been proposed; one that is very well accepted considers the optimum growth of the micro-organisms at different salt concentrations. Thus, Kushner & Kamekura (1988) defined several categories of micro-organisms on the basis of their optimal growth: non-halophiles are those that grow best in media containing less than 0.2 M NaCl (some of which, the halotolerant,

can tolerate high salt concentrations), slight halophiles (marine bacteria) grow best in media with 0.2 to 0.5 M NaCl, moderate halophiles grow best with 0.5 to 2.5 M NaCl and extreme halophiles show optimal growth in media containing 2.5 to 5.2 M (saturated) NaCl.

In hypersaline habitats, especially in those in which salinities exceed 1.5 M (about 10%), the two main groups of micro-organisms that predominate are the moderately halophilic bacteria and the extremely halophilic micro-organisms (archaea and bacteria). Archaea have been associated with extreme environments and, although today they are also recognized as normal inhabitants of other non-extreme environments, they constitute a large proportion of the microbial biota of hypersaline environments. Most are haloarchaea, but some methanogenic species have also been described from these environments.

Halophiles are found in many saline environments; the most important are hypersaline waters and soils, but the latter are much less studied. They can also be isolated from salt or salt deposits and from a variety of salted products, from salted fish or meats to fermented foods, as well as other materials such as salted animal hides. Hypersaline waters, those with higher concentrations of salt than sea water, can be divided into thalassohaline, which have a marine origin, if they have a composition similar to that of sea water, or athalassohaline, if their composition reflects the composition of the surrounding geology, topography and climatic conditions, often particularly influenced by the dissolution of mineral deposits; thus the composition of such waters varies widely (Rodríguez-Valera, 1988; Grant, 1990). Typical examples of thalassohaline water systems are solar salterns, used for the natural evaporation of sea water for the production of salt. They are excellent models for the study of halophiles, providing a series of ponds with different salinities, from sea water to salt saturation (Rodríguez-Valera, 1988; Grant, 1990). Typical examples of athalassohaline waters that have been studied in more detail are the Dead Sea, Great Salt Lake, some cold hypersaline lakes in Antarctica or alkaline lakes, particularly East African lakes, like Lake Magadi or the lakes of Wadi Natrun (Rodríguez-Valera, 1988; Javor, 1989; Grant, 1990).

In this chapter, I will review the micro-organisms that inhabit hypersaline environments and the features that make them unique. Some have very special characteristics that will be emphasized in this review. I will devote special attention to some recent studies on the square archaea and the extremely halophilic bacterium *Salinibacter*.

EXTREMELY HALOPHILIC ARCHAEA

The haloarchaea (also designated halobacteria) constitute a large group of extremely halophilic, aerobic archaea that are placed in the order *Halobacteriales*, family *Halo-*

bacteriaceae (Grant *et al.*, 2001). Classically, and for a long period of time, they were easily differentiated microscopically as rods or cocci that were respectively included within the genus *Halobacterium* or *Halococcus*, with a very limited number of species (Gibbons, 1974), probably due to the homogeneity of the isolation techniques and culture media in use as well as the limited number of hypersaline environments studied. When different approaches were used and many new hypersaline habitats were studied, the diversity found within this microbial group increased considerably. In fact, they are currently represented by 20 different genera and a large number of species, summarized in Table 1. Several other genera have recently been reported: *Halovivax asiaticus* (Castillo *et al.*, 2006) and '*Halostagnum larsenii*' (A. M. Castillo, M. C. Gutierrez, M. Kamekura, Y. Xue, Y. Ma, D. A. Cowan, B. E. Jones, W. D. Grant and A. Ventosa, manuscript in preparation) were isolated from a saline lake in Inner Mongolia, China. In addition to morphological and phenotypic features and comparison of 16S rRNA gene sequences, as is current practice in the systematics of other prokaryotic micro-organisms, the taxonomy of haloarchaea is also largely based on chemotaxonomic features: the polar lipid composition has proven to be an important marker for differentiation at the genus level (Grant *et al.*, 2001).

Haloarchaea require at least 1.5 M NaCl for growth and most species grow optimally in media with 3.5–4.5 M NaCl; many are able to grow in saturated NaCl (5.2 M) (Grant *et al.*, 2001). However, haloarchaea isolated from coastal salt-marsh sediments able to grow at lower salinities (around that of sea water) have been reported (Purdy *et al.*, 2004). They produce red- to pink-pigmented colonies due to the presence of bacterioruberins, C₅₀ carotenoids that are partially responsible for the typical colouration of many natural environments in which they may develop in large numbers. However, there are a few exceptions that are not pink- to red-pigmented, including species of the genus *Natrialba*. Another interesting aspect of the haloarchaea is the presence in some of them of retinal-based pigments, such as bacteriorhodopsin, that act as a proton pump driven by light energy (Lanyi, 1995). They are found in many hypersaline environments, such as salt lakes, soda lakes, salterns, solar salt and subterranean salt deposits and salted foods (Grant *et al.*, 2001).

Haloarchaea have typical archaeal characteristics such as the presence of ether-linked phosphoglycerides that can be easily detected by thin-layer chromatography. 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 (Grant *et al.*, 2001; Kates, 1993). All haloarchaea have diphytanyl (C₂₀C₂₀) glycerol ether core lipids and some may have additional phytanyl-sesterterpanyl (C₂₀C₂₅) glycerol core lipids; furthermore, some haloalkaliphiles have disesterterpanyl (C₂₅C₂₅) glycerol ether lipids (Grant *et al.*, 2001).

Table 1. Validly published genus and species names within the family *Halobacteriaceae*

The first species name listed corresponds to the type species of each genus (updated 31 December 2005). The three-letter genus abbreviations recommended by the ICSP Subcommittee on the taxonomy of the family *Halobacteriaceae* have been used. Basonyms/synonyms of organisms that have been transferred to other genera are not included.

Genus	Reference(s)	Genus	Reference(s)
<i>Halobacterium</i>	Elazari-Volcani (1957); Grant (2001a)	<i>Halobrubrum</i> (cont.)	
<i>Hbt. salinarum</i>	Ventosa & Oren (1996); Grant (2001a)	<i>Hrr. distributum</i>	Zvyagintseva & Tarasov (1987); Oren & Ventosa (1996)
<i>Hbt. noricense</i>	Gruber et al. (2004)	<i>Hrr. lacusprofundi</i>	Franzmann et al. (1988); McGenity & Grant (1995)
<i>Halalkalicoccus</i>	Xue et al. (2005)	<i>Hrr. sodomense</i>	Oren (1983); McGenity & Grant (1995)
<i>Hac. tibetensis</i>	Xue et al. (2005)	<i>Hrr. tebenquichense</i>	Lizama et al. (2002)
<i>Haloarcula</i>	Torreblanca et al. (1986)	<i>Hrr. terrestre</i>	Ventosa et al. (2004)
<i>Har. vallismortis</i>	González et al. (1979); Torreblanca et al. (1986)	<i>Hrr. tibetense</i>	Fan et al. (2004)
<i>Har. argentinensis</i>	Ihara et al. (1997)	<i>Hrr. trapanicum</i>	Petter (1931); McGenity & Grant (1995)
<i>Har. hispanica</i>	Juez et al. (1986)	<i>Hrr. vacuolatum</i>	Mwatha & Grant (1993); Kamekura et al. (1997)
<i>Har. japonica</i>	Takahama et al. (1990)	<i>Hrr. xinjiangense</i>	Feng et al. (2004)
<i>Har. marismortui</i>	Oren et al. (1990)	<i>Halosimplex</i>	Vreeland et al. (2002)
<i>Har. quadrata</i>	Oren et al. (1999)	<i>Hsx. carlsbadense</i>	Vreeland et al. (2002)
<i>Halobaculum</i>	Oren et al. (1995)	<i>Haloterrigena</i>	Ventosa et al. (1999)
<i>Hbl. gomorrhense</i>	Oren et al. (1995)	<i>Htg. turkmenica</i>	Zvyagintseva & Tarasov (1987); Ventosa et al. (1999)
<i>Halobiforma</i>	Hezayen et al. (2002)	<i>Htg. saccharovitans</i>	Xu et al. (2005c)
<i>Hbf. haloterrestris</i>	Hezayen et al. (2002)	<i>Htg. thermotolerans</i>	Montalvo-Rodriguez et al. (2000)
<i>Hbf. lacsalsi</i>	Xu et al. (2005b)	<i>Natrialba</i>	Kamekura & Dyll-Smith (1995)
<i>Hbf. nitratireducens</i>	Xin et al. (2001); Hezayen et al. (2002)	<i>Nab. asiatica</i>	Kamekura & Dyll-Smith (1995)
<i>Halococcus</i>	Schoop (1935); Grant (2001b)	<i>Nab. aegyptia</i>	Hezayen et al. (2001)

<i>Hcc. morrhuae</i>	Kocur & Hodgkiss (1973)	<i>Nab. chahannaensis</i>	Xu et al. (2001)
<i>Hcc. dombrowskii</i>	Stan-Lotter et al. (2002)	<i>Nab. hulunbeirensis</i>	Xu et al. (2001)
<i>Hcc. saccharolyticus</i>	Montero et al. (1989)	<i>Nab. magadii</i>	Tindall et al. (1984); Kamekura et al. (1997)
<i>Hcc. salifodinae</i>	Denner et al. (1994)	<i>Nab. taiwanensis</i>	Hezayen et al. (2001)
<i>Haloferax</i>	Torreblanca et al. (1986)	<i>Natrinema</i>	McGenity et al. (1998)
<i>Hfx. volcanii</i>	Mullakhanbhai & Larsen (1975); Torreblanca et al. (1986)	<i>Nnm. pellirubrum</i>	McGenity et al. (1998)
<i>Hfx. alexandrinus</i>	Asker & Ohta (2002)	<i>Nnm. altunense</i>	Xu et al. (2005a)
<i>Hfx. denitrificans</i>	Tomlinson et al. (1986); Tindall et al. (1989)	<i>Nnm. pallidum</i>	McGenity et al. (1998)
<i>Hfx. gibbonsii</i>	Juez et al. (1986)	<i>Nnm. versiforme</i>	Xin et al. (2000)
<i>Hfx. lucentense</i>	Gutierrez et al. (2002)	<i>Natronobacterium</i>	Tindall et al. (1984)
<i>Hfx. mediterranei</i>	Rodriguez-Valera et al. (1983); Torreblanca et al. (1986)	<i>Nbt. gregoryi</i>	Tindall et al. (1984)
<i>Hfx. sulfurifontis</i>	Elsahed et al. (2004)	<i>Natronococcus</i>	Tindall et al. (1984)
<i>Halogeometricum</i>	Montalvo-Rodriguez et al. (1998)	<i>Ncc. occultus</i>	Tindall et al. (1984)
<i>Hgm. borinquense</i>	Montalvo-Rodriguez et al. (1998)	<i>Ncc. amyolyticus</i>	Kanai et al. (1995)
<i>Halomicrobium</i>	Oren et al. (2002a)	<i>Natronolimnobius</i>	Itoh et al. (2005)
<i>Hmc. mukohataei</i>	Ihara et al. (1997); Oren et al. (2002a)	<i>Nlh. baerhuensis</i>	Itoh et al. (2005)
<i>Halorhabdus</i>	Wainø et al. (2000)	<i>Nlh. innermongolicus</i>	Itoh et al. (2005)
<i>Hrd. utahensis</i>	Wainø et al. (2000)	<i>Natronomonas</i>	Kamekura et al. (1997)
<i>Halorubrum</i>	McGenity & Grant (1995)	<i>Nnm. pharaonis</i>	Soliman & Trüper (1982); Kamekura et al. (1997)
<i>Hrr. saccharovororum</i>	Tomlinson & Hochstein (1976); McGenity & Grant (1995)	<i>Natronorubrum</i>	Xu et al. (1999)
<i>Hrr. alkaliphilium</i>	Feng et al. (2005)	<i>Nrr. bangense</i>	Xu et al. (1999)
<i>Hrr. coriense</i>	Kamekura & Dyall-Smith (1995); Oren & Ventosa (1996)	<i>Nrr. tibetense</i>	Xu et al. (1999)

Their absolute requirement for NaCl and their optimal growth at high NaCl concentrations are the most typical features of haloarchaea. The question of their mechanisms of haloadaptation has attracted much attention and, in contrast to most other prokaryotes, which accumulate intracellular organic compounds called compatible solutes, haloarchaea compensate for the high salt concentration in the environment by accumulating mainly KCl, up to 5 M (Grant *et al.*, 2001).

As mentioned previously, haloarchaea are found in many hypersaline environments; they are the predominant microbial biota of saturated ponds of salterns and salt lakes. Several species are haloalkaliphiles, being able to grow optimally at alkaline pH, that also inhabit soda lakes. Ecological studies demonstrate that they may reach high cell densities ($>10^7$ cells ml⁻¹). Traditional studies based on cultivation of viable cells suggested that the predominant species found in most neutrophilic hypersaline environments were related to the genera *Halobacterium*, *Halorubrum*, *Haloferax* and *Haloarcula* (Rodríguez-Valera *et al.*, 1985; Rodríguez-Valera, 1988; Benlloch *et al.*, 2001). However, more recent molecular ecological studies based on cultivation-independent methods indicate that, at least in most environments studied, members of these genera constitute small proportions of the microbial community. Several recent studies carried out in hypersaline environments have allowed some general conclusions to be drawn. Square haloarchaea are very abundant, but they have not been isolated until recently; many environmental clones within the haloarchaeal group are also obtained that are not phylogenetically closely related to previously described species. Some clones related to *Halorubrum*, *Haloarcula*, *Natronobacterium* and *Natronomonas* are observed (Benlloch *et al.*, 2001, 2002; Burns *et al.*, 2004b). Recent studies on a saltern in Slovenia showed that the haloarchaeal community in the crystallizer was strongly dominated by two groups of *Halorubrum*-related environmental phylotypes. In addition, members of four other haloarchaeal genera and two groups of environmental phylotypes were observed. However, the square haloarchaeal morphotype was not observed (Pasic *et al.*, 2005). Another recent study of a saltern in San Diego showed that the predominant haloarchaea were members of *Halobacterium*; the presence of *Haloarcula* and *Halobacterium* was also detected as well as two novel lineages not closely related to any haloarchaeal species (Bidle *et al.*, 2005).

Haloarchaea are used in many studies as archaeal models since they can be easily grown under laboratory conditions. In contrast to other archaeal extremophiles, which require special culture conditions, haloarchaea grow well in complex media and in some cases in minimal media under aerobic conditions by using the standard procedures for growing other prokaryotes. They can also be genetically manipulated; genetic exchange mechanisms are well established and methods for their genetic manipulation in the

laboratory are available (Robb *et al.*, 1995). The complete genome sequences of quite a few haloarchaea have been reported, including *Halobacterium salinarum* NRC-1 (2571 kb) (Ng *et al.*, 2000), *Haloarcula marismortui* ATCC 43049^T (4274 kb) (Baliga *et al.*, 2004) and *Natronomonas pharaonis* DSM 2160^T (2749 kb) (Falb *et al.*, 2005). Several other genome sequencing projects are in progress: a second strain of *Halobacterium salinarum* and strains of *Halobaculum gomorrense*, *Halobiforma lacisalsi*, *Haloferax volcanii*, *Halorubrum lacusprofundi* and *Natrialba asiatica*. Clearly, more effort would be necessary in this field in order to have genomic information available that reflects the diversity found within the haloarchaea.

Besides their use as excellent models to study the molecular biology of archaea and their mechanisms of adaptation to extreme environments and the important ecological roles that they play in hypersaline habitats, haloarchaea have very interesting biotechnological applications. Bacteriorhodopsin in the form of purple membrane patches produced by *Halobacterium salinarum* is commercially available; other compounds of industrial interest produced by haloarchaea are extracellular hydrolytic enzymes, exopolysaccharides, polyhydroxyalkanoates (PHAs) used as bioplastics and the halocins (antimicrobial compounds produced by haloarchaea). These and many other potential applications have been reviewed in detail elsewhere (Ventosa & Nieto, 1995; Margesin & Schinner, 2001; Mellado & Ventosa, 2003).

FROM 'SQUARE BACTERIA' TO 'SQUARE HALOARCHAEA'

In 1980, A. E. Walsby described the abundant presence of 'square bacteria' in a small, saturated brine pool, or sabkha, in the Sinai Peninsula (Walsby, 1980). These micro-organisms were collected from the surface of the pool, had a large number of gas vesicles and presented unique morphologies never observed previously in the microbial world. The cells are squares and very thin, with sizes from 1.5 to 11 µm and a thickness of about 0.2 µm (Fig. 1). Division planes were observed, with an arrangement indicating that division occurred in two planes alternating at right angles, so that each square grows to a rectangle which then divides into two equal squares, producing sheets divided like postage stamps (Walsby, 1980; Parkes & Walsby, 1981). Also, different gas vesicles were observed, from spindle-shaped to cylindrical with conical ends and, in many cases, they were concentrated at the cell periphery. Both forms may occur within the same cell, as had been observed previously in *Halobacterium* (Parkes & Walsby, 1981). Similar observations were made from other hypersaline environments in different geographical areas, from salt ponds located in Baja California, Mexico, or San Francisco Bay, California (Stoeckenius, 1981). Ultrastructure studies confirmed that the square cells observed by Walsby were micro-organisms with a typical prokaryote structure (Stoeckenius, 1981; Kessel & Cohen, 1982).

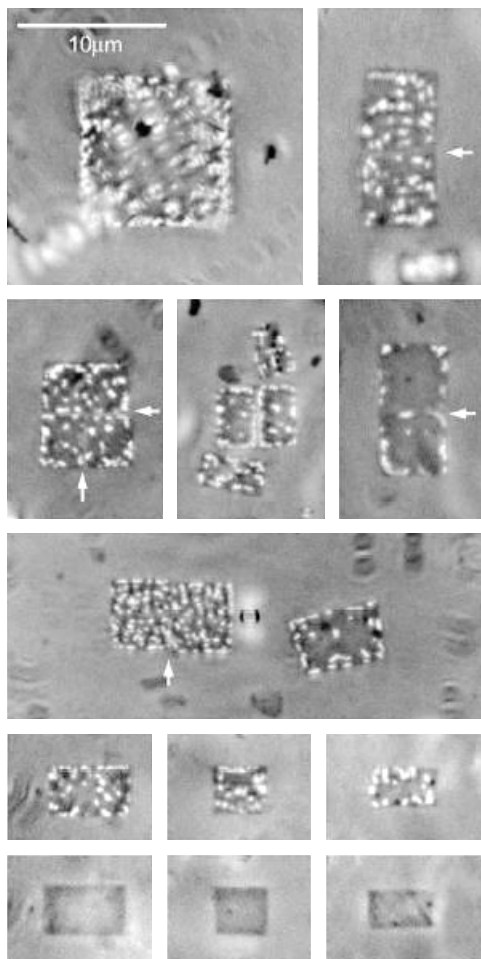


Fig. 1. The square archaeon from the Sinai. Phase-contrast light micrographs of the original square haloarchaeon discovered in a brine sample from a sabkha near Nabq, Sinai. Division lines (arrows) are visible in some of the cells but not in the largest (top left). Gas vesicles show as bright refractile granules in all cells except those in the bottom row, which have been exposed to pressure (the same cells as shown in the row above). Bar, 10 μm . Reproduced from Walsby (2005) with permission from Elsevier.

Direct observations of brines of salterns and other hypersaline environments suggested that these square cells were very abundant in such habitats, especially in the most concentrated ponds with salinities higher than 3–4 M NaCl, and they have been reported to occur in many geographical locations (Oren, 1999). Oren *et al.* (1996) suggested on the basis of polar lipid analysis that they could be archaea not related to the genera *Halobacterium* or *Haloarcula*. When samples from a crystallizer pond of a saltern in Eliat, Israel, with large numbers of gas-vacuolated square cells (representing about

55 % of the total population) were studied with respect to polar lipid composition, Oren *et al.* (1996) observed that the most frequent lipids present were typical of halophilic archaea, members of the family *Halobacteriaceae*, but that the square archaea may belong to a new genus. More recently, molecular techniques revealed that they may represent a large proportion of the microbial population in many hypersaline environments (Benlloch *et al.*, 1996, 2001, 2002; Antón *et al.*, 1999; Burns *et al.*, 2004b).

Many attempts have been made to isolate these square micro-organisms, but only recently have two independent studies reported their isolation and cultivation (Bolhuis *et al.*, 2004; Burns *et al.*, 2004a). Previous studies based on the isolation of pure cultures from different hypersaline environments reported the presence of halophilic archaea with morphologies ranging from squares to discs and triangles, and not the typical rod or spherical cell shapes that are observed within other bacterial groups. An early study describing 'box-shaped' halophiles (Javor *et al.*, 1982) did not permit the isolation of square archaea, since the cells were pleomorphic and lacked the gas vesicles observed in Walsby's square cells. Most isolates studied in recent years that show pleomorphic cells have been placed in the genera *Haloferax* (Ventosa, 2001b) or *Haloarcula* (Ventosa, 2001a). Most species of the genus *Haloarcula* have unique morphologies, such as *Haloarcula japonica*, which typically shows many triangular and rhomboid cells (Takashina *et al.*, 1990), or *Haloarcula quadrata* (Oren *et al.*, 1999). The latter species was described based on a motile, square prokaryote isolated from the Gavish Sabkha in the Sinai Peninsula. The square morphology and interesting mode of motility were reported by Alam *et al.* (1984). The cells have a single or several flagella anchored from a single or several locations that form a bundle. As in the case of other haloarchaea, this species requires high salt concentrations for growth and for stabilization of the cells. Optimal growth is obtained in the presence of 3.4–4.3 M NaCl and 0.1–0.5 M Mg²⁺. It is interesting to note that at least 50–100 mM Mg²⁺ was required for maintenance of the square morphology (Oren *et al.*, 1999). However, its lack of gas vesicles and a more variable morphology suggest that it does not correspond to the 'square bacteria' described by Walsby (1980).

The recent isolation of Walsby's square haloarchaea, 25 years after their original description, by two independent groups of microbiologists can be considered as an important event. It required the use of new approaches that differed from traditional culture methods, as well as extraordinary persistence.

Burns *et al.* (2004b) studied water samples from a crystallizer pond of a saltern located in Geelong, Victoria, Australia, in which they observed by microscopy the abundance of square cells. They used a combination of serial dilutions of the samples in liquid media with different compositions (extinction cultures), and growth of the square

haloarchaea was determined by PCR screening. When conventional media were used, cells were similar to *Halorubrum* species. However, when a medium composed of filtered and sterilized natural saltern water supplemented with 50 μM amino acids mixture and 0.5 % pyruvate was used, square cells were detected both by light microscopy and PCR screening after 3 weeks of incubation in three dilution cultures. The best-growing isolate, culture C23, was grown further and reached cell densities of about 10^7 cells ml^{-1} , at which point cultures were slightly turbid by eye. However, it does not grow on solid media. Further studies with isolate C23 determined its doubling time at around 1.5 days. A faster growth rate was observed when a rich medium (composed of 23 % salt water plus 0.1 % yeast extract and 0.5 % peptone) was supplemented with 0.5 % pyruvate, obtaining final cell densities of 10^8 cells ml^{-1} . Microscopy studies showed that the cells of isolate C23 were thin, square (mean of 2.6 μm) or rectangular (mean 2.3 \times 3 μm). Apart from occasional triangular cells, isolate C23 showed a homogeneous morphology. Furthermore, it contained gas vesicles that were easily collapsed by pressure and poly- β -hydroxybutyrate granules of about 0.3 μm diameter; in both cases, they were often found near the periphery of the cells (Fig. 2). Comparison of the complete 16S rRNA gene sequence with sequences of all members of the *Halobacteriaceae* and clone sequences available from the databases confirmed that isolate C23 is closely related to the 'square haloarchaea group' clones, showing more than 99 % sequence similarity. However, the closest 16S rRNA gene sequence similarity with any of the cultured species was about 91 %, with the sequence of *Halogeometricum borinquense*, a very pleomorphic haloarchaeon, showing different shapes (from rods to triangles, squares or oval cells) but not the typical square of Walsby's archaea (Montalvo-Rodríguez *et al.*, 1998).

Bolhuis *et al.* (2004) studied samples obtained from salt-saturated crystallizer ponds of a solar saltern in Alicante, Spain, in which square cells were observed microscopically, and they were inoculated into different culture media. When media composed of hypersaline artificial sea water supplemented with high concentrations of yeast extract, peptone or tryptone and glycerol or other alternative carbon sources were used, rapid growth of halophilic prokaryotes was obtained but square cells were not observed under the microscope. Only in media with very low concentrations of yeast extract and glycerol (less than 0.5 g l^{-1} in both cases) and after long incubation periods of 1–2 months were the square cells enriched, but they did not grow on solid media when agar was added as a solidifying agent. After several further enrichments in these media for a period of 2 years, they obtained a culture of square cells that was not pure. Finally, in a medium with a low concentration of yeast extract (0.1 g l^{-1}) supplemented with sodium pyruvate (1 g l^{-1}) they obtained an enrichment with square cells that were plated in the same medium with agarose (10 g l^{-1}) instead of agar as solidifying agent. The first pure culture of a square isolate that contained gas vesicles was designated strain HBSQ001.

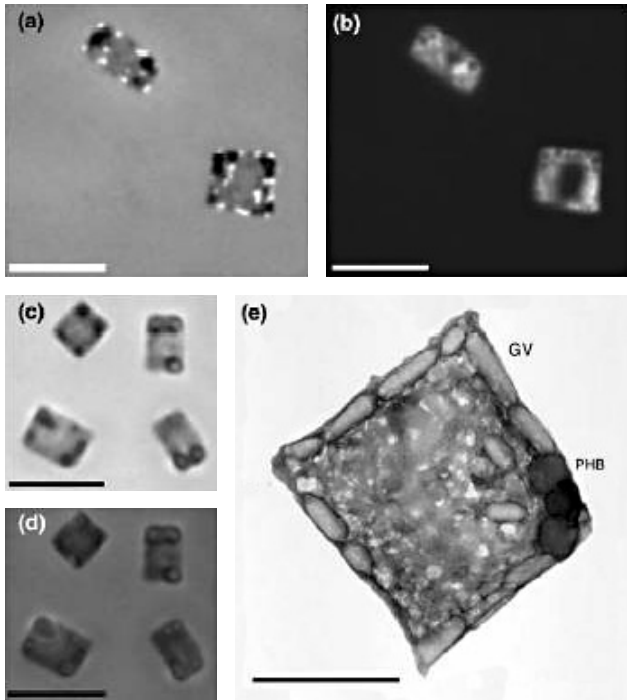


Fig. 2. Cells of isolate C23 viewed by light and electron microscopy. (a) Cells examined by phase-contrast microscopy; (b) the same field viewed by epifluorescence microscopy after acridine orange staining; (c) cells fixed and stained with Nile blue A (a poly- β -hydroxybutyrate-specific fluorescent stain) and viewed by phase-contrast microscopy; (d) the same field as in (c) viewed by epifluorescence, and the image superimposed over that of (c) to show the cell borders relative to the red-fluorescing granules. Bars, 5 μm (a–d). (e) Uranyl acetate- (2 %) stained preparation examined by transmission electron microscopy. Bar, 1 μm . Reproduced from Burns *et al.* (2004a) with permission from Elsevier.

The cells were 2–5 μm wide and about 0.1–0.5 μm thick; in fresh cultures they observed very large cells (20 \times 20 to 40 \times 40 μm and 0.1–0.5 μm thick) in which division walls were not observed. This isolate required at least 3 M NaCl and showed an elevated tolerance of high concentrations of MgCl_2 , being able to grow in media containing over 2 M MgCl_2 and 3.3 M NaCl.

When the 16S rRNA gene sequence of strain HBSQ001 was determined, phylogenetic study showed that it was closely related to the SPbT sequence (99 % similarity) that had been observed previously as related to Walsby's square archaeon. Only 93 % 16S rRNA gene sequence similarity was obtained with the haloarchaeon T1.3, a strain isolated from an ancient salt deposit (McGenity *et al.*, 2000), and lower values were seen with haloarchaeal species. This low 16S rRNA gene sequence similarity suggests that the new isolate would represent a new genus of the family *Halobacteriaceae*. Burns and co-

workers proposed the new name '*Haloquadratum walsbyi*' if this isolate were to constitute a new taxon (Bolhuis *et al.*, 2004). However, a complete taxonomic characterization would be required, including phenotypic as well as chemotaxonomic data (such as polar lipid analysis). Furthermore, it would be interesting to determine the sequence similarity between the two novel square archaea, strains HBSQ001 and C23, in order to determine whether they are closely related, and to carry out a comparative study to determine whether they are members of the same genus and species.

The reasons for the difficulties in isolating the square archaea can be attributed to several factors, such as their sensitivity to agar and to high concentrations of yeast extract, their slow growth rate (doubling time of 1–2 days under optimal conditions), which permits rapid growth of other extremely halophilic micro-organisms in the enriched cultures, and probably their requirement for relatively high concentrations of $MgCl_2$ to maintain the square morphology.

The isolation by two independent research groups of the square archaea introduces a new methodology that permits the isolation of new fresh isolates of square archaea, allowing us to establish whether they are represented by a single or several novel species. Furthermore, it will be possible to determine their roles in the natural hypersaline environments in which, on the basis of molecular ecology techniques, they represent a large proportion of the microbial population, and to study other physiological or molecular aspects such as their high tolerance of $MgCl_2$ or the unique square morphology. The complete sequence of the genome of these micro-organisms will also constitute a highlight for comparative purposes with other organisms.

Recently, Walsby (2005) reviewed the features of the square archaea and the biological significance of the square shape. This shape depends on the lack of turgor; most cells are distended by turgor pressure, generated by the difference in water potential between the external medium and the cell. In spherical or cylindrical cells, the pressure produces stress in the cell wall. Square cells, like other haloarchaea, have no turgor pressure (Walsby, 1980) and are free of stress in the cell wall, thus they need less complex cell walls than other prokaryotes and might, in theory, adopt any shape. The shape of the cell might be dependent on the shape and arrangement of the wall subunits. The cell wall of square archaea is composed of regularly arranged subunits, forming a dominantly hexagonal lattice (Walsby, 1980; Parkes & Walsby, 1981; Stoeckenius, 1981; Kessel & Cohen, 1982). The advantages of the thinness of the square cells could be related to the large surface area, which would permit the uptake of nutrients as well as more efficient light absorption, since it has been shown that the square archaea contain purple membranes that are used for phototrophic growth. Furthermore, the gas vesicles offer another clear advantage: they permit the cells to concentrate and rest parallel in a

horizontal position on the water surface. This movement to the surface has less energetic cost in cells with gas vesicles than with flagella and could explain the slow growth strategy and their dominance in saturated hypersaline environments (Walsby, 2005).

SALINIBACTER AND OTHER EXTREMELY HALOPHILIC BACTERIA

The genus *Salinibacter*, with the single species *Salinibacter ruber*, was proposed by Antón *et al.* (2002) for red-pigmented, motile, rod-shape extremely halophilic bacteria isolated from saltern crystallizer ponds in Alicante and Mallorca (Spain). It grows optimally between 20 and 30 % salt and requires at least 15 % salts. Therefore it is as halophilic and salt-dependent as most extremely halophilic archaea belonging to the family *Halobacteriaceae* (Grant *et al.*, 2001). It is phylogenetically related to the phylum *Bacteroidetes* but the closest cultivated relative is *Rhodothermus marinus* (showing only about 89 % 16S rRNA gene sequence similarity), a thermophilic and slightly halophilic bacterium. This bacterium constitutes a major component of the community of hypersaline environments and was previously known only by its phylo-type (designated 'Candidatus *Salinibacter*') (Antón *et al.*, 2000). By using fluorescent oligonucleotide probes, this organism has been shown to be abundant in the crystallizer ponds of salterns, representing between 5 and 25 % of the total prokaryotic community (Antón *et al.*, 2000). They may be also responsible for the red colouration of saltern crystallizer ponds (Oren & Rodriguez-Valera, 2001). However, their presence has not been observed in other salterns studied recently (Bidle *et al.*, 2005).

Despite its relatively recent isolation and description, new data about this interesting bacterium, which can be considered as one of the most halophilic organisms belonging to the *Bacteria*, have been accumulated in recent years. *S. ruber* has an extremely high intracellular potassium content but very low concentrations of organic osmotic solutes (glutamate, glycine betaine and *N*- α -acetyl lysine) (Oren *et al.*, 2002b). Thus, it uses a similar mode of haloadaptation to that of the haloarchaea, which also have high intracellular K⁺ concentrations, and does not accumulate organic osmotic solutes such as those used by most halophilic or halotolerant bacteria (Ventosa *et al.*, 1998; Oren *et al.*, 2002b). Amino acid analysis of bulk protein of *S. ruber* showed a high content of acidic amino acids, a low proportion of basic amino acids, a low content of hydrophobic amino acids and a high abundance of serine (Oren & Mana, 2002). When these authors determined the levels of activity of several cytoplasmic enzymes at different KCl and NaCl concentrations, they showed that these enzymes are adapted to function in the presence of high salt concentrations (Oren & Mana, 2002). However, it seems that the behaviour of each enzyme towards salt varies considerably. Thus, while some enzymes are truly halophilic and require salt for activity and stability, such as the NAD-dependent isocitrate dehydrogenase (Oren & Mana, 2002) or the fatty acid synthetase

complex (Oren *et al.*, 2004), other enzymes may function just as well in the presence of high salt or in its absence, like the NADP-dependent isocitrate dehydrogenase, or may even be inhibited by high salt concentrations (Oren & Mana, 2002). Two glutamate dehydrogenases have also been detected in *S. ruber*; they show differences in their affinity for substrates, in their pH and salt dependence for activity and stability and in their regulation by different effectors. While one glutamate dehydrogenase depends on high salt concentrations for both activity and stability, the other shows a strong dependence on high salt concentrations for stability but not for activity (Bonete *et al.*, 2003).

In a recent study, the malate dehydrogenase of *S. ruber* was purified and characterized in detail, comparing it with those produced by extremely halophilic archaea or non-halophilic micro-organisms (Madern & Zaccai, 2004). In contrast to most other halophilic enzymes, which unfold when incubated at low salt concentrations, this enzyme is completely stable in the absence of salt; its amino acid composition does not display the strong acidic character specific to halophilic proteins. Its activity is reduced by high salt concentrations, but remains sufficient for the enzyme to sustain catalysis at approximately 30 % of its maximal rates in 3 M KCl (Madern & Zaccai, 2004). The differences in the behaviour of the enzymes of *S. ruber* pose questions about the origins of the genes encoding such proteins and the possibility of horizontal gene transfer from other bacteria or archaea that might inhabit the same environments. Sequencing of the complete genomes of two different strains of *S. ruber* is in progress and will allow comparative analysis at the molecular level of the features of this interesting micro-organism, not only concerning its physiological and biochemical behaviour but also other basic and applied aspects in comparison with the extremely halophilic archaea, with which it must compete in natural environments.

The bright-red pigmentation of *S. ruber* is due to a novel carotenoid that has been designated salinixanthin, which constitutes more than 96 % of the total carotenoid content. The chemical structure of this C₄₀ carotenoid has been reported (Lutnaes *et al.*, 2002), being different from the C₅₀ carotenoids of the bacterioruberin group present in haloarchaea. It has been proposed that salinixanthin would provide protection from photodamage and stabilize the cell membrane (Lutnaes *et al.*, 2002). However, it has recently been established that salinixanthin is not the only pigment in the *S. ruber* cell membrane; it also contains an unusual retinal protein that uses salinixanthin to harvest light energy and then uses it for transmembrane proton transport. This light-driven proton pump, similar to bacteriorhodopsin or the archaeorhodopsins but with two chromophores, has been designated xanthorhodopsin (Balashov *et al.*, 2005). The membrane lipids of *S. ruber* have also been analysed recently. Apart from the phospholipids typical of the bacterial domain, a novel sulfonolipid

that represents about 10 % of total cellular lipids has been identified (Corcelli *et al.*, 2004). It has been suggested that this novel sulfonolipid could be used as a chemotaxonomic marker for the detection of *Salinibacter* within the halophilic microbial community in hypersaline environments (Corcelli *et al.*, 2004).

S. ruber is not the only extremely halophilic bacterium that has been described. Several other examples of extremely halophilic bacteria are *Halorhodospira halophila* (formerly *Ectothiorhodospira halophila*), a phototrophic bacterium that grows optimally in media with about 25 % salts (Hirschler-Rea *et al.*, 2003), *Halanaerobium lacusrosei*, a strictly anaerobic bacterium isolated from hypersaline sediments of Lake Retba in Senegal (Cayol *et al.*, 1995), *Acetohalobium arabaticum* (Zhilina & Zavarzin, 1990) and *Halobacteroides lacunaris* (Zhilina *et al.*, 1991), two anaerobic organisms isolated from hypersaline lakes, and the actinomycete *Actinopolyspora halophila* (Gochnauer *et al.*, 1975).

MODERATELY HALOPHILIC BACTERIA

Moderately halophilic bacteria are a group of micro-organisms that grow optimally in media containing 0.5 M (3 %) to 2.5 M (15 %) NaCl (Ventosa *et al.*, 1998). They are represented by a large number of species belonging to many different genera. In 1980, when the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) were published, only six species were described: *Salinivibrio costicola* (formerly *Vibrio costicola*), a vibrio motile by a single sheathed polar flagellum, that is frequently isolated from salted meats and other food products and is present in salt lakes and in ponds of salterns of intermediate salinities (Garcia *et al.*, 1987), *Nesterenkonia* (*Micrococcus*) *halobia*, *Halomonas* (*Paracoccus*) *halodenitrificans*, *Halomonas* (*Flavobacterium*) *halmophila*, *Marinococcus* (*Planococcus*) *halophilus* and *Spirochaeta halophila*. Most of them were isolated from salted foods or as laboratory contaminants and their taxonomic descriptions were very incomplete. The role(s) that these bacteria played in natural habitats was unknown. However, extensive studies carried out over the last 25 years have given us a better knowledge of their biodiversity, phylogenetic relationships, physiological and haloadaptive mechanisms and, more recently, biotechnological potential. Currently, the number of bacterial species with a moderately halophilic response is very large and they are represented in many different bacterial phyla, especially the Gram-positives (low- and high-G+C content), spirochaetes and *Bacteroidetes*, but most of them belong to the *Proteobacteria*. Table 2 shows some representative genera that include moderately halophilic bacterial species. In many cases they are members of genera that include both moderately halophilic and other non-halophilic or halotolerant species, but in most cases they are included in genera with only moderately halophilic representatives. However, it cannot be disputed that other non-halophilic species could be also included in the near future within these genera. The

Table 2. Representative genera that include moderately halophilic species

Gram-positive	Gram-negative	
<i>Alkalibacillus</i>	<i>Algoriphagus</i>	<i>Psychrobacter</i>
<i>Bacillus</i>	<i>Alteromonas</i>	<i>Rhodospirillum</i>
<i>Clostridium</i>	<i>Arhodomonas</i>	<i>Rhodothalassium</i>
<i>Gracilibacillus</i>	<i>Chromohalobacter</i>	<i>Rhodovibrio</i>
<i>Halobacillus</i>	<i>Desulfocella</i>	<i>Roseisalinus</i>
<i>Lentibacillus</i>	<i>Desulfohalobium</i>	<i>Salegentibacter</i>
<i>Marinococcus</i>	<i>Desulfovibrio</i>	<i>Salinimonas</i>
<i>Nocardiopsis</i>	<i>Halanaerobacter</i>	<i>Salinisphaera</i>
<i>Pontibacillus</i>	<i>Halanaerobium</i>	<i>Salinivibrio</i>
<i>Prauserella</i>	<i>Halochromatium</i>	<i>Salipiger</i>
<i>Saccharomonospora</i>	<i>Haloincola</i>	<i>Selenihalanaerobacter</i>
<i>Salinibacillus</i>	<i>Halomonas</i>	<i>Spirochaeta</i>
<i>Streptomonospora</i>	<i>Halorhodospira</i>	<i>Sporohalobacter</i>
<i>Tenuibacillus</i>	<i>Halospina</i>	<i>Staleyia</i>
<i>Tetragenococcus</i>	<i>Halothermothrix</i>	<i>Sulfitobacter</i>
<i>Thalassobacillus</i>	<i>Halothiobacillus</i>	<i>Thiohalocapsa</i>
<i>Virgibacillus</i>	<i>Halovibrio</i>	
<i>Yania</i>	<i>Idiomarina</i>	
<i>Microbacterium</i>	<i>Marinicola</i>	
<i>Filobacillus</i>	<i>Marinobacter</i>	
<i>Dietzia</i>	<i>Methylarcula</i>	
<i>Marinibacillus</i>	<i>Methylohalobius</i>	
<i>Desulfotomaculum</i>	<i>Muricauda</i>	
<i>Salinicoccus</i>	<i>Natroniella</i>	
<i>Nesterenkonia</i>	<i>Nitricola</i>	
<i>Jeotgalicoccus</i>	<i>Orenia</i>	
<i>Jeotgalibacillus</i>	<i>Palleronia</i>	
<i>Natronincola</i>	<i>Pseudoalteromonas</i>	
<i>Sporosarcina</i>	<i>Psychroflexus</i>	

close phylogenetic relationship of many moderately halophilic and non-halophilic bacteria indicates that they are not evolutionary descendents of a single lineage and that they evolved as extremophilic micro-organisms adapted to high saline environments by developing haloadaptation mechanisms that are similar to those of halo-tolerant bacteria.

The biology of moderately halophilic bacteria has been reviewed elsewhere (Ventosa *et al.*, 1998), with special emphasis on their taxonomy, ecology, physiology and metabolism, genetics, as well as biotechnological applications. Most studies carried out with laboratory cultures have used a limited number of species, especially members of the genera *Halomonas*, *Chromohalobacter* and *Salinivibrio* (Ventosa *et al.*, 1998). However, most species have been described very recently and, besides their taxonomic description and in some cases a few other data concerning their physiological adaptation to hypersaline environments, little is known about them. It would be necessary to carry out extensive studies over the next few years in order to know in more detail whether the moderately halophilic bacteria constitute a homogeneous group with respect to their mechanisms of haloadaptation and other molecular-biological aspects, or there may be interesting new aspects to be explored. To date, only one genome has been sequenced, from the heterotrophic Gram-negative bacterium *Chromohalobacter salexigens*, but this sequence is still not completely available, and this is a field in which more efforts are clearly necessary.

The most interesting features of moderately halophilic bacteria are their haloadaptation mechanisms and, since they may grow over a wide range of salt concentrations, their ability to adapt to changes in environmental osmolarity. Their main strategy for coping with osmotic stress caused by changing salinity of the surrounding medium is based on the intracellular accumulation of organic compounds, compatible solutes, which function as osmoprotectants. These compatible solutes can be taken up from the external medium if they are present, as in the cases of the betaines or choline, or can be synthesized by the cells, like ectoine or β -hydroxyectoine (Ventosa *et al.*, 1998; Nieto & Vargas, 2002).

The ecological distribution of moderately halophilic bacteria based on culture-dependent methods was already established (Rodriguez-Valera *et al.*, 1985; Rodriguez-Valera, 1988; Ventosa *et al.*, 1998). Recent studies based on molecular ecology techniques have been carried out (Bowman *et al.*, 2000; Benlloch *et al.*, 2002; Ma *et al.*, 2004; Rees *et al.*, 2004; Bidle *et al.*, 2005). However, new approaches would be required in order to define their ecological role and relationships with haloarchaea and halo-tolerant bacteria in hypersaline environments.

The moderately halophilic bacteria have very interesting industrial applications and they show many potential biotechnological uses, but most of them need to be studied in more detail. Their ability to produce organic compounds as compatible solutes is one of the best-known industrial applications. Several compatible solutes such as ectoine or hydroxyectoine are commercially available and are used in the cosmetics industry. Since many of them can grow well under low (0.5 % NaCl) or high (20–25 % NaCl) salinities,

they are used for the biological treatment of saline industrial waste effluents and for the biodegradation of toxic compounds (Ventosa & Nieto, 1995; Mellado & Ventosa, 2003). More recently, they are being explored for the production of several extracellular enzymes (amylases, proteases, lipases) (Mellado *et al.*, 2004).

VIRUSES

Few studies have been carried out with respect to the presence of viruses in hypersaline environments and their ecological roles. Most studies have focused on haloarchaeal viruses (haloviruses). Surprisingly, considering the presence of bacteria in such environments, information concerning bacteriophages is almost non-existent. Three phages, designated F9-11, F5-4 and F12-9, from lysogenic strains of *Halomonas halophila* (formerly *Deleya halophila*) have been isolated and characterized (Calvo *et al.*, 1988, 1994); they have isometric heads and non-contractile tails. The salt concentration for optimal adsorption and phage production is between 2.5 and 7.5%. Another bacteriophage specific for a moderately halophilic bacterium was described by Kauri *et al.* (1991). The first haloarchaeal virus was reported by Torsvik & Dundas (1974), but the most extensive studies were carried out by Wolfram Zillig and colleagues at the Max Planck Institute in Munich. They described and studied several haloviruses, particularly the halovirus ϕ H, that infect *Halobacterium salinarum* (Schnabel *et al.*, 1982). More recently, Mike Dyall-Smith and co-workers, from the University of Melbourne, have greatly contributed to our knowledge of the diversity and molecular characteristics of haloviruses. Several recent articles have reviewed them in detail and can be consulted for information in more depth (Dyall-Smith *et al.*, 2003, 2005; Tang *et al.*, 2004a).

Considering that virus populations in natural environments are assumed to be greater than those of their prokaryotic hosts and that each host species is susceptible to infection by several different viruses, it may be deduced that a great diversity of such viruses must exist in hypersaline environments. However, to date, only about 15 haloviruses have been described and very few have been studied in detail. Few attempts have been made to isolate viruses from hypersaline environments, and most studies used *Halobacterium salinarum* as host archaeon, a species that is not predominant in most hypersaline habitats studied.

The halovirus ϕ H is the most intensively studied halovirus. It is a temperate, head–tail virus with a genome of about 59 kb linear double-stranded (ds) DNA. Apart from a few methylase genes, it shows little sequence similarity to the genomes of bacteria, bacteriophages or eukaryotic viruses. At the level of replication, morphology and control of lysogeny, it shows similarities to P-type coliphages, such as P1 (Stolt & Zillig, 1994a, b). Another temperate, head–tail halovirus, ϕ Ch1, with a linear dsDNA genome

of 55 kb, has been studied. Its host is the haloalkaliphilic archaeon *Natrialba magadii*. It shows extensive similarity to halovirus ϕ H, but they have several differences that reflect their different hosts and ecosystems (Klein *et al.*, 2002).

Two head–tail haloviruses, HF1 and HF2, isolated from crystallization ponds of an Australian saltern, have also been studied (Tang *et al.*, 2004a). Unlike the previously reported viruses, they are lytic and infect a wide range of haloarchaea (*Halobacterium*, *Haloferax*, *Haloarcula*, *Natrialba*, *Haloterrigena* and *Halorubrum*). The two haloviruses have similar features, like a head-and-tail morphology and linear dsDNA genomes with similar sizes. The complete sequences of the HF1 and HF2 genomes have recently been completed (Tang *et al.*, 2002, 2004b). They are the largest archaeal virus genomes sequenced; HF1 is 75.9 kb (Tang *et al.*, 2004b) and HF2 is 77.7 kb (Tang *et al.*, 2002). Comparison of the two genomes showed that they are 94.4 % identical. Except for a single base change, HF1 and HF2 are identical in sequence over the first 48 kb, but they are very different in the other 28 kb region, suggesting a recent recombination event between either HF1 or HF2 and other HF-like haloviruses. This example suggests that there is a high level of recombination among viruses that live in hypersaline environments (Tang *et al.*, 2004b).

Very recently, a new halovirus, designated SH1, has been isolated from a hypersaline lake in Australia. It has a spherical morphology and infects *Haloarcula hispanica* and a *Halorubrum* isolate. It is an icosahedral, dsDNA virus with morphology that suggests the presence of a membrane underneath the protein capsid (Porter *et al.*, 2005). Bamford *et al.* (2005) have determined the complete sequence of the SH1 genome (31 kb) and identified genes for 11 structural proteins. The SH1 genome is unique and, except for a few open reading frames, shows no detectable similarity to other sequences from databases, but the overall structure of the SH1 virion and its linear genome with inverted terminal repeats is reminiscent of lipid-containing dsDNA bacteriophages like PRD1 (Bamford *et al.*, 2005).

In summary, knowledge of the diversity of haloviruses has increased, particularly in recent years, but is still in its infancy when compared to knowledge of bacteriophages. Their isolation from hypersaline waters was probably biased by the use of host strains that perhaps are not the predominant microbiota in natural environments. Direct electron microscopy of hypersaline waters shows that they maintain high levels of virus-like particles (about 10 times higher than the cell population), with recognizable morphotypes including head–tail and lemon-shaped particles (Guixa-Boixereu *et al.*, 1996; Oren *et al.*, 1997; Pedrós-Alió, 2004). Guixa-Boixereu *et al.* (1996) determined the abundance of viruses in two different salterns in Spain and they observed that the number of viruses increased in parallel to that of prokaryotes, from 10^7 ml⁻¹ in the

lowest salinity ponds to 10^9 ml⁻¹ in the most concentrated ponds (crystallizers), thus maintaining a proportion of 10 virions per prokaryotic cell throughout the salinity gradient. A lemon-shaped virus was found infecting square archaea; its abundance increased along the salinity gradient together with the abundance of the square archaea. In addition, many square cells were infected by viruses with other morphologies. Two additional studies (Díez *et al.*, 2000; Sandaa *et al.*, 2003) carried out in the salterns of Alicante (Spain) determined the genetic diversity of the viruses by pulsed-field gel electrophoresis. Sandaa *et al.* (2003) detected an increase in diversity from sea water to intermediate salinity (15 %), followed by a decrease at higher salinities. Oren *et al.* (1997) observed the presence of large numbers of virus-like particles by electron-microscopy techniques in water samples of the Dead Sea. Up to 10^7 virus-like particles ml⁻¹ were detected, showing a variety of morphologies, from spindle-shaped to polyhedral and tailed phages. The recent culture under laboratory conditions of the square haloarchaea will permit the isolation of haloviruses that infect them and will increase our knowledge of virus diversity in hypersaline environments. Further studies are necessary in order to understand their role in the ecology and evolution of haloarchaea.

OTHER HALOPHILIC ORGANISMS

According to the information given earlier in this chapter, the organisms that inhabit hypersaline environments are predominantly prokaryotes, but other eukaryotic organisms may also be present, especially in habitats with lower salinities. They include different species that are adapted to the high salt concentrations or may just survive under these extreme conditions, such as algae, diatoms, protozoa or fungi. Several publications have reviewed their biodiversity in hypersaline environments (Rodríguez-Valera, 1988; Javor, 1989; Pedrós-Alió, 2004; Gunde-Cimerman *et al.*, 2005a, b). In saltern ponds, the change in species composition is associated with salinity and species halotolerance or salt requirements. Thus, halotolerant species can be expected to be progressively replaced by halophilic organisms as salinity increases; the presence of different eukaryotic species and their abundance decrease continuously with increasing salinity. Primary production is due to cyanobacteria and green algae; *Dunaliella* is the unicellular green algae responsible for most of the primary production in hypersaline environments. It is considered an excellent model organism for the study of salt adaptation in algae. Besides, some *Dunaliella* strains can accumulate very large amounts of β -carotene; this is produced commercially and constitutes a good example of biotechnological applications of halophilic micro-organisms (Oren, 2005). Rodríguez-Valera *et al.* (1985) and Rodríguez-Valera (1988) reported high productivity values in saltern ponds between 10 and 30 % salinity, with a maximum at around 25 % salts, which corresponded to the highest densities of *Dunaliella*. In ponds of salterns

and saline lakes with salinities higher than 10–15 %, large organisms disappear and the brine shrimp *Artemia salina* and the larvae of the brine fly *Ephedra* are the only macroscopic organisms that are observed.

Gunde-Cimerman and colleagues, from the University of Ljubljana, Slovenia, have carried out several studies focused on the isolation and characterization of fungi isolated from salterns in Slovenia and other geographical locations (Gunde-Cimerman *et al.*, 2004, 2005b). They observed a surprisingly rich diversity of fungi. Enumeration of fungi in these habitats revealed their presence in relatively large numbers (up to $4 \times 10^4 \text{ ml}^{-1}$), but the biodiversity appears to be limited to a small number of fungal genera. The melanized fungi were represented by a yeast-like fungus called black yeast and the related genus *Cladosporium*. Among the non-melanized fungi most frequently observed were species of the genera *Aspergillus*, *Penicillium* with teleomorphic stages and *Wallemia*, *Scupolariopsis* and *Alternaria*. Most species are halotolerant, but recent data support the existence of halophilic species (Gunde-Cimerman *et al.*, 2004; Kogej *et al.*, 2005). The black yeasts that were detected with the highest frequency just before the increase in NaCl for the crystallization process were *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and *Aureobasidium pullulans*. Since *Hortaea werneckii*, *Phaeotheca triangularis* and *Trimmatostroma salinum* are not known outside saline environments, it is assumed that hypersaline waters are their natural habitat (Gunde-Cimerman *et al.*, 2000, 2004). More recently, non-melanized yeasts have been isolated from several salterns worldwide and some salt lakes (Dead Sea, Great Salt Lake). Among the species isolated from these environments were *Pichia guilliermondii*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Candida parapsilosis*, well-known contaminants of low-water-activity food products, as well as other species not previously related to hypersaline habitats nor known for their halotolerance, such as *Rhodosporidium sphaerocarpum*, *Rhodosporidium babjevae* and *Rhodotorula larynges* (Butinar *et al.*, 2005).

Independently, an exhaustive study carried out by Buchalo *et al.* (2000) permitted the taxonomic characterization of filamentous fungi isolated from the Dead Sea. They included 26 species representing 13 different genera of Zygomycotina (*Absidia glauca*), Ascomycotina (most representative were species of *Aspergillus*, *Chaetomium*, *Cladosporium*, *Penicillium* and *Eurotium*, as well as a new species of the genus *Gymnascella*, designated *Gymnascella marismortui*) and mitosporic fungi (four species belonging to the genera *Acremonium*, *Stachybotrys* and *Ulocladium*). It must be pointed out that most fungal species isolated from the Dead Sea waters are common soil fungi and are probably contaminant halotolerant organisms that may be adapted to live in this hypersaline environment or are present as dormant spores (Buchalo *et al.*, 2000).

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