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Archaeal Diversity in the Dead Sea: Microbial Survival under Increasingly Harsh Conditions

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ABSTRACT

The Dead Sea is rapidly drying out. The lake is supersaturated with NaCl, and precipitation of halite from the water column has led to a decrease in sodium content, while concentrations of magnesium and calcium greatly increased, making the lake an ever more extreme environment for microbial life. In the past decades, blooms of algae (*Dunaliella*) and halophilic Archaea were twice observed in the lake (1980–1982 and 1992–1995), triggered by massive inflow of freshwater floods, but no conditions suitable for renewed microbial growth have occurred since. To examine whether the Dead Sea in its current state (density 1.24 g ml^{-1}, water activity about 0.67) still supports life of halophilic Archaea, we collected particulate matter from a depth of 5 m at an offshore station by means of tangential filtration. Presence of bacterioruberin carotenoids, albeit at low concentrations, in the particulate material showed that members of the *Halobacteriaceae* were still present in the lake's water column. Amplification of 16S rRNA genes from the biomass yielded genes with less than 95% identity with environmental sequences reported from other environments and only 89-95% identity with cultivated *Halobacteriaceae.* It is thus shown that the Dead Sea, in spite of the ever more adverse conditions to life, supports a unique and varied community of halophilic Archaea. We have also isolated a number of strains of *Halobacteriaceae* from the samples collected, and their characterization is currently in progress.

INTRODUCTION

During the nearly three decades that have passed since the start of a systematic monitoring program of the biological communities and processes in the Dead Sea, the lake has proven to be an ever changing environment, requiring constant adaptation of the microorganisms inhabiting its waters. Occasional rainy winters bring large amounts of floodwaters diluting the upper meters of the water column, triggering dense microbial blooms. When this happens, the density of red halophilic Archaea can be so high that the entire lake obtains a red color. On the other hand, the lake is now drying out at an ever increasing rate, and this had important implications for the salt concentration and the ionic composition of its waters.

While many halophilic and halotolerant microorganisms are known to inhabit NaCl-dominated ('thalassohaline') brines up to halite saturation, only few organisms can live in the 'athalassohaline' environment of the Dead Sea, dominated by divalent cations. Studies in the past have shown that quantitatively the most important inhabitants of the water column are the unicellular green alga *Dunaliella*–the sole primary producer in the lake, and red extremely halophilic Archaea of the family *Halobacteriaceae* (Kaplan & Friedmann 1970; Oren 1988). Species first reported from the Dead Sea include *Haloferax volcanii* (Mullakhanbhai & Larsen 1975), *Haloarcula marismortui* (Oren et al. 1990), *Halorubrum sodomense* (Oren 1983a), and *Halobaculum gomorrense* (Oren et al. 1995a). A recent addition to the list is *Haloplanus natans*, an unusual flat, gas-vesicle-containing organism isolated from an experimental mesocosm at Sedom (Elevi Bardavid et al. 2007; see also Oren et al. 2004, 2005). Other organisms have been obtained from the Dead Sea as well, including colorless members of the domain Bacteria, protozoa, and fungi (Oren 1988, 2003), as well as viruses (Oren et al. 1997). The quantitative importance of these components of the biota in governing the biological properties of the lake is as yet unclear.

In the years 1980–1982 and 1992–1995, dense blooms of microorganisms were observed in the entire Dead Sea. In both cases these blooms occurred following exceptionally rainy winters that caused the formation of a pycnocline at a depth varying between 5 and about 15 m, turning the holomictic regime of the lake into a meromictic one (Gavrieli et al. 1999; Gavrieli & Oren 2004). During these blooms the density of the biota was very high: up to 9 x 10^3 and 1.5 x 10^4 *Dunaliella* cells ml^{-1} and up to 2 x 10^7 and 3.5 x 10^7 prokaryote cells ml^{-1} were counted in 1980 and 1992, respectively (Oren & Shilo 1982; Oren 1983a, 1985, 1988, 1993; Oren & Gurevich 1993, 1995; Oren et al.

1995b, Oren 1997, 1999a, 1999b, 2000, 2002). These archaeal blooms imparted a red color to the entire lake. The blooms ended with the termination of the meromictic state and the renewed overturn of the water column (Oren 1985; Anati et al. 1995; Oren & Anati 1996). Field observations combined with laboratory simulations have shown that two conditions must be fulfilled for a microbial bloom to occur in the Dead Sea: the salinity of the upper water layers must become reduced to a sufficient extent, and phosphate, the limiting nutrient in the lake, must be available (Oren & Shilo 1985; Oren et al. 2004, 2005).

The overall negative water balance of the lake has caused a drop in water level, in the past decade over a meter per year (Yechieli et al. 1998; Oren & Gavrieli 2002; Gavrieli & Oren 2004). The water column is now supersaturated with NaCl, and large amounts of halite have precipitated to the bottom. As a result, the ionic composition of the brines has changed dramatically: the Na^+ concentration decreased from 1.73 M in 1977 to 1.54 M in 2007, while Mg^{2+} increased from 1.81 M to 1.98 M. The lake's chemistry is thus becoming increasingly dominated by divalent cations (Table 1). The water activity (a_w) of the brines is now around 0.67, near the lowest level known to support life (Oren 2008). Due to the precipitation of halite, the total salt concentration has remained approximately constant at around 340 g l^{-1}. The pH of the brine is about 6.

Table 1–Changes in the ionic composition of the Dead Sea waters, 1977–2007. Data for 1977 were derived from Beyth (1980); data for 1996 and 2007 were provided by the Geological Survey of Israel and the Dead Sea Works Ltd. All concentrations are in mol l^{-1}.

	1977 (average)	1996 (average)	2007 (average)
Ion			
Na^+	1.730	1.590	1.540
K^+	0.180	0.200	0.210
Mg^{2+}	1.810	1.890	1.980
Ca^{2+}	0.430	0.440	0.470
Cl^-	6.340	6.340	6.480
Br^-	0.070	0.070	0.080
SO_4^{2-}	0.005	0.005	0.004

As conditions suitable for renewed microbial growth have not occurred since the 1992–1995 microbial bloom, and as conditions in the lake have since become ever more extreme due to the continuing drying out accompanied by a dramatic increase in the divalent/monovalent cation ratio, the question should be asked whether the Dead Sea may already now have become too extreme even for the most salt-tolerant and salt-requiring microorganisms, or whether this unusual lake does still support different forms of life (Oren 1999b). We have therefore initiated a study, involving culture-independent molecular microbial ecology approaches as well as classic culture-dependent techniques, to examine the present state of the Dead Sea biota. It is our goal to compare the biological properties of the current Dead Sea both with the properties of the lake in earlier times when conditions were less extreme than presently, and with other, thalassohaline lakes dominated by halophilic Archaea. We here report the first data on our characterization of the *Halobacteriaceae* present in the water column in the beginning of 2007 and show that the Dead Sea still supports a small, but apparently unique community of halophilic Archaea adapted to life in this unusual lake.

MATERIALS AND METHODS

Sampling of Dead Sea and Concentration of the Biomass by Tangential Filtration

Samples were collected on February 5, 2007 and March 8, 2007 at a station 4 km east of Ein Gedi, at the location of a moored meteorological station (31° 25' N, 35° 26' E), where the depth of the lake is about 100 m. Water pumped through a hose from a depth of 5 m was immediately diluted with 10% (vol/vol) of filter-sterilized distilled water to prevent clogging of the tangential filtration filters due to the crystallization of halite from the NaCl-supersaturated brine. During the February 5, 2007 cruise, the water was prefiltered through glass fiber filters (Millipore AP2514250; nominal pore size 0.8-8 µm, diameter of the filtered area 11 cm) before it was fed into a tangential filtration system with a Millipore (Billerica, MA) Pellicon® 2 unit equipped with a C screen 0.22 µm Durapore® 0.5 m^2 cartridge. About 200 l of water could be passed through such a glass fiber filter before it became clogged with brownish material. After it had become apparent that the glass fiber prefilter effectively removed all prokaryotes from the water and that little material was subsequently collected by the tangential filtration setup, no prefiltration was used during the March 8, 2007 cruise, and the Dead Sea water, diluted as above with distilled water, was directly pumped into the tangential filtration unit.

Molecular Characterization of Archaeal 16S rRNA Genes from DNA Collected from Prefilters and Ultrafiltration Retentate

DNA was extracted according to the method of Massana et al. (1997). Archaeal 16S rRNA genes were amplified by PCR from DNA collected from the prefilters as well as from the concentrated Dead Sea water. For Archaea we used two specific primers: Ar20-F (5'-TTC CGG TTG ATC CYG CCR G-3') (DeLong et al. 1999) and Arch958R (5'- TCC GGC GTT GAM TCC ATT-3') (DeLong 1992).

Microscopic Estimation of Prokaryote and Algal Densities

Prokaryotic cells (Archaea and Bacteria combined) in Dead Sea water were enumerated microscopically using a Petroff-Hauser counting chamber after 380-fold concentration by centrifugation (40 min, 12000 x g). The relative accuracy of prokaryotic cell counts was estimated at ± 20%.

For the enumeration of *Dunaliella* cells, 5 ml portions of fixed samples were supplemented with 0.1 ml of 0.1 N iodine to stain intracellular starch. The samples were then filtered through Millipore filters (25 mm diameter, 5 µm mean pore size, cat. no. SMWP-25). Filters were placed on microscope slides, and cells were counted under a 16x objective as outlined by Oren & Shilo (1982) and Oren et al. (1995b).

Pigment Determinations

The content of chlorophyll and carotenoids collected on the glass fiber prefilters was determined on sections (4.5-11 cm^2) cut out of the filters following overnight extraction in 3 ml methanol/acetone (1:1, by volume). The extracts were cleared of particles by centrifugation, and their absorption spectra (400-700 nm) were measured in a Cary Varian model E1 scanning spectrophotometer, using the solvent as a blank. Archaeal bacterioruberin pigments were quantified based on an $E^{1\%}_{1}cm$ value of 2540 at 496 nm for α-bacterioruberin. As no significant amount of chlorophyll was detected in the samples, it was not necessary to apply a correction for the contribution of algal pigments to the total absorbance at this wavelength, as outlined in Oren & Gurevich (1995).

Isolation of Dead Sea Archaea on Hypersaline Growth Media

Material from collected on the glass fiber prefilter and ultrafiltration retentate was inoculated on a variety of growth media suitable for cultivation of a variety of halophilic Archaea. Best results in terms of colony recovery were obtained on a medium designed for halophilic Archaea from the Dead Sea (Oren 1983a; Oren et al. 1995a), which contained (g l^{-1}): NaCl, 125; $MgCl_2.6H_2O$, 160; K_2SO_4, 5; $CaCl_2.2H_2O$, 0.1; yeast extract, 1; peptone or casamino acids, 1; starch, 2; pH 7.0. The medium was solidified with 20 g l^{-1} agar. Plates were incubated at 35°C.

RESULTS

To obtain an upper estimate of the numbers of prokaryotic cells in the February 15, 2007 sample, we concentrated the particles suspended in the water by centrifugation and enumerated these microscopically. This same technique had been used to monitor the rise and decline of archaeal blooms in the Dead Sea in the past (Oren 1983b, 1985, 1993; Anati et al. 1995; Oren & Gurevich 1995; Oren & Anati 1996). We counted 4.5 x 10^5 'bacteria-like particles' per ml in the water sample collected from 5 m depth. For comparison, numbers up to 2 x 10^7 and 3.5 x 10^7 were observed during the 1980 and the 1992 archaeal blooms (Oren 1983b; Oren & Gurevich 1995). The number of 4.5 x 10^5 'bacteria-like particles' ml^{-1} may overestimate the true number of prokaryotic cells present in the sample as not all particles observed may have been prokaryotic cells. After filtration of 200 l of Dead Sea brine, the glass-fiber prefilters were stained brown rather than pink-red or purple as were expected when all particles were halophilic Archaea.

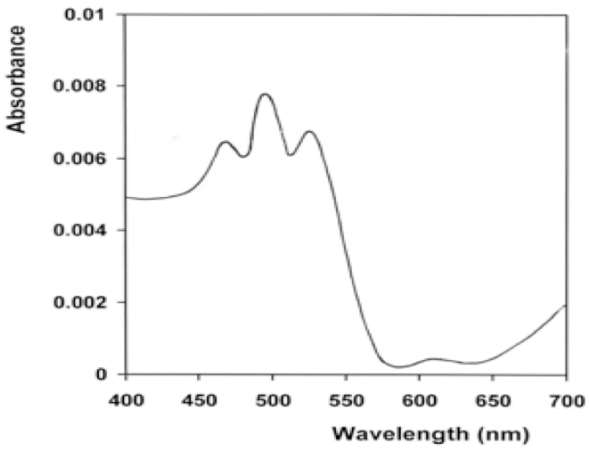

Figure 1–Presence of bacterioruberin carotenoids in the Dead Sea particulate material collected on February 15, 2007. A 4.5 cm^2 piece was cut out of a glass fiber prefilter (AP2514250; effective filtration diameter 11 cm) after filtration of 200 liter of diluted Dead Sea brine, extracted overnight with 3 ml of methanol/acetone 1:1 (vol/vol), and the absorption spectrum of the extract was recorded using the solvent as a blank.

The presence of members of the *Halobacteriaceae* on these prefilters was unequivocally proven when pieces of these prefilters were extracted in methanol/acetone 1:1 and the absorption spectra of the extracts recorded (Figure 1). The peaks at 496 and 530 nm and the shoulder at 470 nm are characteristic of the bacterioruberin carotenoids of the halophilic Archaea. No peaks of chlorophyll and algal carotenoids were found that would have indicated presence of *Dunaliella* or other phototrophs in the water column. Based on data including the volume of Dead Sea water that had passed through the filter, the filter area extracted and the specific absorbance of α-bacterioruberin, we estimated

the bacterioruberin content at 0.011 µg l^{-1}. This value should be compared to the peak value of 14 µg l^{-1} observed during the 1992 bloom when total cell counts were 3.5 x 10^7 ml^{-1}. When assuming a similar carotenoid content per cell in both communities, about 3 x 10^4 red, carotenoid-containing archaeal cells may be calculated to have been present per ml brine in early 2007. Taking all uncertainties and limitations of the approach into account, our calculation shows that in 2007 a small community of *Halobacteriaceae* was still found in the Dead Sea water column.

To obtain information on the nature of the Archaea present in the samples collected, and especially on the phylogenetic affiliation of the organisms, we amplified and sequenced archaeal 16S rRNA genes from DNA isolated from the glass fiber prefilters (February 15, 2007) and ultrafiltration retentate (March 15, 2007). Table 2 presents a representative selection of the sequences retrieved and their similarity with published 16S rRNA sequences of cultured haloarchaeal species and of 'environmental sequences'. The data presented suggest that the community of halophilic Archaea was diverse. Similarity values with 16S rRNA sequences of the type strains of the published species within the family *Halobacteriaceae* showed low values only (87-96%); for the 14 sequences shown, the closest phylogenetic affiliation was found with the species *Halorhabdus utahensis* (4x), *Halosimplex carlsbadense* (3x), *Halomicrobium mukohataei* (3x), *Halogeometricum borinquense* (1x), *Haloplanus natans* (1x), *Natronomonas pharaonis* (1x), and *Halalkalicoccus tibetensis* (1x). In some cases a somewhat higher similarity existed with 'environmental sequences' recovered from a variety of hypersaline environments worldwide, but still the Dead Sea sequences appear to be unique and do not closely resemble sequences recorded from other hypersaline habitats.

We also succeeded in isolating a number of colonies of red halophilic Archaea and other prokaryotes from the samples retrieved during the February-March 2007 cruises. For isolation of *Halobacteriaceae*, the high-magnesium (0.8 M) –low nutrient medium described in the Materials and Methods section proved to be superior to all other media tested. Use of similar media had in the past led to the isolation of *Halorubrum sodomense* and *Halobaculum gomorrense* (Oren 1983a; Oren et al. 1995a). On other, less saline media richer in organic nutrients a number of halophilic or halotolerant Bacteria were recovered, including members of the genus *Halobacillus* and the *Halomonas/Chromohalobacter* group. The isolates are currently being subjected to a taxonomic evaluation.

Table 2–Representative archaeal 16S rRNA sequences retrieved from the glass fiber prefilters (February 15, 2007) and ultrafiltration retentate (March 8, 2007) with prokaryote biomass collected from the Dead Sea, and their similarity with published 16S rRNA sequences of cultured haloarchaeal species and of environmental sequences.

	Sequenced Length (bp)	Closest Related Named Species and Other Cultured Strains	Closed Related Environmental Sequence
February 15, 2008			
1	755	*Halorhabdus utahensis* (94%); Ancient salt deposit isolate (McGenity et al. 2000) (95%)	Tuz Lake, Turkey (Burçin et al. unpublished) (97%)
2	908	*Halosimplex carlsbadense* (91%)	Tuz Lake, Turkey (Burçin et al. unpublished) (92%)
3	757	*Halogeometricum borinquense* (93%)	Australian saltern (Burns et al. 2004) (95%)
4	413	*Halorhabdus utahensis* (95%); Ancient salt deposit isolate (McGenity et al. 2000) (99%)	Saline soil (Walsh et al. 2005) (97%)
5	753	*Haloplanus natans* (96%)	
6	755	*Halomicrobium mukohataei* (89%); Ancient salt deposit isolate (McGenity et al. 2000) (99%)	Tuz Lake, Turkey (Burçin et al. unpublished) (92%)
7	541	*Natronomonas pharaonis* (89%); Ancient salt deposit isolate (McGenity et al. 2000) (99%)	Saltern, Korea (Park et al. unpublished) (92%)
March 8, 2007			
1	850	*Halosimplex carlsbadense* (90%)	Evaporite crust (Sahl et al. unpublished) (90%)
2	850	*Halosimplex carlsbadense* (90%)	Saline soil (Walsh et al. 2005) (90%)
3	790	*Halomicrobium mukohataei* (89%)	Alkaline, saline soil, Mexico (Valenzuela-Encinas et al. unpublished) (95%)
4	850	*Halalkalicoccus tibetensis* (87%)	Alkaline, saline soil, Mexico (Valenzuela-Encinas et al. unpublished) (95%)
5	1030	*Halomicrobium mukohataei* (92%)	Saline soil (Walsh et al. 2005) (93%)

DISCUSSION

The Dead Sea is a far more extreme environment for life than all 'thalassohaline', NaCl-dominated salt lakes. The a_w value for a saturated NaCl solution is about 0.75, while the a_w value of the Dead Sea waters is now as low as 0.67 (Oren 2008). Not only the low water activity makes the Dead Sea hostile to life, also the specific ionic composition is continuously becoming more difficult to tolerate by microorganisms. Divalent cations such as Mg^{2+} have a destabilizing, 'chaotropic' effect on proteins and other cellular components. Therefore the concentrations at which such ions are present, as well as the concentration ratio of such destabilizing ions and stabilising ('kosmotropic') ions such as Na^+, determines whether or not microbial life may be possible. In the Na^+-saturated Dead Sea, large amounts of NaCl have precipitated to the bottom in the past 25 years, leading to an ever increasing ratio of Mg^{2+}/Na^+. As shown in the present paper, a small community of Archaea and other microorganisms still survives in the lake, but conditions have probably become too extreme for active growth, and the alga *Dunaliella* has not been seen in the water column during the past 12–13 years.

One of the indications for the continued presence of halophilic Archaea in the Dead Sea was the finding of bacterioruberin carotenoid pigments (Figure 1). It should be noted that the spectrophotometric pigment assay following extraction in organic solvents did not enable qualitative or quantitative assessment of the presence of retinal pigments that some members of the *Halobacteriaceae* possess and that enable the cells to use light energy to pump out protons and produce ATP (bacteriorhodopsin) or pump chloride ions into the cells (halorhodopsin). Such pigments may help the cells survive in situations in which the availability of organic substrates is limited, as is surely the case in the Dead Sea at times *Dunaliella*, the sole primary producer known from the lake, is absent from the water column. During the 1980–1981 bloom, bacteriorhodopsin was present in the archaeal community (Oren & Shilo 1981) and the pigment was shown to contribute to the energy metabolism of the cells (Oren 1983c). Whether such retinal pigments are also currently present in the Dead Sea and may contribute to the survival of the microbial communities cannot be assessed on the basis of the information obtained. Cultivation experiments using a variety of media have yielded a number of isolates, Archaea as well as Bacteria, and these are currently being characterized. It will be of special interest to see whether one or more of the archaeal isolates obtained may harbor a 16S rRNA gene that is identical to one of the genes directly amplified from the community DNA (Table 1). In this way it can be assessed to what extent the isolates may or may not be representative for the total archaeal community. We also isolated a number of Bacteria (*Halobacillus* and organisms affiliated to the *Halomonas/Chromohalobacter* group). Based on the first results of our analyses of fosmid clones prepared from the community DNA of the March 8, 2007 sample (see below), it appears that most genes recovered are probably archaeal, and that the Bacteria are present at a lower level in the overall community. These data will be documented elsewhere.

Analysis of the 16S rRNA genes amplified from the community DNA yielded sequences affiliated with the genera *Halorhabdus*, *Halosimplex*, *Halomicrobium*, *Halogeometricum*, *Haloplanus*, *Natronomonas*, and *Halalkalicoccus*. It is interesting to note that none of the sequences showed presence of close relatives of those genera that have earlier been recovered from the Dead Sea: *Haloarcula*, *Haloferax*, *Halorubrum* and Halobaculum. There is no indication that organisms isolated and described from the Dead Sea such as Har. marismortui, *Hfx. volcanii*, *Hrr. sodomense* and *Hbc. gomorrense* were ever numerically dominant in the lake. It is more probable that these isolates happened to grow quite easily in enrichment media and on agar plates, so that they could be conveniently isolated and characterized. Biomass material from the 1992–1993 archaeal bloom in the Dead Sea has been preserved, and the application of a culture-independent, 16S rRNA-gene-based approach toward the characterization of the bloom community may in the future show to what extent the dominant type(s) of Archaea in that bloom have or have not already been cultured. Recovery of a 16S rRNA gene from the 2007 community with a relatively high (96%) similarity to *Haloplanus natans* is interesting. *Hpl. natans* (Elevi Bardavid et al. 2007) is an organism recently discovered (as a minor component of the community) in a red archaeal bloom that had developed in a 0.9 m^3 mesocosm on the grounds of the Dead Sea Works Ltd. near the southern end of the lake. This mesocosm contained a mixture of Dead Sea water and Red Sea water, and had been supplemented with phosphate to stimulate growth of *Dunaliella* (Oren et al. 2004, 2005). The finding of 16S rRNA sequences affiliated with the genera *Natronomonas* and *Halalkalicoccus* was completely unexpected, as these genera consist thus far of alkaliphilic organisms only, while the Dead Sea has a slightly acidic pH.

We are now expanding the molecular study of the 2007 Dead Sea microbial community beyond the 16S rRNA gene characterization. A fosmid library has been prepared of the community DNA, and we are now in the process of sequencing as much DNA from this library as possible to obtain information not only on the phylogenetic affiliations of the organisms present in the community but also on their metabolic potential. We thus also hope to obtain an insight into the special adaptations that may be connected with life and survival in the present-day Dead Sea, one of the harshest environments known to support life.

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