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SALT LAKE RESEARCH

Microbial communities and processes within a hypersaline gypsum crust in a saltern evaporation pond (Eilat, Israel)

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Abstract Gypsum crusts containing multicolored, stratified microbial communities develop in the evaporation ponds of a commercial saltern in Eilat, Israel at salt concentrations between 190 and 240 g l^{-1}. The upper 0.5–2 cm of the crust is densely populated by orange-brown unicellular cyanobacteria. Below, a layer of green-colored filamentous cyanobacteria is found. Underneath, a bright purple layer of anoxygenic phototrophs is present, below which a reduced black layer is found. We have investigated the biological properties of this crust using a wide variety of techniques, and we here review the results of these interdisciplinary studies. The tests performed included microscopic examination of the biota, phylogenetic analyses based on 16S rRNA gene clone libraries and denaturing gradient gel electrophoresis, fatty acid analysis, light intensity and light quality measurements, microelectrode studies of oxygen profiles and oxygen evolution, determination of sulfate reduction using radioisotope methods, and measurement of methane evolution. The stable vertical stratification in the system enabled separate analyses of the different layers with a high spatial resolution. It was therefore possible to combine the different approaches and obtain information

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on the activities of the different types of oxygenic and anoxygenic phototrophs, dissimilatory sulfate reducers and methanogens in the different layers, as well as phylogenetic information on the nature of the microorganisms responsible for these processes. The gypsum crust thus becomes a paradigm for the study of a wide variety of microbial processes and their interrelationships in the presence of high salt concentrations.

Keywords Hypersaline · Salterns · Gypsum · Halophilic · 16S rRNA gene sequences

Introduction

When seawater is evaporated in saltern ponds for the production of NaCl, sequential precipitation of different salts occurs. The first salt to precipitate is $CaCO_3$ (calcite or aragonite). This is followed by gypsum ($CaSO_4 \cdot 2H_2O$), which accumulates on the bottom of the evaporation ponds at salt concentrations above 140–150 g l^{-1}. When the salt concentration has reached values above 300 g l^{-1}, NaCl precipitates as halite crystals, leaving a concentrated solution of magnesium, potassium, chloride, and sulfate ions behind (Javor, 1989; Oren, 2002).

The gypsum crusts accumulating in salterns of intermediate salinity are often characterized by development of multicolored stratified microbial communities. Different types of cyanobacteria, purple sulfur bacteria, and other pigmented microorganisms arrange themselves according to the gradients of light intensity, oxygen, and sulfide concentrations present at different depths in the crust. Such layered prokaryotic communities within gypsum crusts have been documented from saltern ponds at different geographic locations: Spain (Cornée, 1984), France (Caumette, 1993; Caumette et al., 1994), Italy (Margheri et al., 1987), and from other hypersaline evaporite systems (Rothschild et al., 1994; Airs & Keely, 2003; Spear et al., 2003).

A particularly well-developed gypsum crust with brightly colored layers of microorganisms is found in a commercial saltern in Eilat, Israel, at salt concentrations between 190 and 240 g l^{-1} (Fig. 1). The upper 0.5–2 cm of the crust is densely populated by orange-brown unicellular *Halothece*-type cyanobacteria. Below, a layer of green-colored *Phormidium*-type filamentous cyanobacteria is found with occasional representatives resembling *Halospirulina* spp. (Nübel et al., 2000a). The brown and the green layers are sometimes separated by a 2–8-mm thick white zone that appears to be devoid of phototrophs. Underneath the green layer a bright purple layer of anoxygenic phototrophs is present, below which a reduced black layer is found. A thin, olive-green layer is sometimes present at the bottom of the phototrophic community (Oren, 2000, 2006; Sørensen et al., 2005).

We have investigated the biological properties of this crust during the last decade, using a variety of

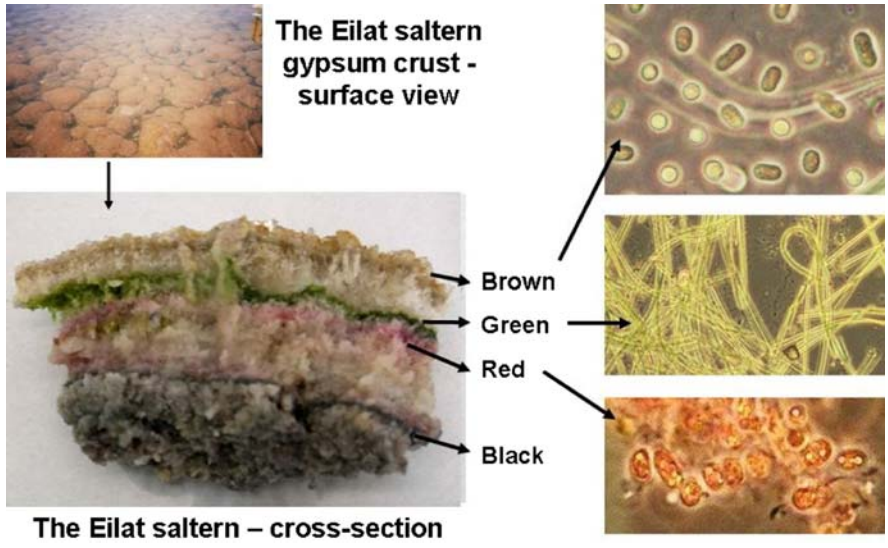

Fig. 1 The gypsum crust on the bottom of a saltern evaporation pond in Eilat, Israel: surface view, cross-section, and microscopic view of the organisms dominating in the *brown*, the *green*, and the *red* layer (Color figure online)

techniques. These include microscopic examination of the biota (Oren et al., 1995; Oren, 2005; Sørensen et al., 2005), analysis of photosynthetic pigments (Oren et al., 1995) and of UV-absorbing pigments and their function (Oren, 1997), phylogenetic analyses based on 16S rRNA gene clone libraries and denaturing gradient gel electrophoresis (DGGE) (Sørensen et al., 2005), fatty acid analysis (Oren et al., 2005; Ionescu et al., 2007), light intensity and light quality measurements (Oren et al., 1995), microelectrode studies of oxygen profiles and oxygen evolution, determination of sulfate reduction using radioisotope methods and measurement of methane formation, and examination of the effect of salinity variations on the different processes (Canfield et al., 2004; Sørensen et al., 2004). Most samples were collected between 0.5 and 1 m from the shore line; the depth of brine over the crust varied from 5 to 10 cm. The analysis of the different types of data collected in the course of these studies provides a unique opportunity to obtain an in-depth understanding of the biota present within the gypsum crust and their activities, as determined by their position within the gradients of light, oxygen, and sulfide concentrations, as well as by the high salinity of the system. We here review the data obtained and provide an integrative synthesis of the wealth of information collected on the properties of the Eilat gypsum crust.

Oxygenic photosynthesis and the organisms performing it

The upper brown layer and the green layer below it are dominated by unicellular cyanobacteria and filamentous cyanobacteria, respectively (Fig. 1). Both cyanobacterial layers, and especially the upper brown one, are characterized by large amounts of slimy extracellular polysaccharides in which the cells are embedded. Microscopically, the most prominent organisms in the brown layer are unicellular *Halothece*-like cyanobacteria (Garcia-Pichel et al., 1998), while the green layer is dominated by filaments. Most of these belong to the *Phormidium*-type, but occasionally helically wound *Halospirulina*-like filaments (Nübel et al., 2000a) were encountered as well. Cyanobacterial filaments containing heterocysts were never observed. The cyanobacteria showed red autofluorescence in the epifluorescence microscope due to the presence of chlorophyll *a*. All these organisms consist of large cells, so that these cyanobacteria dominate in terms of biovolume, as well as by their conspicuous pigmentation. However, counts of DAPI-stained cells in the epifluorescence microscope showed that oxygenic phototrophs were greatly outnumbered by the other constituents of the community: the cyanobacteria constituted no more than 12% of the cell counts in the brown layer and 27% in the green layer (Sørensen et al., 2005). The occurrence of a white layer, devoid of phototrophs, separating the brown and the green cyanobacterial layers, may well be due to local nutrient depletion, when the brown layer absorbs nutrients from the overlaying water, and the green layer obtains its nutrients from anaerobic degradation processes in the lower layers.

Molecular surveys of the communities were made, based on sequencing of clone-libraries of PCR-amplified genes for 16S rRNA. Genomic DNA was extracted from samples of the different layers by three cycles of freezing/thawing, proteinase K treatment, and extraction in phenol/chloroform/isoamylalcohol. The DNA was precipitated with ethanol and purified. PCR reactions were performed with primers bac8f (5′-AG(A/G)GTTTGATCCTGGCTCAG-3′) and bac1492r (5′-CGGCTACCTTGTTACGACTT-3′) for amplification of bacterial genes for 16S rRNA, or arc8f (5′-TCCGGTTGATCCTGCC-3′) and arc1492r (5′-GGCTACCTTGTTACGACTT-3′) for archaeal genes (Teske et al., 2002). PCR products were cloned and representative clones were sequenced. In addition, rRNA gene fragments of about 150 base pairs were amplified using primers bac341f (5′-CCTACGGG(A/G)GGCAGCAG-3′) and bac521r (5′-ACCGCGGCTGCTGGCAC-3′) for Bacteria and arc344f (5′-ACGGGG(C/T)GCAGCAGGCG-3′) and arc518r (5′-GGT(A/G)TTACCGCGGCGGCTG-3′) for Archaea. Both forward primers were supplied with a GC-clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) at the 5′-end. The PCR products were separated by DGGE. Individual bands were excised, reamplified, and sequenced (Sørensen et al., 2005).

Molecular surveys based on sequencing of clone-libraries of PCR-amplified genes for 16S rRNA showed that oxygenic phototrophs were outnumbered by the other constituents of the community (Sørensen et al., 2005). The cyanobacteria were even underrepresented among the phylotypes found in the clone

libraries compared with the in situ abundance of cyanobacterial cells. Possible explanations are differences in the number of rRNA operons in each prokaryotic cell and/or bias introduced by differences in DNA extraction efficiency and subsequent PCR amplification. Three cyanobacterial phylotypes were detected (Sørensen et al., 2005). Two of these are affiliated (97–99% identity) with the *Halothece*-cluster of unicellular and halophilic cyanobacteria (Garcia-Pichel et al., 1998). The third 16S rRNA gene sequence amplified was distantly related (93%) to *Microcoleus chthonoplastes*. Similar sequences have been recovered from hypersaline microbial mats elsewhere, such as, e.g., the salterns of Guerrero Negro, Mexico (Nübel et al., 2000b).

For pigment analyses, samples were extracted with acetone or with methanol-acetone 1:1 (by volume) to extract chlorophylls, carotenoid pigments, as well as mycosporine-like amino acids (MAAs). Absorption spectra were recorded between 300 and 800 nm. Pigments were separated by high performance liquid chromatography, using a reverse phase column (Merck LiChrospher RP 18) eluted with a water—acetonitrile/methanol/tetrahydrofuran gradient for chlorophyll and carotenoid analysis or isocratic elution with 0.1% acetic acid or with 6% methanol + 0.2 acetic acid for MAA analysis. Pigments were detected with a diode array detector or a spectral analyzer based on a rotating prism (Oren et al., 1995).

The color of the brown *Halothece*-containing layer was caused by a high content of carotenoids (mainly myxoxanthophyll and echinenone) and a low content of chlorophyll *a*, as expected for cells exposed to high light intensities. Typical irradiance levels in Eilat in summer are around 2,000 μmol quanta m^{-2} s^{-1}, and this intensity is only little attenuated by the thin layer (about 10 cm) of clear water overlaying the gypsum crust. Measurements of scalar irradiance level at different depths within the crust, using microsensors (Lassen et al., 1992), showed that the intensity of the 620–675 nm light reaching the top of the green layer was about 1–2% of that at the surface. Most of the wavelengths below 550 nm had been filtered out by the carotenoid pigments in the upper brown layer. The remaining wavelengths between 550 and 700 nm were effectively absorbed by the pigments in the green layer: a high content of chlorophyll *a* and the blue phycocyanin (Oren et al., 1995).

Extracts of the upper brown layer also showed a very high absorbance of ultraviolet light, the absorbance at 331 nm typically being 8–10 times as high as that of the carotenoid maximum at 450 nm (Oren, 1997). The cyanobacterial cells contain two UV-absorbing pigments belonging to the group of MAAs, one with a maximum absorbance at 331 nm and the other at 365 nm. The first compound has now been identified as mycosporine-2-glycine (Kedar et al., 2002); the second is a novel compound, and its structure was recently elucidated (Volkmann et al., 2006). One of the functions of these MAAs is surely the absorption of harmful UV-B radiation. However, there are also indications that these MAAs are present within the cells dissolved in the cytoplasm at such high concentrations that they may also contribute to osmotic balance within the cells which must withstand the very high salt concentrations in their environment (Oren, 1997, 2006).

Photosynthetic activities were estimated based on measurements of oxygen evolution, using a specially constructed Clark-type oxygen microelectrode with a guard cathode (Revsbech, 1989). The electrode was inserted into a 0.8-mm (outer diameter) hypodermic needle with the sensor tip just protruding at the bottom of the beveled edge. The electrode was mounted in a micromanipulator on an electrode stand and connected to a pico-amperemeter. Since the crust was impossible to penetrate without breaking the needle electrode, a pre-drilled hole of the same size as the electrode needle was used for the measurements of oxygen profiles. Photosynthetic rates were measured by the light/dark shift method (Revsbech et al., 1981, 1983): the electrode was placed at the desired position in the crust and allowed to equilibrate, light was then shielded off by covering the electrode setup with a black felt cloth, and the decrease in oxygen concentration was monitored during 15–45 s.

Microelectrode studies to monitor the levels of oxygen at different depths within the gypsum crust showed, as expected, pronounced diel variability. Oxygen was found to penetrate down to 2-cm depth (the bottom of the green layer) during daytime. Two separate peaks in oxygen concentration developed during the day, corresponding to the depths housing the two prominent cyanobacterial populations. In the most active crusts examined, fourfold oxygen supersaturation was reached in the upper brown layer

around noon. Although there was some evidence early in the morning for oxygen production within the cyanobacterial green layer, oxygen production within this layer was not intense, and elevated oxygen concentrations did not persist through the day. Instead, anaerobic conditions prevailed within this layer for part of the day and all of the night (Canfield et al., 2004).

Based on the observation that cyanobacteria make up the greatest part of the biovolume of the brown and green layer, we analyzed the fatty acid composition of the biomass in these layers to obtain further information about the way of life of the different types of cyanobacteria. Lipids were isolated from crust samples using a modified Bligh and Dyer extraction. Total lipids or the isolated phospholipid fraction were saponified and the released fatty acids esterified in a one-step process by heating in the presence of 2% sulfuric acid in methanol. Fatty acid methyl esters were then extracted with hexane—methyl-*tert*-butyl-ether (1:1, v:v), separated by gas chromatography—mass spectrometry, and identified according to their elution time and their mass spectra, based on comparison with using authentic standards (Ionescu et al., 2007).

The upper brown layer contained significant amounts of polyunsaturated fatty acids (21% and 7%, respectively, of the total fatty acids on the average being 16:2 cis 7,10 and 18:2 cis 9,12) (Oren et al., 2005; Ionescu et al., 2007). Polyunsaturated fatty acids are rarely found in prokaryotes, but they do occur in many cyanobacteria. Unicellular cyanobacteria can be divided into groups that contain only saturated and mono-unsaturated fatty acids, others that also contain di-unsaturated fatty acids, and some others that have tri-unsaturated fatty acids as well. Polyunsaturated fatty acids can only be synthesized in an oxygen-dependent biosynthetic pathway, and the presence of such fatty acids in the community points to an aerobic life style, as expected for oxygenic phototrophs.

In contrast, the green layer was found devoid of polyunsaturated fatty acids. This was to some extent unexpected, as a survey of the fatty acid content of different types of filamentous cyanobacteria shows di-, tri- and even tetra-unsaturated fatty acids to be common. Abundant amounts of 18:2 and to a lesser extent 16:2, as well as the large amounts of 16:1 cis 9 in hypersaline (70–100 and 100–140 g l^{-1} salts, respectively) cyanobacterial mats from Spain, containing *Phormidium* or *Microcoleus* as main primary producers, suggest the use of the oxygen-dependent pathways for the synthesis of unsaturated fatty acids (Grimalt et al., 1992). Moreover, the mono-unsaturated fatty acids present in the green layer differed in structure from those in the upper brown layer: 16:1 cis 7 and 18:1 cis 9 dominated in the green layer, while in the upper brown layer the dominant positional isomer of 16:1 was 16:1 cis 9 (Oren et al., 2005; Ionescu et al., 2007). Occurrence of these fatty acids points to the use of the "anaerobic", oxygen-independent pathway of fatty acid biosynthesis. The key reaction in this pathway is the dehydration of an intermediate β-hydroxydecanoyl-ACP (acyl carrier protein).

There are a few precedents in the literature for the occurrence of the oxygen-independent biosynthetic pathway for mono-unsaturated fatty acids, combined with lack of polyunsaturated fatty acids in some filamentous cyanobacteria, and these all relate to the organisms from low-oxygen environments, often occurring in the presence of high sulfide concentrations and adapted to an anaerobic way of life in which sulfide is used as alternative electron donor in photosynthesis (Jahnke et al., 1989; Oren et al., 1995).

To assess the availability of sulfide within the green cyanobacterial layer, we used a microelectrode sensitive to H_2S gas. H_2S diffusing through the electrode tip is converted to HS^- in the alkaline ferricyanide solution behind the tip. The HS^- is immediately oxidized by the ferricyanide producing S° and ferrocyanide. The ferrocyanide is reoxidized at the anode of the sensor, generating a current. The sulfide microelectrode was placed in the micromanipulator setup next to the oxygen electrode with the sensing tips of both electrodes positioned at the same level. Total sulfide concentrations were calculated on the basis of the H_2S concentration measured and the pH, measured independently by means of a pH microelectrode. These measurements showed that indeed the green layer, although obviously capable of photosynthetic oxygen evolution, is found during a large part of the day under anaerobic conditions in the presence of sulfide. We have shown that the cyanobacterial community in the green layer is capable of CO_2 photoassimilation in the presence of sulfide and DCMU, an inhibitor of photosynthetic electron flow

between photosystem II and photosystem I in oxygenic photosynthesis (Oren et al., 2005). Such an ability to perform anoxygenic photosynthesis has been documented as well in other cyanobacteria growing under anaerobic conditions (Cohen et al., 1975). The ability of the cyanobacteria in this layer to use sulfide as electron donor in an anoxygenic type of photosynthesis, as well as the lack of polyunsaturated fatty acids and the production of mono-unsaturated fatty acids in an oxygen-independent pathway, may all be considered as adaptations toward a largely anaerobic mode of life of these filamentous cyanobacteria.

In order to assess the effect of the salinity of the brine on the photosynthesis rates in the brown and the green cyanobacterial layer, we equilibrated cores or slurries prepared from the different layers with brines of different salt concentrations and quantified photosynthesis rates both on the basis of oxygen production as measured with microelectrodes, as well as on the basis of $^{14}CO_2$ photoassimilation rates. Such measurements were made by incubation of the samples in stoppered-glass vials in the presence of $NaH^{14}CO_3$ in the light and in the dark, collection of the microorganisms on glass fiber filters, acidification to remove any remaining radiolabeled inorganic carbon, and liquid scintillation counting. These experiments indicated that both the unicellular and the filamentous cyanobacteria metabolized at near-optimum rates at the in situ salinity. The slurry experiment indicated that the unicellular cyanobacteria in the brown layer were well adapted to the hypersaline conditions and were growing near their optimal salinity between 180 and 220 g l^{-1}. A second peak in carbon fixation appeared at low salinities (<100 g l^{-1}) in the slurry experiments (Sørensen et al., 2004). A similar pattern had been observed for evaporites from Guerrero Negro, Mexico containing *Halothece*-like cyanobacteria (Rothschild et al., 1994), excepting that there the maxima were shifted toward higher salt concentrations, with the minimum occurring at 230 g l^{-1}. It may also be noted that our culture experiments showed that the unicellular cyanobacteria isolated from the brown layer in Eilat grew optimally at relatively low salt concentrations (30–50 g l^{-1}). Photosynthesis in the green layer was strongly inhibited at salinities above 230 g l^{-1}. Oxygen formation in the microelectrode experiment peaked at 198 g l^{-1} and decreased only slightly at lower salinities. The slurry experiment indicated a broad range of optimum salinities for CO_2 photoassimilation from 80 to 230 g l^{-1}.

Photosynthetic purple bacteria

The purple layer found directly beneath the filamentous green cyanobacteria was densely populated by single-celled phototrophs containing sulfur globules, and resembling *Chromatium*/*Halochromatium*-type purple sulfur bacteria (Fig. 1). Motile spiral-shaped bacteria resembling *Ectothiorhodospira*/*Halorhodospira* were also often encountered. This layer varied in thickness from 0.2 to 2 cm (Oren et al., 1995; Sørensen et al., 2004). Similar layers of purple bacteria have been described from hypersaline gypsum crusts in salterns in France (Caumette, 1993; Caumette et al., 1994) and in Spain (Cornée, 1984). Microscopic counts showed that the *Halochromatium*-type cells with internal sulfur granules constituted 6% of the total prokaryote cell counts (Sørensen et al., 2005). As in the case of the cyanobacteria, their contribution to the biomass/biovolume is much greater as the cells are much larger than those of most other prokaryotes present: the purple bacteria typically measure 5–7 × 2–3 μm (Oren et al., 1995).

In view of the reasonably large abundance of the *Halochromatium*-type cells in the biomass, it is surprising that no sequences affiliated with phototrophic *Gammaproteobacteria* were encountered in our clone libraries constructed from genomic DNA. The reasons for our failure to find the expected sequences may be similar to those given above for the cyanobacteria. We did, however, find a number of sequences that may be affiliated with other types of phototrophic proteobacteria. A phylotype encountered often (>30% of the total number of clones in the red layer, virtually absent in the other layers) was 97% identical to *Roseospira* (*Alphaproteobacteria*). Sequences with a high similarity to *Rhodovibrio salinarum*, *Roseovarius tolerans*, and *Rhodovulum* sp. were occasionally detected (Sørensen et al., 2005).

The *Chromatium* group (purple sulfur bacteria; *Gammaproteobacteria*) does not possess any distinctive fatty acids that may be used as biomarkers: 16:0, 16:1, and 18:1 are the most common fatty acids in these organisms. We did find between 4% and 6% 19:0 cyclo 11–12 in the red-purple layer. The fatty

acid 19:0 cyclo was reported to occur in the *Ectothiorhodospiraceae* (purple sulfur bacteria; *Gammaproteobacteria*) in proportions varying from 0% to 38%, depending on the species and culture conditions. Whether indeed the 19:0 cyclo detected in the gypsum crust was derived from either *Ectothiorhodospira* or *Halorhodospira* could not be ascertained, as this fatty acid was also found below the red layer, sometimes at even higher concentrations. The *Chromatiaceae* and the *Ectothiorhodospiraceae* were reported to contain the hydroxy fatty acids 10:0 3-OH, 12:0 3-OH, and 14:0 3-OH. Unfortunately, our chromatographic system was not able to detect such hydroxy fatty acids at a satisfactory sensitivity.

Bacteriochlorophyll *a* was the photosynthetically active pigment detected in the red gypsum layer. In extracts, an absorption peak was found at 755 nm, while in vivo the pigment had a dual peak in the infrared range, between 800 and 860 nm. Spectral analysis of the light penetrating down to the red layer, using the scalar irradiance microsensors, showed that despite the deep burial, about 1% of the near infrared radiation penetrated below the green layer, and was rapidly absorbed in the red layer (Oren et al., 1995).

We have made estimations of the potential photosynthesis rate in the red layer, based on the assumption that sulfide produced by the lowermost black layers serves as electron donor for the purple bacteria and is reoxidized to sulfate (Canfield et al., 2004). Sulfate reduction measurements (see below) allowed us to estimate a flux of sulfide in the order of 0.2–0.3 nmol cm^{-2} min^{-1}. Oxidation of this sulfide by a 0.3–0.4-cm thick layer of purple bacteria gives rates of 0.5–1.0 nmol cm^{-3} min^{-1}, equivalent to 1–2 nmol CO_2 fixed cm^{-3} min^{-1}, or cell-volume specific rates of CO_2 fixation ranging from 0.2 to 0.4 nmol mm^{-3} min^{-1}. These rates are in the same range as those that can be calculated for the green cyanobacterial layer above. It suggests that the purple layer, in spite of being so deeply buried in the crust and in spite of the low ambient light intensities, is highly active in photosynthesis.

Slurry experiments to evaluate the effect of salinity on CO_2 fixation rates by the purple bacteria in the red layer showed optimal activity at 120 g l^{-1} salt, similar to the optimum of 100–120 g l^{-1} reported for *Halochromatium salexigens* isolated from the gypsum-depositing ponds at Salin-de-Giraud, France (Caumette et al., 1988). At the in situ salinity around 200 g l^{-1}, the rate was about half of the optimum (Sørensen et al., 2004).

Green filamentous anoxygenic phototrophs

An up to 2-mm thick olive-green layer was encountered below the layer of purple sulfur bacteria in part of the samples. Microscopic examination showed filamentous microorganisms of different types. Some showed a strong red autofluorescence, indicating presence of chlorophyll *a* (Sørensen et al., 2004). These were possibly cyanobacteria that had become buried as the crust expanded through precipitation and growth of crystals. There were also long slender filaments, much thinner than those of the *Phormidium* type cyanobacteria in the green layer, and these did not show red fluorescence. Morphologically, these filaments resembled members of the *Chloroflexus* group, and closely resembled "*Candidatus* Chlorothrix halophila", an obligatory anaerobic sulfide-dependent phototrophic green non-sulfur bacterium that was isolated from a hypersaline microbial mat, but still has not been brought into pure culture (Klappenbach & Pierson, 2004). *Chloroflexus*-like organisms are often encountered in the lower layers of hypersaline mats of phototrophic microorganisms (Pierson et al., 1994; Nübel et al., 2001). The presence of *Chloroflexus*-like organisms associated with the purple layer was confirmed from our molecular studies: a phylotype showing 98% identity with "*Candidatus* Chlorothrix halophila" was frequently recovered from this layer (15 out of the 29 bacterial clones sequenced) (Sørensen et al., 2005). Filamentous green non-sulfur bacteria are among the most abundant organisms in both oxic and anoxic layers of microbial mats from a variety of environments, but the physiological properties and the ecological role of the group are still poorly understood (Pierson et al., 1994; Nübel et al., 2001; Jonkers et al., 2003). The single fatty acid profile obtained for the olive-green layer was highly complex and differed greatly from that of the other layers, with a high content of 19:0 cyclo 11–12 and 15:0 anteiso (12.7% and 7.3%, respectively) (Ionescu et al., 2007). It remains to be determined whether these fatty acids were mainly derived either from the *Chloroflexus*-type filaments or from other, unidentified components of the biota in the layer.

Dissimilatory sulfate reduction

Different phylotypes of *Deltaproteobacteria* were detected in the crust that indicated affiliation with sulfate reducers of the "Desulfovibrionales" and the *Desulfobacterales*. These phylotypes constituted 1%, 7%, and 5% of clone libraries from the white, the green, and the lower purple + olive-green layers, respectively. The most encountered phylotype had 88% sequence similarity with *Desulfovibrio bastinii*, and we also found a clone 92% similar to *Desulfohalobium retbaense*, the most halophilic of all cultured dissimilatory sulfate-reducing bacteria (Sørensen et al., 2005).

Rates of dissimilatory sulfate reduction were determined by injecting ^{35}S-labeled sulfate into gypsum crust cores or sediment slurries under anaerobic conditions in the dark. After 12–24 h incubation at the in situ temperature, the cores were frozen and sawed in 1-cm slices that were immediately fixed with zinc acetate. The samples were distilled by the one-step distillation technique of Fossing & Jørgensen (1989), in which HCl and Cr^{2+} are added to release all reduced sulfur as H_2S, which is then trapped in Zn-containing traps and quantified by liquid scintillation counting.

Sulfate reduction rates in the dark, as measured in such radiotracer experiments, peaked in the depth interval between 1 and 2 cm, which was above the permanently anoxic zone. The rate of sulfate reduction in this layer was between 0.36 ± 0.04 and 0.72 ± 0.20 μmol cm^{-3} day^{-1}, and the rate decreased rapidly in the crust below to less than 0.2 μmol cm^{-3} day^{-1}. Integration of the sulfate reduction rates in the upper 7 cm of the crust yielded a depth-integrated rate of 1.9 μmol cm^{-2} day^{-1} or 0.022 nmol cm^{-2} s^{-1} (Sørensen et al., 2004) [data for March 2002; in the experiments performed a year earlier, 4–7 times higher rates were measured (Canfield et al., 2004)]. Compared with the photosynthesis rate at noon of 0.08 nmol O_2 cm^{-2} s^{-1} measured in the same crust sample, this shows that sulfate reduction is one of the major mineralization processes in the crust. The error margin of these measurements was rather large in view of the low counts of radiolabeled sulfide obtained in many cases, caused also by the high sulfate concentrations in the crust (Sørensen et al., 2004).

Sediment slurry experiments in which sulfate reduction rates were measured at different salinities showed optimum sulfate reduction rates at salt concentrations between 80 and 130 g l^{-1}, and a strong inhibition at the in situ salinity of 215 g l^{-1} (Sørensen et al., 2004). A similar behavior has been reported for halophilic sulfate- reducing bacteria in culture, such as *Desulfovibrio oxycinae*, *Desulfovibrio halophilus*, *Desulfohalobium retbaense,* and *Desulfobacter halophilus*. It may thus be assumed that the community of sulfate-reducing bacteria in the Eilat gypsum crust is growing at a considerable salinity stress. Measurements of sulfate reduction in slurries of sediments from Great Salt Lake showed a salinity response very similar to the one measured in the Eilat gypsum crust (Brandt et al., 2001).

We have no information as yet on the nature of the electron donors used by the community of sulfate reducers in the gypsum crust. Thermodynamic considerations have suggested that in salt-stressed systems, "incomplete oxidizers" such as *Desulfovibrio*, that grow on lactate or on hydrogen as electron donors, are likely to be able to make a living, while "complete oxidizers" such as *Desulfobacter*, that oxidize acetate, will be much more severely stressed (Oren, 1999). In fact, the most salt-tolerant, complete oxidizer isolated, *Desulfobacter halotolerans* from the sediments of the Great Salt Lake, Utah, grows optimally at 10–20 g l^{-1} salt only, which does not tolerate salt concentrations exceeding 130 g l^{-1} (Brandt & Ingvorsen, 1997).

Microscopically, most sulfate-reducing bacteria are not distinctive. Fatty acid profiles of the layers in which sulfate reduction occurs also do not necessarily provide much information about the presence or absence of certain types of sulfate reducers, as there is no a priori reason to assume that sulfate-reducing bacteria contribute the bulk of the lipids extracted from this layer. Still, fatty acid analysis may provide some information about the types of sulfate reducers encountered. The acetate-oxidizing genus *Desulfobacter* characteristically contains 16:0 10-methyl and other 10-methyl fatty acids. We detected 16:0 10-methyl in the black layer, as well as in the purple and in the olive-colored layers. Whether this fatty acid might have been derived from *Desulfobacter* could not, however, be ascertained. *Desulfobacter* also contains large amounts of the cyclopropyl fatty acid 17:0 cyclo. We did not detect this fatty acid, but the long acid methylation step employed in the FAME preparation may well have destroyed such compounds.

Methanogenesis

We limited the examination of the role of methanogenesis in the gypsum crust to measurements of methane concentrations, methane formation rates, and the search for archaeal phylotypes associated with the methanogens. Additional possible approaches such as the qualitative and quantitative assay of archaeal lipids and direct microscopic detection of methanogenic Archaea on the basis of F_{420} autofluorescence have not yet been applied in the study of the Eilat gypsum crust.

The only phylotype encountered in our clone libraries, which was closely affiliated with known methanogens (97% similar to *Methanohalophilus mahii* and *M. halophilus*) was found in three out of the 53 archaeal sequences obtained (Sørensen et al., 2005). Members of the genus *Methanohalophilus* grow by disproportionation of methyl substrates, as in methylamines and methanol, to methane and CO_2; they do not use H_2, formate, or acetate, thus avoiding competition for substrates with sulfate-reducing bacteria (Boone, 2001). In addition, a single sequence was found to be affiliated with the yet-uncultured MSBL1 group, hypothesized to be responsible for methanogenesis in hypersaline marine basins (van der Wielen et al., 2005). However, direct evidence for the existence of methanogenic strains within the group is still lacking.

The in situ concentrations of methane were about two orders of magnitude lower than the sulfide concentrations. The mean concentration was between 2 and 5 μM, with lower values in the upper layer of the crust. The concentration profiles showed little variation with the time of the day. Based on the vertical concentration gradient of methane measured in the crust, we estimated the total flux of methane from the sediment to be approximately 1.6×10^{-5} nmol cm^{-2} s^{-1}. Based on the earlier documented absence of significant methane-oxidizing activity in this environment (Conrad et al., 1995), this value should represent methane production. This low value, being less than 0.1% of the sulfate-reduction rate in the crust, suggests that activity of methanogens contributes only little to anaerobic mineralization in this ecosystem.

In slurry incubation experiments, in which sediment samples were incubated under a gas phase of nitrogen in sealed vials and the accumulation of methane was measured in the gas phase, the maximum rates of methanogenesis obtained (0.12–0.14 μM day^{-1}; both with and without molybdate addition—see below) were much lower than the rates of sulfate reduction at the same salinities (2.0–9.5 μM day^{-1}) (Sørensen et al., 2004).

Sediment slurry incubation experiments showed little inhibition of the methanogenic activity at the in situ salt concentration: rates of overall methanogenesis were similar over the salinity range between 80 and 220 g l^{-1} when these measurements were performed in the presence of molybdate, an inhibitor of dissimilatory sulfate reduction. In the absence of molybdate, methanogenesis rates sharply decreased below 180‰ (Sørensen et al., 2004). This suggested that the gypsum crust may contain two different communities of methanogenic Archaea, using different substrates. The community active in the lower salinity range apparently used substrates more successfully exploited by the sulfate reducers (hydrogen and acetate being the obvious candidates), while the community active at the in situ salinity appears to specialize in "non-competitive" substrates that are not used by sulfate reducers. Such substrates are, for example, trimethylamine and other methylated amines, as well as dimethylsulfide. These compounds are well known to be used by the most halophilic among the methanogenic Archaea characterized, and also energetically the use of these substrates is more favorable than that of the "competitive" substrates, hydrogen and acetate (Oren, 1999). In fact, halophilic or halotolerant methanogens that use hydrogen or acetate as energy sources at salt concentrations above 100–150 g l^{-1} have yet to be found. The most halotolerant isolate that grows on $H_2 + CO_2$ is *Methanocalculus halotolerans* obtained from an oil well, and growing up to 12% NaCl with an optimum at 50 g l^{-1} (Ollivier et al., 1998).

Aerobic chemoautotrophic sulfur bacteria

Microscopic observations have shown the presence of prominent spherically shaped colorless bacteria with intracellular globules, presumably of elemental sulfur, associated with the layer of red-purple bacteria or immediately above it (Oren et al., 1995). Morphologically, these organisms resemble the genus *Achromatium*, and based on the presence of intracellular sulfur granules and apparent lack of pigmentation,

these yet-to-be-isolated organisms presumably make a living by chemoautotrophic oxidation of sulfide with oxygen as an electron acceptor.

In our 16S rRNA gene clone library, we found one phylotype affiliated with a known chemolithotrophic sulfide oxidizer, a sequence with 97% identity to *Thioalkalivibrio jannaschii* (*Gammaproteobacteria*; family *Ectothiorhodospiraceae*). This sequence was encountered eight times, and only from the white layer between the two pigmented cyanobacterial layers, a layer shown to undergo daily fluctuations in oxygen and sulfide content (Sørensen et al., 2005). The genus *Thioalkalivibrio* consists of sulfur-oxidizing organisms that use nitrate or oxygen as electron acceptors, and it seems likely that the phylotype detected in the crust share this metabolism. No such organisms have yet been isolated from the saltern gypsum crust.

Heterotrophic microorganisms

Based on the microscopic counts, only a small percentage of the microorganisms present in the colored layers within the gypsum crust actually belonged to pigmented phototrophs. Most prokaryotes present are probably heterotrophs that degrade organic material produced by the photosynthetic communities. Most of the 16S rRNA gene sequences recovered from the different layers are associated with known genera of heterotrophs, belonging to different groups of Bacteria (Sørensen et al., 2005). Most abundant were representatives of the *Bacteroidetes* (75 clones). These included a large number of sequences affiliated with *Salinibacter*, a recently discovered genus of extremely halophilic Bacteria from hypersaline environments (Antón et al., 2002), members of which have already been isolated from the crystallizer ponds of the Eilat salterns (Elevi Bardavid et al., 2007). We also found numerous types of *Alphaproteobacteria*, *Gammaproteobacteria*, and others. A complete account of the phylotypes detected and their possible affiliations has been given by Sørensen et al. (2005). 16S rRNA gene sequences belonging to the *Bacteroidetes* and to different groups of *Alphaproteobacteria* and *Gammaproteobacteria* were also the most frequently encountered in a gypsum-encrusted community in the Salin-de-Giraud salterns at the Mediterranean coast of France (Mouné et al., 2003).

Within the saltern ecosystem, halophilic Archaea of the family *Halobacteriaceae* are most abundantly found in the salt-saturated environment of the crystallizer ponds. However, salinities such as encountered in the gypsum crust are also conducive for growth of archaeal halophiles; in fact, most isolates grow optimally in the laboratory at salt concentrations around 200 g l^{-1}. Therefore it was not surprising to find 16S rRNA gene clones amplified from the gypsum crust using archaeal primers. Out of the 53 archaeal sequences obtained, 10 were affiliated with the *Halobacteriaceae*. Four out of these showed moderate (91%) sequence similarity with *Haloferax lucentense*, one was 89% similar to *Haloferax volcanii*, and one was 99% similar to the 16S rRNA gene sequence of *Haloquadratum walsbyi*, the square halophilic archaeon that is so abundant in crystallizer ponds, and has recently been brought into culture (Bolhuis et al., 2004; Burns et al., 2004) and described as a new species (Burns et al., 2007).

Epilogue

The Eilat gypsum crust forms an ideal system to study many different aspects both of microbial diversity at extremely high salt concentrations and of activities of different metabolic groups in a stratified system with sharp dynamic gradients of oxygen, sulfide, and light intensity. The stable vertical stratification of the microorganisms embedded between the gypsum crystals allows analyses of the different layers with a high spatial resolution. It was therefore possible to combine a wide range of different experimental approaches, including in situ measurements, rate determination of processes in laboratory incubations, microscopy, and a molecular phylogenetic analysis of the diverse microbial communities in the different layers. This enabled us to obtain detailed information on the activities of the diverse types of oxygenic and anoxygenic phototrophs, dissimilatory sulfate reducers, and methanogens in the different layers, combined with information on the nature of the microorganisms responsible for these processes. In addition, these studies shed considerable light on the microbial diversity at high salinities. The Eilat saltern gypsum crust thus serves as a paradigm for the study of a wide variety of microbial processes and their interrelationships in the presence of extremely high salt concentrations.

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