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Diversity of halophilic archaea in the crystallizers of an Adriatic solar saltern

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Abstract

Haloarchaeal diversity in the crystallizers of Adriatic Sečovlje salterns was investigated using gene fragments encoding 16S rRNA and bacteriorhodopsin as molecular markers. Screening of 180 clones from five gene libraries constructed for each gene targeted revealed 15 different 16S rRNA and 10 different bacteriorhodopsin phylotypes, indicating higher haloarchaeal diversity than previously reported in such hypersaline environments. Furthermore, results of rarefaction analysis indicated that analysis of an increasing number of clones would have revealed additional diversity. Finally, most sequences from the crystallizers grouped within the *Halorubrum* branch, whereas square-shaped '*Haloquadratum*' relatives, repeatedly reported to dominate crystallizer communities, were rare. Presence of such special and diverse haloarchaeal community could be attributed to the Sečovlje salterns rare continuous short-cycling salt production mechanism.

Keywords: 16S rRNA; Bacteriorhodopsin; *Haloarchaea*; Saltern; Hypersaline; Halophiles

1. Introduction

Hypersaline waters of a saltern crystallizer at salinities at or near saturation are one of the most extreme environments with respect to the sodium chloride concentration. The dominant microorganisms in such systems are members of the halophilic archaeal family *Halobacteriaceae* [1–3], co-existing with a small percentage of halophilic bacteria, such as the recently described *Salinibacter ruber* [4], halophilic fungi and protists.

The microbial diversity of this type of hypersaline environments has been extensively studied, focusing on the use of both molecular ecological [1,5–8] and cultivation-based methods [9,10]. All these efforts have been concentrated on crystallizers of solar salterns located in areas with Mediterranean or arid climate, allowing sufficient solar energy for year-round solar salt production with one to two salt harvests per year.

In areas with no dry season, solar salt production is enabled by modifications to an almost universal solar salt production technology. To date, the effect of such changes on microbial assemblages remains unknown. Thus, our objective was to investigate the abundance and diversity of halophilic archaea in the crystallizers of this type of solar salterns and make comparisons with studies of geographically different saltern crystallizers. To this aim, we have chosen the most northerly located

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solar salt production system on the Adriatic Sea coast. Slovenian Sečovlje solar salterns exhibit several interesting features. Dating from the 12th century, the salterns are located in southeast part of Piran Bay along the estuary of the Dragonja River. The salterns receive enough solar energy for salt production from May to October. During the salt harvesting season the evaporation of seawater is strongly enhanced by offshore winds, which cool the evaporating surface so that the brine temperature rarely exceeds 32 °C [11]. The crystallizer sedimentation surface is stabilized with a firm stromatolitic mat, allowing only manual salt collection. Here, the brine is led from the concentrators to crystallizers once or twice a day. Unlike in other salterns studied, the salt is harvested almost continuously as soon as it starts to crystallize, as opposed to one or two yearly harvests. Such continuous short-cycling technology is enabled by keeping the crystallizer brine levels extremely shallow, measuring less than 10 cm in depth. The crystallizers do not have the typical red brine coloration.

Sequencing of PCR-amplified 16S rRNA genes from DNAs extracted from environmental samples has proven to be a powerful and very reliable means of describing microbial community structure. Recently, the studies of genes encoding relevant ecological activities came in focus gaining insight into ecological functions of groups under investigation [12,13]. To further assess haloarchaeal diversity, we have chosen to investigate – in parallel – the diversity of bacteriorhodopsins, a wide and diverse group of proteins occurring almost ubiquitously within haloarchaea [14]. Containing retinal as a chromophore, bacteriorhodopsins function as proton pumps within the membrane, generate an electrochemical gradient and are assumed to have a role in a bioenergetics of the cell, providing a light-driven energy source.

2. Materials and methods

2.1. Saltern and biomass collection

Water samples were collected from the crystallizer pond at the Sečovlje solar saltern located in Sečovlje (near Portorož), Slovenia (E 13°36′, N 45°28′), in August 2003. The water was kept in 0.5-l sterile flasks for 2–3 hours until further processing in the laboratory. Samples (5 μl) were analyzed using a Zetopan Binolux microscope (Reichert, Germany). For cell counts, cells were stained with acridine orange (Sigma–Aldrich Chemie, GmbH, Steinheim, Germany) and 20–30 microscopic fields were analyzed.

The following physicochemical parameters were determined: pH (ISO 10523: 1994E, electrometric method), temperature and water activity (a_w) of the sample (CX-1 system, Campbell Scientific Ltd.). Concentrations of nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were analyzed using standard colorimetric procedures [15]. Extraction of total microbial community DNA was performed as described earlier [16]. Samples were stored in Tris–EDTA buffer, pH 8 at 4 °C.

2.2. Molecular techniques

16S rRNA PCR amplifications were performed using *Taq* DNA polymerase (Fermentas), primers D30 (5′-ATT CCG GTT GAT CCT GC) and D56 (5′-GYT ACC TTG TTA CGA CTT) from Arahal et al. [17] and the following program: 94 °C (2 min), followed by 30 cycles of 94 °C (45 s), 50 °C (45 s) and 72 °C (90 s), with an additional 5 s added for each cycle and a final 10 min extension step of 72 °C.

The second set of 16S rRNA PCR amplifications was performed using primers Arch21F (5′-TTC CGG TTG ATC CYG CCG GA) and Arch958R (5′-YCC GGC GTT GAM TCC AAT T), following the protocol described by DeLong [18].

Rhodopsins were PCR-amplified using primers bop1 (5′-GAC TGG YTG TTC ACS ACR CC) and bop2 (5′-ASG TCR AKS ACC ATG AA) as described earlier [19]. PCR products were then cloned using the pGEM-T Easy cloning kit (Promega) according to the manufacturer's directions and five clone libraries were constructed for each gene targeted. Clones positive for inserts were then sequenced using Perkin Elmer 377 DNA sequencer and edited using Sequencher (Gene Codes, Ann Arbor, USA). For bidirectional sequencing of 16S rRNA fragments, the additional primer B99 (5′-GTG TTA CCG CGG CTG CTG) was used [17]. Clone sequences have been deposited into GenBank under Accession numbers DQ071586–DQ071610.

2.3. Sequence comparisons and rarefaction analysis

Relevant sequences were obtained from GenBank (www.ncbi.nlm.nih.gov) using BLASTN and BLASTP. Alignments of the 16S rRNA region corresponding to nucleotides 74–710 (*Escherichia coli* numbering, [20]) and the bacteriorhodopsin region corresponding to Helix C to Helix G [14] were created using ClustalX [21]. The stability of the alignments has been assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP [22]. This conservative approach assumes that confidence in homology assessment increases with stability to variation in alignment parameters, and only unaffected positions across the spectrum of settings are considered to be unambiguously aligned and are kept for phylogenetic analysis. In the 16S rRNA analysis, gap penalties were incrementally adjusted from 7 to 17