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Research in Microbiology 159 (2008) 609–627

www.elsevier.com/locate/resmic

Prokaryotic community profiles at different operational stages of a Greek solar saltern

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Received 10 July 2008; accepted 24 September 2008
Available online 14 October 2008

Abstract

A combination of culture-dependent and independent approaches was employed to identify the microbial community structure in a Greek solar saltern. A total of 219 and 132 isolates belonging, respectively, to *Bacteria* and *Archaea*, were recovered. All bacterial isolates were phylogenetically related to 43 members of *Actinobacteria*, *Firmicutes* and *γ-Proteobacteria*. The archaeal isolates were placed within the *Halobacteriaceae*. At least four groups of isolates represented novel species among the *Bacteria*. High bacterial diversity, consisting of 417 subfamilies, was revealed using a high-density oligonucleotide microarray (PhyloChip). At the four stages of saltern operation analyzed, the archaeal community consisted of both *Crenarchaeota* and *Euryarchaeota*, except for the sediment where *Crenarchaeota* were not detected. The bacterial community in sediment consisted mainly of *γ-Proteobacteria* and *Actinobacteria*, while, in hypersaline water, it was restricted to a few representatives of *Bacteria*. Members of *α-Proteobacteria* were the main constituents in saturated brine and crude salt, followed by *γ-Proteobacteria*, *Actinobacteria* and *Firmicutes*. A large *Bacteroidetes* and *Verrucomicrobia* diversity was identified in saturated brine, while *δ-Proteobacteria* and *Cloroflexi* were abundant in crude salt. Significant changes in the microbial community structure were detected during a short time period, denoting a rapidly adaptive dynamic ecosystem and viable diversity. Prokaryotic members reported for the first time in solar salterns were identified.

Keywords: Halophiles; Prokaryotic diversity; Hypersaline; PhyloChip; MikroBioKosmos

1. Introduction

Hypersaline environments such as subterranean evaporate deposits of seawater, brines located in arid, coastal and deep-sea areas, naturally occurring and artificial solar salterns, like the Dead Sea and hypersaline lakes, are inhabited by both halotolerant and halophilic microorganisms which can adapt to a wide range of salt concentrations [45,46].

Solar salterns are well known as a habitat for halophiles [46,63] and an understanding of the microbial isolates in such hypersaline environments is highly desirable due to their potential applications [45]. Artificial solar salterns designed to harvest NaCl from seawater are found worldwide and consist of a set of shallow ponds connected by canals in which seawater is gradually driven to ponds of greater salinities, ranging from that of seawater to sodium chloride saturation and sometimes even beyond [52].

By a gradual salinity increase, moderate halophiles outcompete marine bacteria, which almost disappear above 15%

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doi:10.1016/j.resmic.2008.09.007

NaCl [63]. In higher salinities, halophilic archaea, which are able to grow up to NaCl saturation and survive even in salt crystals for a long time, appear to be dominant [40]. As salinity increases, progressive restriction of several physiological groups, such as acetoclastic methanogens and autotrophic nitrite oxidizers, is observed [58]. The remaining community mainly consists of oxygenic and anoxygenic phototrophs, aerobically respiring archaea and bacteria, and denitrifiers, which are able to operate at salt concentrations up to saturation [58,59]. In anaerobic hypersaline sediments, obligatory anaerobic, fermentative, and sulfate-reducing bacteria are reported to be the dominant microorganisms [41].

Both classical and molecular techniques, such as microscopy and flow cytometry [20], community level physiological profiles [36], polar lipid and pigment profiles [20,37], molecular fingerprinting [13], analysis of bacteriorhodopsin gene fragments as a molecular marker [48,50], fluorescence in situ hybridization (FISH) and 16S rRNA gene sequencing [6,35,68] have been used for analyzing the microbial community in solar salterns.

MikroBioKosmos (www.MikroBioKosmos.org) is the Hellenic National Initiative for the exploration and commercial exploitation of microbial national resources. The present study is part of this initiative and aims to determine prokaryotic diversity at the four different functional stages of an annual operation of a saltern located in the region of Messolonghi (the main sea-salt-producing area in Greece) using: (i) a culture-dependent approach and (ii) an innovative high-density oligonucleotide microarray that permits first-ever simultaneous monitoring of the population dynamics of almost 9000 distinguishable prokaryotic taxa/operational taxonomic units (OTUs). This is the first study in which a newly designed oligonucleotide microarray which can identify various microorganisms simultaneously by targeting unique regions of the 16S rRNA gene was employed in samples obtained from a solar saltern located in a geographic area that has not been previously studied. The salinity tolerance of isolated strains was determined in order to define relationships among selected groups and to obtain information concerning the influence of increased salinity to microbial communities. Novel taxa, which deserve further taxonomic analysis, were identified.

2. Materials and methods

2.1. Sampling

The Messolonghi-Tourlis saltworks, located in the western part of the city of Messolonghi (38°22′5″ N, 21°25′45″ E), western Greece, have been operating since 1902, producing annually up to 15,000 tons of sea salt. Four samples were taken from the Messolonghi saltworks during the functional cycle of a solar saltern, in a period of a year: sediment, hypersaline water, saturated brine and crude salt. Herein we use the term ''sediment'' (SD) to refer to the sample that was taken 3 cm from the surface of an empty evaporation pan in March 2005 (pH and electrical conductivity were 8.1 and 0.6 mS cm^{-1} at 10^{-2} dilution, temperature of 14 °C). By ''hypersaline water'' (HW), we refer to water that was collected from the solar saltern in July 2005 after 1 month of evaporation of the seawater in the pond (temperature, pH and salinity of 35.2 °C, 7.0 and 26%, respectively). ''Saturated brine'' (SB) refers to water (saturation point) that was obtained in October 2005 (pH 7.0 at 10^{-2} dilution, temperature of 30.8 °C). ''Crude salt'' (SA) is salt (pH 7.9 at 10^{-2} dilution and temperature of 10 °C) that was collected (from 15 cm depth of the bulk) in December 2005. Each of the above samples consisted of pooled subsamples obtained from at least five different representative sites.

2.2. Isolation of bacteria and archaea

Isolation was performed using high-substrate-concentration complex media based on findings of Burns et al. [12]. To isolate bacteria and archaea, 100 g (for crude salt and sediment) or 100 ml (for saline water samples) were mixed (up to 1 l) with sterile crude salt or brine solutions of appropriate concentrations (the same as salt concentrations used in isolation media). The mixtures were shaken for 3 h at 150 rpm and plated after a series of tenfold dilutions. From each dilution, 0.2 ml of the suspensions were aliquoted onto three replicate plates of appropriate isolation media. The following isolation media were used: (i) a slightly saline medium consisting of 40 g l^{-1} NaCl, 10 g l^{-1} glucose, 5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 0.1 mM $MgSO_4$ and phosphate buffer at pH 7; and (ii) saline media, consisting of 3 g l^{-1} peptone, 2.5 g l^{-1} yeast extract and 2 g l^{-1} KCl in the presence of: (a) 120 g l^{-1} NaCl plus 5 g l^{-1} $MgCl_2 \cdot 6H_2O$; (b) 120 g l^{-1} NaCl plus 50 g l^{-1} $MgCl_2 \cdot 6H_2O$; (c) 250 g l^{-1} NaCl plus 5 g l^{-1} $MgCl_2 \cdot 6H_2O$; or (d) 250 g l^{-1} NaCl plus 50 g l^{-1} $MgCl_2 \cdot 6H_2O$, respectively. One-fourth of the volume in each medium consisted of seawater. All media were solidified with 15 g l^{-1} agar. Plates were incubated at three different temperatures, 25 °C, 37 °C and 50 °C, for 3 months. Colonies were isolated and the purity of cultures obtained was checked routinely under a phase-contrast light microscope. Liquid cultures of all isolates obtained were maintained as glycerol stocks (20% v/v) at −80 °C.

2.3. PCR amplification and SSU rRNA gene sequencing of prokaryotic isolates

The nearly full-length 16S rRNA gene was amplified for all isolates by colony PCR using universal primers for bacteria and archaea (Table 1). A reaction mixture (20 μl) containing 10× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, the appropriate primers at 0.3 mM each and 1 U *Taq* polymerase (Minotech, Greece) was prepared. A PCR reaction was performed by an initial step at 94 °C for 10 min in order to lyse bacterial and archaeal cells and to denature DNA, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 52 °C and 90 s DNA chain extension at 72 °C. The PCR was completed by a final extension at 72 °C for 10 min. PCR products were purified by PEG precipitation. Sequencing reactions were performed by Macrogen (South Korea). The 16S rRNA gene