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New insights into the archaeal diversity of a hypersaline microbial mat obtained by a metagenomic approach

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ABSTRACT

A metagenomic approach was carried out in order to study the genetic pool of a hypersaline microbial mat, paying more attention to the archaeal community and, specifically, to the putatively methanogenic members. The main aim of the work was to expand the knowledge of a likely ecologically important archaeal lineage, candidate division MSBL1, which is probably involved in methanogenesis at very high salinities.

The results obtained in this study were in accordance with our previous report on the bacterial diversity encountered by using a number of molecular techniques, but remarkable differences were found in the archaeal diversity retrieval by each of the procedures used (metagenomics and 16S rRNA-based methods). The lack of synteny for most of the metagenomic fragments with known genomes, together with the low degree of similarity of the annotated open reading frames (ORFs) with the sequences in the databases, reflected the high degree of novelty in the mat community studied. Linking the sequenced clones with representatives of division MSBL1 was not possible because of the lack of additional information concerning this archaeal group in the public gene repositories. However, given the high abundance of representatives of this division in the 16S rRNA clone libraries and the low identity of the archaeal clones with known genomes, it was hypothesized that some of them could arise from MSBL1 genomes. In addition, other prokaryotic groups known to be relevant in organic matter mineralization at high salinities were detected.

Introduction

Microbial mats are one of the oldest, most diverse and highly productive microbial ecosystems known [38]. The prokaryotic communities thriving in these habitats are fueled by oxygenic photosynthesis within the surface mat layers that support anaerobic assemblages in the underlying organic horizons [38]. Although largely recognized for their potential contribution to early oxygenation of the Earth, Archean microbial mats were also a likely source of reduction gases (including H_2, CH_4, CO and methyl sulphides), as demonstrated by a number of studies [3,6,15,20,56].

In a previous study, we investigated the prokaryotic abundance and diversity of a microbial mat formed at the bottom of a crystallizer in a multi-pond solar saltern in the south of Mallorca Island, and a highly diverse and dense benthic prokaryotic community was found to be thriving in such salt-saturated sediments [28]. One of the most remarkable findings was the higher abundance and diversity of bacterial types compared to their archaeal counterparts. In fact, all the 16S rDNA archaeal clones retrieved affiliated with a single phylogenetic group, the candidate division MSBL1. This division was proposed as comprising the most abundant clones retrieved in a Mediterranean deep-sea hypersaline anoxic basin [55], for which no cultured representatives had ever been obtained. In a similar way, when molecular techniques were applied in order to explore the autochthonous microbiota of the brines of the crystallizers, the dominance of a phylotype was reported that had never been obtained previously by culturing techniques [4]. One decade later, *Haloquadratum walsbyi* was isolated, brought into pure culture, and described as one of the most ecologically relevant microorganisms in its habitat [5,25]. On the basis of the phylogenetic relatedness of MSBL1 to methanogens and the lack of other detected groups that might be responsible for methane production in the Mediterranean deep-sea brines, it was speculated that MSBL1 members might be involved in methanogenesis at very high salinities [55]. The 16S rRNA signature from very similar organisms was also found in the anoxic hypolimnion of a shallow hypersaline Solar Lake

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in Egypt [8], and sediments of hypersaline Lake Chaka in China [19].

Methane is a major end product of anaerobic biomass degradation in anoxic environments where the concentrations of sulphate, nitrate, Mn(IV) or Fe(III) are low, since in their presence methanogenesis is normally outcompeted by anaerobic respiration, mainly for thermodynamic reasons [52]. Most of the methanogens live in environments of low ionic strength, or in marine biotopes, but some genera are able to grow in salt concentration s of up to 4 M NaCl [27]. In the upper layer of marine sediments, where the sulphate concentration is usually high, methanogenesis is restricted to substrates that cannot be metabolized by sulphate-reducing bacteria (SRB), such as the methylamines originating from the breakdown of osmoregulatory amines. In hypersaline ecosystems, the important source of these non-competitive substrates is the N-trimethylated amino acid glycine betaine, which is an osmoregulator widespread among eukaryotes and prokaryotes living in hypersaline conditions [54]. Upon release from the cell, fermentative microorganisms can convert glycine betaine to methylamines, which provides an energy rich compound that can be used by many halophilic and halotolerant methanogens, but not by sulphate-reducing bacteria. Hydrogenotrophic methanogenesis could also be an important process in hypersaline environments since, contrarily to marine sediments, there are unusually high hydrogen partial pressures in hypersaline mats within the photosynthetically active surface layers of the mat [15,16]. Such conditions create favorable environments for hydrogenotrophic methanogenesis, since it alleviates the competition of methanogens with hydrogen-scavenging sulphate-reducing bacteria [6,15].

Despite their relevance in carbon cycling within hypersaline habitats, few halophilic methanogens have been isolated recently, and none has been taxonomically described in the last decade. However, although recent studies highlight the presence of numerous *Euryarchaeota* that may be halophilic methanogens, they still have not been cultivated [32]. To understand carbon cycling better in extremely saline environments, as well as to understand how methanogenic microorganisms cope with desiccation in these habitats, it is important to study the diversity of halophilic methanogenic representatives, and attempt to culture representatives of this group. The lack of information about archaeal diversity in salt-saturated sediments, and especially about hypersaline methanogens, helped form the basis of this study that aimed to extend our knowledge of the genetic and metabolic diversity of the archaeal assemblage in a highly saline microbial mat of a Mediterranean multi-pond solar saltern (Mallorca, Spain), as well as to attempt to culture methane-producing members of the community. In addition, other prokaryotic groups known to be relevant in organic matter mineralization at high salinities (i.e. *Firmicutes* and sulphate-reducing bacteria [36]) were also considered.

Materials and methods

Sample site and sample collection

Sediment samples were taken from the Mediterranean S'Avall solar salterns located on the south-east coast of Mallorca Island at 39°32′N; 002°99′E. The sampling site, which had been previously characterized and named as Station E [28], is located in a crystallizer pond from which the salt is periodically harvested. Between 5 cm and 10 cm of the soft sediment were sampled in June 2009, prior to salt harvesting. At this time, the development of a microbial mat in the salt crust overlaying the black sediment was observable (Fig. 1). Thus, the microbial mat in the salt crust was taken as the superficial sample (sample ERV), and the underlying sediment as the second subset sample (sample EN). Samples were extracted in methacrylate cores and, once homogenized, subsamples were taken with a sterile plastic syringe.

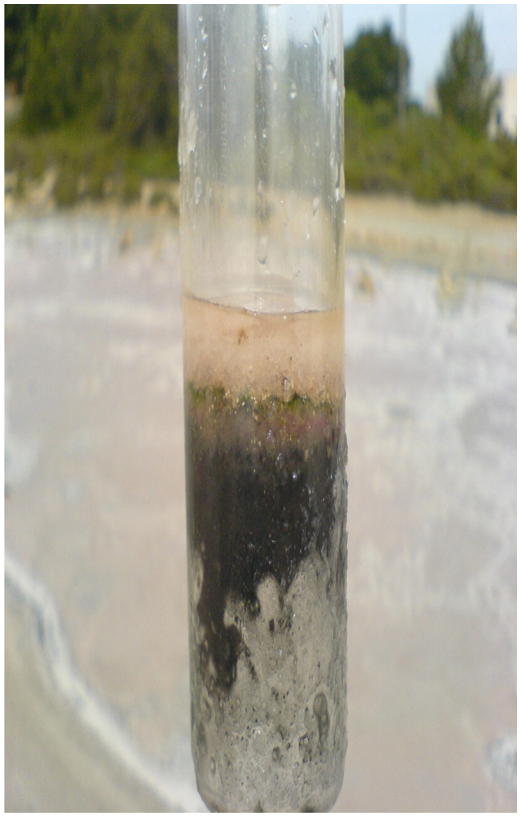

Fig. 1. Extracted core from station E, showing the well-developed microbial mat at the sampling time (early summer).

Retrieval of microbial biomass

Once taken, samples were immediately transported to the laboratory for processing. Due to sample manipulation difficulties encountered during DNA extractions, the cells were recovered from the solid phase. Briefly, homogenized subsamples (20 mL) were introduced into 50 mL Falcon tubes and mixed with 20 mL of PBS 4X by vigorous shaking. Cells were detached from sediments by immersing the tubes in an ultrasound bath (Selecta) for 15 min, mixing them every 3–4 min. The suspensions were centrifuged for 1 min at 2500 rpm and 14 °C. The washing/ultrasound step was repeated four times (or until a clean supernatant was obtained). Supernatants were recovered and kept on ice in 50 mL Falcon tubes. A centrifugation step of 2 min at 2500 rpm was performed to remove any remaining sediment particles. The supernatant was transferred to a new 50 mL Falcon tube and centrifuged for 10 min at 10,000 rpm at 14 °C. The segregation of cells from sediment does not significantly reduce the recovery of biomass, although it decreases the background and any contaminants [28]. The pellet was stored at −20 °C for subsequent DNA extractions. Highly purified suspensions of microorganisms were obtained by density gradient centrifugation with Nycodenz (Axis-Shield PoC), as described previously [13].

Nucleic acid extractions

The pellets obtained were resuspended in 5 mL saline buffer (1 M NaCl; 50 mM Tris–HCl), and the suspension was divided into 1 mL aliquots and introduced into 1.5 mL Eppendorf tubes. Tubes