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Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence in situ hybridization

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Abstract The combination of fluorescence in situ hybridization and microautoradiography (FISH-MAR approach) was applied to brine samples of a solar saltern crystallizer pond from Mallorca (Spain) where the simultaneous occurrence of *Salinibacter* spp. and the conspicuous square *Archaea* had been detected. Radioactively labeled bicarbonate, acetate, glycerol, and an amino acid mixture were tested as substrates for the microbial populations inhabiting such brines. The results indicated that hitherto uncultured 'square *Archaea*' do actively incorporate amino acids and acetate. However, *Salinibacter* spp. only showed amino acid incorporation in pure culture, but no evidence of such activity in their natural environment could be demonstrated. No glycerol incorporation was observed for any component of the microbial community.

Keywords Fluorescence in situ hybridization · Halophiles · In situ physiology · Microautoradiography · *Salinibacter* · Square archaea · Uncultured microorganisms

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Introduction

Molecular techniques applied to the understanding of the complexity and dynamics of natural microbial communities have led to the knowledge of the real extent of a biological diversity traditionally hindered by the use of culture-dependent techniques. Methods based on small subunit rRNA sequence analysis, which allow the phylogenetic identification and in situ detection of individual microbial cells (Amann et al. 1995), have been particularly successful. With such approaches, a vast number of hitherto uncultured prokaryotes have been discovered, and their morphologies, abundance, and population dynamics have been reported. However, one of the main challenges to contemporary microbial ecologists is the attribution of metabolic properties to those microorganisms never brought to pure culture. Moreover, even when an organism can be cultivated, properties determined in the laboratory may not necessarily reflect the activities and physiology of their counterparts in the environment (Gray and Head 2001). Knowing the metabolic properties that microorganisms exhibit in their natural environment is essential to understand their role and importance in a particular ecosystem. Linking the genetic identity of uncultured cells to their function in the environment is still in a very primitive stage, although several newly developed approaches allow us to obtain some initial insights into the ecological role of environmentally relevant prokaryotes (Gray and Head 2001).

Recently, the combined use of microautoradiography (MAR) and fluorescence in situ hybridization (FISH) has opened the door to the understanding of the nutritional requirements of hitherto uncultured microorganisms (Lee et al. 1999). Radiolabeled substrate uptake of single cells is followed by MAR (Brock and Brock 1966), and its combination with FISH identification with general or specific probes may reveal the metabolic capabilities of single identified cells or clusters (Lee et al. 1999). This technique, known as FISH-MAR, or STAR-FISH (substrate tracking autoradiographic fluorescent

in situ hybridization) has been successfully applied to samples of very different origin such as activated sludge (Lee et al. 1999; Nielsen et al. 1999; Daims et al. 2001), freshwater (Gray et al. 2000), and marine waters (Cottrell and Kirchman 2000; Ouverney and Fuhrman 1999, 2000). These studies demonstrate that FISH-MAR is a powerful and promising technique that may give some first insights into the ecological function of single individuals in their natural ecosystems.

The composition and ecological aspects of extreme halophilic communities have been exhaustively studied both by culture-dependent and culture-independent approaches, leading to a good knowledge of their biodiversity (Antón et al. 1999; 2000; Oren 1994; Casamayor et al. 2002; Benlloch et al. 2002). Microbial communities of these brines with salinities above 30% are endowed with a very low diversity of prokaryotic organisms with a rather limited number of metabolic types and a very simple ecosystem structure (Oren 2002). The most abundant organism inhabiting crystallizer brines is a conspicuously square-shaped archaeon (Walsby's "square bacterium"; Walsby 1980) for which affiliation with an uncultured phylotype (Rodríguez-Valera et al. 1999) has recently been demonstrated (Antón et al. 1999). Although data regarding the composition and ecology of this prokaryote are available (Stoeckenius 1981; Oren et al. 1996; Antón et al. 1999), it has never been brought into pure culture despite many attempts at isolating it (Oren 2002). Until fluorescence in situ hybridization (FISH) was applied to brine samples at salt concentrations close to saturation (Antón et al. 1999, 2000), there was a common agreement that *Archaea* were the only ecologically relevant prokaryotes and that *Bacteria* might sporadically be represented (Oren 1994). However, it has recently been demonstrated that a single genus of *Bacteria*, already brought into pure cultures and named *Salinibacter* spp. (Antón et al. 2002), could represent as much as 30% of the total prokaryotic community inhabiting the brine (Antón et al. 2000). The presence of significant amounts of *Bacteria* with ecological relevance had been overlooked because the metabolic state of the community measured with radiotracers firmly indicated an archaeal predominance on the heterotrophic metabolism (Oren 1990a, 1990b, 1990c, 2002; Pedrós-Alió et al. 2000). Additionally, the shape and color of *Salinibacter* spp. colonies are identical to those of extremely halophilic *Archaea* (Antón et al. 2002).

We already know that *Archaea* and *Bacteria* coexist in crystallizer ponds of solar salterns, and that cultured members of the *Salinibacter* genus also have an heterotrophic metabolism (Antón et al. 2000, 2002). However, nothing is known about the metabolic capabilities of the 'square archaeon' since, as explained above, it has never been brought into laboratory culture. Here we present the application of FISH-MAR to brine samples of the solar salterns of S'Avall (Mallorca, Spain). The aim was to study some of the in situ nutritional requirements of the microbial community (mainly composed of square *Archaea* and *Salinibacter* spp.) by incubating it with five different types of radioactive substrates (acetate, amino acids, bicarbonate, and glycerol with two different types of labeling) under conditions very similar to those found in the solar salterns.

Methods

Brine samples from a crystallizer pond of the S'Avall solar saltern (Mallorca, Spain) were collected on 4 June 2002. On the sampling day, the water temperature was 25°C and the brine was salt saturated (above 35% NaCl). DAPI and FISH results with general and specific probes (Antón et al. 1999, 2000) revealed that the microbial community was composed of a dense population ($4.5\times10^7 \pm 1.2$ cells ml^{-1}) of microorganisms consisting of 62% *Archaea* ($2.8\times10^7 \pm 0.7$ cell ml^{-1}) and 29% *Bacteria* ($1.3\times10^7 \pm 0.4$ cell ml^{-1}). The archaeal phylotype corresponding to the SPhT clone (detected with the probe CS1337; Antón et al. 1999) was the most abundant archaeon in the sample ($2.0\times10^7 \pm 0.4$ cells ml^{-1}, 72% of *Archaea*, detected with a mixture of probes ARCH915 (Stahl and Amann 1991) and ARC344 (Raskin et al. 1994). However, there were also square morphotypes (28% of the *Archaea*) that were not detected by the CS1337 probe. When using the probes directed toward members of the genus *Salinibacter* (Antón et al. 2000) we could observe that such cells constituted 72% of the total *Bacteria*, and the phylotype EHB-1 (detected with the probe EHB586) dominated ($6.11\times10^6 \pm 0.3$ cells ml^{-1}) over EHB-2 ($3.2\times10^6 \pm 0.2$ cells ml^{-1}, detected with probe EHB1451). Cells not detected when using either EUB338 (Amann et al. 1990) or the mix of ARCH915 and ARC344 accounted for 9% of DAPI numbers.

The brine solution was incubated in five parallel experiments, using five radioactive substrates, namely sodium [^{14}C]-bicarbonate (Amersham Biosciences, UK) with a specific activity of 59 mCi mmol^{-1}, 2-[^{3}H]-glycerol (Amersham Biosciences, UK) with a specific activity of 1.00 Ci mmol^{-1}, [U-^{14}C]-glycerol (Amersham Biosciences, UK) with a specific activity of 142 mCi mmol^{-1}, acetic acid sodium salt (methyl-^{3}H, Moravek Biochemicals CA) with a specific activity of 15 Ci mmol^{-1}, and [^{3}H]-amino acid mixture (Amersham, UK) composed by the following amino acids L-leucine (161 mCi mmol^{-1}), L-lysine (79 mCi mmol^{-1}), L-phenylalanine (120 mCi mmol^{-1}), L-proline (102 mCi mmol^{-1}), and L-tyrosine (97 mCi mmol^{-1}). The experiments were performed by incubating 2 ml of the brine solution with 15 μCi of each radioactive substrate in 10-ml sterile serum tubes sealed with rubber stoppers for three different time periods (2, 24, and 48 h, respectively) at the optimal growth temperature for halophiles, 37°C (Antón et al. 2002) in a shaking water-bath. The samples were fixed with 4% formaldehyde (Merck, Darmstadt) for 16 h at 4°C (Antón et al. 1999), washed three times with PBS-buffer and finally transferred to an ice-cold 50% PBS-EtOH solution for final storage at −20°C. In order to exclude adsorption of substrates to sample material as a cause of MAR signals, brine samples pretreated either by formaldehyde-fixation or by a combination of heat (pasteurization, 10 min at 80°C) and metabolic inhibition (with 2 mM Na-azide) were incubated as mentioned above. In this regard, it is noteworthy that pasteurization and metabolic inhibition by azide treatment did not completely hinder biological uptake, and that only formaldehyde fixation effectively killed all cells (data not shown). Additionally, control experiments with a pure culture of *Salinibacter ruber* strain M31 (DSM 13855^T) growing in exponential phase (culture conditions as previously reported; Antón et al. 2002) were performed by using the five radioactive substrates, respectively.

The combination of FISH and MAR on fixed samples after incubation with the radioactive substrates was performed as described previously (Lee et al. 1999), using the phylogenetic probes mentioned above, and DAPI or SYBR-Green I (using a 10,000× dilution of the stock solution from Biowhittaker Molecular Applications, Rockland, ME, USA) as a general nucleic acid

counterstain of all cells. The samples were exposed to the autoradiographic film on gelatin-coated cover slips for 3 –20 days at 4°C before final development, as described by Lee et al. (1999).

Results and discussion

For the crystallizer samples, radiolabel incorporation was observed both when the amino acid mixture or the acetate were used as substrates (Fig. 1A, B), after 2, 24, and 48 h of incubation. However, neither incorporation of glycerol (any of either radiolabeled [U-^{14}C]-glycerol and 2-[^{3}H]-glycerol) nor of HCO_3^- was detected for any of the prokaryotic cells of the samples even after 48 h incubation and a prolonged exposure time up to 20 days

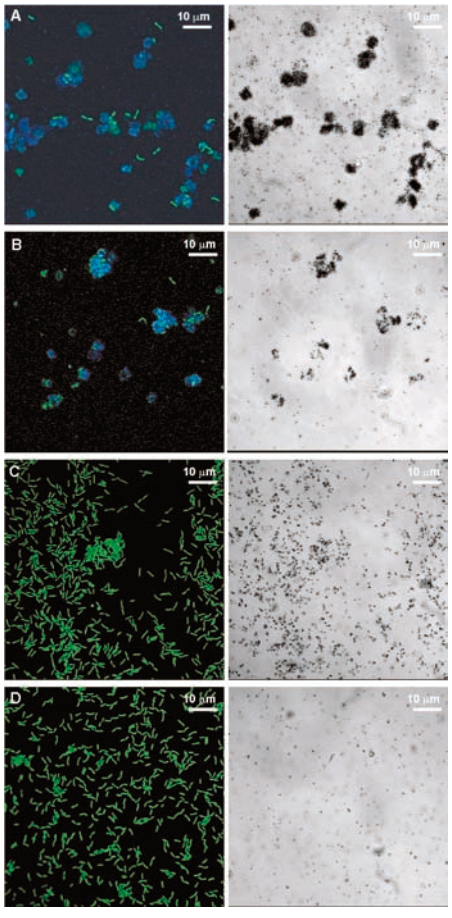

Fig. 1A–D Epifluorescence micrographs (*left panels*) and their corresponding microautoradiographies (*right panels*) of brine samples and pure cultures of *Salinibacter ruber* strain M31. **A** Brine sample incubated for 24 h with the radioactive amino acid mixture, square and pleomorphic-shaped cells correspond to the archaeal population (blue stain with the mixture of probes ARCH915 and ARC344 Cy5 labeled) and rod-shaped cells correspond to the bacterial (*Salinibacter* ssp.) populations (green stain with probe EUB338 fluosprime labeled). **B** Brine sample incubated for 24 h with radiolabeled acetate, morphotypes and stains as described above. **C** Pure culture of strain M31 incubated with the radioactive amino acid mixture (cells were stained with SYBR-Green I). **D** Pure culture of strain M31 incubated with radioactive acetate (cells stained with SYBR-Green I)

(the minimal exposure time needed for detection of incorporation of the radioactive amino acid mixture as well as the radioactive acetate was 3 days). In the brine samples, uptake of radiolabeled substrates, either acetate or amino acids, was only observed in square shaped cells, all of them belonging to the domain *Archaea*. Amino acids were assimilated by most of the square *Archaea* (95% after 2 hours of incubation, and 99% after 24 hours of incubation). Acetate uptake was observed by 81% of the FISH detectable archaeal cells after 24 h incubation. However, when compared to amino acid incorporation, a less homogeneous pattern of signal distribution per cell cluster was observed (Fig. 1B). We could not observe in any case members of the *Bacteria*, and thus *Salinibacter*, showing incorporation of any of the tested radiolabeled substrates even after 48 h of incubation and prolonged exposure (20 days) to the autoradiographic film. MAR experiments performed with pure cultures of *Salinibacter ruber* strain M31 and radiolabeled amino acids after 24 h incubation resulted in positive signals after only 3 days of exposure (Fig. 1C), while these cells did not take up acetate under any of the different incubation and exposure conditions investigated (Fig. 1D).

The present study shows the potential of the application of the FISH-MAR technology to the understanding of the microbial functions in extreme environments such as brines of crystallizer ponds from solar salterns. Here we give a first insight into the metabolic requirements of the hitherto uncultivated square *Archaea* and explicitly demonstrate its heterotrophic metabolism that had been postulated by indirect evidence (Oren 1994).

The prokaryotic community composition of the brine sample investigated is consistent with previous observations for similar sites in the western Mediterranean (Antón et al. 2000). *Archaea* dominated the brine community, whereas bacteria made up a significant part of the prokaryotic population, with percentages close to 30% of the total prokaryotic population. As previously indicated (Antón et al. 1999), there were also squared morphotypes that did not hybridize with the CS1337 probe; thus, not all squared cells corresponded to a single archaeal population. However, CS1337 hybridizing cells clearly dominated the archaeal population and accounted for up to 72% of the cells which were affiliated with this phylum.

Among the four substrates used, only amino acids and acetate yielded conclusive results. No autotrophy or glycerol uptake could be demonstrated with our experiments. The fact that only the archaeal population in the crystallizer sample did incorporate amino acids is in accordance with previous observations (Oren 1990a, 1990b; Pedrós-Alió et al. 2000). These former experiments suggested a negligible metabolic importance of *Bacteria* in crystallizer ponds (Oren 1994), since the analyses on metabolic activity (based on metabolic inhibition experiments using either radioactive amino acid mixtures or [^{3}H]-leucine) at high salinity was

entirely due to *Archaea*. However, our results indicate that amino acid incorporation is insufficient to globally measure the heterotrophic metabolism of the crystallizer community and thus suggest that additional measures need to be taken. However, the finding that no bacterial cell showed uptake of amino acids is surprising because they are the preferred growth substrate for the pure cultures of *Salinibacter ruber* (Antón et al. 2002; Fig. 1C). The absence of detectable amino acid uptake by *Bacteria* in the brine sample in situ thus either demonstrates that they do not incorporate amino acids at all in the environmental sample, or that they take up these substrates at a very slow rate. This could represent one more piece of evidence that laboratory growth observations might not reflect the real metabolic state of microorganisms in their natural environment, but only potential metabolic characteristics (Gray and Head 2001).

Acetate incorporation gave similar insights as those observed from amino acid uptake. However, in this case, not all archaeal cells incorporated acetate at the same rate. Heterogeneity in substrate uptake among morphologically and/or phylogenetically indistinguishable cells present in the same sample is likely to occur (Nielsen et al. 1998). Thus, the differences that we see in the amount of silver granules per cell cluster, which mirror the amount of substrate assimilation, might be correlated to different metabolic states of the cells (Ouverney and Fuhrman 1999). The absence of acetate uptake in the bacterial population is in accordance with the results from pure culture experiments (Fig. 1D) and laboratory growth observations (Antón et al. 2002).

The absence of autotrophy among the brine prokaryotic community was not surprising since these organisms have always been regarded as heterotrophic (Oren 1994). However, the absence of uptake of radioactive glycerol was quite unexpected. Glycerol has been regarded as the key substrate for the prokaryotic community in crystallizers (Oren 1994), because it is produced in these systems in considerable amounts. The most important primary producer in such environments, single cell algae of the genus *Dunaliella*, synthesizes glycerol as compatible solute for osmotic purposes and it is accumulated in very high concentrations in its cytoplasm (Oren 1993). We used [U-^{14}C]-glycerol and 2-[^{3}H]-glycerol and neither of them gave intracellular radioactive accumulation, which is at variance with the accumulation of radiolabeled glycerol previously observed in bulk experiments in crystallizer samples from Eilat, Israel (Oren 1993).

Here we have demonstrated that the archaeal population does accumulate substrates that clearly suggest a heterotrophic metabolism. However, we obtained no conclusive results about the metabolic requirements of the bacterial population in the brine. One could argue that this bacterial population was not active in the analyzed brine since it did not incorporate any of the tested radiolabeled substrates. However, the FISH signal for the bacterial community revealed a high number of ribosomes per cell, which has been consistently regarded as a sign of activity (e.g., Rosselló-Mora et al. 1999), although high ribosome numbers per cell have previously also been observed for physiologically completely inhibited autotrophic ammonia oxidizers (Wagner et al. 1995). The fact that *Salinibacter* is an extreme halophile (Antón et al. 2002) and a numerically important part of the autochthonous community of the crystallizers (Antón et al. 2000) suggests, however, that bacterial cells should thrive in these systems. The absence of radiolabel incorporation by the bacterial cells might not necessarily imply a lack of uptake, since substrates that are metabolized and entirely transformed in soluble products will not be detectable by FISH-MAR (Oren 1993). Therefore, the bacterial cells in the crystallizer might transform amino acids but will definitively require other substrates for growth.

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