DIVERSITY OF CULTURABLE HALOPHILIC ARCHAEA ISOLATED FROM RAMBLA SALADA, MURCIA (SPAIN)

Luque, R.[a], González-Domenech, C. M.[a,b], Llamas, I.[a], Quesada, E.[a], Béjar, V.[a]

[a]Department of Microbiology, Faculty of Pharmacy, University of Granada, Granada, Spain.

[b]Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, Valencia, Spain.

Keywords: biodiversity; halophiles; archaea; hypersaline habitat; taxonomy; Rambla Salada.

Abstract

We have studied the diversity of culturable halophilic *Archaea* at Rambla Salada, Murcia (south-eastern Spain). We made 8 samplings at different places in this habitat during the years 2006 and 2007 and isolated a total of 49 strains, which were identified by means of phenotypic tests and the hypervariable V1-V3 region of the 16S rRNA sequences (around 500 bp) .The ribosomal data showed that the isolates belonged to 12 genera within the *Halobacteriaceae* family*, Haloferax* and *Natrinema* being the most abundant. Five strains showed less than 97% sequence identity with validly described species and may well represent new taxa. All the strains grew best with around 25% w/v salts, required high concentrations of NaCl and magnesium and produced red to pink colonies. They were facultative anaerobes with both respiratory and fermentative metabolisms. The diversity of the archaeal community was analysed with the MOTHUR package. We identified 14 OTUs at the 3% genetic distance level and found quite high diversity. Rarefaction curves and diversity indexes demonstrated that our collection of isolates adequately represented the archaeal community at Rambla Salada. This is the first report on the culturable archaea at Rambla Salada, an area of considerable ecological interest.

Introduction

Rambla Salada is a hypersaline “rambla” (a steep-sided river bed, normally dry but subject to flash flooding) located in Murcia (south-eastern Spain). Rambla Salada has been declared a protected area by the Murcian regional government (BORM 10/09/1998), a place of community interest (LIC) by the European Union and a protected wildfowl zone (ZEPA). It is an athalassohaline habitat and includes areas of soils, waters and sediments with different salt contents, deriving mainly from Miocene gypsiferous marls in the Fortuna basin (Muller and Hsü 1987). Nowadays the habitat is seriously threatened by human activities that induce changes in the natural hydrology and salinity levels: inputs of freshwater, nutrients, pesticides and other pollutants are dramatically changing its biodiversity.

Velasco and co-workers (Velasco et al. 2006) first studied the primary producers and macro-invertebrates at Rambla Salada and demonstrated that their community composition is closely linked to salinity. Nevertheless, to our knowledge there have been no ecological studies so far describing the population of microorganisms that live in Rambla Salada, although our group has in the past discovered two new halophilic bacterial species there: *Idiomarina ramblicola* (Martínez-Cánovas et al. 2004) and strain R53 of *Halomonas cerina* (González-Domenech et al. 2008).

Thus we undertook an analysis of the community of prokaryotes that live in various environments in the different areas of Rambla Salada. The aims of this study were firstly, to quantify the archaeal community, secondly, to isolate a significant number of archaeal strains of those that represent the community of these organisms living in Rambla Salada, and finally, to identify them and ascertain their diversity.

Materials and methods

Sample collection and physical-chemical determinations

We took samples from four different zones in Rambla Salada (Murcia, south-eastern Spain): soil, sediment and water at the Finca de la Salina (site 1, 38º 07´ 34.44´´ N 1º 07´ 11.13´´ W), water and sediment from a saline groundwater spring (site 2, 38º 07´ 29.09´´ N 1º 07´ 42.15´´ W), soil from the Humedal de Derramadores (site 3, 38º 10´ 24.96´´ N 1º 05´ 38.73´´ W) and water and sediment from the Tajo-Segura interconnecting canal (site 4, 38º 07´ 30.23´´ N 1º 07´ 42.22´´ W). We collected a total

of 32 samples over two years (February and June in 2006, and February and November in 2007). The samples were taken aseptically and stored at 4°C until study in the laboratory (always within 24 hours). The soils and sediments were suspended in sterile 25% w/v NaCl solution (1 g in 9ml), thoroughly homogenized by agitation and then serially diluted (ranged from 10^{-1} to 10^{-6}). The waters were directly diluted in sterile 25% w/v NaCl solution. 100µl of of dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were surface-plated on MY medium (Moraine and Rogovin 1966) supplemented with 30% w/v sea-salt solution (Rodríguez-Valera et al. 1981) and incubated at 41°C for three weeks.

pH and conductivity were measured at each sampling point. Conductivity was determined with an ECmeter (TetraConR 325), which automatically calculates salinity.

Counts and selection of the strains

Counts were made in those plates containing between 30 and 300 colonies. A collection of 50 colonies, chosen on the basis of their different appearances, were re-isolated by streaking on two fresh media following the recommendations of Oren in 2006 (Tindall and Collins 1986 and Soliman and Trüpper 1982) and grown at the same temperature for the same length of time. The sites where each strain was isolated are shown in Table 1.

DNA extraction, PCR amplification of 16S rRNA genes and sequencing

Genomic DNA was extracted from log-phase cells according to Marmur (1961) with the modification developed by Martín-Platero et al. (2007). The hypervariable V1-V3 regions of the 16S rRNA sequences (around 500 bp) were then determined using the method described in Burns et al. (2004) with the specific primers for *Archaea*: F1 as forward primer (5´- ATTCCGGTTGATCCTGC-3´) (Ihara et al. 1997) and 1492R as reverse primer (5´-ACGGHTACCTTGTTACGACTT´-3´) (Grant et al. 1999). PCR amplifications were made using 50 µl reaction mixtures containing 20–100 ng of template DNA, 10 pmol of each primer (Sigma®), 0.2 mM of dNTP mix (Bioline®), 2 mM of $MgCl_2$, 5X PCR buffer (Bioline®) and 1.25 U of BioTaqTM DNA polymerase (Bioline®). Amplified PCR products were purified with the Illustra® GFX DNA and Gel Band Purification kit (GE Healthcare®) and sequenced directly.

Sequence analysis

The sequences obtained were preliminary identified by similarity-based search through the EzTaxon server 2.1. (http://147.47.212.35:8080/index.jsp) (Chun et al. 2007). Thereafter, the sequences were aligned using Clustal X (Thompson et al. 1997). To study the phylogenetic relationship among the isolates and other species of *Halobacteriaceae,* we applied neighbour-joining (NJ) and maximum parsimony (MP) criteria using the MEGA version 4 software (Tamura et al. 2007). Confidence levels for the phylogenetic trees were assessed by bootstrapping with 1000 replicates. The sequence of the type strain of *Methanospirillum hungatei* JF-1^T was used as outgroup.

Phenotypic characterization

We carried out the phenotypic tests described by Oren et al. (1997), which are the minimal standards for the description of new taxa in the order *Halobacteriales*.

Diversity measures and rarefaction analysis

Sequence alignments of the 16S rRNA genes allowed us to construct a distance matrix using MOTHUR (http://www.mothur.org/) (Schloss et al. 2009), a software package integrating an improved version of DOTUR (Schloss and Handelsman 2005). Once the matrix was generated, weconducted an OTU-based analysis to study archaeal diversity. The clustering algorithm was furthest neighbour. We carried out rarefaction studies taking the default value of 1000 as the number of randomizations. We also calculated the Shannon (H´) diversity, the reciprocal of Simpson´s indexes (Simpson 1949; Magurran 1996) and Chao 1 and ACE species-richness estimators (Chao 1987).

Nucleotide sequence accession numbers

The sequences reported in this study have been submitted to the GenBank database under accession numbers HQ659121 to HQ659169.

Results

Physical-chemical measurements

Salinity in the different zones (sites 1-4) and samples (water, soil and sediment) taken at Rambla Salada ranged from 1.6% to 8% w/v in 2006 and from 1.2% to 3.4% w/v in 2007 with the exception of the water sampled at site 2 (natural groundwater spring), the

salt content of which remained at around 15% w/v. The pH was ranged from 6.3 to 8.3. In the case of water and sediment sampled at site 4, the pH ranged from 8.1 to 9.7 (see Table 1).

Microbial counts and selection of the archaeal strains

Microbial counts (UFC/ml or UFC/gram) revealed values of around 10^4 (1.2 x 10^4 - 4.3 x 10^4) in February 2006 and around 10^6 (1.2 x 10^6 – 2.6 x 10^6) in June 2006 and February and November 2007. We chose 50 isolates on the basis of the different appearances of their colonies. Phenotypic tests and ribosomal data (see below) proved that 49 of these 50 isolates were strains of archaea. This result suggested that the microbial counts could be attributed almost entirely to archaeal strains. The great majority of the colonies were red to pink, which is the norm among these microorganisms.

Phylogenetic analyses

The use of specific primers for the hypervariable region of the 16S rRNA gene of *Archaea* and subsequent sequencing of the PCR product allowed us to determine a preliminary phylogeny of the isolates. Both NJ and MP methods gave similar clusters, supported by bootstrap values above 70% (Fig.1). The sequences for each phylogenetic reconstruction (around 500 bp) were compared to the same region from reference 16S rRNA gene sequences retrieved from the GenBank database and subject to EzTaxon pairwise comparison results. Phylogenetic analyses indicated that all the strains were related to different genera within the *Halobacteriaceae*, *Haloferax* and *Natrinema* being predominant with 10 representatives each. In addition, the 16S rRNA genes of strains M2-2d, M2-7b, M3-1c, M4-6a and M4-6b showed a similarity of less than 97% with other archaeal species, which leads us to surmise that they probably constitute new taxa (Table 2).

Phenotypic characterization

We used a total number of 78 phenotypic tests to characterize the strains, in accordance with the minimal standards for the description of new taxa in the order *Halobacteriales* (Oren et al. 1997). The results are shown in Table S1. All the strains were either Gram-negative rods or pleiomorphic, and extremely halophilic, growing best with 25% w/v sea salt. They required magnesium. They grew best between 37º C

and 41ºC. Colonies ranged from pink to red in colour. They were facultative anaerobes. All fermented glucose and arginine and some of them respired with nitrate. They were resistant to ampicillin, chloramphenicol, erythromycin, nalidixic acid, penicillin, and tetracycline.

Diversity measures and rarefaction analyses

Using the clustering algorithm implemented in the MOTHUR package we identified 14 OTUs at the 3% distance level.

We used rarefaction curves to compare the relative richness between the archaeal population from each sampling season, 2006 and 2007. The rarefaction analyses at 97% grouping stringency revealed that diversity was higher in 2006 than in 2007 (Fig. 2). Furthermore, Chao1 and ACE richness estimate rarefaction's curves tends to be parallel to the x-axis, indicating a representative sampling under the conditions used (Fig. 3). The Chao1 and ACE estimators predicted between 15-17 species at 97% grouping stringency: at a 95% confidence interval the values for Chao1 ranged between 14.18 and 26.47 and for ACE between 14.51 and 29.16, taking the whole sampling area into account (Fig. 3). Thus, the values of predicted number of OTUs are quite close to the observed number of OTUs (see above).

In addition, we also assessed diversity by means of Simpson´s and Shannon´s diversity indexes by the MOTHUR program, obtaining values of 0.09 (reciprocal value of Simpson´s index, 10.05) and 2.35 for the total sampling area (Table 2). These values reflect a reasonably high archaeal diversity, higher in 2006 than in 2007, as seen before. In comparison to other studies of archael population from hypersaline habitats (Clementino et al., 2008; Baati et al., 2008, 2010; Pašić et al., 2005), we found similar values for these diversity indexes, more even, independently if such studies are or not culturing approach (Table 2).

Discussion

All extremely-halophilic archaea (also known as haloarchaea) cultured to date belong to the *Halobacteriaceae* family, within the order of *Halobacteriales* in the phylum *Euryarchaeota*. They are found extensively in such saline environments as salt lakes and saltern-crystallizer ponds and also in saline soils (Oren 1994; Grant et al. 2001; Maturrano et al. 2006; Pašić et al. 2005; Dave and Desai 2006). In recent years some

publications have also described their presence in medium-to-low-salinity environments or even non-saline habitats (Aller and Kemp 2008; Cambon-Bonavita et al. 2009). Furthermore, new molecular ecology techniques have found archaea belonging to the phylum *Crenarchaeota* in saline habitats, but they remain to be cultured.

Our study has demonstrated that Rambla Salada is host to a considerable density and diversity of culturable halophilic archaea belonging to the *Halobacteriaceae*, even in zones of low and medium salinity (see Table 2) and that they represent a very diverse group of taxa belonging to a large number of genera and species.

As far as the total counts are concerned, they were high and quite similar to those obtained in other hypersaline habitats, such as solar salterns in Alicante (Spain) (Rodríguez-Valera et al. 1981; 1985) and in San Francisco (C.A. USA) (Litchfield et al. 1999), where the values were 10^4 and 10^5-10^6 UFC/ml respectively.

Salinity is one of the most important driving forces of diversity for both macro- and microorganisms (Auguet et al. 2010; Lozupone and Knight 2007; Tamames et al. 2010). According to Velasco et al. (2006), this is reflected in the composition of the communities of primary producers and macro-invertebrates at Rambla Salada. Our work has also demonstrated that the diversity of archaea is affected to some extent by this factor.

In our study, salinity was automatically calculated from the conductivity measurements made *in situ* during each sampling season. Generally the salinity gradient reached its highest values in 2006. At site 2, a natural spring, the salinity values were practically constant throughout the sampling period, which might be expected from a permanent flow of saline groundwater. We found the highest biodiversity in 2006, which, according to the physical-chemical parameters measured, could be ~~seems to be~~ related to higher salinity. Thus, in 2006 we identified 12 genera (*Haladaptatus, Haloarcula, Halococcus, Haloferax, Halogeometricum, Halomicrobium, Halorhabdus, Halorubrum, Halostagnicola, Haloterrigena, Natrialba* and *Natrinema*) while in 2007 only 8 (*Haladaptatus, Halococcus, Haloferax, Halomicrobium, Halostagnicola, Haloterrigena, Natrialba* and *Natrinema*). As shown in Table 3, this also is the trend observed in other hypersaline habitat of archaeal population according to their measured diversity indexes.

1 Rarefaction analyses suggest that the total number of sequences studied within the
2 area of Rambla Salada represent a reasonable initial coverage of the culturable
3 archaeal diversity in it; in other words, we have isolated most of the main
4 representative groups of culturable archaea inhabiting this environment with the
5 conditions chosen. Although Chao1 and ACE estimators normally underestimate true
6 richness when sample sizes are small (Hughes et al. 2001), we found in our study that
7 the estimated value was quite similar to that observed.
8
9 In general terms, the diversity of haloarchaea at Rambla Salada was quite similar to
10 that in other saline environments (Oren 2002; Burns et al., 2004; Baati et al. 2008;
11 2010; Clementino et al. 2008; Ozcan et al. 2007). Nevertheless, the predominant taxa
12 were different. In solar salterns, one of the most thoroughly studied types of
13 hypersaline habitat, the predominant population is made up of strains belonging to the
14 genera *Haloferax, Halorubrum, Halococcus, Haloterrigena, Haloarcula, Natrialba* and
15 *Halobacterium* (Oren 2002). In saltern crystallizers the predominant archaea are
16 *Halorubrum* and *Haloquadratum* (Oren 2002). In Rambla Salada, however, we isolated
17 more strains belonging to the genera *Natrinema* and *Haloferax*, this latter often being
18 found in habitats with low salinity, although it grows in media containing 1.0 to 5.1M
19 NaCl and grows best at 2.5M NaCl (Oren 2011)..
20
21 We also found several taxa that were isolated only during a certain sampling period.
22 Thus, *Halorubrum aidingense, Haloarcula argentinensis, Haloarcula quadrata,*
23 *Halogeometricum borinquense* and *Halorhabdus tiamatea* were isolated in 2006, whilst
24 *Halomicrobium mukohataei, Haloferax prahovense, Halococcus hamelinensis* and
25 *Haladaptatus paucihalophilus* were collected in 2007.
26
27 Oh the other hand, it is important to note that we have not isolated any of our samples
28 the extreme halophilic bacterium *Salinibacter* which most significant features are
29 similar to archaea.
30
31 The present study is the first to describe the culturable halophilic-archaeal community
32 at Rambla Salada. Our results confirm the validity of our sampling strategy and the
33 high biodiversity and density of archaea in this environment. In addition we have
34 discovered a number of strains that may well constitute new taxa and are being subject
35 to further scrutiny in our laboratory
36
37

Acknowledgements

The authors thank Josefa Velasco and Andrés Millán for their assistance with field work and zone selection for sampling. Thanks also go to Miguel Angel Núñez of the Explanation Centre at Rambla Salada for his willing assistance in the field. This research was supported by grants from the *Dirección General de Investigación Científica y Técnica* (CGL2005-05947; CGL2008-02399), from the *Plan Andaluz de Investigación* and *Plan Propio de la Universidad de Granada*, Spain. We also thank our colleague Dr J. Trout for revising our English text.

References

Aller JY, Kemp PF (2008) Are *Archaea* inherently less diverse than *Bacteria* in the same environments? FEMS Microbiol Ecol 65:74-87

Auguet JC, Barberan A, Casamayor EO (2010) Global ecological patterns in uncultured Archaea. *ISME J* 4:182-190

Baati H, Guermazi S, Amdouni R, Gharsallah N, Sghir A, Ammar E (2008) Prokaryotic diversity of a Tunisian multipond solar saltern. Extremophiles 12:505-518

Baati H, Guermazi S, Gharsallah N, Sghir A, Ammar E (2010) Novel prokaryotic diversity in sediments of Tunisian multipond solar saltern. Res Microbiol 161:573-582

Burns DG, Camakaris HM, Janssen PH, Dyall-Smith M.L (2004) Combined Use of Cultivation-Dependent and Cultivation-Independent Methods Indicates that Members of Most Haloarchaeal Groups in an Australian Crystallizer Pond Are Culturable. Appl Environ Microbiol 70:5258-5265

Cambon-Bonavita MA, Nadalig T, Roussel E, Delage E, Duperron S, Caprais JC, Boetius A, Sibuet M (2009) Diversity and distribution of methane-oxidizing microbial communities associated with different faunal assemblages in a giant pockmark of the Gabon continental margin. Deep Sea Res Part II: Topical Studies in Oceanography 56:2248-2258

Chao A (1987) Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43:783–791

Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57:2259-2261

Clementino MM, Vieira RP, Cardoso AM, Nascimento AP, Silveira CB, Riva TC, González AS, Paranhos R, Albano RM, Ventosa A, Martins OB (2008)

1 Prokaryotic diversity in one of the largest hypersaline coastal lagoons in the world. Extremophiles 12:595-604
2
3 Dave SR, Desai HB (2006) Microbial diversity at marine saltern near Bhavnagar, Gujarat, India. Curr Sci 90:497–500
4
5 Felsenstein J (1993) Phylogeny Inference Package (PHYLIP). Version 3.5, University of Washington, Seattle
6
7 González-Domenech CM, Martínez-Checa F, Quesada E, Béjar V (2008) *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. Int J Syst Evol Microbiol 58:803-809
8
9
10 Grant S, Grant WD, Jones BE, Kato C, Li L (1999) Novel archaeal phylotypes from an East African alkaline saltern. Extremophiles 3:139-145
11
12 Grant WD, Kamekura M, McGenity TJ, Ventosa A (2001) Order I. Halobacteriales. In: Garrity GM (ed.) Bergey's Manual of Systematic Bacteriology V.I, The Archaea and Deeply Branching and Phototrophic Bacteria, 2nd Ed. Springer, New York.
13
14
15 Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 67:4399-4406
16
17
18 Ihara K, Watanabe S, Tamura T (1997) *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. Int J Syst Bacteriol 47:73-77
19
20
21 Litchfield CD, Irby A, Vreeland RH (1999) The microbial ecology of solar salt plants. In: Oren A. (ed.). Microbiology and biogeochemistry of hypersaline environments. CRC Press, Boca Ratón, pp 39-52
22
23
24 Lozupone CA, Knight R (2007) Global patterns in bacterial diversity. Proc Natl Acad Sci USA 104:11436–11440
25
26 Magurran AE (1996) Ecological diversity and its measurement. Chapman and Hall, London, pp 178
27
28 Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:207-208
29
30 Martínez-Cánovas MJ, Béjar V, Martínez-Checa F, Páez R, Quesada E (2004) *Idiomarina fontislapidosi* sp. nov. and *Idiomarina ramblicola* sp. nov., isolated from inland hypersaline habitats in Spain. Int J Syst Evol Microbiol 54:1793-1797
31
32
33 Martín-Platero AM, Valdivia E, Maqueda M, Martínez-Bueno M (2007) Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. Anal Biochem 366:102-104
34
35
36
37

Maturrano L, Santos F, Rosselló-Mora R, Antón J (2006) Microbial diversity in maras salters, a hypersaline environment in the Peruvian Andes. Appl Environ Microbiol 72:13887-3895

Moraine RA, Rogovin P (1966) Kinetics of polysaccharide B-1459 fermentation. Biotechnol Bioengin 8:511-524

Muller DW, Hsü KJ (1987) Event stratigraphy and paleoceanography in the Fortuna basin (Southeast Spain): a scenario for the Messinian salinity crisis. *Paleoceanography* 2:679-696

Oren A (1994) The ecology of the extremely halophilic archaea. FEMS Microbiology Reviews 13:415-440

Oren A (2002) Halophilic microorganisms and their environments. Kluwer Academic Publishers, Netherlands

Oren A (2011) Diversity of halophiles. In: Extremophiles Handbook. Vol. 1. Horikoshi K (ed) Springer, Tokyo, pp 309-325

Oren A, Ventosa A, Grant WD (1997) Proposed minimal standards for description of new taxa in the order *Halobacteriales*. Int J Syst Evol Microbiol 47:233-238

Ozcan B, Ozcengiz G, Coleri A, Cokmus C (2007) Diversity of halophilic archaea from six hypersaline environments in Turkey. J Microbiol Biotechnol 17:985-992

Pasić L, Bartual SG, Ulrih NP, Grabnar M, Velikonja BH. (2005) Diversity of halophilic archaea in the crystallizers of an Adriatic solar saltern. FEMS Microbiol Ecol. 54:491-8.

Rodríguez-Valera F, Ventosa A, Juez G, Imhoff J (1985) Variation of the environmental features and microbial population with salt concentrations in a multipond saltern. Microbiol Ecol 11:107-115.

Rodríguez-Varela F, Ruiz-Berraquero F, Ramos-Cormenzana A (1981) Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentration. Microbiol Ecol 7:235-243

Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501–1506

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing MOTHUR: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541

Simpson EH (1949) Measurement of diversity. Nature 163:688

1 Tamames J, Abellán JJ, Pignatelli M, Camacho A, Moya A (2010) Environmental
2 distribution of prokaryotic taxa. BMC Microbiol 10:85
3 Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics
4 analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
5 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The
6 CLUSTAL_X windows interface: flexible strategies for multiple sequence
7 alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
8 Tindall BJ, Collins MD (1986) Structure of 2-methyl-3-VIII-dihydrooctaprenyl-1,4-
9 naphthoquinone from *Halococcus morrhuae*. FEMS Microbiol Lett 37:117–119
10 Velasco J, Millán A, Hernández J, Gutiérrez C, Abellán P, Sánchez D, Ruíz M (2006)
11 Response of biotic communities to salinity changes in a Mediterranean
12 hypersaline stream. Saline Systems 2:12

Legends to Figures

Figure 1. Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of the archaeal isolates with respect to other members of the family Halobacteriaceae. The sequence of the type strain of Methanospirillum hungatei JF-1T was used as outgroup. Bar: 1% sequence divergence. Common clusters in both Neighbour-Joining and Maximum-Parsimony methods show bootstrap values at the corresponding nodes (in such order). The 5 strains with less than 97% of similarity to validly described species are shaded in grey. GenBank/EMBL/DDBJ accession numbers are given in parenthesis**.**

Figure 2. Comparative rarefaction curves representing observed archaeal diversity from the totality sampling area of Rambla Salada and during each season with clusterization stringency at 97%.

Figure 3 Estimated OTU richness and diversity of archaea *vs* sample size from the whole sampling area in Rambla Salada. Estimated OTU richness is plotted for Chao1 (◊) and ACE (■) estimators.