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Intraspecific comparative analysis of the species *Salinibacter ruber*

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Abstract *Salinibacter ruber* is the first extremely halophilic member of the *Bacteria* domain of proven environmental relevance in hypersaline brines at or approaching NaCl saturation, that has been brought to pure culture. A collection of 17 strains isolated from five different geographical locations (Mallorca, Alicante, Ebro Delta, Canary Islands, and Peruvian Andes) were studied following the currently accepted taxonomic approach. Additionally, random amplification of genomic DNA led to the phenetic analysis of the intraspecific diversity. Altogether the taxonomic study indicated that *S. ruber* remained highly homogeneous beyond any geographical barrier. However, genomic fingerprints indicated that populations from different isolation sites could still be discriminated.

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Introduction

Salinibacter ruber (Antón et al. 2002) is the first extremely halophilic member of the *Bacteria* domain of proven environmental relevance (Antón et al. 2000) that has been cultured in the laboratory. Members of this species occur naturally in hypersaline brines at or approaching NaCl saturation. The use of molecular techniques allowed the discovery of this microorganism (Antón et al. 2000) and, shortly thereafter, its isolation and characterization (Antón et al. 2002). *S. ruber* affiliates as a deep branch with the *Bacteroidetes-Chlorobi* superphylum, together with *Rhodothermus marinus* (Antón et al. 2002), and it has been classified within the Phylum BXX, *Bacteroidetes*, Class III *Sphingobacteria*, Order I *Sphingobacteriales*, Family V *Crenotrichaceae*, as a single new genus (Genus IV) and species (Garrity et al. 2003).

Five strains were first isolated from the brines of solar salterns in Alicante and Mallorca that were the basis for the classification of the new genus and species (Antón et al. 2002). Since then, new information obtained from these isolates has shown a surprising similarity to the extremely halophilic *Archaea*. For example, like haloarchaea, *S. ruber* strains accumulate high intracellular amounts of potassium and chloride (Antón et al. 2002; Müller and Oren 2003; Oren et al. 2002). This feature has only been previously found in *Bacteria* for the members of the *Haloanaerobiales* and this is reflected by the high proportion of acidic amino acids in *S. ruber* proteins, another typical feature of haloarchaea (Oren and Mana 2002). Additionally, the proteins analysed show a low proportion of hydrophobic amino acids that are compensated for by the increase of serine (Oren and Mana 2002). Such modifications in amino acid composition directly influence the requirements for inorganic anions. Several enzymes have been tested and most of them require chloride that has to be pumped into the cells (Müller and Oren 2003). The high concentrations of chloride in haloarchaea are achieved by cotransport with

sodium ions and/or using the light driven primary chloride pump halorhodopsin (Müller and Oren 2003). The presence of a homologous gene to halorhodopsin has in fact been found in several strains of *S. ruber* (J.Antón et al. in preparation). Additionally, features like the high G+C content of the genome (Antón et al. 2002) and pigmentation (Oren and Rodríguez-Valera 2001) clearly indicate that these organisms share many characteristics with the known haloarchaea.

Although the first five isolates were obtained from solar salterns in Alicante and Mallorca (Antón et al. 2002), molecular microbial ecology methods showed that *S. ruber* thrives in a broader geographical region that covers the whole Mediterranean (Antón et al. 2000). The extreme nature of the environments that *S. ruber* inhabits and the distant geographical position of such sampled sites are adequate conditions for allopatric speciation (Staley 2004). The aim of the present study was to isolate new organisms from new sampling sites to understand the geographical distribution and intraspecific diversity of *S. ruber*. We have characterised a collection of 17 strains (including the five original strains used for species description) by studying several biochemical traits and chemotaxonomical markers such as fatty acid profiles, the quinone system, lipopolysaccharide and polar lipids, as well as the two genetic typing strategies randomly amplified polymorphic DNA (RAPD; Sikorski et al. 1999) and pulsed field gel electrophoresis analysis (PFGE; Grothues and Tummler 1991).

Methods

Strains, media and culture conditions

New *S. ruber* isolates were obtained from brine samples collected from crystallizer ponds of salterns located at La Palma (Canary Islands, Spain), San Carles de la Ràpita (Ebro Delta, Spain) and Maras (Peruvian Andes) during the years 2000–2002. All isolates were obtained by plating samples or dilutions on salt solution 25% sea water (SW), containing (in gram per litre) NaBr, 0.65; $NaHCO_3$, 0.167; KCl, 5; $CaCl_2$, 0.723; $MgSO_4{\cdot}7H_2O$, 49.492; $MgCl_2{\cdot}6H_2O$, 34.567; NaCl, 195; with 0.2% yeast extract (the pH was adjusted to 7.2 with NaOH prior to autoclaving), followed by incubation at 37°C. After growth, cells from colonies were examined microscopically, with rods being selected and inoculated again on the same medium. In all cases, agar plates (SW supplemented with yeast extract and 1.5% agar), or liquid media (with shaking) were incubated at 37°C for at least 14 days to obtain sufficient biomass for further experiments.

Screening and selection of *Salinibacter* isolates

The universal primers used for PCR amplification of the bacterial and archaeal 16S rDNA were a forward primer 27f for *Bacteria*, (Lane 1991), or a forward primer 21F for *Archaea* (DeLong et al. 1992) and universal reverse primer 1492r (Lane 1991) for both *Bacteria* and *Archaea*. PCR was carried out as described previously (Acinas et al. 1999). Isolates belonging to the domain *Bacteria* were submitted to a second PCR amplification with the specific primers for *Salinibacter* spp. EHB4F (5′-ACACCCCTATGGGGCGTA-3′, Antón et al. 2002), and EHB9R (5′-AGCGTCGAGCCTAGGTA-3′). Alternatively, strains were identified as *Salinibacter spp*. by fluorescence in situ hybridisation (FISH) using the specific probe EHB412 labelled with the fluorochrome CY3 (5′-TACGCCCCATAGGGTGT-3′, Antón et al. 2000). Both the FISH probe and the amplification primers targeted specifically all those hitherto known sequences of the genus *Salinibacter* which include sequences of all strains of *S. ruber* and that of the hitherto uncultured EHB-2.

DNA isolation, DNA base composition and DNA-DNA hybridisation

Genomic DNA was prepared following the method of Marmur (1961). G+C content was analysed by hydrolysis of DNA to its nucleosides and quantified by HPLC following a modification of the method of Tamaoka and Komagata (1984). The experiments were carried out as follows: 10 µl containing about 1 µg of DNA were denatured by incubating 5 min at 100°C and immediately ice chilled. Denatured DNA was then mixed with 10 µl of nuclease buffer (sodium acetate 40 mM; $ZnSO_4$ 2 mM; pH 5.3) containing 0.1 mg/ml of nuclease P1 (SIGMA), and incubated for 2 h at 50°C. Once the DNA was digested, 10 µl of dephosporylation buffer (Tris-HCl 0.1 M pH 8.1) containing 3 U of alkaline phosphatase (Roche) were added to the mixture and incubated for 2 h at 37°C. Those samples were then directly injected into the flow. Nucleoside chromatography was carried out with a Waters-Millipore HPLC apparatus, using a C18 reversed-phase column (NOVA-PAK, Waters-Millipore). Nucleoside were eluted by using $NH_4H_2PO_4$ 0.1 M pH 4: acetonitrile (20:1, v:v), with a flow of 1 ml/min at room temperature. Chromatography was completed after 10 min. Nucleosides were detected by UV absorbance at 270 nm. Relative amounts of G+C were calculated by the use of the following standard DNAs: *Staphylococcus aureus* (strain CECT239, G+C: 34%; Holländer and Pohl 1980); *Shewanella putrefaciens* (CECT5346, G+C: 43.9; Owen et al. 1978); *Escherichia coli* B (strain CECT101, G+C: 52%; Holländer and Pohl 1980); *Pseudomonas aeruginosa* (strain DSMZ 50071; G+C: 67.2%; Palleroni 1984); and *Agrococcuss jenensis* (strain DSMZ 9580; G+C: 74%; Groth et al. 1996). DNA–DNA hybridisation experiments were carried out following a microtiter plate non-radioactive method (Ziemke et al. 1998).

Sequencing

In order to sequence the almost complete 16S rRNA gene, three primers were used: 533R (Amann et al. 1995) EHB4F (Antón et al. 2002) and 1492r (Lane 1991). PCR products were purified with the Concert Rapid PCR Purification System (Promega) according to the manufacturer's protocol. The nucleotide sequences of the products were determined using the Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 310 DNA sequencer (Applied Biosystems). Complete 16S rRNA sequences were compared initially with reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST (Altschul et al. 1997) and they were subsequently aligned with 16S rRNA reference sequences in the ARB package (Ludwig et al. 2004).

PCR genomic fingerprints

In order to randomly amplify genomic DNA, eight primers were used, six of them especially designed for RAPD analysis of *Pseudomonas* strains, with additional ERIC and BOX primers (Sikorski et al. 1999). The following primers were used: RAPD1 (5′-TGCGAACTGTTGGGAAGGG-3′), RAPD2 (5′-CGAGCTTCGC GTACCACCCC-3′), RAPD3 (5′-CGCTGCGGTTG CGCGCCGCC-3′), RAPD4 (5′-CTCAATGGCAGCG-GCTATGG-3′), RAPD5 (5′-GTTTCGCTCGATG-CGCTACC-3′), RAPD6 (5′-CGGCACACTG TTCC-TCGACG-3′), BOX (5′-CTACGGCAAGGCGAC-GCT-3′), ERIC1R (5′-ATGTAAGCTCCTGGG GA-TTCAC-3′) and ERIC2 (5′-AAGTAAGTGACT GGGGTGAGCG-3′). Both ERIC primers (ERIC1R and ERIC2) were simultaneously used for amplification. Thermocycling conditions were identical to those previously published (Sikorski et al. 1999). A Mastercycler Personal (Eppendorf) was used for amplifications: four cycles at 94, 40, and 70°C for 5 min each followed by 30 cycles at 94°C and 55°C for 1 min each and at 70°C for 2 min with a final primer extension at 70°C for 5 min. Samples were run onto 1.5% agarose (SIGMA) gels, and the 100 base-pair ladder (Amersham Biosciences) was used as a molecular weight standard. Ethidium bromide stained DNA gels were documented with the Gel Printer Super II (T.D.I. S.A., Spain). Digitalized pictures were analysed with the Lane Manager 2.2 software for monodimensional gel analysis (T.D.I. S.A., Spain). All positions where at least one band was present in any of the lanes analysed were counted as single covariant characters. For each amplification experiment, a single binary matrix was constructed. Dendrograms were drawn from the distance matrices using the unweighted pair group method of analysis (UPGMA) as contained in the Treecon software package (Van der Peer and De Wachter 1994).

Pulsed field gel electrophoresis (PFGE)

Twenty milliliters of cultures were grown at 37°C and 170 rpm until an OD_{600} of 0.5–0.6 was reached, and were then centrifuged for 10 min at 3939 g. Cell pellets were washed and resuspended in 200 μL of buffer A (10 mM Tris, 3 M NaCl). The cell suspension was equilibrated at 37°C and mixed with an equal volume of melted 1.6% low-melting-point agarose (Pronadisa). The mixture was distributed in 0.1 ml plastic moulds (BioRad) and allowed to solidify at room temperature for 15 min. Agarose blocks were removed from the moulds and incubated overnight at 50°C in ESP (0.5 M EDTA, pH 9–9.5; 1% N-laurylsarcosine; 0.5 mg/ml proteinase K). Proteinase K was inactivated with Pefabloc (Roche) according to the manufacturer's instructions. Restriction with endonucleases (*Xba*I from Roche, and *Dra*I from Gibco) was carried out as previously described (López-García et al. 1994). Contour-clamped homogeneous electric field electrophoresis of digested DNA was carried out in a CHEF-DR III System (BioRad). Yeast Chromosome PFGE Marker and Low Range PFGE marker (New England BioLabs), and *Hansenula wingei* and *Schizosaccharomyces pombe* CHEF DNA size markers (BioRad) were used as linear size standards. Samples were loaded in 1% LE agarose (FMC Bioproducts) gels in 0.5× TBE. Electrophoreses of DNA digestions were carried out in 0.5× TBE at 14°C, 6 V/cm, 120° field angle. For *Xba*I digestions, samples were run for 24 h with a 1–10 s pulse linear ramp. *Dra*I restriction products were separated using two linear ramps: 1–17 s for 24 h followed by 1–8 s for 20 h. The dendrogram was produced with the same procedure used for genomic fingerprints.

Southern analysis

Gels were transferred onto positively charged nylon membranes (Hybond Amersham) as previously described (Smith et al. 1988). PCR amplified (see above) 16S rDNA from *S. ruber* M31 was labelled and hybridised against the blotted gels using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) according to the manufacturer's protocol.

Inter-spacer

The internal region DNA sequences between the 16S rRNA and 23S rRNA gene sequences were amplified, digested, and sequenced as previously described by Guasp et al. (2000). Complete and partial sequences were aligned with a selection of gene spacer regions compiled in the RISSC database (García-Martínez et al. 2001; http://www.miracle.umh.es/rissc/). Alignment of the sequences, tree reconstructions, and similarity cal-

Table 1 Strains used, their origin and some of the determined genetic and phenotypic properties

Origin[a]	M1 1	M8 1	M31 1	A1 2	A7 2	P13 3	P18 3	C 4 4	C 5 4	C 9 4	C 12 4	C 16 4	C 25A 4	E 3 5	E 7 5	E 11 5	PR 1 6
Halorhodopsin gene present	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No
G+C mol %	69.8 (66,3)[b]	69.6 (67.0)	70.2 (66.5)	70.3 (66.5)	70.3 (67.7)	70.2	69.5	68.3	69.5	68.3	69.6	69	67.9	69.2	68.6	68.4	68.7
Reassociation to M31	92.5	88.5	100	94.3	96.5	80.8	87.3	85.5	94.3	83.4	76.4	89.6	88.5	87	89.6	84.7	100
16S rDNA accession number	AF323499	AF323501	AF323500	AY529491	AY531224	AF323503	AF323502	AY531225	AY531234	AY531230	AY531226	AY531229	AY531227	AY531232	AY531233	AY531235	AY531231
Intergenic spacer accession number	AJ605300	–	–	AJ605294	AJ605296	AJ605296	AJ605293	AJ605298	AJ605301	–	–	–	–	–	–	–	AJ605297
Quinone system	MK-7 (99.8%) MK-8 (0.2%)	MK-7	MK-7 (98.2%) MK-8 (1.8%)	MK-7	MK-7	MK-7 (99.4%) MK-8 (0.6%)	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7
Polar lipids	DPG, PE, PL, GL1-3 L1-5, L7	DPG, PE, PL, GL1-3 L1-4, L6-7	DPG, PE, PL, GL1-3 L2-4, L7	DPG, PE PL, GL1-3 L1-3, L6-7	DPG, PE PL, GL1-3 L1, L3, L7	DPG, PE PL, GL1-3 L2-4, L7	DPG, PE PL, GL1-3 L1-4, L7	DPG, PE PL, GL1-3 L2-3, L7	DPG, PE PL, GL1-3 L1-3, L7	DPG, PE PL, GL1-3 L1-3, L6, 7	DPG, PE PL, GL1-3 L1-3, L7	DPG, PE PL, GL1-3 L1-3, L6, L7	DPG, PE PL, GL1-3 L1-3, L6, L7	DPG, PE PL, GL1-3 L1, L7	DPG, PE PL, GL1-3 L1, L7	DPG, PE PL, GL1-3 L1-4, L6- L7	DPG, PE PL, GL1-3 L1-4, L7
Hydrolysis of [c]																	
*o*NP-*β*-D-Galactopyranoside	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
*p*NP-*β*-D-Glucopyranoside	–	+ + +	+ + +	+ + +	–	+ + +	+ + +	+ +	+ +	+ + +	–	–	+ +	–	–	–	–
*o*NP-*β*-D-Xylopyranoside	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L-Glutamate-*γ*-*p*NA	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +	+ +	–
L -Glutamate-*γ*-3-carboxy-*p*NA	+ +	+ +	+ + +	+ +	+ +	+ +	+ + +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	–
*p*NP-*α*-D-Glucopyranoside	+ +	+ +	+ + +	+ +	+ +	+ +	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	–
*p*NP-*α*-D-Mannopyranoside	+ + +	+ + +	+ + +	+ + +	+ +	+ +	–	+ +	+ +	+ + +	+ +	+ +	+ +	+ +	+ + +	+ +	–
*p*NP-*β*-D-Glucopyranoside	–	+ + +	+ + +	+ + +	-	+ + +	+ + +	+ +	+ +	+ + +	-	–	+	–	–	+ + +	–
*p*NP-beta-L-Fucopyranoside	+ + +	+ +	+ + +	+ + +	+	+ + +	+ + +	+	+	+ + +	+ +	+ +	+	+ +	+ +	+ +	–
*p*NP-N-acetyl-*β*-D-Glucosaminide	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	–	+ +

[a] Origin of the strains: 1 Mallorca, Levante salterns; 2 Mallorca, s'Avall salterns; 3 Alicante; 4 Canary Islands; 5 Ebro Delta; 6 Peru

[b] in brackets GC content measured previously (Antón et al. 2002)Positive for all tests were: L-Leucine-*p*NA, L-Glycine-*p*NA, L-Proline-*p*NA, *p*NP-*α*-D-Maltoside, *p*NP-phenyl-phosphonate, *p*NP-phosphate, *p*NP-Phosphorylcholine, *p*NP-Thymidine-5′-monophosphate ester

[c] + + +, strong positive results, + + positive results; + weak positive results; − negative result

Negative for all tests were: mNP-*α*-D-Galactopyranoside, *p*NP-*α*-L-Arabinopyranoside, *p*NP-*α*-L-Fucopyranoside, *p*NP-*α*-L-Rhamnoside, *p*NP-*β*-D-Galactopyranoside, *p*NP-*β*-D-Glucuronide, *p*NP-*β*-D-Lactopyranoside, *p*NP-*β*-D-Xylopyranoside, *p*NP-N-acetyl-*α*-D-Glucosaminide

*p*NP, *para*-nitrophenyl; *p*NA, *para*-nitroanilide; oNP (ortho-nitrophenyl-); mNP (meta-nitrophenyl); DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; PL, unknown phospholipid; GL1-3, unknown glycolipids; Lx, unknown lipids (these unnown lipids were only present as minor compounds. Strains that lack one or the other may have them but they could not be detected because the concentrations may have been too low

culations were performed by the use of the ARB program (Ludwig et al. 2004). Gene alignments were performed by the ClustalW tool contained in the ARB package. Tree reconstructions were performed by the use of subsets of the imported database, with neighbour joining, parsimony, and maximum likelihood algorithms using the same package. The presence and the position of putative tRNAs within the sequences were checked by the use of the tRNAscan-SE (Lowe and Eddy 1997; http://www.genetics.wustl.edu/eddy/tRNAscan-SE).

Biochemical tests with chromogenic substrates

The chromogenic substrates (2 mM) were dissolved in 5 ml of SW medium and the pH adjusted to 7.0. The completed media were sterilized by filtration. Aliquots of 50 µl were filled into microtiter plates. Pre-inocula were grown in the SW medium (with 0.2% yeast extract, pH 7.2) until turbidity was visible. Fifty microliters of these suspensions were added to the wells of the prepared microtiter plates and the plates were incubated at 36°C for 24 h. Development of a yellow colour indicated positive results.

Chemotaxonomic markers

Quinone systems were extracted and analysed as previously described (Tindall 1990; Altenburger et al. 1996). Polar lipids were extracted and analysed by two-dimensional TLC according to Ventosa et al. (1993). Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID) after cultivation of the strains on SW agar. Extracts were analysed using a Hewlett Packard model HP6890A GC equipped with a FID, an automatic sampler, an integrator and a computer, as described previously (Kämpfer and Kroppenstedt 1996). Cell envelope lipopolysaccharides were extracted following previously published protocols (Westphal and Jann 1963) with some modifications as recommended by Bengoechea et al. (1996). Polyamines were analysed as described recently (Altenburger et al. 1997).

Results

Strain isolation and identification

In addition to the already studied strains M1, M8, M31, P13 and P18, 12 new strains were isolated from crystallizer ponds of several geographical origins (Table 1): Mediterranean (strains A1, A7, E3, E7 and E11), Atlantic (strains CAN4, CAN5, CAN9, CAN12, CAN16 and CAN25A) and even an Andean saltern (strain PR1). All strains were motile, straight or slightly curved rods, and growth on agar plates was shown in all cases to be pigmented as previously reported for other members of the species (Antón et al. 2002). The identification of the strains as putative members of *Bacteria* was achieved after PCR amplification with domain specific primers (pair 27f-1492r), and *Salinibacter spp.* (pair EHB4F-EHB9R) primer set.

Genetic traits

The 16S rRNA gene sequences of all new isolates were analysed and they were shown to be identical to those of the already sequenced *Salinibacter ruber*. No representative of the second *Salinibacter* spp. phylotype EHB-2 was obtained. The 16S-23S rRNA gene spacer regions were amplified for all strains and enzymatically digested with *Taq* I. In all cases, the digestion patterns were identical for all tested strains. The same region of nine selected strains was sequenced to compare them with the type strain M31 (see accession numbers in Table 1). Altogether, the sequences were very similar with values always higher than 97% sequence identity. With these

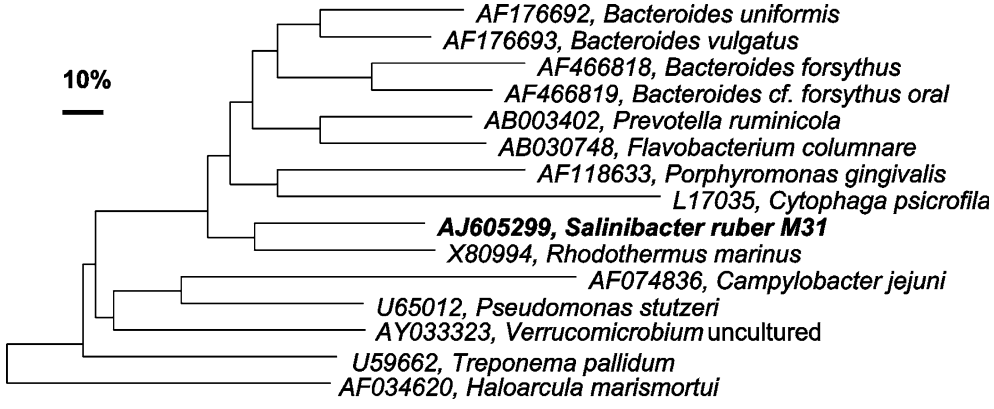

Fig. 1 Maximum parsimony phylogenetic reconstruction of the 16S-23S rRNA gene spacer region of a selection of sequences available at the RISSC web site (García-Martínez et al. 2001). This tree is in accordance with the various reconstructions carried out by the use of different subsets of data, and the three different algorithms neighbour joining, maximum parsimony, and maximum likelihood as contained in the ARB package (Ludwig et al. 2004).

similarities, no phylogenetic reconstruction could be performed to infer reliable genealogies within *S. ruber*. Additionally, the phylogeny of the gene spacer region of M31 was reconstructed with respect to a selection of the available homologous regions (Fig. 1). The closest relative sequence was that of *Rhodothermus marinus* (sequence X80994) in accordance with the 16S rRNA gene sequence reconstructions (Antón et al. 2002). Blotted profiles of enzymatically restricted fragments of genomic DNA separated by PFGE were hybridized with a 16S rDNA probe to reveal the number of rDNA operons. In all cases a single copy of the rDNA operon was found. The G + C mol% of the strains was quite homogeneous and the values ranged from 67.9% to 70.3% (Table 1), which was slightly higher (maximum of 3.4%) than those calculated previously (Antón et al. 2002). DNA-DNA hybridisation experiments gave reassociation values always higher than 76.4% with the genomic DNA of the type strain of the species.

Phenotypic traits

A total of 27 different chromogenic substrates were tested for all strains (Table 1). Cleavage of L-leucine-*p*NA, L-glycine-*p*NA, L-proline-*p*NA, *p*NP-α-D-maltoside, *p*NP-phenyl-phosphonate, *p*NP-phosphate, *p*NP-phosphorylcholine and *p*NP-thymidine-5′-monophosphate ester resulted positive for all strains tested. No strain rendered cleavage of *m*NP-α-D-galactopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-L-fucopyranoside, *p*NP-α-L-rahmnoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-glucuronide, *p*NP-β-D-lactopyranoside, *p*NP-β-D-xylopyranoside and *p*NP-n-acetyl-α-D-glucosaminide. Strain PR1 was the most divergent strain since, apart from the common positive traits of the species, only *p*NP-n-acetyl-β-D-glucosaminide was cleaved by this strain. Apart from PR1, all strains showed cleavage of l-glutamate-γ-*p*NA, l-glutamate-γ-3-carboxy-*p*NA, *p*NP-α-D-glucopyranoside and *p*NP-beta-l-fucopyranoside. Strain A1 showed a weak reaction of *o*NP-β-D-galactopyranoside and *o*NP-β-D-xylopyranoside for which the remaining strains were negative. The only traits that could serve for discriminative intraspecific purposes were *o*NP-β-D-glucopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-β-D-glucopyranoside and *p*NP-n-acetyl-β-D-Glucosaminide. With the exception of strain PR1 the isolates showed very similar physiological reactions.

Chemotaxonomic markers

All strains showed menaquinone 7 as the single or major quinone component (Table 1). Only strains M1, M31 and P13 showed traces of menaquinone 8 in amounts of up to 1.8% of the total quinone present. *S. ruber* has a membrane lipid composition comprising glycerophospholipids containing ester-linked fatty acyl chains that are typical of *Bacteria* (Harwood and Russell 1984). The major polar lipids in *S. ruber* were diphosphatidylglycerol and the unknown polar lipid L7. Phospatidylethanolamine, as well as three unknown glycolipids, several

Table 2 Fatty acid profiles of whole cell hydrolysates (%)

Strain	M1	M8	M31	A1	A7	P13	P18	C 4	C 5	C 9	C 12	C 16	C25A	E3	E7	E11	PR 1
Fatty acid unknown 13.565[a]																	2.4
14:0																0.6	
15:0 ISO	29.7	33.1	34.6	29	23.5	32.9	25.6	30	29.4	25.3	32.6	33.6	30.8	38	33	23.3	25.9
15:0 ANTEISO	6.5	7.4	8	9.3	6.7	8.7	8.1	5.7	5.5	6	6.5	7	6	7.6	7.3	5.3	4.9
15:1 ω6c														1.1			
15:0	1.3	1.5	2.1		1.5			1.5	1.5	1.1	1.4	1.3		2.2		1.1	1.9
16:0 ISO	2.1	3.2	3.9	5.3	4.4	5	2.7	3.9	3.7	2.4	2.9	3.5	3.3	1.8	3	1.6	2.6
Sum in feature [b]																	
16: 1 ω7c/15 iso 2OH	30	33.3	27.5	35	35	35	38	33.3	33.9	30.5	28.6	26.2	35.8	29.6	31.1	37	30
16:1 ω5c							1.1									0.9	
16:0	5	5	5.2	5.7	3.9	3.8	2.7	4.1	4.5	5.8	4.2	5.4	4.3	3.3	4.8	3.7	6.3
17:1 ω9c ISO	1.1						1.2	1.4	1.3	1	1.3	2.1		0.8			
17:0 ANTEISO	0.9							1.3	1	1.2	1.1	1.2					
17:1 ω6c	0.9							1.2	1.3	0.9				1		0.9	
16:0 ISO 3OH							1.1										
16:0 3OH																0.7	
16:0 10 methyl					1.4												
17:0 CYCLO					1.8												
18:1 ω7c	14.8	12	12.1	15.7	16.1	9.9	13.8	12.7	12.9	19.8	14.6	10.8	15.1	8	15.4	20.5	23.4
17:0 ISO 3OH	5.8	3	4.1		4	2.8	4.2	3.9	4.1	3.8	5.1	5.1	4.6	5.1	5.4	3.4	2.8
17:0 2OH	1.8		1.6		1.6		1.6	1	1.1	1.3	1.7	1.5		1.6			
Total	99.9	99.9	100	99.9	100	100	100	100	100	100	100	91.8[c]	99.9	100	99.7	100	98.4

[a] This fatty acid could not be identified by the MIDI system
[b] These two fatty acids could not be separated on the basis of the analytical procedure
[c] Isolate Can16 contained several fatty acids in minor amounts not identified by the MIDI system

polar lipids, and one phospholipid were usually found from moderate to minor amounts. Contrarily to previous observations (Oren et al. 2004) no phosphatidylglycerol was detected in any of the strains. The unknown polar lipid L3 displayed a chromatographic behaviour most similar to that expected for phosphatidylglycerol but L3 did not react with the spray reagent specific for phosphorus groups. Thus, we assumed that this unknown L3 was not phosphatidylglycerol. Phosphatidylcholine was also not detected in any of the extracts analysed contrarily to previous observations (Oren et al. 2004). Phospholipid PL, such as phosphatidylcholine, could be stained with the specific Dragendorff reagent, but the chromatographic behaviour and shape of the corresponding spot differed from the egg-like shape of the phosphatidylcholine. Consequently, the unknown PL might be a derivative of phosphatidylcholine that differs in structure from the authentic compound. The whole cell fatty acid profiles of all strains were mainly composed of 15:0 Iso (23.3–38%), 16:1 ω7c/15:0 iso 2OH (26.2–38%) and 18:1 ω7c (8–23.4%). In addition, the fatty acids 16:0, (3.3–6.3%), 15:0 anteiso (4.9–9.3%), and 17:0 iso 3OH (2.8–5.8%) were detected in minor amounts (Table 2). No pronounced differences in the fatty acid profiles were found between the strains. Polyamine patterns were analysed for strains A1, A7 and M8. All three strains contained putrescine, spermidine and *sym*-homospermidine in extremely low concentrations that were slightly above the detection limit of our system (0.2 µmol g^{-1} dry weight). Lipopolysaccharide profiles were identical for all studied strains (data not shown) and no significant differences could be seen between the isolates.

Fingerprinting with PFGE and randomly amplified genomic fragments

Two dendrograms were produced after UPGMA analysis, one for the PFGE patterns and one as a result of the combination of eight independent random amplifications with the primers cited. PFGE patterns resulted in about 30 different independent positions where a single band (i.e. digestion product) was found in at least one profile. In the derived dendrogram shown in Fig. 2 two clusters were found with more than 55% banding pattern similarity. Cluster 1 harboured all the isolates from the Mallorca and Ebro Delta salterns (M1, M8, M31, A1, A7, E3, E7 and E11), whereas cluster 2 harboured all strains isolated from Alicante and the Peruvian Andes (P13, P18 and PR1). Strains isolated from the Canary Islands appeared to be spread across both clusters (C25a and C4 in cluster 1, and C5, C9, C12 and C16 in cluster 2). Single randomly amplified genomic DNA patterns rendered between 20 (ERIC) and 48 (RAPD4) independent positions (i.e. each single random amplification product) that could be used for the analysis. In Fig. 3, the eight independent analyses are shown, and a single dendrogram based on 260

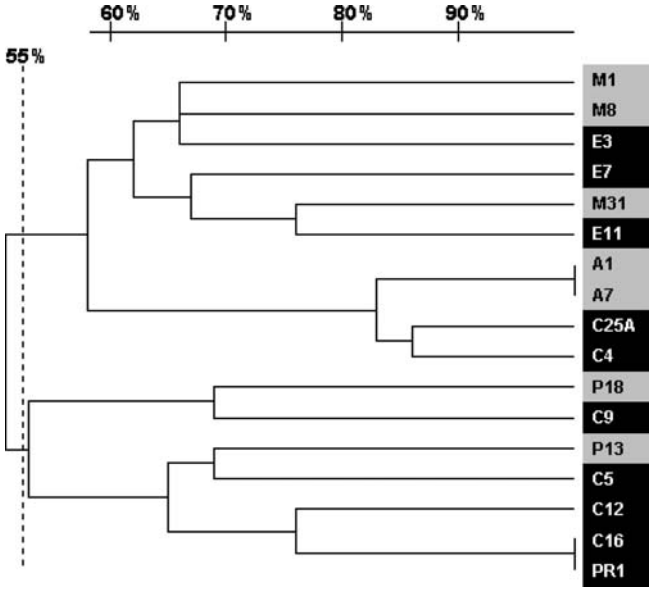

Fig. 2 Dendrogram generated after UPGMA analysis of PFGE patterns. The analysis was carried out after a set of about 30 covariant positions defined as the presence of at least one band in one of the analysed lanes. Black boxed strains are those not harbouring halorhodopsin

independent positions resulted from the combination of all amplification experiments. In the dendrogram based on the combined results, one single independent cluster of seven strains sharing at least 85% pattern similarity could be found. All strains isolated from the Alicante and Mallorca salterns were grouped within the same cluster, whereas the rest of the strains appeared to be spread in four additional clusters. Additionally, this unique cluster harboured all strains for which a copy of a homologous halorhodopsin gene was found (J. Antón et al., in preparation; M31 halorhodopsin accession number AY667579). No single independent amplification fingerprinting gave results similar to the similarity matrix generated after the combination of all experiments.

Discussion

Shortly after the discovery of the existence and ecological relevance of *Salinibacter* spp. in brines close to saturation (Antón et al. 2000), members of this genus were isolated leading to the classification of the new genus and species *S. ruber* (Antón et al. 2002). The first description was made after five organisms that were isolated from solar salterns of Alicante and Mallorca. Since then, the occurrence of this genus in brines of geographically distant sites has become more evident, and this led us to compile a larger strain collection from different locations in Europe and South America. The collection of isolates studied here includes organisms occurring in brines from the Canary Islands, Ebro Delta and the Peruvian Andes, in addition to the first sites

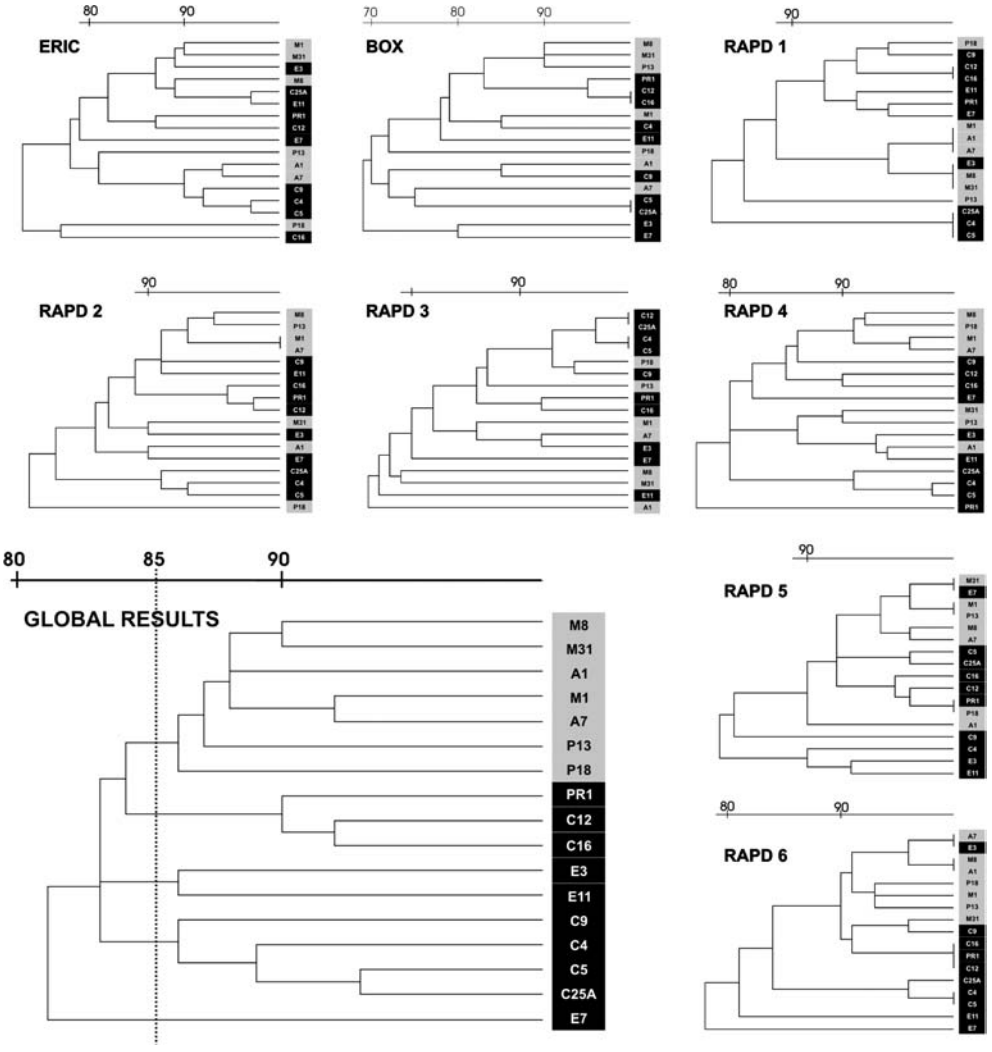

Fig. 3 Dendrograms generated after UPGMA analyses of each single random amplification profile. The number of covariant positions varied between the different fingerprints as follows: ERIC 20 positions; BOX 31 positions; RAPD1 25 positions; RAPD2 41 positions; RAPD3 38 positions; RAPD4 48 positions; RAPD5 30 positions; RAPD6 27 positions. The resultant global dendrogram was produced after the combination of all single results into a single similarity matrix; the number of covariant characters was 260. Results are expressed in percentages of similarity. Black boxed strains are those not harbouring halorhodopsin

sampled. Despite the isolation efforts, all new isolates were identified as *S. ruber*, and no isolation of a single member of the EHB-2 phylotype was successful. The lack of success on the isolation of the hitherto uncultured EHB-2 phylotype might be due to either the culture media used that do not satisfy their growth requirements, or to the relative low abundance of this phylotype in natural brines. In all cases studied EHB-2 showed abundances lower than 2.4% of the total prokaryotic population, whereas the phylotype EHB-1 corresponding to *S. ruber* were always at least five fold higher (Antón et al. 2000).

In a different study (J. Antón et al., in preparation), a homologous gene to the archaeal halorhodopsin could be detected in the type strain of the genus after the annotation of the open reading frames detected during a partial genome-sequencing program. The presence of this gene was investigated for the same collection of isolates and an identical copy was found in the genomes of a subset of the collection, but not in all of them (see Table 1). The seven halorhodopsin-harbouring strains were isolated from three different crystallizer ponds, one situated in Alicante and two in Mallorca. In principle, it seems that the presence of such a gene is related to the origin of isolation. However, halorhodopsin-harbouring strains in the Canary Island samples have been found recently (data not shown). Mallorca, Alicante and the Ebro Delta are geographically equidistant, but this gene has not been found yet in the strains from the Ebro Delta. The so called 'polyphasic taxonomy' (Vandamme et al. 1996) may reveal whether the geographical isolation of these extreme sites determines allopatric speciation phenomena (Staley, 2004).

S. ruber seems to be very homogeneous from the taxonomic point of view, as indicated both by genomic and phenotypic data. We could not find solid trait differences to discriminate between the isolates from distant geographical zones. All strains showed a single rRNA operon with identical 16S rRNA gene sequences and nearly identical inter-spacer sequences. G+C mol percent, and reassociation values were very similar to the type strain of *S. ruber*, consistent for closely related strains of a single species, and close enough to be considered as a single genomovar within the species (Rosselló-Mora and Amann 2001). Despite DNA-DNA reassociaton values ranged from 76.4% to 100% with

the type strain of the species, 16S rDNA sequences were identical for all isolates, and this is another example of the lack of resolution power of this gene to discriminate strains at the species level (Rosselló-Mora and Amann 2001). The phylogenetic reconstruction made from the inter-spacer region of the rRNA operons was in accordance with previous reconstructions based on 16S rRNA gene sequences, with *Rhodothermus marinus* being the closest relative to *S. ruber* (Antón et al. 2000). Lipopolysaccharide profiles, the quinone system and polar lipid profiles, despite some minor differences, were also identical for all strains. Some metabolic differences were found among the isolates, and only the strain from Peru (PR 1) could be discriminated. However, such discrimination is made upon 6 out of 27 characters, and such differences are taxonomically not significant. Fatty acid profiles were also similar among the strains studied and resulted in agreement with previously published patterns for other members of the same phylum (Moore et al. 1994), and they were similar to their closest relative genus *Rhodothermus* (Silva et al. 2000). However, *Rh. marinus* contains higher amounts of 15:0 anteiso and also 17:0 anteiso branched fatty acids, while no hydroxylated fatty acids have been detected (Silva et al. 2000). As in representatives of the genera *Bacteroides*, *Prevotella*, and *Porphyromonas*, 17:0 Iso 3OH was also found in significant amounts (Moore et al. 1994). Extremely low polyamine concentrations in the three analysed strains were in agreement with the observations for extremely halophilic *Archaea*, where the total cellular polyamine content is at least two orders of magnitude lower than for non-halophilic *Bacteria* (Hamana et al. 1985). Excretion of polyamines in order to compensate for an increased intracellular cation concentration is considered to be a response to osmoadaptation (Wood 1999). The high K^+ concentrations in cells of *S. ruber* (Oren et al. 2002) might be responsible for the low concentration of polyamines observed in our analyses. Altogether, the taxonomic approach showed that *S. ruber* remains homogeneous beyond any geographical barrier.

Significant intraspecific differences could only be found by the use of DNA fingerprinting approaches such as RAPD (Sikorski et al. 1999) and PFGE that are considered to be more discriminative (Vandamme et al. 1996). As shown in Fig. 2, PFGE profiles rendered two different clusters that slightly reflected the origin of isolation except for the strains isolated from brines in the Canary Islands. The clustering approach was different when analysing the genomic fingerprinting shown in Fig. 3. In this case, the combined analysis of the eight different amplification patterns produced finer tuned results in comparison to any single one of the UPGMA analyses, in accordance with previous observations for strains of *Pseudomonas stutzeri* (Sikorski et al. 1999). Global results of the combined similarity matrices produced a single cluster with 85% similarity where all strains harbouring the halorhodopsin gene were included. The strains not harbouring a homologous gene appeared unclustered. The combined approach may resolve natural relationships better simply because the predictability of phenetic analysis is directly influenced by the amount of covariant independent traits (Sneath and Sokal, 1973). In this case, the randomly amplified fingerprinting dendrogram produces nearly one order of magnitude more independent positions than by PFGE, thus the influence of homoplasic (false homologies, i.e. non-homologous DNA fragments with similar electrophoretic migration) traits is minimized (Rosselló-Mora and Kämpfer 2004).

As expected (Vandamme et al. 1996), the results of the phenetic analyses of genomic fingerprints defined the intraspecific diversity more accurately than those of a standard taxonomic approach. However, due to the extreme nature of the environments where *Salinibacter spp*. thrive, the reduced dimensions of the salterns, and the geographical distances between them it would not be expected to observe such genomic and phenotypic homogeneity (Whitaker et al. 2003). Nevertheless, both DNA-based phenetic analyses indicate that there is a correlation between the geographical isolation source and the genotype of the cultured strains of *S. ruber*. PFGE discriminates between Mallorca and the Ebro Delta, and the Alicante and Peru isolates, whereas random amplifications discriminate those harbouring halorhodopsin. Although the number of isolates per site is not high, the results obtained indicate that the species remains highly coherent in many genomic and phenotypic traits, but populations from different isolation sites can still be discriminated. Finally, there should be unknown dispersal mechanisms that might not act randomly because strains harbouring identical halorhodopsin genes were isolated from distant locations. The use of other approaches, such as multilocus sequence typing, that have been very useful for discriminating endemic populations of extremophiles (Whitaker et al. 2003), and the inclusion of new isolates may help to understand such mechanisms.

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