

Biodata of **Josefa Antón**, author of “*Salinibacter ruber*: genomics and biogeography”

Josefa Antón is associate professor of microbiology at the University of Alicante, Spain. In the summer of 1998 she joined for three months the group of Prof. Rudolf Amann at the Max-Planck Institute for Marine Microbiology in Bremen, to try to develop a FISH protocol to study microorganisms in hypersaline environments, in collaboration with Dr. Ramon Rosselló-Mora (at that time, postdoctoral researcher at MPI and currently at IMEDEA in Mallorca, Spain). The development of these techniques allowed the unexpected discovery of *Salinibacter* in the salterns. Lately, this bacterium was isolated and described in collaboration with Profs. Oren and Rodríguez-Valera. Since the discovery of *Salinibacter*, JA team works together with Drs. Amann and Rosselló-Mora in the ecology, biogeography and genomics of *Salinibacter ruber*.

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SALINIBACTER RUBER: GENOMICS AND BIOGEOGRAPHY

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1. Introduction

Salinibacter ruber is an extremely halophilic bacterium that was first isolated from crystallizer ponds of solar salterns located in Alicante and Mallorca, Spain. These environments have a very high salt concentration (from 25% to saturation) and are populated by two types of microorganisms: the numerically dominant square Archaea and different phylotypes of *Salinibacter* (Antón et al., 2000; Øvreås et al., 2003). Until recently, no representatives of these two majority populations had been brought into pure culture, while a wide collection of haloarchaea had been isolated from the very same environments, showing that even in a very low diversity environment, culture techniques can be biased towards minority microorganisms. *S. ruber* was first described as "*Candidatus Salinibacter*" based on molecular ecology techniques and was finally isolated in 2002, enabling validation of the species name (Antón et al., 2002). More extensive documentation has recently become possible by the study of a wider collection of strains isolated around the world (Peña et al., in press). On the other hand, Walsby's square archaeon has been reluctant to cultivation, although many researchers had attempted different isolation approaches for years. However, many data about the squares are available from structural and molecular techniques (Antón et al., 1999; Kessel and Cohen, 1982; Oren et al., 1996), that identify them as Archaea belonging to the family *Halobacteriaceae*. More specifically they are related to SPhT (or Susana's phylotype, named after its discoverer, Susana Benlloch), a 16S rDNA sequence that had been recovered from different salterns around the world (Rodríguez-Valera et al., 1999). Very recently, Mike Dyal Smith and coworkers have finally isolated the square archaeon (Burns et al., 2004). Now, the two main players in crystallizer ponds are available for complete characterization.

S. ruber was thus the first aerobic extremely halophilic member of the domain Bacteria which ecological relevance in crystallizer ponds. This was unexpected, since hypersaline environments had been described as almost monospecific cultures of

halophilic Archaea (Guixa-Boixareu et al., 1996). Surprisingly, *S. ruber* was very easy to isolate: only a lower nutrient concentration had to be used in the very same medium used for isolating haloarchaea and, after screening of the red colonies (very much haloarchaeal like), a high percent of them turned out to be members of this new genus. Once isolated in pure culture, it was evident that the similarity of *S. ruber* and haloarchaea could have hampered the isolation of the bacterium since both types of prokaryotes are extremely similar at the phenotypic level: both are extreme halophiles, aerobes, heterotrophs and pigmented by carotenoids (Lutnaes et al., 2002; Oren and Rodríguez-Valera, 2001). More detailed studies showed that *S. ruber*, like haloarchaea, accumulates high concentrations of K^+ to counterbalance the osmotic pressure of the medium (Antón et al., 2002; Oren et al., 2002), has a high proportion of acidic amino acids in its proteins, as well as enzymes functional at high salt concentrations (Oren and Mana, 2002), and even has a high proportion of G+C in its genome (65-70% for *S. ruber*, in the range of 59-70 for haloarchaea; Oren, 2002). All these similarities, together with the fact that *S. ruber* and haloarchaea share the same habitat in which prokaryotic density is very high, poses interesting questions about the evolutionary processes leading to a high degree of similarity between these two groups of organisms. As pointed out by Oren and Mana (2002), a comparative analysis of *Salinibacter* and haloarchaea would be of great interest to understand the mechanisms of this apparent convergent evolution and to ascertain the extent of lateral gene transfer between these members of the two prokaryotic domains.

2. Genomic and Proteomic Analysis of *Salinibacter ruber*

A few years ago we undertook a low coverage shotgun sequencing of *S. ruber* M31^T genome for data mining purposes. Besides, we have been isolating *S. ruber* strains from salterns around the world (from the Peruvian Andes to Eilat in Israel) to ascertain the degree of intraspecific diversity using different genetic and phenotypic tools, including the distribution of "interesting" genes newly found when sequencing the M31 genome.

S. ruber M31 has a genome of 3.4 ± 0.2 Mbp of which we have sequenced approximately 60% and assembled into contigs ranging from 100 to 16,000 bp. *S. ruber* has a typically prokaryotic ribosomal operon 16S-tRNA(Ile-GAU)-tRNA(Ala-UGC)-23S-5S that, according to our hybridization experiments, is present in a single copy. Among the genes identified in the sequence we have found many *che* and *fla* genes (EM studies had previously revealed the presence of flagella in *S. ruber*) as well as a high quantity of membrane transporters, including haloarchaeal-like K^+ transporters and, most interestingly, a homologue to the precursor of light-driven chloride pump halorhodopsin (Antón et al., in preparation). Besides, as found in the genome of the haloarchaeon *Halobacterium* NRC-1 (Ng et al., 2000), the genome of *S. ruber* shared a large number of homologues with the radiation-resistant bacterium *Deinococcus radiodurans*.

Comparing the complete proteomes available at the time of analysis (16 from Archaea and 43 from Bacteria), we found that 6% of the proteins coded in the genes in our library had a best match with archaeal proteins (considering only BLAST hits with E-values lower than e^{-20}). However, when the more restrictive conditions of Gophna et al. (2004) were used, only 1% of the proteins we had annotated could be of archaeal origin. Obviously, since we only have partial data, these results are not conclusive.

One very interesting question was whether *Salinibacter* proteins shared indeed the characteristics of haloarchaeal proteins, something that had already been studied for some *Salinibacter* enzymes and for bulk proteins (Oren and Mana, 2002). In order to address this point we have randomly chosen several proteins (more precisely, predicted gene products) from *Salinibacter* and compared their isoelectric points and their contents of acidic and basic amino acids and serine with their homologues of haloarchaeal origin, as well as from some non extremely halophilic Bacteria and Archaea. Results are shown in Fig. 1 and Table 1. All the proteins were of "bacterial origin", except the Na⁺/Pro symporter and PyrE, that were of "archaeal origin" according to Gophna et al. (2004). Except for RecA, in every case analyzed, *S. ruber* proteins had isoelectric points closest to their haloarchaeal homologues and always lower than those for the rest of archaeal and bacterial proteins analyzed. *Halobacterium* NRC-1 proteome analysis had shown an average pI of approximately 5. Acidic proteomes were also predicted from partial genome sequences of *Haloarcula marismortui* and *Haloferax volcanii* (DasSarma, 2004). Most of the selected *Salinibacter* proteins resemble proteins from haloarchaea since they contain more acidic amino acids (and less basic amino acids) than their homologues from non halophilic Archaea and Bacteria. The evolutionary modifications required to re-engineer a protein so that it becomes halophilic appear to involve the introduction of additional acidic residues (Dennis and Shimmin, 1997). This seems also to be the case in the analyzed *S. ruber* proteins, although the genealogies of these proteins do not relate them with halophilic Archaea. However, we could not see a clear trend regarding the serine content. The amount of serine in bulk proteins from *Salinibacter* has been shown to be higher than that of proteins from the non-halophilic *Escherichia coli* and the moderately halophilic *Halomonas elongata* (Oren and Mana, 2002). However, the amount of serine in bulk protein is obviously influenced by the relative amounts of the individual cellular proteins, that are not considered in our analysis.

3. Halorhodopsin of *Salinibacter ruber*

As mentioned above, one of the more interesting findings in the *S. ruber* genome was the presence of a homologue to the haloarchaeal halo-opsin (called SalHO, for *Salinibacter* halo-opsin), the precursor of the retinal derivative light-driven chloride pump halorhodopsin. Together with the light-driven proton pump bacteriorhodopsin and two sensory rhodopsins involved in light sensing for phototaxis (Oren, 2002), halorhodopsin is one of the four type-1 retinal pigments that until recently were believed to be unique for the halophilic Archaea. However, homologues for bacteriorhodopsin and sensory rhodopsin have been found among members of the Bacteria, and even in the eukaryotic domain (Bieszke et al., 1999; Gärtner and Losi, 2003). In fact, the presence of a wide variety of proteorhodopsins, the bacterial homologue of bacteriorhodopsin, as a way of using solar energy seems to be a very common feature in prevalent components of marine microbiota (Béjà et al., 2001; De la Torre et al., 2003; Venter et al., 2004). So far, no halorhodopsin homologues had been found in bacterial genomes.

The halo-opsin homologue was found in a contig containing other genes of putative archaeal origin, as shown in Fig. 2A. Most likely, these different archaeal-like genes had a different evolutive history, since they are not simultaneously present in all the *S. ruber* strains from our collection (Peña et al., in preparation). A phylogenetic tree reconstructed

by comparing the amino acid sequence of the translated SalHO gene with microbial rhodopsins (Fig. 2B) shows that SalHO is clearly affiliated with the archaeal halorhodopsins. As previously described (Ihara et al., 1999), four clusters were observed in the tree corresponding to four different functional groups (i.e. H⁺ pumps, Cl⁻ pumps, sensory rhodopsin I and sensory rhodopsin II).

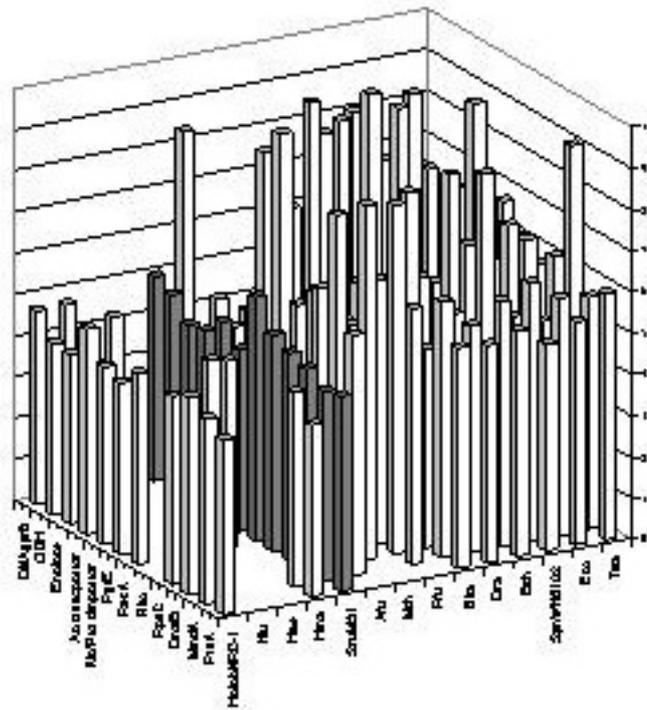


Figure 1. Calculated pI of proteins predicted from gene sequences: *Halobacterium* sp. NRC-1 ATCC 700922 (HalobNRC-1), *Haloferax lucentense* (Hlu), *Haloferax mediterranei* (Hme), *Haloarcula marismortui* ATCC 43049 (Hma), *Haloferax volcanii* (Hvo), *Salinibacter ruber* DSM 13855 (SruM31), *Archaeoglobus fulgidus* DSM 4304 (Afu), *Methanothermobacter thermautotrophicus* Δ H (Mth), *Pyrococcus furiosus* DSM 3638 (Pfu), *Bacillus halodurans* C-125 (Bha), *Deinococcus radiodurans* R1 (Dra), *Bacteroides thetaiotaomicron* VPI-5482 (Bth), *Synechococcus* sp. WH8102 (SynWH8102), *Escherichia coli* K12-MG1655 (Eco), *Thermotoga maritima* MSB8 (Tma). The proteins investigated were DNA gyrase B (DNAgyrB), glutamate dehydrogenase (GDH), enolase, the Na⁺/proline symporter (Na⁺/Pro symporter), orotate phosphoribosyltransferase (PyrE), recombination protein A (RecA), transcription termination factor (Rho), DNA-directed RNA polymerase beta' subunit (RpoC), replicative DNA helicase (DnaB), methylmalonyl-CoA decarboxylase alfa subunit (MmdA), and gamma-glutamyl phosphate reductase (ProA).

TABLE 1. Calculated percent of acidic amino acids (%ac), basic amino acids (%ba) and serine (%Ser) of predicted proteins shown in Fig. 1.

	DNAglyB	GDH	Enolase	Aa transporter	Na ⁺ /Pro symporter	PyE
	%ac / %ba / %Ser	%ac / %ba / %Ser				
SruM31	16.41 / 10.65 / 6.8	14.28 / 9.44 / 3.2	17.21 / 6.55 / 5.2	4.38 / 9.92 / 4.55	7.01 / 5.17 / 6.6	19.05 / 8.57 / 5.7
HalobNRC1	17.83 / 10.47 / 4.7	17.78 / 7.45 / 4.8	17.58 / 8.29 / 3.0	4.99 / 10.30 / 7.42	10.45 / 4.51 / 5.2	19.34 / 7.55 / 6.1
Hlu	18.46 / 10.64 / 4.7					
Hme		17.00 / 8.61 / 5.4				
Hma						19.52 / 8.57 / 6.7
Afu	18.14 / 12.82 / 4.9		16.29 / 10.09 / 5.0	4.35 / 12.26 / 6.13	6.38 / 3.19 / 6.4	
Mth			16.82 / 7.93 / 5.5	8.46 / 8.83 / 9.24	4.71 / 6.22 / 4.1	16.41 / 14.36 / 5.1
Pfu			16.12 / 11.33 / 4.0		3.99 / 6.84 / 5.7	16.11 / 13.89 / 6.1
Bha	15.07 / 12.40 / 5.0	10.91 / 10.04 / 6.3		8.61 / 5.85 / 6.40	7.40 / 9.13 / 5.7	18.13 / 15.39 / 3.3
Dra	14.03 / 12.67 / 3.9	12.26 / 10.14 / 5.4	15.16 / 10.18 / 6.4	9.01 / 5.00 / 6.09	4.73 / 7.10 / 7.1	13.33 / 10.00 / 8.1
Bth	15.00 / 11.48 / 5.5	12.61 / 11.94 / 4.7	15.15 / 12.12 / 6.3	7.68 / 5.91 / 6.14	5.59 / 6.88 / 4.5	11.34 / 9.28 / 5.7
SynWH8102	15.04 / 12.64 / 5.3		13.48 / 7.90 / 7.0	9.15 / 4.98 / 6.71		12.50 / 11.50 / 4.0
Eco	15.04 / 12.81 / 5.2	11.18 / 9.84 / 4.9	13.65 / 11.11 / 5.1		5.96 / 5.96 / 5.5	10.03 / 12.60 / 6.9
Tma	16.67 / 15.88 / 5.0	13.46 / 12.26 / 3.6				13.90 / 13.90 / 5.9

	Rec A	Rho	Rpoc	DnaB	MmdA	ProA
	%ac / %ba / %Ser					
SruM31	14.81 / 14 / 7.1	16.5 / 13.25 / 6.5	15.61 / 11.74 / 5.8	17.82 / 12.52 / 6.4	17.42 / 10.41 / 4.2	16.43 / 9.8 / 4.9
HalobNRC1	17.84 / 9.63 / 4.8		18.85 / 10.09 / 3.8	17.42 / 11.69 / 5.6	18.44 / 10.15 / 5.1	17.18 / 5.93 / 5.5
Hlu						
Hme						
Hma	18.36 / 9.32 / 6.4				16.70 / 10.82 / 5.3	18.09 / 6.86 / 6.0
Afu	18.5 / 7.6 / 6.6		14.99 / 14.42 / 4.4	16.82 / 17.44 / 6.6	13.73 / 12.02 / 3.1	
Mth	13.76 / 11.96 / 5.4		16.20 / 13.10 / 4.9	14.49 / 15.11 / 7.0		
Pfu	16 / 11 / 6.7		15.22 / 14.55 / 3.2	15.42 / 16.34 / 6.0	12.64 / 11.88 / 3.3	
Bha	14.34 / 13.12 / 6.4	16.35 / 14.74 / 6.4	13.93 / 14.43 / 4.5	16.52 / 11.89 / 5.3	12.60 / 11.63 / 4.8	13.94 / 10.82 / 6.2
Dra		14.3 / 13.14 / 4.2	15.72 / 12.48 / 5.4	13.84 / 10.94 / 6.2	13.27 / 11.73 / 5.4	12.27 / 10.19 / 4.4
Bth	12.94 / 11.84 / 5.5	15 / 15.4 / 4.6	13.81 / 13.6 / 5.0	12.58 / 13.65 / 6.1	12.26 / 11.28 / 5.1	12.95 / 10.79 / 6.2
SynWH8102	14.24 / 12.79 / 5.8		15.03 / 11.44 / 6.4	13.59 / 11.04 / 7.4	12.63 / 11.60 / 5.8	11.83 / 8.12 / 4.4
Eco	12.23 / 11.34 / 4.8	13.76 / 13.54 / 5.4	13.51 / 13.22 / 5.0	15.71 / 11.68 / 7.2	14.42 / 13.17 / 5	12.71 / 10.55 / 5.3
Tma	13.76 / 13.2 / 6.2	14.75 / 13.81 / 5.4	15.09 / 14.32 / 4.7	15.39 / 16.77 / 6.0	13.59 / 11.65 / 4.1	16.39 / 15.18 / 4.8

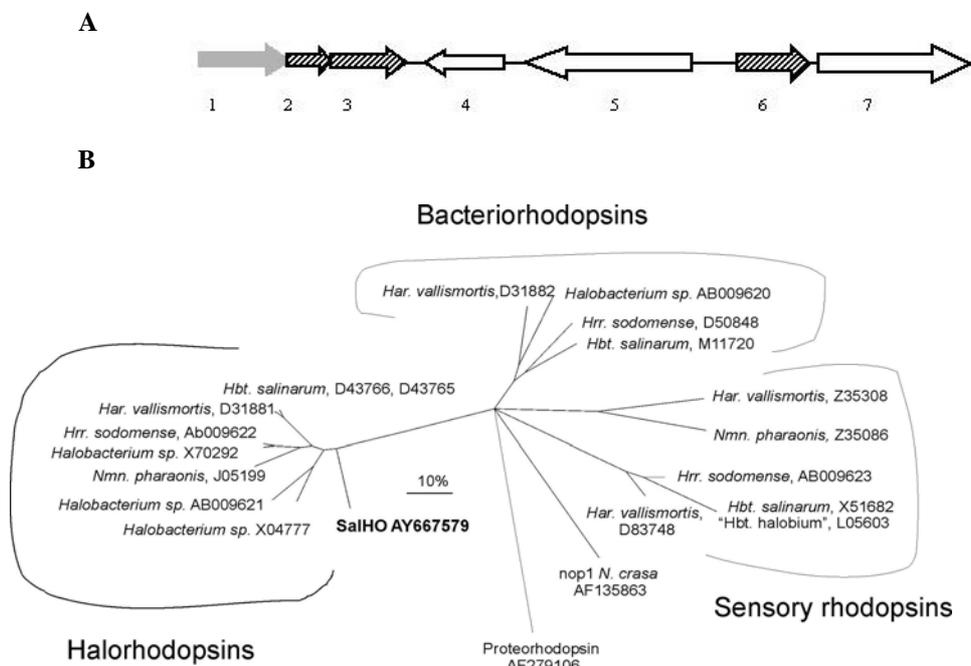


Figure 2. A. Description of the contig where the halo-opsin homologue was found: hypothetical (1), hypothetical (2), short chain alcohol dehydrogenase of unknown specificity (3), halo-opsin (4), sodium:proline symporter (proline permease) (5) oxidoreductase, short chain dehydrogenase/family (6), hypothetical (7). Putative origin of the ORFs: unknown (grey arrows), archaeal (white), bacterial (black bars). B. Phylogenetic tree based on amino acid sequences of all homologous archaeal rhodopsins, *Neurospora crassa nop-1* gene product, and bacterial proteorhodopsins available in public databases. The tree shown is based on neighbour joining approach by using the Kimura algorithm. Results were simultaneously evaluated by the maximum parsimony and maximum likelihood treeing approaches using the same data and subsets thereof. Multifurcations show such positions where the branching order could not be resolved. The SalHO sequence appeared consistently in the position shown in the tree independently of the algorithm or the dataset used. However, the position of the proteorhodopsin branch remained unstable and its position was dependent on the approach used. Fig. 2B was derived from Antón et al. (in preparation). *Har.* = *Haloarcula*; *Hbt.* = *Halobacterium*; *Hrr.* = *Halorubrum*; *Nmn.* = *Natronomonas*.

However, there must be another system for chloride transport since, as explained below, not all the *Salinibacter* strains harbour SalHO genes in their genomes. In fact, the extremely halophilic archaeon *Halobacterium salinarum* possesses both halorhodopsin and a light-independent chloride transport system which probably acts by coupling the inward transport of chloride with the influx of Na^+ (Duschl and Wagner, 1986).

4. Biogeography of *Salinibacter*

Since the description of *S. ruber* species, we have been isolating different strains (now, up to 50) from crystallizer ponds around the world. A collection of 17 strains (including the five used for species description: M1, M8 and M31 from Mallorca, Pola13/P13 and Pola 18/P18 from Alicante) was used to take a look at the intraspecific diversity (Peña et al., in press). Several techniques were used for this study, including sequencing of 16S rDNA and the 16S-23S rRNA gene spacer regions, as well as genomic fingerprinting with randomly amplified polymorphic DNA (RAPD; Sikorski et al., 1999) and pulsed field gel electrophoresis analysis (PFGE; Grothues and Tummeler, 1991).

The 16S rRNA gene sequences of all new isolates were found to be identical to those of the already sequenced *S. ruber* strains. No representative of the second *Salinibacter* spp. phylotype EHB-2 (Antón et al., 2000) was obtained. The 16S-23S rRNA gene spacer regions were amplified for all strains and enzymatically digested with *TaqI*. In all cases, the digestion patterns were identical for all tested strains. Accordingly, their sequences were also very similar with values always higher than 97% sequence identity. With these similarities, no phylogenetic reconstruction could be performed to infer reliable genealogies within *S. ruber*. When the phylogeny of the gene spacer region of M31 was reconstructed with respect to a selection of the available homologous regions, we found that the closest relative sequence was that of *Rhodothermus marinus* in accordance with the 16S rRNA gene sequence reconstructions (Peña et al., in press).

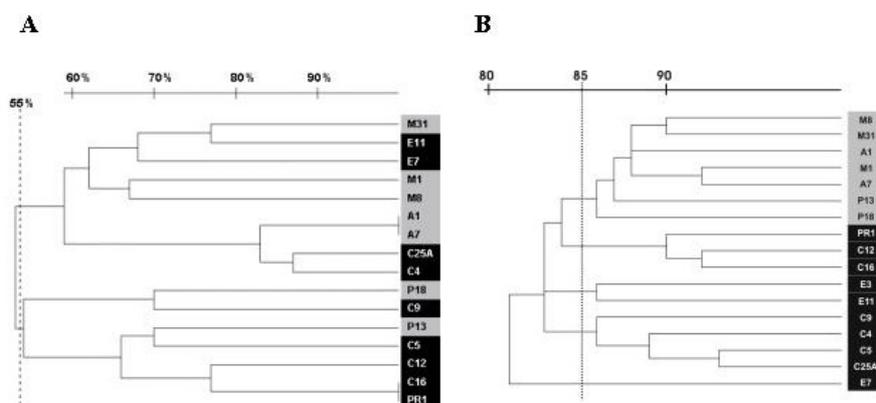


Figure 3. Dendrograms generated after UPGMA analyses of PFGE patterns (A) and RAPD profiles (B) from 17 *S. ruber* isolates. Source of isolation: S'Avall salterns, Mallorca, Spain (A1 and A7); Salinas de Levante salterns, Mallorca, Spain (M1, M8 and M31); Bras del Port salterns, Alicante, Spain (P13 and P18); Canary Islands salterns, Spain (C4, C5, C9, C12, C16, and C25); San Carles de la Rápita salterns, Ebro Delta, Spain (E7 and E11); Maras salterns, Peruvian Andes (Pr1). Black boxes indicate strains harbouring SalHO genes. From Peña et al., in press.

Genomic fingerprinting could however give a more detailed picture of intraspecific diversity. According to PFGE separation of genomic macrorestriction products, all the strains grouped in two clusters with more than 55% banding pattern similarity (Fig. 3).

Cluster 1 harboured all the isolates from the Mallorca and Ebro Delta salterns, whereas cluster 2 harboured all strains isolated from Alicante and the Peruvian Andes. Strains isolated from the Canary Islands appeared to be spread across both clusters. On the other hand, a dendrogram was constructed based on the analysis of 260 independent positions resulting from 8 different RADP analyses. One single independent cluster of 7 strains sharing at least 85% pattern similarity was found. All strains isolated from the Alicante and Mallorca salterns were grouped within the same cluster, whereas the rest of the strains were spread in 4 additional clusters. Interestingly, this unique cluster harboured all strains for which a copy of a *SalHO* gene was found. This result could indicate that the presence of a *SalHO* is related to the origin of isolation, although we have recently isolated some *S. ruber* strains from Canary Island that harbor this gene in their genomes.

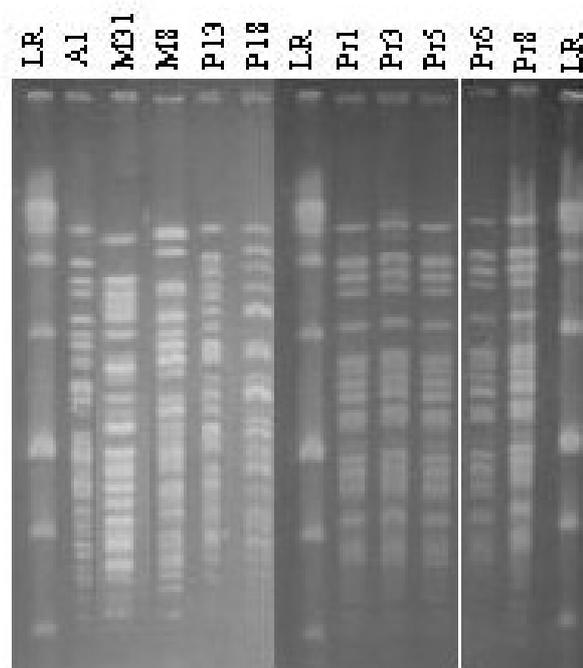


Figure 4. *XbaI* digestion of agarose embedded genomic DNA from different *S. ruber* strains. Source of isolation: S'Avall salterns, Mallorca, Spain (A1); "Salinas de Levante" salterns, Mallorca, Spain (M31 and M8); Bras del Port salterns, Alicante, Spain (P13 and P18); Maras salterns, Peruvian Andes (Pr1, Pr3, Pr4, Pr6, Pr8). LR: Biorad low-range marker for PFGE.

From the PFGE analysis of the different strains, it seems that *S. ruber* populations present different degrees of homogeneity depending on the location of isolation. This is particularly noticeable for strains isolated from a Peruvian saltern located at 3000 m of altitude in the Andes. In this environment, *S. ruber* can not be detected by FISH and

therefore must be present in very low numbers, although it can be readily isolated (Maturrano et al., in preparation). When genomic macrorestriction products of five Peruvian strains (isolated by different members of our group in different years) were compared by PFGE (Fig. 4), their patterns were found to be identical. This finding was in contrast with the diversity of PFGE patterns displayed by the otherwise very similar strains isolated from other salterns. There are thus many unanswered questions about how this species diversify in the environment and why very closely related, but not identical, strains thrive sharing the same habitat. Again, comparative genomics could help to understand how and why intraspecific diversity arises. For this purpose, we continue now to explore genomic differences of representatives of *S. ruber*, both uncultured and cultured. Hopefully, these studies will tell us more about the meaning of microdiversity in hypersaline environments.

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