

***Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the *Bacteria* from saltern crystallizer ponds**

Josefa Antón,¹ Aharon Oren,² Susana Benlloch,³ Francisco Rodríguez-Valera,³ Rudolf Amann⁴ and Ramón Rosselló-Mora⁵

Author for correspondence: Josefa Antón. Tel: +34 965903870. Fax: +34 965909569. e-mail: anton@ua.es

¹ División de Microbiología, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Apto 99, 03080 Alicante, Spain

² Division of Microbial and Molecular Ecology, The Institute of Life Sciences and The Moshe Shilo Minerva Center for Marine Biogeochemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

³ División de Microbiología, Universidad Miguel Hernández, Campus de San Juan, 03550 San Juan, Alicante, Spain

⁴ Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen, Germany

⁵ Laboratori de Microbiologia, Departament de Biologia, Facultat de Ciències, Universitat de les Illes Balears, 07071 Palma de Mallorca, Spain

Five brightly red-pigmented, motile, rod-shaped, extremely halophilic bacteria were isolated from saltern crystallizer ponds in Alicante (two strains) and Mallorca (three strains), Spain. They grew optimally at salt concentrations between 20 and 30% and did not grow below 15% salts. Thus, these isolates are among the most halophilic organisms known within the domain *Bacteria*. The temperature optimum was 37–47 °C. A single, yet to be identified pigment was present, with an absorption maximum at 482 nm and a shoulder at 506–510 nm. The G+C content of the DNA was 66.3–67.7 mol% and, together, they formed a homogeneous genomic group with DNA–DNA similarities above 70%. The 16S rRNA gene sequences were almost identical to sequences recovered earlier from the saltern biomass by amplification of bacterial small-subunit rRNA genes from DNA extracted from the environment. This phylotype, earlier described as '*Candidatus Salinibacter*', was shown by fluorescence *in situ* hybridization to contribute between 5 and 25% of the prokaryote community of the saltern crystallizers. We have therefore succeeded in isolating a bacterium from the natural environment that, although being a major component of the community, was previously known by its phylotype only. Isolation of the organism now allows formal description of a novel genus and species, for which we propose the name *Salinibacter ruber* gen. nov., sp. nov. The type strain is strain M31^T (= DSM 13855^T = CECT 5946^T).

Keywords: *Salinibacter*, extreme halophiles, salterns, *Rhodothermus*

INTRODUCTION

Until recently, it was assumed that representatives of the domain *Bacteria* do not play a major role in the microbial community of hypersaline brines at or approaching NaCl saturation. Few heterotrophic bacteria were known to grow at such high salt concentrations and those were outcompeted by halophilic members of the *Archaea* under the conditions prevailing in such environments (Oren, 1994; Rodríguez-Valera *et al.*, 1980).

Indications that saltern crystallizer ponds may harbour significant communities of bacteria that live together with the red archaea were obtained recently using molecular biological approaches. Amplification of small-subunit rRNA sequences from DNA collected from Spanish crystallizer ponds yielded a novel phylotype, clustering near the *Cytophaga–Flavobacterium–Bacteroides* group (Antón *et al.*, 2000). The rRNA sequences most closely related to this phylotype belong to the genus *Rhodothermus*, which consists of slightly halophilic, thermophilic bacteria isolated from marine hot springs (Alfredsson *et al.*, 1988; Sako *et al.*, 1996). Using fluorescent probes designed for the detection of the novel phylotype, it was shown that it belongs to rod-shaped bacteria that are abundant in the Spanish saltern ponds, contributing between 5 and 25% of the total prokaryotic community. Enrichment of the en-

Abbreviations: FISH, fluorescence *in situ* hybridization; SW, sea water.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains M1, M31^T, M8, Pola-18 and Pola-13 are respectively AF323499–AF323502.

environmental samples with low concentrations of yeast extract resulted in an increase in cell numbers harbouring this phylotype. In these experiments, the salt optimum for growth was found to be between 20 and 25%, i.e. in the same range as the most halophilic archaea of the *Halobacteriaceae*. The new type of extremely halophilic bacteria was described on the basis of these environmental studies as '*Candidatus Salinibacter*' (Antón *et al.*, 2000).

We have now isolated a number of extremely halophilic bacteria from saltern ponds in Mallorca and Santa Pola, Alicante, Spain, with 16S rRNA sequences almost identical to those of the '*Candidatus Salinibacter*' phylotype. They are all brightly red-coloured, rod-shaped bacteria. They are among the most halophilic bacteria known, requiring at least 15% salts for growth. The isolation in culture of organisms that correspond to the phylotype '*Candidatus Salinibacter*', their characterization and deposition in culture collections allows the description of *Salinibacter* gen. nov. A formal description of *Salinibacter* gen. nov. with the type species *Salinibacter ruber* sp. nov. is presented below.

METHODS

Source of strains. *Salinibacter* isolates were obtained from brine samples collected from crystallizer ponds of salterns located at Santa Pola (Alicante) and Mallorca. Information on the isolates and their source of isolation is summarized in Table 1.

Media, isolation and culture conditions. Samples or dilutions thereof were plated on different hypersaline agar media. Strain Pola-18 was isolated from an agar plate of R2A medium (Difco), supplemented with 25% crude solar salt, on which 0.1 ml of a 10^{-2} dilution of the sample in 80% autoclaved brine was spread, followed by incubation at 37 °C. Isolate Pola-13 was derived from a red colony that appeared on a plate of R2A agar supplemented with 20% NaCl, 2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.2% bentonite and inoculated with 0.1 ml of a 10^{-5} dilution of the same brine. The Mallorca isolates all came from agar plates [salt solution 25% sea water (SW) containing 1^{-1} : 195 g NaCl, 34.6 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 49.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.25 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 g KCl, 0.25 g NaHCO_3 , 0.625 g NaBr] with 0.1% yeast extract (referred to below as medium B) streaked directly with water from the crystallizer pond. After growth, cells from colonies were examined microscopically, rods were

selected and inoculated again on the same medium. Strains Pola-18 and Pola-13 were recognized as members of the *Bacteria* based on polar lipid analysis, while the Mallorca strains were identified as *Salinibacter* by fluorescence *in situ* hybridization (FISH) using the specific FISH probe EHB412 (Antón *et al.*, 2000).

The *Salinibacter* strains were routinely grown in liquid culture in medium of the following composition (referred to below as medium A) (1^{-1}): 200 g NaCl, 20 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g KCl, 0.3 g KH_2PO_4 , 0.5 g yeast extract, 0.5 g Bacto-peptone, 0.5 g Casamino acids, 0.5 g glucose, 0.5 g starch and 0.3 g sodium pyruvate. The pH was adjusted to 7.2 with NaOH prior to autoclaving and readjusted after autoclaving. Another suitable medium was medium B (see above). For solid media, agar was added at 20 g 1^{-1} .

Growth conditions. For each of the five strains, cultures were prepared in medium B with different salt concentrations (5, 10, 15, 20, 25, 30% SW and 30% SW plus NaCl up to saturation), pH values (5.5, 6, 7, 8 and 9), yeast extract concentrations (0.1, 0.2, 0.5 and 1%) and incubation temperatures (27, 32, 37, 42, 47, 52 and 57 °C). The influence of Mg^{2+} concentration on the growth rate was checked using modified medium B (1^{-1} : 195 g NaCl, 1.25 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 g KCl, 0.25 g NaHCO_3 , 0.625 g NaBr, 0.1% yeast extract) supplemented with increasing concentrations of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.05, 0.1, 0.2, 0.4 and 0.6 M). For pH-controlled experiments, different buffers [MES (pH 5–6), Tris (pH 7–9)] were added at a concentration of 20 mM. Cells were grown in 15 ml volumes in 50 ml tubes or in 50 ml volumes in 250 ml Erlenmeyer flasks with shaking (150–200 r.p.m.) at 37 °C, unless specified otherwise. Growth was monitored by measuring optical density at 600 nm throughout the growth curve until stationary phase was reached or for at least 14 days.

Microscopy. Cell morphology and motility were examined using a Zeiss standard microscope equipped with phase-contrast optics. For photography, drops of culture were mixed on a microscope slide with an equal volume of melted 2% (w/v) agar containing 20% (w/v) NaCl and covered with a cover slip. Gram stains were prepared as described by Dussault (1955). For scanning electron microscopy examination, 1 ml samples were fixed overnight at 4 °C by adding formaldehyde to a final concentration of 7%. Nine millilitres PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.2) was added to the samples, which were then filtered through 0.2 μm GTTP Millipore filters and washed with PBS. The filters were then serially dehydrated in 25, 50, 70 and 100% ethanol solutions (three times for 10 min at each stage), critical-point dried, mounted on scanning electron microscope stubs, sputter-coated with gold and viewed on a JEOL JSM 840 scanning electron microscope.

Table 1. The *Salinibacter* strains isolated, with details of the source of isolation and further identifying information

Strain	Source and date of isolation	G + C content (mol%)	16S rDNA accession no.	DNA–DNA hybridization (%) with:	
				M31 ^T	Pola-18
M1	Mallorca, September 1999	66.3	AF323499	87.7	71.0
M8	Mallorca, September 1999	67.0	AF323501	96.6	84.4
M31 ^T	Mallorca, September 1999	66.5	AF323500	100	85.0
Pola-13	Santa Pola, May 1999	66.5	AF323503	99.2	78.5
Pola-18	Santa Pola, May 1999	67.7	AF323502	80.5	100

Estimation of cell-associated potassium. *Salinibacter ruber* strains M31^T and Pola-18 were grown in medium B. *Halobacterium salinarum* R1 was grown in medium containing (l⁻¹) 250 g NaCl, 5 g KCl, 5 g MgCl₂ · 6H₂O, 5 g NH₄Cl and 10 g yeast extract, pH 7.0. Cells from the mid-exponential growth phase were collected by centrifugation (1 min, 10000 g) in 1.5 ml plastic centrifuge tubes. Cell pellets, containing between 0.23 and 0.68 mg protein, were washed once with a solution of 250 g NaCl l⁻¹, 20 g MgCl₂ · 6H₂O l⁻¹, pH 7.0. Pellets were then extracted with 0.2 ml 10% perchloric acid for 2 h and extracts were diluted with distilled water to obtain K⁺ concentrations between 0.2 and 0.5 mM. K⁺ concentrations were determined by flame photometry. Cellular protein was determined in identically prepared cell pellets using the Lowry method (Lowry *et al.*, 1951). All assays were performed with three or four replicates.

Biochemical tests. Most tests were performed as outlined by Holding & Collee (1971), in most cases using medium A. Appropriate positive and negative controls were included in all experiments. Nitrate reduction was tested in liquid media supplemented with 5 or 10 g NaNO₃ l⁻¹. The formation of nitrite was monitored colorimetrically and formation of gaseous products from nitrate was detected by the presence of gas bubbles in Durham tubes. Hydrolysis of starch was examined on agar plates supplemented with 2.5 g soluble starch l⁻¹. Starch hydrolysis was detected by flooding the plates with iodine solution. Gelatin hydrolysis was determined by growing colonies on agar plates amended with 0.4% gelatin and flooding the plates with a solution of 15% (w/v) HgCl₂ in 20% (w/v) HCl after growth was obtained (Gutiérrez & González, 1972). Hydrolysis of Tweens 20 and 80 was tested as outlined by Gutiérrez & González (1972); Tween was added at a concentration of 1 ml l⁻¹ to the autoclaved medium, in the case of medium A supplemented with 0.2 g CaCl₂ · 2H₂O l⁻¹. Indole production was detected with Kovacs' reagent after having grown the cells in medium supplemented with 0.1 g L-tryptophan l⁻¹.

To test for acid production from sugars, media were supplemented with 5 or 10 g of the sugars tested l⁻¹ and the pH of the cultures was measured periodically with a pH electrode. When medium A was used in these experiments, glucose was omitted.

The presence of catalase was tested by adding a 1% (v/v) H₂O₂ solution to colonies on plates. The presence of oxidase was determined with tetramethyl *p*-phenylenediamine hydrochloride (Holding & Collee, 1971) or with Oxoid BR64A indicator sticks.

Sensitivity to antibiotics was tested in 30-ml liquid cultures or in 2.5 ml volumes in 12 5-ml-well microtitre plates. The following antibiotics and antibacterial compounds were used, each at a concentration of 50 µg ml⁻¹: penicillin G, ampicillin, streptomycin, novobiocin, bacitracin, chloramphenicol, kanamycin, tetracycline, ciprofloxacin, rifampicin, colistin, anisomycin and aphidicolin (the last added from a solution in DMSO).

Pigments were extracted from cell pellets with methanol/acetone (1:1, v/v) and absorption spectra were recorded against the solvent in a Hewlett Packard model 8452A diode array spectrophotometer.

DNA base composition and DNA-DNA hybridization. The G+C content of the DNA was determined by HPLC and DNA-DNA hybridizations were performed by using a non-radioactive method as described by Ziemke *et al.* (1998).

Phylogenetic reconstruction. PCR-mediated amplification of the 16S rDNA and purification of the PCR products were carried out as described previously (Benlloch *et al.*, 1995, 1996). Template DNA was extracted from colonies of *Salinibacter* by using the Instagene matrix (Bio-Rad) according to the manufacturer's recommendations. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced using the ABI Prism Terminator cycle sequencing ready reaction kit and ABI Prism 310 automated sequencer (Perkin Elmer) according to the manufacturer's instructions. For sequencing both strands of the complete 16S rDNA, six primers were used: Ant 1 (Benlloch *et al.*, 1995), S (Benlloch *et al.*, 1995), EHB4F (5'-ACACCCCTATGGGGCGTA-3'), EHB4R, (5'-TACGCCCCATAGGGGTGT-3'), EHB 9F (5'-TACCTAGGCTCGAACGCT-3') and EHB (5'-AGCCTTGCTCGGTAGGTA-3'). Primers with the prefix EHB, specific for '*Candidatus Salinibacter*' sequences, were designed by comparing the EHB clones obtained from direct amplification of bacterial 16S rDNA from water from different solar salterns (Antón *et al.*, 2000). New sequences were added to an alignment of about 18000 homologous bacterial 16S rRNA primary structures (Maidak *et al.*, 2000; <http://www.mikro.biologie.tu-muenchen.de>) by using the aligning tool of the ARB program package (Ludwig & Strunk, 1998). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied as implemented in the ARB software. Phylogenetic trees were reconstructed by using subsets of data that included complete or almost complete sequences of representative members of the *Bacteria*. Topologies were evaluated by using the different approaches to elaborate a consensus tree (Ludwig *et al.*, 1998).

RESULTS AND DISCUSSION

Enrichment and isolation

In the course of this study, we have isolated five strains of halophilic red bacteria (Table 1), all very similar in their phenotypic properties and 16S rRNA gene sequences. They were selected from colonies that developed on agar plates inoculated with environmental samples on the basis of FISH with a specific probe (Antón *et al.*, 2000) or on the basis of the presence of bacterial rather than archaeal polar lipids. For the Mallorca sample, 70 colonies grown on solid

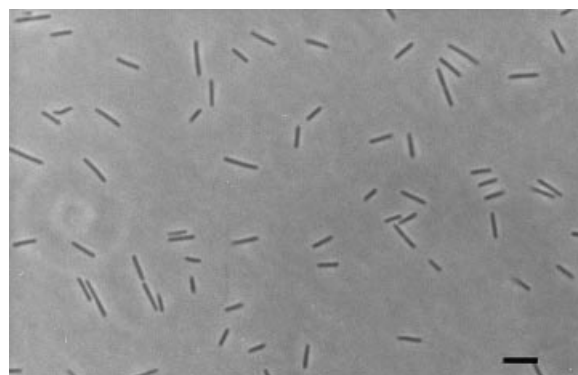


Fig. 1. Phase-contrast micrograph of *Salinibacter ruber* strain M31^T. Bar, 5 µm.

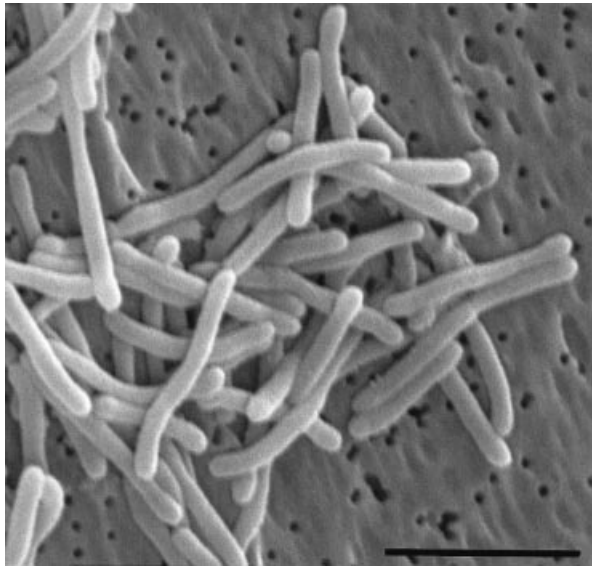


Fig. 2. Scanning electron micrograph of *Salinibacter ruber* strain M31^T. Bar, 2.5 μm .

medium were analysed and 23 gave positive FISH signals with the specific probe.

Morphology, fine structure and pigmentation

The bacteria were motile, straight or slightly curved rods, measuring $2\text{--}6 \times 0.4 \mu\text{m}$ (Figs 1 and 2). Flagella were observed under the electron microscope after negative staining with phosphotungstic acid (data not shown), but no definitive information could be obtained on the number or localization of these flagella. After fixation with acetic acid (Dussault, 1955), the cells stained Gram-negative. Colonies on agar were red, about 1 mm in diameter, circular and convex with an entire margin. Pigment extracts in methanol/acetone (1:1, v/v) showed an absorption maximum at 478 nm and a shoulder at 506–510 nm. Similar spectral data were reported for the (yet to be chemically characterized) pigment of *Rhodothermus marinus* (Alfredsson *et al.*, 1988). It remains to be determined whether this pigment is a carotenoid, possibly a flexirubin-like pigment common in the *Cytophagales* (Reichenbach, 1992), or may belong to a novel class of bacterial pigments.

It is noteworthy that bright-red pigmentation is common in micro-organisms inhabiting salt lakes and saltern ponds. Members of the *Halobacteriaceae* possess C-50 carotenoids of the bacterioruberin group. The role of this pigmentation in protecting against the harmful intensities of sunlight to which the cells are exposed in their natural environment was shown many years ago (Dundas & Larsen, 1962). Most *Dunaliella* sp. alga cells found in salterns are similarly pigmented red due to a high content of β -carotene. The nature of the red pigment of the novel bacterium is yet to be determined. The red colour of most members of the

Halobacteriaceae has been used in the past as an easily recognizable character to discriminate between archaeal and bacterial members of the prokaryote community (Rodríguez-Valera *et al.*, 1981). It is now becoming increasingly clear that colony colour itself is not a reliable trait to judge the phylogenetic affiliation of halophilic prokaryotes. Not only have colourless members of the *Halobacteriaceae* been isolated (e.g. *Natrialba asiatica*; Kamekura & Dyll-Smith, 1995), but the presently described extremely halophilic member of the *Bacteria* produces colonies as brightly red as those of the typical red halophilic members of the *Archaea*.

Growth and physiology

The *Salinibacter* strains were extremely halophilic. They grew optimally at 20–30% total salts. The strains isolated from Mallorca did not grow at 15% SW while the Santa Pola strains were somewhat less halophilic, since they grew at this salt concentration. All the strains could grow in solutions saturated with NaCl. Near-optimal growth rates were obtained over the whole range of Mg^{2+} concentrations from below 0.1 to 0.6 M for the Santa Pola strains, while strains isolated from Mallorca needed 0.2 M to grow optimally and did not grow at 0.05 M. High salt concentrations were not required for the maintenance of cell shape and cells did not lyse when suspended in distilled water. The optimal pH range for growth was 6.5–8.0. The optimum temperature for growth was 32–47 °C. At 27 °C, growth was very slow, and no growth was observed at temperatures above 52 °C. Doubling times for the five strains in medium B incubated at 37 °C ranged from 14 to 18 h, according to data obtained from three independent experiments for each strain.

The *Salinibacter* strains were chemo-organotrophic and strictly aerobic. Oxidase and catalase reactions were positive. Nitrate was not reduced to nitrite or to gaseous products. High nutrient levels did not increase growth rates; in fact, very long lag times (up to more than 10 d) were observed when the yeast extract concentration of medium B (see above) was increased to 5 or 10 g l^{-1} . Amino acids appeared to be the preferred nutrients for growth. Our attempts to design a defined medium were unsuccessful. Simple sugars and organic acids (acetate, succinate) did not support growth as sole carbon and energy sources. Addition of sugars and related compounds (glucose, glycerol, sucrose, ribose, fructose, xylose, lactose, mannitol, galactose, sorbitol, maltose) at concentrations of 5 g l^{-1} to medium A did not stimulate growth greatly and did not result in acid production. All isolates hydrolysed gelatin. Starch was hydrolysed by most strains. Tween 80 was not hydrolysed, but a very weak hydrolysing activity towards Tween 20 was sometimes observed. No indole was produced from L-tryptophan.

The isolates proved sensitive to penicillin G, ampicillin, chloramphenicol, streptomycin, novobiocin, rifampicin and ciprofloxacin. No inhibition by kanamycin,

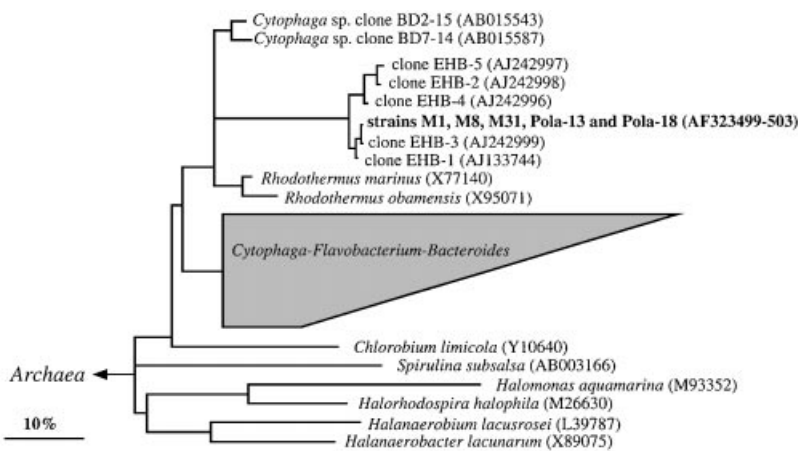


Fig. 3. Phylogenetic reconstruction based on 16S rDNA sequences from strains and clones affiliated to *Salinibacter*. The tree is based on the results of a parsimony analysis including only complete or almost-complete 16S rDNA sequences of all sequences available of the phylum *Cytophaga-Flavobacterium-Bacteroides* (over 400) and representatives of the domain *Bacteria* (Maidak *et al.*, 2000). The topology of the tree was corrected according to the results of distance-matrix, maximum-parsimony and maximum-likelihood analyses of various datasets as recommended by Ludwig *et al.* (1998). Multifurcations indicate where a topology could not be resolved unambiguously. Bar, 10% estimated sequence divergence. Accession numbers of individual sequences are shown on the tree.

bacitracin, tetracycline, colistin, anisomycin or aphidicolin was observed.

Cells of *Salinibacter* contained extremely high concentrations of K⁺ ions in their cytoplasm. In strain M31^T, the level of K⁺ was 11.4 ± 1.1 μmol K⁺ (mg protein)⁻¹ (n = 6, derived from two independent experiments). In strain Pola-18, it was 15.2 ± 0.6 μmol K⁺ (mg protein)⁻¹ (n = 3). These values are in the same range as those detected in *Halobacterium salinarum* R1 [12.0 ± 0.7 μmol K⁺ (mg protein)⁻¹ (n = 7), based on two independent experiments], an organism that uses KCl osmotically to balance the high NaCl concentration in its surrounding medium. It is thus suggested that *Salinibacter* uses a similar strategy of osmotic adaptation.

Phylogenetic reconstruction and genomic characteristics

Comparative analysis of the 16S rDNA sequences of the *Salinibacter* isolates resulted in a reconstructed phylogeny very similar to that observed previously when using 16S rDNA clones retrieved directly from the environment (Fig. 3). All sequences were affiliated with the two *Salinibacter* branches represented by EHB-1 and EHB-2 (Antón *et al.*, 2000). All the strains analysed in this work had 16S rDNA sequences that were nearly identical (lowest similarity of 99.6%) to sequence EHB-3, corresponding to a 16S rDNA clone retrieved from a solar saltern in Ibiza. Together with this EHB-3 sequence, these strains clustered with sequence EHB-1, which was shown by FISH (Antón *et al.*, 2000) to be the most abundant bacterial phylotype in crystallizer solar salterns in Santa Pola (Alicante, Spain). The strains showed sequence similarity values that ranged from 100 to 99.7%. Pola-13 and Pola-18 shared identical sequences.

The closest cultivated relative of our isolates is *Rhodothermus marinus*, a species of slightly halophilic (optimum 0.5–2% NaCl), thermophilic (optimum

65–70 °C, maximum around 77 °C) bacteria isolated from marine hot springs (Alfredsson *et al.*, 1988; Sako *et al.*, 1996; Silva *et al.*, 2000). The 16S rRNA sequence similarity between *Rhodothermus* and our isolates (about 89%) is low enough to warrant classification of a new genus and to formalize the description of *Salinibacter* gen. nov.

The five isolates formed a homogeneous genomic group and DNA–DNA similarities among the strains were above 71% (Table 1). The G + C content of the strains ranged between 66.3 and 67.7 mol% (Table 1). The pooled standard deviations of both experiments were 1.2% (similarity) and 1.8 mol% (G + C content).

Conclusion

We have been successful in isolating five strains that correspond to a previously uncultured bacterial community that has been shown by molecular techniques to be abundant in saltern crystallizer ponds. The 16S rRNA gene sequences of the isolates obtained were nearly identical to sequences recovered earlier from the saltern biomass. The rod-shaped morphology of the isolates was also very similar to that observed in the natural samples by using specific fluorescent oligonucleotides (Antón *et al.*, 2000).

When examining their physiology, there is a surprising similarity between the newly isolated extremely halophilic bacterium and the archaea of the family *Halobacteriaceae*. Both groups are aerobic heterotrophs, many members of the *Halobacteriaceae* also have complex growth requirements and both maintain high intracellular K⁺ concentrations. The isolates described here are among the most halophilic organisms known within the domain *Bacteria*; they require high salt concentrations, with optimum growth in the range 15–25% total salts. The five isolates exhibit a bright-red pigmentation that is also common in halophilic archaea and even the G + C content of their DNA is similar.

The relatively slow growth rate of *Salinibacter* together with the remarkable similarity of their colonies to those produced by halophilic archaea explains why these organisms have previously escaped isolation. It is even probable that bacteria of the type described here have been isolated many times in the past but, because of the similarity of their colony morphology, they have mistakenly been considered as members of the *Archaea*.

The isolation of organisms harbouring 16S rRNA sequences and morphology nearly identical to those of 'Candidatus *Salinibacter*' allows a formal description of the genus *Salinibacter* gen. nov. The five isolates studied are sufficiently similar to be classified in a single species and we propose the name *Salinibacter ruber* sp. nov. as the type species of the novel genus. The type strain is strain M31^T (= DSM 13855^T = CECT 5946^T), isolated from Mallorca.

Description of *Salinibacter* gen. nov.

Salinibacter (Sa.li.ni.bac'ter. L. fem. pl. n. *salinae* salterns, salt-works; N.L. masc. n. *bacter* masc. equivalent of the Gr. neut. n. *bakterion* a rod; N.L. masc. n. *Salinibacter* a rod from salt-works).

Rod-shaped or curved bacteria, phylogenetically loosely affiliated with the phylum *Cytophaga-Flavobacterium-Bacteroides*. Gram-negative. Aerobic, heterotrophic. Oxidase- and catalase-positive. Extremely halophilic, requiring at least 150 g salt l⁻¹ for growth. Habitat: salt lakes and saltern ponds. The G+C content of the type species is 66.5 mol%. The type species is *Salinibacter ruber*.

Description of *Salinibacter ruber* sp. nov.

Salinibacter ruber (ru'ber. L. adj. *ruber* red).

Cells are motile, straight or slightly curved rods, measuring 2–6 × 0.4 µm. Colonies on agar are red, about 1 mm in diameter, circular and convex with an entire margin. Pigment extracts in methanol/acetone (1:1, v/v) show an absorption maximum at 478 nm and a shoulder at 506–510 nm. Extremely halophilic, growing optimally at total salt concentrations of 150–300 g l⁻¹ and requiring at least 150 g salt l⁻¹ for growth. The optimal pH range for growth is 6.5–8.0 and no growth is observed below pH 6.0 or above pH 8.5. Temperature optimum 37–47 °C. No growth above 52 °C. Nitrate is not reduced. No acid produced from sugars. Starch and gelatin are hydrolysed. Tween 80 is not hydrolysed and Tween 20 is hydrolysed weakly or not at all. No indole is produced from L-tryptophan. Sensitive to penicillin G, ampicillin, chloramphenicol, streptomycin, novobiocin, rifampicin and ciprofloxacin. Insensitive to streptomycin, kanamycin, bacitracin, tetracycline, colistin, anisomycin and aphidicolin. The G+C content of the DNA is 66.5 mol% (HPLC).

The type strain is strain M31^T (= DSM 13855^T = CECT 5946^T), isolated from a saltern crystallizer pond in Mallorca, Balearic Islands, Spain.

ACKNOWLEDGEMENTS

We are grateful to Mr Miguel Cuervo for allowing access to the Santa Pola salterns and to Salinas de Levante S.A. for providing crystallizer samples. A.O. thanks Nona Dinevich for her help with the phenotypic characterization. J.A. thanks Arantxa Peña, Fernando Santos, Marina Torreblanca and Andrés Amorós for technical assistance. This study was supported by a European Commission grant MAS3-CT-97-0154, UMH.DCET.DM.B, MIDAS project. Further support was obtained from the Israeli Ministry of Science and Technology (MOST) and the Spanish Ministry of Foreign Affairs – the General Directorate of Cultural and Scientific Relations (to A.O. and F.R.V.), the Israel Science Foundation, founded by the Israel Academy of Sciences and Humanities (to A.O.), the interchange program Acciones Integradas HA1999-0013 CICYT-DAAD (to R.R.M. and R.A.) and the Spanish Ministry of Science and Technology grants BOS2000-1123C02-01 and BOS2000-1123C02-02 (to R.R.M. and J.A.).

REFERENCES

- Alfredsson, G. A., Kristjansson, J. K., Hjörleifsdóttir, S. & Stetter, K. O. (1988). *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *J Gen Microbiol* **134**, 299–306.
- Antón, J., Rosselló-Mora, R., Rodríguez-Valera, F. & Amann, R. (2000). Extremely halophilic bacteria in crystallizer ponds from solar salterns. *Appl Environ Microbiol* **66**, 3052–3057.
- Benlloch, S., Martínez-Murcia, A. J. & Rodríguez-Valera, F. (1995). Sequencing of bacterial and archaeal 16S rRNA genes directly amplified from a hypersaline environment. *Syst Appl Microbiol* **18**, 574–581.
- Benlloch, S., Acinas, S. G., Martínez-Murcia, A. J. & Rodríguez-Valera, F. (1996). Description of prokaryotic biodiversity along the salinity gradient of a multipond solar saltern by direct PCR amplification of 16S rDNA. *Hydrobiologia* **329**, 19–31.
- Dundas, I. D. & Larsen, H. (1962). The physiological role of the carotenoid pigments of *Halobacterium salinarum*. *Arch Mikrobiol* **44**, 233–239.
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Gutiérrez, C. & González, C. (1972). Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. *Appl Microbiol* **24**, 516–517.
- Holding, A. J. & Collee, J. G. (1971). Routine biochemical tests. *Methods Microbiol* **6A**, 1–32.
- Kamekura, M. & Dyal-Smith, M. L. (1995). Taxonomy of the family *Halobacteriaceae* and the description of two new genera *Halorubrobacterium* and *Natrialba*. *J Gen Appl Microbiol* **41**, 333–350.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Ludwig, W. & Strunk, O. (1998). ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich, Germany. <http://www.mikro.biologie.tu-muenchen.de>
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K.-H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.

- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 9 other authors (2000).** The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- Oren, A. (1994).** The ecology of the extremely halophilic archaea. *FEMS Microbiol Rev* **13**, 415–440.
- Reichenbach, H. (1992).** The order Cytophagales. In *The Prokaryotes: a Handbook on the Biology of Bacteria – Ecophysiology, Isolation, Identification, Applications*, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1980).** Behaviour of mixed populations of halophilic bacteria in continuous cultures. *Can J Microbiol* **26**, 1259–1263.
- Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1981).** Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentrations. *Microb Ecol* **7**, 235–243.
- Sako, Y., Takai, K., Ishida, Y., Uchida, A. & Katayama, Y. (1996).** *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *Int J Syst Bacteriol* **46**, 1099–1104.
- Silva, Z., Horta, C., da Costa, M. S., Chung, A. P. & Rainey, F. A. (2000).** Polyphasic evidence for the reclassification of *Rhodothermus obamensis* Sako *et al.* 1996 as a member of the species *Rhodothermus marinus* Alfredsson *et al.* 1988. *Int J Syst Evol Microbiol* **50**, 1457–1461.
- Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.