Salicola marasensis gen. nov., sp. nov., an extremely halophilic bacterium isolated from the Maras solar salterns in Peru

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Six strains of extremely halophilic bacteria were isolated from several crystallizer ponds of the Maras solar salterns in the Peruvian Andes. On the basis of 16S rRNA gene sequence similarity, G + C contents and DNA–DNA hybridization results, the six isolates constituted a genomically homogeneous group affiliated with the *Gammaproteobacteria*. The closest relatives were members of the halophilic genera *Halovibrio* and *Halospina*, which showed 16S rRNA gene sequence similarities below 97 % and whole-genome hybridization levels below 33 % for the type strain, 7Sm5^T. From the genomic and phenotypic properties of the six novel isolates and phylogenetic reconstruction based on 16S rRNA gene sequence analysis, they can be considered to represent a novel genus within the *Gammaproteobacteria*. On the basis of the taxonomic study, a novel genus, *Salicola* gen. nov., is proposed containing the single species *Salicola marasensis* sp. nov., which is the type species. The type strain of *Salicola marasensis* is 7Sm5^T (= CECT 7107^T = CIP 108835^T).

Until the description of the genus *Salinibacter*, only haloarchaea were considered to be of ecological relevance in solar salterns (Antón *et al.*, 2000). The classification of *Salinibacter ruber* as a novel species within the phylum *Bacteroidetes* (Antón *et al.*, 2002) extended the short list of extremely halophilic members of the *Bacteria*. There are not many characterized members of this domain able to grow under such extreme conditions [only some members of the family *Ectothiorhodospiraceae* of the *Proteobacteria*, some members of the *Firmicutes* (*Halanaerobiaceae* and *Bacillaceae*) and some members of the *Actinobacteria*], but, in all, no more than 11 genera are represented (Oren, 2002). However, only species of the genus *Salinibacter* have been reported to thrive abundantly in crystallizer ponds of solar salterns (Antón *et al.*, 2002).

Hypersaline environments can be classified as thalassohaline or athalassohaline: thalassohaline water is concentrated seawater with NaCl as the major salt, while athalassohaline water is saline water that is rich in anions other than chloride

and/or cations other than sodium (Oren, 2002). The Maras solar salterns are located at 3380 m above sea level in the heart of the Peruvian Andes (Cusco Region), and they have been exploited for more than 500 years (Basadre, 1969). Brines are produced from the dissolution of the surrounding rocks of the Maras formation, and their composition can be considered as thalassohaline (Maturrano *et al.*, 2006). These salterns have been studied using culture-independent molecular methods to reveal the structure of the indigenous microbial community. Additionally, enrichment cultures of heterotrophic halophilic micro-organisms have been performed in order to obtain more information about the heterotrophic culturable fraction of the microbial population (Maturrano *et al.*, 2006).

During the enrichment and isolation procedure of several Maras brine samples, six Gram-negative, heterotrophic strains were isolated. Here, we report the taxonomic characterization of these six isolates, which could be considered as relevant representatives of the bacterial fraction of the natural microbial community thriving in these environments (Maturrano *et al.*, 2006). Together, the results showed that the six strains formed a monophyletic and genomically and phenotypically coherent group. Their closest relatives are the members of the genera *Halovibrio* and *Halospina* (Sorokin *et al.*, 2006) and strain DSM 3050, which was described as the type strain of *Pseudomonas*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 7Sm5^T, 2Sb7, 7Sa10, 7Sm7, 7Mb1 and 5Ma3 are DQ019934, DQ019935 and DQ087259–DQ087262, respectively.

Details of the reference sequences used in the construction of Fig. 1 are available as supplementary material in IJSEM Online.

halophila (Fendrich, 1988). The position of strain DSM 3050 is currently being re-evaluated because this strain may answer the description of *Halovibrio variabilis* (Sorokin & Tindall, 2006) rather than its original description (Fendrich, 1988). On the basis of the results of our taxonomic study and previous characterizations of the most closely related genera, we consider that the six Peruvian strains should be included within a novel genus and species within the *Gammaproteobacteria*.

For the isolation of halophilic prokaryotes, two different media were used: SW medium (containing the following, in g l⁻¹: NaBr, 0.65; NaHCO₃, 0.167; KCl, 5.0; CaCl₂, 0.723; MgSO₄·7H₂O, 49.492; MgCl₂·6H₂O, 34.567; NaCl, 195.0; pH 7.0); and MS medium, designed for this work (containing the following, in g l⁻¹: NaBr, 0.128; NaHCO₃, 0.14; KCl, 0.95; CaCl₂, 3.32; NaCl, 234.0; pH 7.2). In both cases, the medium was supplemented with different concentrations of yeast extract (0.1, 0.2 and 0.5 %) and incubated at 37 °C for at least 4 weeks. Solid media were prepared by adding agar to a final concentration of 1.5 % prior to sterilization. Brines were plated directly onto the different media without prior enrichment.

To determine pH, salinity and temperature growth ranges, SW medium with 0.2 % yeast extract was used. Isolates were grown, in triplicate, with increasing levels of NaCl (0, 5, 10, 15, 20, 25, 30 % and up to saturation), at increasing pH (pH 5.0, 6.0, 6.5, 7.0, 8.0 and 9.0) and at increasing temperatures (15, 20, 25, 30, 35, 40, 45 and 50 °C). Temperature and pH tests were performed in SW medium containing 15 % salt (15 % SW) and 0.2 % yeast extract. In all cases, growth was assessed by observing increases in optical density at 600 nm up to the stationary phase.

Cell biomass for enzyme testing and macromolecule extraction was also obtained after growth on SW medium containing 1.5 % salt and 0.2 % yeast extract. Gram staining was performed using a protocol adapted for halophilic organisms (Dussault, 1955). Catalase activity was determined by adding 10 % (v/v) H₂O₂ and observing its hydrolysis and the consequent gas formation (Bradshaw, 1992). Oxidase activity was determined with Oxoid BR644 indicator sticks. Denitrification was analysed by growing the strains in SW medium containing 15 % salt and 0.2 % yeast extract and supplemented with 0.5 % NaNO₃. Gas formation was observed from its accumulation in Durham tubes. Nitrite formation, gelatinase, amylase, casein hydrolysis and the presence of poly-β-hydroxybutyrate were assayed according to the protocols of Smibert & Krieg (1981). Enzyme assays for arginine dihydrolase, lysine decarboxylase, urease and ornithine decarboxylase were performed by following the protocols of Baumann & Baumann (1981). Voges-Proskauer and methyl red assays were performed by modifying the medium by using a salt concentration of 15 % (Smibert & Krieg, 1981). Indole production was assayed in 15 % SW supplemented with tryptophan and by adding Kovács' indole reagent (Holding & Colle, 1971). Motility and H₂S production were assayed

on Oxoid SIM medium. Glucose fermentation, gas production, aesculin hydrolysis and β-galactosidase were determined using the API 20NE system (bioMérieux). Carbohydrate utilization was analysed in triplicate by means of growth in 15 % SW supplemented with each of the carbohydrates at different concentrations (0.5, 1.0 and 5.0 g l⁻¹), as described by Fendrich (1988). Cellular fatty acid profiles were estimated by GC performed at the Identification Service Laboratories of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were grown in SW medium containing 15 % salt and with 0.2 % yeast extract as the sole carbon and energy source. Cell morphology, size and flagellation were determined by observation by electron microscopy after negative staining of cells with 1 % phosphotungstic acid (Rosselló-Mora *et al.*, 1993).

Complete 16S rRNA gene sequences were compared initially with reference sequences of the nr (formerly 'non-redundant') database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using BLAST (Altschul *et al.*, 1997). New 16S rRNA gene sequences were then added to an alignment of about 50 000 homologous bacterial 16S rRNA gene primary structures as implemented by the ARB software package, and corresponding to the released database available at <http://www.arb-home.de> (Ludwig *et al.*, 2004). Phylogenetic analyses were performed by the use of different datasets and by the use of the three different algorithms (neighbour joining, maximum parsimony and maximum likelihood) as implemented by the ARB software package. Finally, a consensus tree was constructed after the evaluation of the trees resulting from the use of different algorithms and datasets, as previously recommended (Ludwig *et al.*, 1998).

Genomic DNA was prepared according to the method of Marmur (1961). The G + C content was analysed by hydrolysis of DNA to its nucleosides, and was quantified by HPLC according to the method reported by Peña *et al.* (2005). DNA-DNA hybridization experiments were carried out by using a non-radioactive, microtitre plate method (Ziemke *et al.*, 1998).

Samples from three different crystallizer ponds were inoculated onto two different media (SW and MS) with increasing yeast extract concentrations (0.1, 0.2 and 0.5 %). Of all the colonies that grew on these plates, six pure cultures were selected for taxonomic characterization because of their phylogenetic coherence and their relevance to the environment in which they thrived. The phenotypic properties shared by the six isolates and details of the isolation media are listed in Table 1. In all cases, colonies were non-pigmented (slightly beige) and mucoid with smooth edges when grown on 15 % SW with 0.2 % yeast extract. The cells of all isolates were rods about 1 µm long and 0.5 µm wide and were motile by means of single polar flagella. All of the isolates were extremely halophilic: no growth was observed in culture media with NaCl concentrations below 10 %. Strains were able to grow at NaCl concentrations up to 30 %, on Oxoid SIM medium.

Table 1. Differential phenotypic characteristics among the six novel Peruvian strains (*Salicola* gen. nov.)

All strains showed a strict dissimilatory metabolism with oxygen as the sole electron acceptor, whereas no fermentation or nitrate respiratory pathway was detected. All were Gram-negative and motile by means of single polar flagella. All strains were positive for cytochrome *c* oxidase, catalase, urease, gelatin liquefaction, phenylalanine deaminase, β -galactosidase, arginine dihydrolase and lysine decarboxylase. All were positive for the use of the following substrates as sole carbon and energy sources: fructose, galactose, glucose, arabinose, cellobiose, maltose, mannose, sucrose, trehalose, xylose, acetate, citrate, pyruvate, succinate, propionate, aesculin, ethanol, glycerol, caproate, glutamine, lactose, mannitol and sorbitol. All strains were negative for starch hydrolysis, indole production, sulphide production and ornithine decarboxylase and for growth with salicin, lactate, butyrate, caprylate, formate, malate, pelargonate and valerate as sole carbon sources.

Characteristic	7Sm5 ^T	7Sa10	5Ma3	2Sb7	7Mb1	7Sm7
pH/salinity (% total salts) of crystallizer	7.0/30	7.0/30	7.0/31	6.5/30	7.0/31	7.0/31
Isolation medium*	SW + 0.2 % YE	SW + 0.5 % YE	MS + 0.5 % YE	SW + 0.1 % YE	MS + 0.1 % YE	SW + 0.2 % YE
Aesculin hydrolysis	+	+	+	+	–	+
Casein hydrolysis	+	+	–	+	–	+
Growth with the following as sole carbon source						
Alanine (0.5 g l ⁻¹)	+	+	+	+	+	–
Glycine	+	+	+	–	–	–
Benzoate	+	+	+	–	–	–
DNA G+C content (mol%)	61.6	61.6	61.3	61.9	61.5	61.7
Hybridization with DNA from strain 7Sm5 ^T	100	100	99.7	78.2	64.6	76.1

*YE, Yeast extract.

with an optimum at 15 % (Table 2). The optimal temperature for growth was about 35 °C and the optimal pH was 7.

16S rRNA gene sequence phylogenetic analysis (Fig. 1) showed this set of strains to be a monophyletic group that shared internal sequence similarity values of more than 99 %. The most closely related cultured strains known were *Halospina denitrificans* HGD 1-3^T (DQ072719), *Halovibrio denitrificans* HGD 3^T (DQ072718) and [*P. halophila*] DSM 3050^T (AB021383), with rRNA gene similarity values ranging from 95.5 to 96.9 %. All of them formed a stable cluster of sequences representing halophilic isolates. The whole group of Maras sequences can be regarded as an independent branch within this clade of halophilic species (Fig. 1), since all treeing approaches showed the sequences as a single independent branch. We could not resolve the branching order of the neighbouring genera with respect to the clade of halophiles. However, and in contrast to the tree reported by Sorokin *et al.* (2006), we could not confirm *Marinobacter* as the closest relative. In our case, all of the related genera showed incongruent branching patterns that varied depending on the treeing approach used or the dataset used. The branching orders that could not be resolved were drawn as multifurcations, as recommended by Ludwig *et al.* (1998). However, all of the bifurcations shown in the tree appeared to be stable independently of the algorithm, dataset or mask used in the analysis.

The genomic traits represented by G+C content and DNA–DNA reassociation indicated that the six novel isolates also constituted a homogeneous genomic group (Table 1). The strains had nearly identical G+C contents in the range 61.3–61.7 mol%, and the DNA of each of them reassociated with strain 7Sm5^T at values above 64.6 %. The three most closely related strains, DSM 3050^T, HGD 1-3^T and HGD 3^T, which represented the phylogenetically closest genera, gave hybridization results of 33.1, 31.6 and 25.7 %, respectively. The pooled standard deviation of the experiments was 1 %.

The six extremely halophilic strains from Maras shared a homogeneous phenotype but showed some intraspecific diversity (Table 1). All members of this phylogenetic branch showed positive reactions for cytochrome *c* oxidase, catalase, urease, gelatinase, β -galactosidase and phenylalanine decarboxylase, as well as for a large number of compounds that could serve as primary substrates for growth (Table 1). Additionally, the members of this phylogenetic branch harbouring extreme halophiles could be differentiated from the most closely related genera by means of traits such as type of motility, ability to hydrolyse gelatin and urea, inability to respire nitrate and hydrolyse starch and by the ability to use carbohydrates (Table 2). Intraspecifically, the group of extremely halophilic, Peruvian strains differed in traits such as aesculin and casein hydrolysis and by the use of alanine, glycine and benzoate.

Table 2. Differential phenotypic characteristics of the novel Peruvian isolates (*Salicola* gen. nov.) and members of closely related genera

Data for reference taxa were taken from the following studies: *Halovibrio* [Fendrich, 1988 (isolate III); Sorokin *et al.*, 2006], *Halospina* (Sorokin *et al.*, 2006), *Halomonas* (Vreeland, 2005), *Microbulbifer* (González, 2005), *Marinobacter* (Bowman & McMeekin, 2005) and *Alcanivorax* (Golyshin *et al.*, 2005). ±, Variable; NR, not reported.

Characteristic	<i>Salicola</i>	<i>Halovibrio</i>	<i>Halospina</i>	<i>Halomonas</i>	<i>Microbulbifer</i>	<i>Marinobacter</i>	<i>Alcanivorax</i>
Cell morphology	Rod	Vibrioid or short spirillum	Rod	Rod	Rod	Rod	Rod
Flagella	Polar	Polar	Non-motile	Peritrichous, polar or none	None	None or polar	None or polar
Optimal temperature for growth (°C)	35	33	NR	30	37	30	20–30
Salinity range for growth (%)	10–30	7–27	11.5–29	3–32	0.5–5.8	3–20	1–12.5
Optimal salinity for growth (%)	15	9	14.5–17.5	3–8	0.5–3	3–6	3–5
Denitrification	–	–	+	±	–	+	–
NO ₂ [–] production from NO ₃ [–]	–	+	+	±	–	+	+
Arginine dihydrolase	+	–	NR	–	NR	–	–
Ornithine decarboxylase	–	–	NR	+	NR	–	–
Lysine decarboxylase	+	–	NR	+	NR	–	–
Gelatin liquefaction	+	–	NR	±	+	–	–
Starch hydrolysis	–	NR	NR	–	+	–	–
Urease	+	+	NR	±	NR	–	–
Carbohydrate utilization	+	–	–	+	+	–	–
DNA G+C content (mol%)	61.3–61.9	61–61.8	60	50–61	57.6	53	53–66

Finally, all of the fatty acid profiles of the Peruvian isolates were very similar (Table 3), having C_{12:0} 3-OH, C_{16:0}, C_{18:1}ω9*c* and C_{18:0} 10-methyl as the major components.

The similar features are in accordance with previously reported profiles for closely related strains (Sorokin *et al.*, 2006). However, the Peruvian isolates can be differentiated

Fig. 1. Phylogenetic reconstruction based on a neighbour-joining calculation. The tree was modified after multiple analysis of different datasets and by the use of maximum-parsimony, neighbour-joining and maximum-likelihood algorithms. To reconstruct the phylogeny and resolve branching patterns, the different datasets were analysed by using all homologous positions in the alignment, by using consensus masks that removed all the non-conserved indels of the domain *Bacteria* or by using maximum-frequency masks that removed highly variable positions within the *Gammaproteobacteria*, as implemented by the ARB datafile. The resulting tree shows a consensus topology where multifurcations indicate those branching orders that could not be resolved unambiguously. In this tree, representative members of the genera *Halomonas*, *Carnimonas*, *Zymobacter*, *Alcanivorax*, *Marinobacter*, *Zooshikella*, *Hahella* and *Microbulbifer* appear hidden within their respective phylogenetic clades. Details of the sequences used are given in Supplementary Table S1 in IJSEM Online. Bar, 5% sequence divergence.

Table 3. Fatty acid profiles of the novel Peruvian strains (*Salicola* gen. nov.) and some closely related strains

Data were obtained in this study or were taken from Sorokin *et al.* (2006) (for *Halovibrio denitrificans* HGD 3^T and *Halospina denitrificans* HGD 1-3^T). Values are percentages of total fatty acids; –, not detected.

Fatty acid	7Sm5 ^T	7Sa10	5Ma3	2Sb7	7Mb1	7Sm7	[<i>P. halophila</i>] DSM 3050 ^T	<i>Halovibrio</i> <i>denitrificans</i> HGD 3 ^T	<i>Halospina</i> <i>denitrificans</i> HGD 1-3 ^T
C _{10:0}	3.26	2.99	3.88	4.46	4.19	4.27	7.44	1.92	1.62
C _{12:0}	1.53	1.26	1.18	1.96	1.87	1.84	1.41	–	1.17
C _{12:0} 3-OH	5.77	5.21	7.46	8.29	7.63	7.37	8.95	3.35	3.36
C _{14:0}	–	–	–	–	–	0.38	–	–	–
C _{14:1}	–	–	–	–	–	–	–	0.84	1.23
C _{14:1} 3-OH	–	–	–	–	–	–	–	–	1.06
C _{16:0}	15.81	15.92	16.69	14.68	14.79	15.30	10.06	15.40	7.98
C _{16:1} *	–	–	–	–	–	–	–	–	1.47
C _{16:1} ω9 <i>c</i>	2.26	2.25	2.20	2.66	2.63	2.47	3.32	3.20	–
C _{16:1} ω7 <i>c</i>	–	–	–	–	–	–	–	5.22	–
C _{16:1} ω5 <i>c</i>	3.14	3.06	4.96	2.33	2.38	1.52	0.27	–	–
C _{17:0} iso	–	–	–	–	–	0.34	–	–	–
C _{17:0}	–	–	–	–	–	0.51	–	–	–
C _{18:1} ω9 <i>c</i>	33.41	36.08	33.02	31.68	27.92	27.47	52.74	46.56	65.65
C _{18:1} ω7 <i>c</i>	–	–	–	–	–	–	0.44	10.18	–
C _{18:1} ω5 <i>c</i>	–	–	–	–	–	–	0.19	–	–
C _{18:0}	8.50	8.83	8.21	8.82	8.86	8.46	3.13	3.32	5.45
C _{18:0} 10-methyl	21.55	19.91	18.0	19.25	23.30	24.15	6.49	2.01	–
C _{18:0} 2-OH	–	–	–	–	–	–	0.34	–	–

*The exact location of the double bond was not determined.

from representatives of the most closely related genera (DSM 3050^T, HGD 1-3^T and HGD 3^T) by their relatively high C_{18:0} 10-methyl content.

The six extremely halophilic isolates from Maras were shown to be a monophyletic set of individuals with enough phenotypic and genetic coherence to be considered as an independent taxon with respect to their closest relatives (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). The strains analysed were isolated from different crystallizer ponds, which indicates both that they might correspond to different populations and that they may represent an important fraction of the prokaryotes inhabiting Maras' brines. Indeed, this hypothesis was corroborated by culture-independent techniques, since members of this group were found, by fluorescence *in situ* hybridization analysis, to be the most abundant members of the *Bacteria* in the brines (Maturrano *et al.*, 2006). Although 16S rRNA gene sequence analysis of the novel strains revealed a high degree of sequence similarity, the strains differed from each other in terms of genome content, as was demonstrated by the different DNA–DNA hybridization values obtained. Phylogenetically related strains have been isolated recently in similar environments, but they did not constitute an important fraction in relation to the total microbiota cultured at the same time (Caton *et al.*, 2004). Additionally, it seems that relatives of [*P. halophila*] could enhance growth of Walsby's square archaeon when co-cultured under

laboratory conditions (Bolhuis *et al.*, 2004). All of these data are indications that members of this phylogenetic branch may be relevant in their own environments.

Together, the data indicate that the six extremely halophilic isolates from Maras can be regarded as representing a single species independent of *Halovibrio* and *Halospina* species (Fendrich, 1988; Sorokin *et al.*, 2006). In view of the way in which novel genera have been classified, our observations lead us to include the novel species within a novel genus, *Salicola* gen. nov. We propose the binomial *Salicola marasensis* sp. nov. for the novel group of extremely halophilic organisms isolated from crystallizer ponds of the Maras solar salterns in the Peruvian Andes, as they may be the most abundant and representative bacterial heterotrophs in this Andean environment.

Description of *Salicola* gen. nov.

Salicola (Sa.li'co.la. L. n. *sal*, *salis* salt, salt water, brine; L. masc. suff. *-cola* from L. n. *incola* an inhabitant; N.L. masc. n. *Salicola* an inhabitant of brine).

Rod-shaped bacteria, motile by means of single polar flagella. Phylogenetically affiliated to the *Gammaproteobacteria*. Gram-negative, strictly aerobic, heterotrophic, oxidase-, catalase-, urease-, phenylalanine decarboxylase-, β-galactosidase- and gelatinase-positive and able to use carbohydrates. Inhabitants of highly saline habitats such as salt lakes,

saltern ponds and salt flats; in general, optimum growth occurs at salt concentrations above 14 % (w/v). Major fatty acids of the genus are C_{12:0} 3-OH, C_{16:0}, C_{18:1}ω9c and C_{18:0} 10-methyl. The latter fatty acid distinguishes *Salicola* species from the most closely related genera. The DNA G+C content of members of the genus ranges between 61.3 and 61.9 mol%. The type species of the genus is *Salicola marasensis*.

Description of *Salicola marasensis* sp. nov.

Salicola marasensis (ma.ra.sen'sis N.L. masc. adj. *marasensis* pertaining to Maras, a region of the Peruvian Andes, where the first strains were isolated).

Cells are straight rods, motile by means of single polar flagella. Gram-negative. Extremely halophilic with an optimum NaCl requirement (in medium) of 15 %, though growth has been reported at NaCl concentrations up to 30 %. No growth is observed below 10 % NaCl. The temperature optimum for growth is 35 °C. Strictly aerobic with a chemo-organotrophic metabolism; able to use carbohydrates as sole carbon and energy sources. Members of this species can be distinguished from other related species by their extreme halophily, by their temperature optimum and by their arginine dihydrolase and lysine decarboxylase activities. pH for growth ranges from 6.0 to 8.0, with an optimal pH of 7.0. Among the major fatty acids observed for the genus, C_{16:0} and C_{18:0} 10-methyl predominate over C_{12:0} 3-OH and C_{18:1}ω9c. The DNA G+C content is 61.3–61.9 mol%.

The type strain, strain 7Sm5^T (=CECT 7107^T=CIP 108835^T), was isolated from one of the saltern crystallizer ponds at Maras in the Peruvian Andes, and was one of the most representative inhabitants of these ponds.

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