Salinithrix halophila gen. nov., sp. nov., a halophilic bacterium in the family Thermoactinomycetaceae

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A halophilic actinomycete, strain R4S8T, was isolated from soil of Inche-Broun hypersaline wetland in the north of Iran. The isolate grew aerobically at temperatures of 30–50 °C (optimum 40 °C), pH 6–10 (optimum pH 7.0) and in the presence of 1–15 % (w/v) NaCl (optimum 3–5 %). It formed short and straight to moderately flexuous aerial mycelium without motile elements. The cell wall of strain R4S8T contained meso-diaminopimelic acid as the diamino acid without any diagnostic sugars. The polar lipid pattern consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylmonomethylethanolamine, phosphatidylserine, phosphatidylmonomethylethanolamine two unknown phospholipids and one unknown aminophospholipid. It synthesized anteiso-C₁₅:₀ (44.8 %), iso-C₁₅:₀ (28.8 %) and iso-C₁₄:₀ (8.5 %) as major fatty acids. MK-6 was the predominant respiratory quinone. The G+C content of the genomic DNA was 52.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain R4S8T belongs to the family Thermoactinomycetaceae and showed the closest 16S rRNA gene sequence similarity with Desmospora activa IMMIB L-1269T (95.5 %) and Marininema mesophilum SCB 10219T (95.3 %). On the basis of phylogenetic analysis and phenotypic characteristics, strain R4S8T represents a novel species in a new genus within the family Thermoactinomycetaceae, for which the name Salinithrix halophila gen. nov., sp. nov. is proposed. The type strain of the type species is R4S8T (=IBRC-M 10813T=CECT 8506T).

Members of the family Thermoactinomycetaceae are characterized by the formation of single, sessile spores like bacterial endospores on the aerial and substrate hyphae, or chains of spores on simple or branched sporophores (Tsilinsky, 1899; Lacey & Cross, 1989; Yoon et al., 2005). Except some genera such as Seinonella, Mechercharinycyes, Marininema and Shimazuella, which have mesophilic growth below 45 °C, growth in a thermophilic range is a main feature of the family Thermoactinomycetaceae (Hatayama et al., 2005).

Strain R4S8T was isolated from soil during studies focused on the determination of the biodiversity of the Incheh Broun hypersaline wetland. This wetland is a natural athalassohaline ecosystem, located below the Caspian Sea near the border with Turkmenistan (37° 13′–14′ 57″ N 54° 50′–51′ 57″ E) where an arid–semiarid continental climate dominates. It has an altitude of about 5 m above sea level and an area of about 1000 km². At the time of sampling, the lake possessed a temperature range of 34–37 °C (annual temperature ranges from −2 °C to 40 °C), a salinity of 16–29 % total salts, a pH of 5.5–7.0 and high

Abbreviations: DAP, diaminopimelic acid; ISP, International Streptomyces Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R4S8T is KJ206288.

Two supplementary figures are available with the online Supplementary Material.

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concentrations of Cl\(^{-}\) as major anion and Na\(^{+}\) as major cation, followed by SO\(_4\)\(^{2-}\), and Ca\(^{2+}\) and Mg\(^{2+}\), respectively.

The strain was isolated by diluting the soil sample in sterile 3 % (w/v) NaCl solution, plating on Seawater Nutrient Agar (SNA) consisting of NaCl (2 %, w/v), MgSO\(_4\) \(\cdot\) H\(_2\)O (0.5 %), CaCl\(_2\), 2H\(_2\)O (0.05 %), KCl (0.05 %), Bacto-peptone (0.5 %), yeast extract (0.1 %), meat extract (0.2 %) and agar (1.5 %) and incubating at 35 °C aerobically for 3 weeks. The purified strain was maintained on yeast extract-malt extract agar [International Streptomyces Project (ISP) 2] medium at 4 °C and as glycerol suspensions (20 %, v/v) at −20 °C and −80 °C. Desmospora activa DSM 45169\(^{T}\) and Marinimema mesophilum DSM 45610\(^{T}\) were used as reference strains for comparison in our study and were cultivated under the same growth conditions as strain R4S8\(^{T}\).

Macroscopic morphology and cultural characteristics were examined on media recommended by Shirling & Gottlieb (1966) and Waksman (1961). All media were supplemented with 2 % (w/v) NaCl. The colour of aerial mycelium (spore mass) and substrate mycelium was determined on ISP 2, oatmeal agar (ISP 3) and SNA at 40 °C (spore mass) and substrate mycelium was determined on ISP 6 agar slant medium at 40 °C. Desmospora activa DSM 45169\(^{T}\) and Marinimema mesophilum DSM 45610\(^{T}\) were used as reference strains for comparison in our study and were cultivated under the same growth conditions as strain R4S8\(^{T}\).

The growth temperature range was examined on ISP 2 agar at 10–50 °C (at intervals of 5.0 °C). The pH range for growth was assessed in ISP 2 broth at pH 5.0–10.5 (at 0.5 pH unit intervals) using 50 mM MES (for pH 4–6.5), HEPES (pH 6.5–8) or CHES (pH 8.5–10.5) buffers. Tolerance to NaCl was evaluated on ISP 5 and SNA media containing NaCl concentrations of 1–15 % (w/v), with optimum growth occurring in the presence of 3–5 % (w/v) NaCl. Sodium citrate, D-glucose, D-mannitol, dulcitol and D-ribose were assimilated as sole carbon sources. L-Arginine and L-glycine were consumed as carbon and nitrogen sources. Acid was produced from D-glucose. The strain could not reduce nitrate to nitrite. Casein and urea were hydrolysed but starch was not. Moreover, this strain was not capable of producing H\(_2\)S. The strain was susceptible to neomycin (30 \(\mu\)g), streptomycin (300 \(\mu\)g), tetracycline (30 \(\mu\)g) rifampicin (5 \(\mu\)g), amikacin (30 \(\mu\)g), gentamicin (10 \(\mu\)g), erythromycin (15 \(\mu\)g), chloramphenicol (30 \(\mu\)g) and cephalixin (30 \(\mu\)g). Other phenotypic features are included in the species description and Table 1.

Cell biomass for chemotaxonomic analysis of strain R4S8\(^{T}\) was obtained by cultivation in shaken flasks containing seawater nutrient broth for 5 days. The biomass was harvested by centrifugation at 5000 \(g\) for 20 min, washed twice in distilled water and lyophilized. Isomers of diaminopimelic acid and whole-cell sugars were determined by established TLC methods (Staneck & Roberts, 1974; Lechevalier & Lechevalier, 1970). Cells of strain R4S8\(^{T}\) contained meso-diaminopimelic acid (meso-DAP). No diagnostic sugars were detected in whole-cell-wall hydrolysates. From the standpoint of the presence of meso-diaminopimelic in the cell-wall peptidoglycan as a unique feature, strain R4S8\(^{T}\) and D. activa DSM 45169\(^{T}\) are differentiated from Marinimema mesophilum DSM 45610\(^{T}\). Phospholipids were extracted and identified by the method of Minnikin et al. (1984). Significant quantities of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinerine, phosphatidylmethylthanolamine, two unknown phospholipids and one unknown aminophospholipid were found in the membrane of strain R4S8\(^{T}\) (Fig. S2). The presence of phosphatidylinerine allows the differentiation of strain R4S8\(^{T}\) from D. activa IMMIB L-1269\(^{T}\) and Marinimema mesophilum DSM 10219\(^{T}\). Extraction and purification of menaquinones were done by the method of Collins et al. (1977) at the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The main respiratory menaquinones were MK-6 (87 %) and MK-7 (8 %), while MK-7 has.
Phenotypic differentiation and molecular genetic characteristics of strain R4S8\textsuperscript{T} and related genera of the family \textit{Thermoactinomycetaceae}

Taxa: 1, strain R4S8\textsuperscript{T} (data from this study); 2, \textit{Desmospora activa} DSM 45169\textsuperscript{T}; 3, \textit{Marininema mesophilum} DSM 45610\textsuperscript{T}. Unless otherwise stated all data are from this study. +, Positive reaction; −, negative reaction; DAP, diaminopimelic acid.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour of aerial mycelium</td>
<td>Yellow to strong olive</td>
<td>Yellow</td>
<td>No</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Optimal temperature for growth (°C)</td>
<td>40</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Major menaquinone</td>
<td>MK-6</td>
<td>MK-7\textsuperscript{a}</td>
<td>MK-7\textsuperscript{b}</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0}, iso-C\textsubscript{14:0} (52.6)</td>
<td>iso-C\textsubscript{15:0}, iso-C\textsubscript{17:0}, C\textsubscript{16:0} (49.3\textsuperscript{a})</td>
<td>anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0} (46.5\textsuperscript{b})</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAP isomer</td>
<td>meso-DAP</td>
<td>meso-DAP</td>
<td>LL-DAP</td>
</tr>
<tr>
<td>Phospholipids*</td>
<td>Diphosphatidlyglycerol, phosphatidylmonomethyllethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, two unknown phospholipids and an unknown aminophospholipid</td>
<td>Diphosphatidlyglycerol, phosphatidylglycerol, phosphatidyllethanolamine and phosphatidylmonomethyllethanolamine\textsuperscript{a}</td>
<td>Diphosphatidlyglycerol, phosphatidylmethyllethanolamine, phosphatidylethanolamine, phosphatidylglycerol and five, unknown phospholipids\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\*Data from: a, Yassin et al. (2009); b, Li et al. (2012).

been reported in \textit{D. activa} DSM 45169\textsuperscript{T} and \textit{Marininema mesophilum} DSM 45610\textsuperscript{T} as the major menaquinone. The whole-cell fatty acid composition of strain R4S8\textsuperscript{T} was determined according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; Identification Library TSBA40 4.1; Microbial ID). Cells were harvested in the mid-exponential growth phase. The major fatty acids of strains R4S8\textsuperscript{T} were anteiso-C\textsubscript{15:0} (44.8\%), iso-C\textsubscript{14:0} only in strain R4S8\textsuperscript{T} and iso-C\textsubscript{14:0} and C\textsubscript{16:0} in \textit{D. activa} DSM 45169\textsuperscript{T}. However some differences such as the presence of iso-C\textsubscript{14:0} only in strain R4S8\textsuperscript{T} and iso-C\textsubscript{17:0} and C\textsubscript{16:0} in \textit{D. activa} DSM 45169\textsuperscript{T} can be useful characteristics for differentiating the strains. The genomic DNA G+C content of strain R4S8\textsuperscript{T} was determined by reversed-phase HPLC of nucleosides according to Mesbach et al. (1989). The DNA G+C content was 52.6 mol\%, which was higher than those of other related genera in the family \textit{Thermoactinomycetaceae} (46.5–49.3 mol\%) (Yassin et al., 2009; Li et al., 2012).

Genomic DNA from strain R4S8\textsuperscript{T} was prepared using the modification of salting out procedure described by Pospiech & Neumann (1995). Approximately 50–100 mg biomass of strain R4S8\textsuperscript{T} was taken from nutrient seawater broth medium and transferred to a 150 μl microfuge tube. Biomass was resuspended in 567 μl SET buffer [75 mM NaCl, 25 mM EDTA (pH 8), 20 mM Tris/HCl (pH 7.5)] and mixed with lysis solution buffer which contained 100 μl lysozyme (30 mg ml\textsuperscript{−1} in 10 mM Tris pH 8) and incubated at 37 °C for 1 h. Lysis was accomplished by adding 9 μl proteinase K (600 U ml\textsuperscript{−1}), 100 μl SDS (10%) and 3 μl RNase (10 mg ml\textsuperscript{−1}) followed by brief mixing and incubation at 55 °C for 2 h. The lysate was centrifuged at 10 000 g for 15 min. The resulting preparation was extracted according to the reference protocol (Pospiech & Neumann, 1995). DNA was precipitated with 2-propanol and rinsed with 70% (v/v) ethanol. The pellet was dried at room temperature and the DNA dissolved in 50 μl TE solution.

PCR amplification was carried out using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTAGACTT-3′) universal primers. PCR conditions included three temperature cycles: initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 90 s, then a final extension at 72 °C for 10 min. The 16S rRNA gene of strain R4S8\textsuperscript{T} was sequenced using Applied Biosystems 3730/3730xl DNA Analysers Sequencing, Bioneer, Korea. Identification of phylogenetic neighbours of strain R4S8\textsuperscript{T} (1509 nt) was carried out by BLAST (Altschul et al., 1997) and MEGA BLAST (Zhang et al., 2000) programs against the database of type strains of species with validly published prokaryotic names (Kim et al., 2012). Multiple alignments of sequences...
obtained from databases was carried out using CLUSTAL_X (Thompson et al., 1997) and the alignments were subjected to analysis using the software package MEGA version 5 with neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) methods (Fig. 1). Strain R4S8T formed a distinct clade within the family Thermoactinomycetaceae and showed highest 16S rRNA gene sequence similarity with Desmospora activa IMMIB L-1269T (95.5 %) and Marininema mesophilum SCSIO 10219T (95.3 %). This affiliation was assented to the bootstrap value of 98 % in neighbour-joining tree which was also confirmed in trees generated using the minimum-evolution and maximum-parsimony algorithms.

Strain R4S8T is clearly distinct from other reference genera, not only by 16S rRNA gene sequence divergence, the presence of MK-6 as predominant menaquinone and phosphatidylserine in the cell membrane, but also differing amounts of predominant fatty acids and the DNA G+C content can be firm evidence for distinguishing this strain from other related genera (Table 1). In addition, growth of Marininema mesophilum SCSIO 10219T occurred at a mesophilic range, between 25–35 °C (optimum 30 °C) without formation of aerial mycelium while both strain R4S8T and D. activa IMMIB L-1269T revealed aerial mycelium and optimal growth at temperatures above 40 °C. On the basis of these polyphasic taxonomic data, strain R4S8T represents a novel species in a new genus, for which the name Salinithrix halophila gen. nov., sp. nov. is proposed.

**Description of Salinithrix gen. nov.**

Salinithrix (Sa.li’ni.thrix. L. n. salina a saltern; Gr. fem. n. thrix a hair; N.L. Salinithrix a saltern hair).

Cells are Gram-staining-positive, non-acid-fast and strictly aerobic. Endospores are formed on the substrate mycelium. Motile elements are not observed. The cell wall contains meso-DAP as the diagnostic diaminoc acid. The phospholipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylmono-methylethanolamine two unknown phospholipids and an unknown amino-phospholipid. The predominant menaquinone is MK-6. Major fatty acids are anteiso-C15 : 0, iso-C15 : 0 and iso-C14 : 0. The DNA G+C content is 32.6 mol%.

The type species is Salinithrix halophila.

**Description of Salinithrix halophila sp. nov.**

Salinithrix halophila (ha.lo’phi.la. Gr. n. hal-, -los salt; Gr. n. philos friend, loving; N.L. fem. adj. halophila salt-loving).

In addition to the genus features, this species has the following characteristics. Abundant substrate and aerial mycelium are produced. The reverse sides of colonies are

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**Fig. 1.** Neighbour-joining phylogenetic tree calculated with MEGA 5 software, based on almost-complete 16S rRNA gene sequences, showing the relationship of strain R4S8T with other related species. GenBank accession numbers are given in parentheses. The sequence of Micrococcus luteus DSM 20030T (GenBank accession no. AJ536198) was used as an outgroup. Bootstrap values >50 %, based on 1000 replications are shown at the branching points. Filled circles indicate nodes that were also retrieved in maximum-likelihood and minimum evolutionary trees. Bar, 0.02 substitutions per nucleotide position.
pale yellow to moderate olive green. Sessile endospores are formed on substrate mycelium with a diameter of 0.75–1 μm. Grows at 30–50 °C, with 1–15 % (w/v) NaCl and at pH 6–10. Optimal growth occurs at 40 °C, pH 7 and with 3–5 % (w/v) NaCl. Casein is hydrolysed but not arbutin, aesculin, gelatin, Tweenes 20, 40, 60 and 80, starch, tyrosine, hypoxanthine, adenine, DNA or xanthine. Nitrate and nitrite are not reduced. H₂S is not produced. Utilizes D-glucose, sodium citrate, dulcitol, D-mannitol and D-ribose as sole carbon sources, and L-arginine and L-glucose as sole carbon and nitrogen sources. D-Mannose, maltose, sorbitol, sucrose, trehalose, melezitose, D-galactose, lactose, sodium acetate, sodium pyruvate, D-xylene, xylitol, melibiose, L-rhamnose, raffinose, cellulbiose, myo-inositol, D-arabinose, salicin, glucoronic acid potassium salt, L-aspartic acid l-ornithine, l-isoleucine, L-proline, L-threonine, L-cysteine, L-alanine L-serine, L-histidine, L-phenylalaine, L-glutamic acid and L-methionine are not assimilated. Acid is produced from D-glucose.

The type strain is R4S8T (=IBRC-M 10813T=CECT 8506T) isolated from Inche-Broun hypersaline wetland in Iran. The DNA G+C content of the type strain is 52.6 mol% (HPLC).

Acknowledgements

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References


