Salinifilum gen. nov., with description of Salinifilum proteinilyticum sp. nov., an extremely halophilic actinomycete isolated from Meighan wetland, Iran, and reclassification of Saccharopolyspora aidingensis as Salinifilum aidingensis comb. nov. and Saccharopolyspora ghardaiensis as Salinifilum ghardaiensis comb. nov.

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Abstract

A Gram-positive, halophilic actinobacterial strain Miq-12T was isolated from Meighan wetland in Iran. Strain Miq-12T was strictly aerobic, catalase positive and oxidase negative. The isolate grew at 12–25% NaCl, at 30–50°C and pH 5.5–10.5. The optimum NaCl, temperature and pH for growth were 15–20%, 40°C and 7.0–8.0, respectively. The cell wall of strain Miq-12T contained meso-diaminopimelic acid as diagnostic diamino acid and arabinose as whole-cell sugar. The polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine and phosphatidylinositol. It synthesized cellular fatty acids of anteiso and iso-branched types, anteiso-C15:0, iso-C17:0, iso-C15:0, iso-C16:0. The major respiratory quinone was MK-9(H2). The G+C content of its genomic DNA was 72.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that strain Miq-12T belongs to the family Pseudonocardiaceae, constituted a separate clade, and showed the closest phylogenetic similarity to Saccharopolyspora aidingensis TRM 46074T (96.99%) and Saccharopolyspora ghardaiensis CCUG 63370T (96.92%). On the basis of phylogenetic analysis, phenotypic and chemotaxonomic characteristics, a novel genus and species of the family Pseudonocardiaceae, Salinifilum proteinilyticum gen. nov., sp. nov., are proposed. The type strain is Miq-12T (=IBRCM 11033T=LMG 28390T). We also propose that S. aidingensis and S. ghardaiensis should be transferred to this new genus and be named Salinifilum aidingensis comb. nov. and Salinifilum ghardaiensis comb. nov., respectively. The type strain of Salinifilum aidingensis comb. nov. is TRM 46074T (=CCTCCAA 20121014T=JCM 30185T) and the type strain of Salinifilum ghardaiensis comb. nov. is CCUG 63370T (=DSM 45606T=CECT 8304T).

The family Pseudonocardiaceae consists of aerobic, mesophilic or thermophilic, Gram-stain-positive, non-acid fast, catalase-positive and lysozyme resistant actinomycetes [1]. It currently includes the genera Actinoalloteichus [2], Actinopolyspora [3], Amycolatopsis [4], Crossiella [5], Goodfellowia [6], Kibdelosporangium [7], Kutzneria [8], Prauserella [9], Pseudonocardia [1], Saccharomonospora [10], Saccharopolyspora [11], Streptoalloteichus [12], Thermobispora [13] and Thermocrispum [14].

The richness of the microbial community in the saline and hypersaline environments of Iran has permitted us to implement comprehensive programs for discovering new microbial resources. In a previous work on Meighan wetland, a natural ecosystem in the central area of Iran, a novel strain...
was isolated and described as a new genus, *Salininema proteolyticum* strain Miq-4T [15]. In order to discover new strains in this unique region, a research project was established for isolation of new isolates. The aim of this study is taxonomic characterization of the strain Miq-12T, for which we propose its placement as a new genus and species within the family *Pseudonocardiaeae*. The strain Miq-12T was isolated by diluting the soil sample in sterile 10 % (w/v) salts solution, plating on Modified Growth Medium 23 % total salt (MGM 23 %) [16] containing (gram per litre): NaCl, 184; MgCl₂,6H₂O, 23; MgSO₄, 26.83; KCl, 7.76; NaBr, 0.61; peptone, 5.0; yeast extract, 1.0 and agar, 18 (these salts were applied for all media in this study). The pH was adjusted to 7.5 with Tris-HCl. The isolation plates were incubated at 40 °C aerobically for 3 weeks. The strain was purified on yeast extract-malt extract agar medium [17] containing 23 % total salt (gram per litre): NaCl, 184; MgCl₂,6H₂O, 23; MgSO₄, 26.83; KCl, 7.76; NaBr, 0.61; malt extract, 10; yeast extract, 4.0; glucose monohydrate, 4; and agar, 18. The pH was adjusted to 7.5 with Tris-HCl. The purified strain was preserved at 4 °C and as glycerol suspensions (20 %, v/v) at −20 and −80 °C. *Saccharopolyspora aidingensis* TRM 46074T and *Saccharopolyspora ghardaiensis* CCUG 63370T were used for comparative studies as a reference strains. The cultural and morphological properties of the new isolates were examined on the media recommended by Shirling and Gottlieb [17] and Waksman [18]. All culture media were supplemented with 23 % total salt containing (gram per litre): NaCl, 184; MgCl₂,6H₂O, 23; MgSO₄, 26.83; KCl, 7.76; NaBr, 0.61. The colour of aerial (spore mass) and substrate mycelia was determined on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), nutrient agar (Merck) and Czapek’s agar at 40 °C for 3 weeks using colour chips from the ISCC-NBS charts [19]. Spore motility was investigated under light microscopy using sterile distilled water. The ability for production of diffusible pigment was determined on ISP 2, ISP 3, inorganic salts-starch agar (ISP 4) and glycerol-asparagine agar (ISP 5) media at 40 °C for 3 weeks. Melanin production was evaluated on peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7) at 40 °C after 7 days. Spore chain morphology of a 10-days-old culture grown on ISP 2 agar was observed using a scanning electron microscope VEGA3 TESCAN model.

The organism was a Gram-positive, strictly aerobic and non-acid fast actinomycete. The strain formed creamy, irregular, raised, wrinkled and butyrous colonies on ISP 2. The cells were ovoid to branched filamentous rods with non-motile white to pale yellow spores (Fig. 1). Diffusible pigment was not produced on all media.

Physiological and biochemical tests were done using Gordon et al. [20] and Williams [21] at 40 °C, which included catalase and oxidase activities, hydrolysis of, adenine, hypoxanthine, tyrosine, guanine, xanthine, Tweens 20, 40, 60 and 80, starch, casein. Carbohydrates utilisation was determined according to the methods described by Shirling and Gottlieb [17]. The growth temperature range and optimum was examined on ISP 2 agar at 0, 4, 10, 15, 20, 28–37 °C (at intervals of 1.0 °C), 40–60 °C (at intervals of 5.0 °C). The growth pH range and optimum was assessed on ISP 2 broth at pH 4.0–11.0 using 50 mM buffers MES (4–6.5), HEPES (7–8) and CHES (8.5–11) (at 0.5 pH unit intervals). The growth range in the presence of NaCl was assessed on ISP 2 agar medium containing 0, 1, 3, 5, 7.5, 10, 12, 15, 17.5, 20, 23 and 25 % NaCl. Reduction of nitrate and nitrite, and production of melanoid pigment were determined by the method of the ISP [17] and Williams [21] respectively. Production of H₂S was tested by growing strain Miq-12T on ISP 6 agar slant medium at 40 °C. All tests were recorded after 7–21 days.

Strain Miq-12T grew at a temperature range of 30–50 °C (optimum temperature 40 °C), pH 5.5–10.5 (optimum pH 7.0–8.0). The NaCl concentration for growth was 12–25 %, with optimal growth at 15–20 %, Nitrate and nitrite reduction was not observed. Casein was hydrolyzed but starch was not. The production of H₂S was negative. Other phenotypic characteristics of strain Miq-12T, *S. aidingensis* TRM 46074T and *S. ghardaiensis* CCUG 63370T are given in Table 1.

Cell biomass for analysis of the cell wall, isoprenoid quinones and polar lipids were obtained by cultivation in shaken flasks containing ISP 2 broth medium supplemented with NaCl, 184; MgCl₂,6H₂O, 23; MgSO₄, 26.83; KCl, 7.76 and NaBr, 0.61 (The values based gram per litre). The biomass was harvested by centrifugation (Sigma Model 4–16 k) at 5000 g for 20 min, washed twice in distilled water and lyophilized. Isomers of diaminopimelic acid and whole cell
The predominant menaquinone was MK-9(H4) (72.6 mol%) [27]. Approximately 50–100 mg biomass of strain Miq-12T growth was taken from ISP2 broth medium and transferred to a 200 µl microfuge tube. Biomass was resuspended in 567 µl SET buffer ([NaCl 75 mM, EDTA 25 mM (pH 8), Tris-HCl 20 mM (pH 7.5)] and mixed with lysis solution buffer which contained 100 µl lysozyme (30 mg ml−1 in 10 mM Tris pH 8) and incubated at 37°C for one hour. Lysis was accomplished by adding 9 µl proteinase K (600 U ml−1), 100 µl SDS 10 % and 3 µl RNase (10 mg ml−1) followed by brief mixing and incubation at 55°C for 2 h. The lysate was centrifuged at 10 000 g (Sigma Model 1 g) and 4°C for 2 h. The cellu-

**Table 1. Phenotypic and chemotaxonomic characteristics of strain Miq-12T, Saccharopolyspora ghardaiensis CCUG 63370T and Saccharopolyspora aidingensis TRM 46074T**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strain Miq-12T</th>
<th>S. ghardaiensis CCUG 63370T</th>
<th>S. aidingensis TRM 46074T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>30–50</td>
<td>25–45</td>
<td>25–45</td>
</tr>
<tr>
<td>pH range</td>
<td>5.5–10.5</td>
<td>5–8</td>
<td>5–9</td>
</tr>
<tr>
<td>NaCl tolerance (% w/v)</td>
<td>12–25</td>
<td>7–32</td>
<td>8–18</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Utilization as sole carbon source:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-rhamnose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-galactose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Whole sugar content</td>
<td>Arabinose and ribose</td>
<td>Arabinose, galactose and ribose*</td>
<td>Galactose, arabinose, ribose</td>
</tr>
<tr>
<td>Major menaquinone</td>
<td>MK-9(H4)</td>
<td>MK-9 (H4), MK-9 (H8)*</td>
<td>MK-9 (H4), MK-10 (H4), MK-10 (H8)</td>
</tr>
<tr>
<td>Polar lipid Pattern</td>
<td>PC, DPG, PG, PI</td>
<td>PC, DPG, PG, and PI*</td>
<td>PC, DPG, PE, PG, PI</td>
</tr>
<tr>
<td>DNA G+C content (mol %)</td>
<td>72.1</td>
<td>72.6*</td>
<td>70.9</td>
</tr>
</tbody>
</table>

*Data obtained from Meklat et al. [27].

**Table 2. Comparison of the fatty acids profile of strain Miq-12T, Saccharopolyspora ghardaiensis CCUG 63370T and Saccharopolyspora aidingensis TRM 46074T**

Values are percentages of total fatty acids; –, not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain Miq-12T</th>
<th>S. ghardaiensis CCUG 63370T*</th>
<th>S. aidingensis TRM 46074T*</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C15:0</td>
<td>15.1</td>
<td>25.9</td>
<td>20.3</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>4.8</td>
<td>–</td>
<td>6.6</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>13.5</td>
<td>5.9</td>
<td>12.0</td>
</tr>
<tr>
<td>iso ω9C-C17:1</td>
<td>1.8</td>
<td>12.1</td>
<td>4.3</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>18.0</td>
<td>14.6</td>
<td>10.2</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>34.1</td>
<td>27.2</td>
<td>28.8</td>
</tr>
<tr>
<td>C17:0</td>
<td>3.5</td>
<td>–</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Data obtained from Meklat et al. [27].

Genomic DNA G+C content of strain Miq-12T was 72.1 mol%, which was determined by reversed-phase HPLC of nucleosides according to Mesbah et al. [26]. This DNA G+C content is within the range reported for members of the family *Pseudonocardiaecae* and close to that determined for S. aidingensis TRM 46074T (70.9 mol%) and S. ghardaiensis CCUG 63370T (72.6 mol%) [27].

Genomic DNA from strain Miq-12T was prepared using the modification of salting out procedure described by Pospiech [28]. Approximately 50–100 mg biomass of strain Miq-12T growth was taken from ISP2 broth medium and transferred to a 200 µl microfuge tube. Biomass was resuspended in 567 µl SET buffer ([NaCl 75 mM, EDTA 25 mM (pH 8), Tris-HCl 20 mM (pH 7.5)] and mixed with lysis solution buffer which contained 100 µl lysozyme (30 mg ml−1 in 10 mM Tris pH 8) and incubated at 37°C for one hour. Lysis was accomplished by adding 9 µl proteinase K (600 U ml−1), 100 µl SDS 10 % and 3 µl RNase (10 mg ml−1) followed by brief mixing and incubation at 55°C for 2 h. The lysate was centrifuged at 10 000 g (Sigma Model 1 g) for 15 min. The resulting preparation was extracted according to the reference protocol. DNA was precipitated with 2-propanol and rinsed with ethanol 70 % (v/v). The pellet was
dried at room temperature and the DNA dissolved in 50 μl TE solution.

PCR reaction was performed using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTGTAGACTT-3') universal primers using the following conditions: initial denaturation at 94°C (5 min); 25 cycles including denaturation at 94°C (1 min), annealing at 56°C (1 min) and extension at 72°C (90 s); final extension at 72°C (10 min). The purified PCR product was cloned using Thermo Scientific Ins TA clone PCR Cloning Kit and extracted plasmid was sequenced by an ABI 3730/3730XL DNA sequencer at Bioneer (Daejeon, South Korea). The 16S rRNA gene sequence of strain Miq-12T (1512 nt) was used for initial BLAST searches in GenBank and phylogenetic analysis.

Identification of phylogenetic neighbors was initially performed by BLAST [29] and MEGA BLAST [30] programs. The aligned 16S rRNA gene sequences of Miq-12T with the type strain of the most closely related species were analyzed using MEGA version 6 program [31]. The trees construction were based on near full length 16S rRNA gene sequences of type strains of the most closely related taxa to strain Miq-12T and conducted by neighbour-joining [32], minimum-evolution [33] and maximum-likelihood [34] methods to access the support for each node of phylogenetic tree.

The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the ezbiocloud.net database [35]. The 16S rRNA gene sequence analysis showed that strain Miq-12T is a member of the family Pseudonocardiaceae, and was most closely associated with Saccharopolyspora aidencyensis TRM 46074T (96.9 % similarity value) and S. ghardaiensis CCUG 63370T (96.9 % similarity value). Phylogenetic analysis based on maximum-likelihood algorithm revealed that strain Miq-12T along with S. aidencyensis TRM 46074T and S. ghardaiensis CCUG 63370T comprised an independent clade within the family Pseudonocardiaceae (Fig. 2). In contrast with other members of genus Saccharopolyspora, this clade has rooted from a different ancestor and placed in a separate position within the family. The tree topology in this region was also supported by the minimum-evolution and neighbour-joining methods and confirmed the phylogenetic position of strain Miq-12T with respect to S. aidencyensis TRM 46074T and S. ghardaiensis CCUG 63370T and other neighbours in this family.

Based on the phylogenetic approach, we can conclude that strain Miq-12T could constitute a new member of the family Pseudonocardiaceae which is also supported by the phentypic and other taxonomic features (Table 1). In conclusion, the presence of ribose as a diagnostic sugar in the strains Miq-12T, S. aidencyensis TRM 46074T and S. ghardaiensis CCUG 63370T supported that they could not be regarded as the members of the genus Saccharopolyspora [36]. This evidence along with their evolutionary position on the phylogenetic trees satisfy us for regarding them as the members of a new genus in the family Pseudonocardiaceae. So, strain Miq-12T constitutes a new species in a new genus of the family Pseudonocardiaceae, for which the name Salinifilum proteinilyticum gen. nov., sp. nov. is proposed. It is also proposed that Saccharopolyspora aidencyensis TRM 46074T and Saccharopolyspora ghardaiensis CCUG 63370T be transferred to the genus Salinifilum aidencyensis TRM 46074T comb. nov., and Salinifilum ghardaiensis CCUG 63370T comb. nov., respectively.

**DESCRIPTION OF SALINIFILUM GEN. NOV.**

(Sal.i.ni.fí.lúm. L. pl. n. salinae, salt source; L. neut. n. filum, thread; N.L. neut. n. Salinifilum, a thread from a salt source).

Cells are Gram-stain-positive and strictly aerobic, halophilic and non-acid fast. Exhibits type III cell wall composition (meso-diaminopimelic acid) while whole cell sugar pattern consists of arabinose, ribose and galactose. The diagnostic phospholipid is phosphatidylcholine. The principal menaquinone is MK-9 and MK-10. Major cellular fatty acids are anteiso-C17:0, iso-C15:0, iso-C15:0 3-OH, and iso-C16:0 3-OH. The DNA G+C content is between 70–73 mol% (HPLC). Phylogenetically belongs to the family Pseudonocardiaceae. The type species of the genus is Salinifilum proteinilyticum.

**DESCRIPTION OF SALINIFILUM PROTEINILYTICUM SP. NOV.**

Salinifilum proteinilyticum (pro.te.i.ni.ly’tí.cum. N.L. n. proteinum (from Gr. proteus, first), protein; Gr. adj. lytikos, dissolving; N.L. neut. adj. proteinilyticum, protein-dissolving).

Halophilic, strictly aerobic, Gram-stain-positive and non-acid fast. It forms colorless to pale green, creamy, irregular, raised, wrinkled and butyrous colonies on ISP 2. The cells produce pale green and short aerial mycelium with non-motile spores. Strain does not produce diffusible pigment. It grows at a temperature range of 30–50°C (optimum temperature 40°C), pH 5.5–10.5 (optimum pH 7.0–8.0). The NaCl concentration for growth is 12–25 %, with optimal growth at 15–20 %. Reduction of nitrate to nitrite is negative. Able to hydrolyze casein but not starch. Hypoxanthine, tyrosine, Tweens 40 and 80 are hydrolyzed while adenine, guanine, 15-acetyl-l-glutamic acid, trehalose, rhamnose, D-ribose and myo-inositol, but not melibiose, D-galactose, cellobiose, maltose, mannitol, lactose or D-arabinose. The type strain is Miq-12T (=IBRC M 11033T =LMG 28390T) isolated form Meighan wetland of Iran. Its DNA G+C content is 72.1 mol% (HPLC).

**DESCRIPTION OF SALINIFILUM AIDINGENSIS COMB. NOV.**

Salinifilum aidencyensis (ai.ding.en’sis; N.L. fem. adj. aidencyensis of or belonging to Aiding lake, a salt lake in China, where the type strain was isolated).

The description is identical to that given for Saccharopolyspora aidingensis by Xia et al. 2017. The type strain is TRM 46074ᵀ (=CCTCCAA 2012014ᵀ=JCM 30185ᵀ).

**DESCRIPTION OF SALINIFILUM GHARDAIENS COMB. NOV.**

Salinifilum ghardaiens (ghar.da.i.en’sis. N.L. fem. adj. ghardaiensis, pertaining to Ghardaïa, the source of the soil from which the type strain was isolated).
Basonym: Saccharopolyspora ghordaiensis Meklat et al. 2014.

The description is identical to that given for Saccharopolyspora ghordaiensis by Meklat et al. [27].

The type strain is CCUG 63370T (=DSM 45606T=CCUG 63370T=CECT 8304T).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No experimental work with animals or human has been done in this research project.

References


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