Promicromonospora iranensis sp. nov., an actinobacterium isolated from rhizospheric soil

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A novel strain of the genus Promicromonospora, designated HM 792T, was isolated from soil in Fars Province, Iran. On ISP 2 medium, the yellow-pigmented isolate produced long and branched hyphae that developed into a large number of irregularly shaped spores. It showed growth at 25–30 °C and pH 6.0–9.0 with 0–8 % (w/v) NaCl. Chemotaxonomic and molecular characteristics of the isolate matched those described for members of the genus Promicromonospora. Whole-cell hydrolysates of strain HM 792T contained the amino acids D-glutamic acid, L-alanine and L-lysine along with the sugars glucose and ribose. The main polar lipids were dihexadecanoylphosphatidylglycerol, two unknown phospholipids, unknown glycolipids and two unknown phosphoglycolipids, complemented by minor concentrations of phosphatidylglycerol and phosphatidylethanolamine. MK-9(H4) was the predominant menaquinone. The fatty-acid pattern was composed mainly of the saturated branched-chain acids anteiso-C15:0 and iso-C15:0. The 16S rRNA gene sequence analysis showed the highest pairwise sequence identity (96.6–99.0 %) with the members of the genus Promicromonospora. Based on phenotypic and genotypic features, strain HM 792T is considered to represent a novel species of the genus Promicromonospora, for which the name Promicromonospora iranensis sp. nov. is proposed. Strain HM 792T (DSM 45554T = UTMC00792T = CCUG 63022T) is the type strain.

Three supplementary figures are available with the online version of this paper.
Cultural properties of strain HM 792\textsuperscript{T} were evaluated according to the guidelines of the ISP as described by Shirling & Gottlieb (1966), determining the culture colour by comparison with chips from the ISCC-NBS colour charts (Kelly, 1964). The substrate mycelium characteristics were observed on ISP 2 medium after 14 days at 28 °C using the coverslip technique (Kawato & Shinobu, 1959). Micrographs of mycelium grown on ISP 2 medium after 14 days at 28 °C were taken with a field-emission scanning electron microscope (FE-SEM Merlin; Zeiss). Growth rates were determined at 10–50 °C in 5 °C increments and at pH 4–11 (in increments of 1.0 pH units) on modified Bennett agar after 2 weeks of incubation as described by Williams et al. (1989) using a sodium sesquicarbonate buffer system for preparation of alkaline media. Utilization of carbon sources, acid production and other biochemical tests were determined using GEN III Microplates in an Omnilog device (Biolog). The GEN III Microplates were inoculated with a cell suspension made in a 'gelling' inoculating fluid A at a cell density of 98% transmittance, yielding a running time of 4 days in Phenotype Microarray mode at 28 °C. The exported data were further analysed with the opm package for R (Vaas et al., 2012, 2013), using its functionality for merging subsequent measurements of the same plate, estimating parameters statistically from the respiration curves such as the maximum height, and automatically 'discretizing' these values into negative and positive reactions. Strain HM 792\textsuperscript{T} was studied in comparison with reference strains Promicromonaspora kroppenstedtii DSM 19349\textsuperscript{T}, Promicromonaspora sukumoe DSM 44121\textsuperscript{T} and Promicromonaspora vindobonensis DSM 15942\textsuperscript{T} in GEN III Microplates in two independent determinations. Reactions that gave contradictory results between the two repetitions were regarded as ambiguous.

Strain HM 792\textsuperscript{T} developed a branched substrate mycelium with a wrinkly and shining surface, the septate hyphae of which broke, at a later stage, into fragments of various sizes and rod-shaped spore elements (Fig. 1), a feature shared by all members of the genus Promicromonaspora (Krasil’nikov et al., 1961; Schumann & Stackebrandt, 2012). Substrate mycelium pigmentation varied slightly depending on the tested medium. On ISP 2 medium, oatmeal agar (ISP 3 medium), inorganic salts/starch agar (ISP 4 medium) and glycerol/asparagine agar (ISP 5 medium), it displayed a colour between light yellow and yellow. Light-brown substrate mycelium was observed on peptone-yeast extract iron agar (ISP 6 medium) and tyrosine agar (ISP 7 medium). No diffusible pigments were produced on any medium tested. Strain HM 792\textsuperscript{T} revealed optimal growth on ISP 2 and ISP 5 media, moderate growth on ISP 4, ISP 6, and ISP 7 media and poor growth on ISP 3 medium. HM 792\textsuperscript{T} grew at 25–30 °C (optimal growth at 28 °C), but not at 10 °C, a temperature at which \textit{P. vindobonensis} DSM 15942\textsuperscript{T} and \textit{P. sukumoe} DSM 44121\textsuperscript{T} showed weak growth, or 45 °C, in the presence of 0–8% NaCl (optimal range) and at pH 6–9, but not at pH 4 or 11, in contrast to \textit{P. sukumoe} DSM 44121\textsuperscript{T}, which grew at pH 12. Results from phenotype microarray analysis are shown as a heat map in Fig. S1 (available in the online Supplementary Material) in comparison with the other tested type strains of the genus Promicromonaspora. A summary of selected differential phenotypic characteristics is presented in Table 1.

For studies of cell-wall amino acids and sugars, polar lipids and respiratory lipoquinones and peptidoglycan composition, strain HM 792\textsuperscript{T} was cultivated in trypticase soy broth (TSB) for 6 days in shaking cultures at 200 r.p.m., pH 7.2 and 28 °C. Cell biomass was harvested by centrifugation and washed twice with distilled water. Whole-cell sugars were prepared according to Lechevalier & Lechevalier (1970), followed by TLC analysis (Staneck & Roberts, 1974). Polar lipids were extracted, separated by two-dimensional TLC and identified according to procedures outlined by Minnikin et al. (1984) with the modifications proposed by Kroppenstedt & Goodfellow (2006). Respiratory lipoquinones were extracted from freeze-dried cell material using methanol as described by Collins et al. (1977) and analysed by HPLC (Kroppenstedt, 1982). Isolation of the peptidoglycan and elucidation of its structure were carried out according to published protocols (Schumann, 2011). A purified peptidoglycan preparation was obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion. The amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates. Molar ratios of amino acids were determined by GC and GC-MS of \textit{N}-heptafluorobutyryl amino acid isobutyl esters. The amineterminal amino acid of the interpeptide bridge was detected by dinitrophylation. Extraction of cellular fatty acids was carried out from biomass grown on tryptcase soy agar (TSA) tubes held at 28 °C for 3 days. Analysis was conducted using the Microbial Identification System (MIDI) Sherlock version 6.1 (results evaluated against the TSBA40 peak-naming table database) as described by Sasser (1990).

The peptidoglycan of strain HM 792\textsuperscript{T} contained muramic acid and the amino acids lysine, glutamic acid and alanine in the molar ratio 1.1:1.0:1.9:1.7. In addition to these

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**Fig. 1.** Scanning electron micrograph of spore chains of strain HM 792\textsuperscript{T} grown on ISP 2 medium for 14 days at 28 °C. Bar, 1 μm.
Table 1. Differential characteristics of strain HM792T and the type strains of other species of the genus Promicromonospora

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
<td>Growth at 8% (w/v) NaCl</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Fucose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Glycyl l-proline</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>l-Alanine</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>l-Aspartic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>l-Pyroglutamic acid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>l-Serine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-Hydroxyphenylacetic acid</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Citric acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bromosuccinic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell-wall amino acid(s)</td>
<td>Lys, Ala, Glu (A4x)</td>
<td>Lys, Glu, Ala (A4x)</td>
<td>Lys (A3x)</td>
<td>Glu, Gly, Ala, Lys (A3x)</td>
</tr>
<tr>
<td>Cell-wall sugars†</td>
<td>Glc, Rib</td>
<td>Gal, Rha</td>
<td>ND</td>
<td>Rha, Gal, Glc’</td>
</tr>
<tr>
<td>Predominant menaquinone(s)</td>
<td>9(H₄), 9(H₆), 8(H₄), 9(H₂)</td>
<td>9(H₄), 9(H₂), 8(H₄)</td>
<td>9(H₄), 9(H₂), 8(H₄)</td>
<td>9(H₄)</td>
</tr>
<tr>
<td>Polar lipids‡</td>
<td>DPG, 2PL, 2GL, 2PGL, PI, PG</td>
<td>PG, DPG, PI, PGL</td>
<td>PI, DPG</td>
<td>DPG, PG, 3PGL, PL</td>
</tr>
</tbody>
</table>

*Data taken from: a, Alonso-Vega et al. (2008); b, Takahashi et al. (1987); c, Busse et al. (2003).
†Gal, Galactose; Glc, glucose; Rha, rhamnose; Rib, ribose.
‡Components are listed in decreasing order of amount. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; GL, unknown glycolipid; PL, unknown phospholipid; PGL, unknown phosphoglycolipid.

amino acids, the partial hydrolysate (4 M HCl, 0.75 h, 100 °C) contained the peptides L-Ala–D-Glu, L-Ala–l-Lys, l-Lys–D-Ala and l-Ala–l-Lys–D-Ala. Dinitrophenylation revealed that Glu represents the N terminus of the interpeptide bridge. Dinitrophenylated Ala was detected in smaller amounts. From these data, it was concluded that strain HM 792T shows the peptidoglycan type A4 (Schleifer & Kandler, 1972) L-Lys–L-Ala–D-Glu (type A11.59 according to Schumann, 2011), as does Promicromonosteddii (Alonso-Vega et al., 2008). Whole-cell sugar analysis revealed primarily glucose and ribose, but also traces of rhamnose and galactose. HM 792T contained primarily menaquinone MK-9(H₄) (64.3%), but also MK-9(H₆) (11.1%), MK-8(H₄) (9.4%) and MK-9(H₂) (8.4%), in accordance with the respiratory lipoquinone patterns observed for Promicromonosteddii (Alonso-Vega et al., 2008) and Promicromonospora umidemergens (Martin et al., 2010). The polar lipid profile consisted of diphosphatidylglycerol, two unknown phospholipids, two unknown glycolipids and two unknown phosphoglycolipids, complemented by minor concentrations of phosphatidylinositol and phosphatidylglycerol (Fig. S2). Predominant fatty acids were the saturated branched-chain acids anteiso-C₁₅:0 (38.7 ± 0.1%) and iso-C₁₅:0 (26.6 ± 0.0%), with a smaller amount of anteiso-C₁₇:0 (14.2 ± 0.5%), in agreement with the fatty acid patterns obtained for other reference type strains of the genus Promicromonospora investigated in this study (Table 2). This qualitative and quantitative combination of fatty acids is diagnostic for species of the genus Promicromonospora, corresponding to fatty acid pattern 2c *sensu* Kroppenstedt (1985).

Table 2. Cellular fatty acid profiles of strain HM792T and type strains of closely related species of the genus Promicromonospora

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>anteiso-C₁₅:0</td>
<td>38.7 ± 0.1</td>
<td>46.8 ± 0.1</td>
<td>47.8 ± 0.6</td>
<td>44.7 ± 0.8</td>
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<tr>
<td>iso-C₁₅:0</td>
<td>26.6 ± 0.0</td>
<td>30.9 ± 1.3</td>
<td>29.5 ± 0.5</td>
<td>36.1 ± 0.3</td>
</tr>
<tr>
<td>anteiso-C₁₇:0</td>
<td>14.2 ± 0.5</td>
<td>6.5 ± 0.3</td>
<td>7.2 ± 0.2</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>C₁₆:0</td>
<td>2.8 ± 0.1</td>
<td>5.9 ± 0.0</td>
<td>6.3 ± 0.1</td>
<td>2.6 ± 0.0</td>
</tr>
<tr>
<td>iso-C₁₆:0</td>
<td>6.3 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>3.1 ± 0.0</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>iso-C₁₇:0</td>
<td>4.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.0</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>C₁₄:0</td>
<td>TR</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>anteiso-C₁₅:₁ A</td>
<td>3.2 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C₁₅:₁ G</td>
<td>2.2 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td>TR</td>
</tr>
</tbody>
</table>

Data are mean ± SD percentages of the total fatty acids (from two determinations) from this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not shown. All strains were grown on TSA at 28 °C for 3 days. TR, Trace (<1%); ND, not detected.
Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out as described by Rainey et al. (1996). Phylogenetic analyses and the rooting of the resulting trees were conducted as described previously (Montero-Calasanz et al., 2013). Pairwise similarities were calculated as recommended by Meier-Kolthoff et al. (2013). For DNA–DNA hybridization tests, cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

The almost-complete (1514 bp) 16S rRNA gene sequence of strain HM 792T was determined. The 16S rRNA gene sequence showed the highest similarity to sequences of the members of the genus Promicromonospora, especially the type strains of P. vindobonensis (99.0 % similarity), P. krop- penstedtii (98.9 %), P. sukimae (98.8 %), P. umidenemers (98.5 %), P. thailandica (98.5 %), P. aerolata (98.3 %), P. xylanilytica (98.3 %), P. citrea (98.0 %) and P. endophytica (98.0 %). In addition, the type strains of all members of Promicromonospora were placed within the same phylogenetic group, showing the maximum support by both maximum-likelihood and maximum-parsimony estimations (Fig. S3). 16S rRNA gene sequence analysis thus strongly supports the assignment of strain HM 792T to the genus Promicromonospora. However, 16S rRNA gene sequence similarity between HM 792T and closely related type strains indicated the need to prove the genomic distinctness of the type strain as representing a novel species by DNA–DNA hybridization. Strain HM 792T displayed DNA–DNA relatedness of 38.7 ± 1.7 % with P. vindobonensis DSM 15942T, 47.85 ± 1.65 % with P. krop- penstedtii DSM 19349T, 28.75 ± 3.25 % with P. sukimae DSM 44121T and 40.5 ± 4.8 % with P. aerolata DSM 15943T. DNA–DNA hybridizations of strain HM 792T with the type strains of P. umidenemers, P. thailandica, P. xylanilytica, P. citrea and P. endophytica were not conducted based on the observations of Busse et al. (2003) for novel species of the genus Promicromonospora, which may be detected when new isolates show 16S rRNA gene sequence similarity below 98.6 % to existing species, and Stackebrandt & Ebers (2006) and Meier-Kolthoff et al. (2013), that confirmed statistically that such strains showing 16S rRNA gene sequence similarities of 97.0–98.5 % generally result in DNA–DNA hybridization values below the 70 % threshold recommended by Wayne et al. (1987) to confirm the species status of novel strains.

Several phenotypic characteristics apart from the phylogenetic analysis based on 16S rRNA gene sequences support the distinctiveness of strain HM 792T from all other species of the genus Promicromonospora. Based on the phenotypic and genotypic data presented above, we propose that strain HM 792T represents a novel species within the genus Promicromonospora, which we name Promicromonospora iranensis sp. nov.

**Description of Promicromonospora iranensis sp. nov.**

Promicromonospora iranensis (i.ra.nen’sis. N.L. fem. adj. iranensis referring to Iran, the country from where the type strain was isolated).

Aerobic, Gram-staining-positive actinobacterium that forms a well-developed branched mycelium that breaks up into fragments of various sizes and rod-shaped spore elements that turn to an ellipsoid shape when the culture ages. Colonies on ISP media are wrinkled, varying between yellow and light brown in colour. Optimal growth occurs at 28 °C, at pH 6–9 and in the presence of 0–8 % NaCl. Utilizes dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, lactose, methyl β-D-glucoside, D-salicin, N-acetyl-D-glucosamine, D-glucose, D-mannose, D-fructose, D-galactose, L-rhamnose, sodium lactate, D-mannitol, D-arabitol, glycerol, L-glutamic acid, pectin, D-gluconic acid, L-aspartic acid, L-alanine, D-gluconic acid, L-lactic acid, L-malic acid, bromosuccinic acid, L-pyroglutamic acid, p-hydroxyphenylacetic acid, acetic acid, Tween 40, β-hydroxybutyric acid and butyric acid as sole carbon sources for energy and growth, but not stachyose, raffinose, melibiose, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyleneuraminic acid, 3-O-methyl D-glucose, D-fucose, D- or L-serine, D-sorbitol, myo-inositol, D-glucose 6-phosphate, L-fucose, inosine, glycine L-proline, D-fructose 6-phosphate, D-aspartic acid, L-arginine, L-histidine, D-galacturonic acid, L-galactonic acid γ-lactone, gluconamide, mucic acid, quinic acid, D-saccharic acid, D-lactic acid methyl ester, citric acid, α-ketoglutaric acid, D-malic acid, γ-amino-N-butric acid, α-ketobutyric acid or propionic acid. Acid is produced from N-acetyl-D-glucosamine, L-alanine and L-aspartic acid, which can be used as sole nitrogen sources, but not from glycine L-proline, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, D- or L-serine, D-aspartic acid, L-arginine, L-histidine, glucuronamide or γ-amino-N-butyric acid. Positive for degradation of gelatin. Whole-cell hydrolysates contain the sugars glucose and ribose and minor amounts of rhamnose and galactose and the amino acids D-glutamic acid, L-alanine and L-lysine. The predominant menaquinone is MK-9(H4), but significant amounts of MK-9(H6), MK-8(9H2) and MK-9(9H2) are also present. The polar lipid pattern is diphos- phatidylglycerol, two unknown phospholipids, two unknown glycolipids, two unknown phosphoglycolipids and minor concentrations of phosphatidylinositol and phosphatidylgly- cerol. The predominant fatty acids are anteiso-C15:0 and iso- C15:0 with a smaller amount of anteiso-C17:0.

The type strain, HM 792T (=DSM 4554T =UTMC00792T =CCUG 63022T), was isolated from rhizospheric soil collected at 10 cm depth in Fars Province, Iran.
Acknowledgements

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References


