Marininema halotolerans sp. nov., a novel thermoactinomycete isolated from a sediment sample, and emended description of the genus Marininema Li et al. 2012

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A novel Gram-stain-positive bacterium, designated strain YIM M11385T, was isolated from a marine sediment sample collected from the South Bay, Little Andaman Island, India with a salinity of 35 p.p.m., pH 8.5. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM M11385T belongs to the genus Marininema, supported by a bootstrap value of 100%. The taxonomic position of this organism was further established by using a polyphasic approach. Strain YIM M11385T grew optimally at 28°C, pH 7.0 and in the presence of 0–5% (w/v) NaCl. The 16S rRNA gene sequence similarity between strain YIM M11385T and Marininema mesophilum SCSIO 10219T was 98.3%. Strain YIM M11385T exhibited a quinone system with only MK-7, the polar lipid profile included diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as major components, and the major fatty acids were anteiso-C15:0, iso-C15:0, anteiso-C17:0 and iso-C16:0. The level of DNA–DNA relatedness between strain YIM M11385T and Marininema mesophilum SCSIO 10219T was 59.36%. On the basis of genotypic and phenotypic data, it is apparent that strain YIM M11385T represents a novel species of the genus Marininema, for which the name Marininema halotolerans sp. nov. is proposed. The type strain is YIM M11385T (=CCTCC AB 2012052=DSM 45789T). In addition, we propose that the description of the genus Marininema should be further emended based on the results of the present study.

The genus Marininema, belonging to the family Thermoactinomycetaceae, was recently proposed by Li et al. (2012). At the time of writing, the genus comprises only one species with a validly published name, Marininema mesophilum. This species is Gram-stain-positive and does not produce aerial mycelia. During research on marine microbial resources, strain YIM M11385T was shown to represent a potential novel member of the family Thermoactinomycetaceae. On the basis of its 16S rRNA gene sequence, this isolate fell phylogenetically within the family Thermoactinomycetaceae clustered with M. mesophilum SCSIO10219T. In order to determine the phylogenetic and taxonomic relationship of strains YIM M11385T and M. mesophilum SCSIO10219T, these two strains were selected for the determination of their phenotypic and genotypic characteristics.

Strain YIM M11385T was isolated from a sediment sample from South Bay, Little Andaman Island, India, with a salinity of 35 p.p.m., pH 8.5, by the serial dilution technique using an agar medium which contained (per litre): soluble starch 10.0 g; arginine 5.0 g; (NH4)2SO4 2.64 g; KH2PO4 2.38 g; K2HPO4 5.65 g; MgSO4·7H2O 1.0 g; CuSO4·5H2O 0.0064 g; FeSO4·7H2O 0.0011 g; MnCl2·4H2O 0.0079 g; ZnSO4·7H2O 0.0015 g; sea salt

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM M11385T is KC684488.

Two supplementary figures are available with the online version of this paper.
30 g and agar 15.0 g (pH 7.2), supplemented with nalidixic acid (25 mg l\(^{-1}\)) and cycloheximide (50 mg l\(^{-1}\)), which were sterilized by filter membrane before being added to the medium to suppress growth of Gram-stain-negative bacteria and fungi. Incubation for isolation was performed at 28 °C for 25 days. Colonies were selected and pure cultures were obtained by repeated streaking on medium YIM 38 \(\text{containing, per litre: malt extract 5 g, yeast extract 4 g, glucose 4 g, trace salt 1 ml, vitamin mixture (containing 0.5 mg each of thiamine–HCl, riboflavin, niacin, pyridoxine–HCl, inositol, calcium pantothenate and p-aminobenzoic acid and 0.25 mg biotin), agar 15 g, pH 7.2} \) (Jiang et al., 2007) supplemented with 2.5 % (w/v) sea salt at 28 °C. The purified strain YIM M11385\(^{T}\) was maintained on medium YIM 38 slants at 4 °C and as glycerol suspensions (20 %, v/v) at 280 °C.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Li et al. (2007). The resulting 16S rRNA gene sequence of strain YIM M11385\(^{T}\) was aligned with available corresponding sequences of type strains of members of the family *Thermoactinomycetaceae* retrieved from the DDBJ/EMBL/GenBank databases by using the BLAST program and the EzTaxon server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012) to determine an approximate phylogenetic affiliation. Multiple alignments with sequences of the most closely related bacteria were performed by using the CLUSTAL_X 1.8 program (Thompson et al., 1997). The phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms by using the software packages MEGA version 4.0 (Tamura et al., 2007) and PHYLIP version 3.6 (Felsenstein, 2002). Evolutionary distance matrices (distance options according to Kimura’s two-parameter model) were generated as described by Kimura (1980) and the tree topology was assessed by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Genomic DNA for analysis of G+C content was extracted as described by Marmur (1961), and the G+C content of the DNA was determined by HPLC method (Mesbah et al., 1989). Levels of DNA–DNA relatedness of strain YIM M11385\(^{T}\) and its closely related phylogenetic neighbour *M. mesophilum* SCSIO 10219\(^{T}\) were determined according to the method described by Ezaki et al. (1989). The two DNAs for hybridization were labelled while the other was immobilized, and the reciprocal experiments were performed. Six replications for hybridization were performed for each sample and the highest and lowest values in each

**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain YIM M11385\(^{T}\) (1549 bp) and members of the family *Thermoactinomycetaceae*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding branches were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. *Bacillus subtilis* DSM 10\(^{T}\) was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.
sample were excluded. The results of DNA–DNA hybridizations were taken from the rest data.

In the neighbour-joining phylgetic tree, the novel isolate YIM M11385<sup>T</sup> clustered with _M. mesophilum_ SCSIO10219<sup>T</sup> (Fig. 1). This demonstrated that the novel strain belongs to the genus _Marininema_, supported by a bootstrap value of 100%. This relationship was also supported by the trees generated with the maximum-likelihood and maximum-parsimony methods. Although strain YIM M11385<sup>T</sup> shared 98.3% 16S rRNA gene sequence similarity with _M. mesophilum_ SCSIO10219<sup>T</sup>, the level of DNA–DNA relatedness between the two strains was 59.36%, which is below the 70% cut-off point recommended for the delineation of genomic species (Wayne _et al._, 1987; Stackebrandt & Goebel, 1994). The genomic G+C content of the DNA of strain YIM M11385<sup>T</sup> was 46.64 mol%.

Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH lysis test method (Cerny, 1978). Cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium (Leifson, 1960). Cultural characteristics of strain YIM M11385<sup>T</sup> were observed on ISP media 1–5 (Shirling & Gottlieb, 1966), Czapek's agar (Waksman, 1967), trypticase soy agar (TSA) and nutrient agar prepared as described by Dong & Cai (2001) for 7, 14 and 21 days at 28 °C. The colony colours were determined with colour chips from the Inter-Society Color Council–National Bureau of Standards (ISCC–NBS) colour charts (Kelly, 1964) Cell morphology of strain YIM M11385<sup>T</sup> grown on medium YIM 38 for 14 days was examined by light microscopy (BH 2; Olympus) and scanning electron microscopy (Quanta 200; FEI).

Growth at different temperatures (0, 5, 10, 15, 20, 25, 28, 37, 40, 42, 45, 50 and 55 °C) and tolerance of NaCl (0, 1, 2, 3, 4, 5, 7, 10, 12 and 15, w/v) was tested on nutrient agar medium as basal medium by incubating the cultures for 4 weeks. The pH range for growth (pH 4, 5, 6, 7, 8, 9, 10 and 11, using the buffer system described by Xu _et al._, 2005) was examined at 28 °C for 4 weeks by culturing the strain in trypticase soy broth (TSB). Catalase activity was detected by the production of bubbles after the addition of a drop of 3% (v/v) H<sub>2</sub>O<sub>2</sub>; oxidase activity was determined by the oxidation of tetramethyl p-phenylenediamine; gelatin hydrolysis was determined by incubating strain YIM M11385<sup>T</sup> at 28 °C for 3 weeks on peptone-gelatin medium (5 g peptone and 120 g gelatin per litre distilled water, pH 7.2–7.4). Starch hydrolysis, nitrate reduction and urease activities were assessed as described by Smibert & Krieg (1994). Milk coagulation and peptonization were determined by using the KOH lysis test and was confirmed by using the KOH lysis test. However, negative reactions for oxidase, urease, amylase, gelatin liquefaction, H<sub>2</sub>S production, nitrate reduction, hydrolysis of cellulose, Tween 20, Tween 40 and Tween 80. Other physiological and biochemical results are given in Table 1 and in the species description.

The biomass used for chemotaxonomic analyses was obtained from cultures grown in Bacto trypticase soy broth on a rotary shaker at 28 °C for 10 days. The diaminopimelic acid (DAP) isomer in the cell-wall was determined using TLC as described by Staneck & Roberts (1974). Amino acids and sugars in whole-cell hydrolysates were analysed by precolumn derivatization with o-phthalaldehyde (OPA) and 1-phenyl-3-methyl-5-pyrazolone (PMP) respectively by HPLC (model 1100; Agilent) according to the standard methods (Hasegawa _et al._, 1983; Tang _et al._, 2009). Menaquinones were extracted according to the protocol of Collins _et al._ (1977) and separated by HPLC (Tamaoka _et al._, 1983). Polar lipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin _et al._, 1979; Collins & Jones, 1980). Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. The fatty acid methyl esters were analysed by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database: TSBA6).

The cell-wall of the novel isolate YIM M11385<sup>T</sup> contained LL-diaminopimelic acid, and the whole-cell hydrolysates contained glutamic acid, alanine, lysine and glycine as major amino acids, with mannose, ribose, rhamnose and glucose as major sugars. The strain contained only menaquinone, MK-7. Diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and five unknown phospholipids were detected (Fig. S2). Major fatty acids (>10%) of strain YIM M11385<sup>T</sup> were saturated branched-chain fatty acids: anteiso-C<sub>15</sub>:0 (42.14%), iso-C<sub>16</sub>:0 (12.82%), anteiso-C<sub>17</sub>:0 (10.26%) and iso-C<sub>16</sub>:0 (14.31%). Detailed results of chemotaxonomic tests are given in Table 1 and in the species description.

Besides the phylogenetic analysis based on 16S rRNA gene sequences, the chemotaxonomic properties (whole-cell sugars, predominant menaquinone and main fatty acid
type) of strains YIM M11385T and SCSIO 10219T were very similar to each other, both formed yellow–white colonies while aerial mycelia were not produced on any of tested media. It is clear that strain YIM M11385T belongs to the genus *Marininema*. However, strain YIM M11385T can be easily distinguished from the sole species of the genus with a validly published name, *M. mesophilum* SCSIO 10219T, by using a combination of the phenotypic and chemotaxonomic properties. In contrast to SCSIO 10219T, strain YIM M11385T grows well on ISP 2 and ISP 3 and weakly on ISP 5, while strain SCSIO 10219T does not grow on the above three media. Strain YIM M11385T can be differentiated from *M. mesophilum* SCSIO 10219T based on the absence of phosphatidylmethylethanolamine (PME) in the polar lipid profile. The types of major fatty acids (>1%) were significant in distinguishing strain YIM 11385T from *M. mesophilum* SCSIO 10219T, except for anteiso-C15:0; strain YIM M11385T contained iso-C15:0, iso-C16:0 and C16:1 w7c alcohol, while SCSIO 10219T contained iso-C15:0. The level (59.36%) of DNA–DNA relatedness between the two strains confirmed this distinct species status.

It can be concluded from the phenotypic, genotypic and chemotaxonomic data that strain YIM M11385T represents a novel species of the genus *Marininema*, for which the name *Marininema halotolerans* sp. nov. is proposed. In addition, we propose that the description of the genus *Marininema* should be further emended based on the results of the present study.

**Emended description of the genus *Marininema* Li et al. 2012**

The description of the genus *Marininema* is as given by Li et al. (2012), with the following amendment: phosphatidylmethylethanolamine (PME) may be present.

**Description of *Marininema halotolerans* sp. nov.**

*Marininema halotolerans* (ha.lo.to’le.rans. Gr. n. hals haloes salt; L. pres. part. tolerans tolerating, enduring; N.L. part. adj. halotolerans salt-tolerating).

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**Table 1. Differential phenotypic properties between strain YIM M11385T and *M. mesophilum* SCSIO 10219T**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature for growth (°C)</td>
<td>25–42</td>
<td>25–35</td>
</tr>
<tr>
<td>Growth on medium:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ISP3</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ISP5</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of Tween 20</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization as sole carbon or nitrogen source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Lysine</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DL-Sodium malate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Xylitol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Major cellular fatty acids (&gt;1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>1.35</td>
<td>0.78</td>
</tr>
<tr>
<td>C16:0</td>
<td>2.18</td>
<td>1.02</td>
</tr>
<tr>
<td>iso-C14:0</td>
<td>6.54</td>
<td>3.65</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>12.82</td>
<td>33.73</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>14.31</td>
<td>4.68</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>3.96</td>
<td>5.58</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>42.14</td>
<td>44.17</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>10.26</td>
<td>5.24</td>
</tr>
<tr>
<td>C16:1ω7c alcohol</td>
<td>2.77</td>
<td>0.06</td>
</tr>
<tr>
<td>Polar lipid (s)</td>
<td>DPG, PG, PE</td>
<td>DPG, PG, PE, PME</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>46.64</td>
<td>46.5</td>
</tr>
</tbody>
</table>
An aerobic, Gram-stain-positive bacterium that forms yellow–white colonies. The strain has abundant filamentous substrate mycelia and endospores, but no aerial mycelia are produced. Grows well on nutrient agar, ISP media 2 and 3 and TSA. Grows weakly on ISP medium 5 and no growth occurs on ISP medium 4 and Czapek’s agar. Growth occurs at 25–42 °C, pH 6.0–8.0 and 0–10 % NaCl (w/v). The optimal pH and temperature value for growth are pH 7.0 and 28 °C, respectively. Positive for catalase, weakly positive for milk coagulation and peptization. Negative for anylase, gelatin liquefaction, H₂S production, nitrate reduction, oxidase, urease, hydrolysis of cellulose, Tween 20, Tween 40 and Tween 80. Can use alanine, arginine, asparagine, glutamate, glycine, histidine, threonine and valine as sole nitrogen sources, but lysine, methionine, phenylalanine, tryptophan and tyrosine are not used; utilizes celllobiose, DL-sodium malate, trehalose and xylitol as sole carbon sources; D-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, D-ribose, D-xyllose, glycerol, inositol, lactose, L-rhamnose, maltose, raffinose, sodium acetate, sodium pyruvate, sorbitol and sucrose are not utilized. The cell wall contains D(-)-lactobionic acid as the diaminoc acid. Diagnostic whole-cell sugars are mannose, ribose, rhamnose and glucose. MK-7 is the predominant unknown phospholipids. Major fatty acids are anteiso-C₁⁵:₀, iso-C₁₅:₀, anteiso-C₁₇:₀ and iso-C₁₆:₀.

The type strain is YIM M11385T (=CCTCC AB 2012052T = DSM 45789T), isolated from a sediment sample collected from South Bay, Little Andaman Island, India. The DNA G+C content of the type strain is 46.64 mol%.

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References


