Dictyobacter aurantiacus gen. nov., sp. nov., a member of the family Ktedonobacteraceae, isolated from soil, and emended description of the genus Thermosporothrix

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Abstract

A mesophilic, Gram-stain-positive, spore-forming bacterium that formed branched mycelia was isolated from paddy soil in Gunung Salak (Mount Salak), West Java, Indonesia. This strain, designated S-27T, grew at temperatures between 20 and 37°C; the optimum growth temperature was 25 to 30°C, and no growth was observed at 15 or 45°C. The pH range for growth was pH 3.5 to 8.6; the optimum pH was 6.0, and no growth was observed at pH 3.0 or 9.2. Strain S-27T was able to hydrolyse polysaccharides such as starch, cellulose and xylan. The G+C content of the DNA of strain S-27T was 55.7 mol%. The major fatty acids were iso-C17:0 and 16:1 2-0H, and the major menaquinone was MK-9 (H2). The cell wall of strain S-27T contained d-glutamic acid, glycine, L-alanine, d-alanine, L-ornithine and 8-alanine in a molar ratio of 1.0 : 1.6 : 1.4 : 0.6 : 0.9 : 1.1. The polar lipids consisted of phosphatidylglycerol, phosphatidylinositol and two glycolipids. The major cell-wall sugar was arabinose. Detailed phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S-27T belongs to the order Ktedonobacterales and is most closely related to Ktedonobacter racemifer SOSP1-21T (89.6% sequence identity). On the basis of its chemotaxonomic and phenotypic features and phylogenetic position, we concluded that strain S-27T represents a novel genus and species, for which we propose the name Dictyobacter aurantiacus gen. nov., sp. nov. The type strain of Dictyobacter aurantiacus is strain S-27T (=NBRC 109595T=InaCC B312T). Emendation of the description of the genus Thermosporothrix is also provided.

The class Ktedonobacteria, members of which have an actinomycete-like morphology, was first proposed by Cavaletti et al. [1] and was later moved from an unclassified phylum to the phylum Chloroflexi [2]. The class is currently divided into three families, Ktedonobacteraceae [1] and Thermosporothricaceae [2], in the order Ktedonobacterales, and Thermogemmatisporaceae [3], in the order Thermogemmatisporales. The family Ktedonobacteraceae currently consists of only one species with a validly published name: Ktedonobacter racemifer, which was isolated from soil under black locust wood in Italy [1]. The family Thermosporothricaceae contains two species: Thermosporothrix hazakensis, isolated from compost [2], and Thermosporothrix narukonensis, isolated from geothermal soil [4]. The family Thermogemmatisporaceae contains three species: Thermogemmatispora onikobensis and Thermogemmatispora foliorum (isolated from fallen leaves deposited on geothermal soil [3]), and Thermogemmatispora carboxidivorans (isolated from a geothermally heated biofilm [5]). In the order Ktedonobacterales there are 15 cultured isolates that have not yet been characterized taxonomically: the SOP series (strains SOP1-0, 1–1, 1–9, 1–30, 1–52, 1–79, 1–63, 1–85, 1–142 and 1–165), from the same source as K. racemifer SOP1-21T [1], and strains of the genus Thermosporothrix (FA, FB, F7, MYC and I-1), from the same source as

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Keywords: Ktedonobacteria.

Abbreviations: FDLA, N-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S-27T is LC210880.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.
**Thermosporothrix narukonensis** F4<sup>T</sup> [4]. From an examination of 16S rRNA gene sequence identities, Cavaletti et al. [1] deduced that the unclassified SOSP series represent at least four families or higher-rank taxa. In the order Thermogemmatisporales there are six unclassified isolates, namely strains P-352, P-359, T104 and T81 of the genus *Thermogemmatispora* from geothermal soils [6] and strains BPP55 and PM6 of the genus *Thermogemmatispora* from the same source as *Thermogemmatispora carboxidivorans* PM5<sup>T</sup> [5].

All isolates in the class *Ktedonobacteria* are Gram-stain-positive and aerobic and form branched mycelia with spores. Sporulation of all the type strains occurs via the formation of multiple exospores per cell by budding [2, 4, 5, 7]. Although *K. racemifer* SOSP1-21<sup>T</sup> is a mesophilic heterotroph [1], the other species in the class are thermophilic heterotrophs. Their major menaquinone is MK-9 (H<sub>2</sub>), and their cell walls contain at least ornithine, alanine, glutamic acid, serine and glycine. The G+C content of their genomic DNA ranges from 53.9 to 60.2 mol%.

We isolated a *Ktedonobacteria*-like strain from paddy soil in Indonesia. To determine the taxonomic position of the strain, we examined its morphological, physiological, biochemical and chemotaxonomic characteristics and 16S rRNA gene sequences. The results indicated that the strain should be placed in a novel species of a new genus belonging to the family *Ktedonobacteraceae*.

We sampled soil (0 to 10 cm depth) in a waterlogged rice paddy field on the mountainside of Gunung Salak (Mount Salak) in West Java, Indonesia (6° 47’ 5.5” S 106° 42’ 8.4” E; 600 m above sea level), in December 2011. The sample was placed in a black plastic bag, transported to the laboratory and stored at 4°C until use.

We used R2A broth (Wako Pure Chemical Industries) medium (1 : 10 dilution) solidified with gellan gum in the isolation plates. The pH of the medium was about pH 7.0, without adjustment. A soil suspension (100 µl) including approximately 10<sup>3</sup> g of soil was spread on each plate, and the plates were incubated at 30°C for 10 days. One firm, pale-orange colony that seemed to have the morphological features of actinomycetes, which form vegetative mycelia, was identified. The colony was picked and serially diluted in saline solution, and the dilutions were plated on isolation plates. A single colony was purified by being plated three times. The colonies were restreaked, and stock cultures were prepared by inoculating agar slants with colonies from the second plates. The new isolate was designated strain S-27<sup>T</sup>.

The morphology of cells grown on International Streptomyces Project (ISP) 3 agar [8] was examined by scanning electron microscopy (SEM) (SU8000; Hitachi). Suitable agar blocks including colonies of the strain grown at 30°C for 8 weeks on the medium were analysed by SEM as previously described [2].

SEM observation showed that the novel strain formed vegetative and thin aerial mycelium on ISP3. Although spores were observed, they were rare and did not form clusters (Fig. 1a–c). They were spherical, 0.8 to 1.0 µm long and wide, and formed on aerial mycelium (Fig. 1c). We did not observe thick aerial mycelium or budding spores such as those found in other ktedonobacterial type strains [2, 4, 5, 7].

To determine the effects of pH, temperature and NaCl on growth, we cultured strain S-27<sup>T</sup> in R2A liquid media (1 : 10 dilution) for 2 weeks. Tests were performed at 15 to 45°C (pH 6.0), pH 3.0 to 9.2 (30°C) or with 0 to 4% NaCl (30°C, pH 6.0). The pH was adjusted to various values with HCl and NaOH at room temperature. Assimilation of various sole carbon sources (L-arabinose, D-xylose, D-glucose, D-fructose, sucrose, L-rhamnose, raffinose, inositol, D-mannitol and gellan gum) at a concentration of 0.5% (w/v) was assessed on Pridham–Gottlieb agar [8]. Hydrolysis of microcrystalline cellulose (Avicel), carboxymethylcellulose, xylan, chitin, starch and casein was evaluated on R2A agar (1 : 10 dilution) supplemented with each substrate at a concentration of 0.5% (w/v) at 30°C for 14 days. The presence of a clear zone around the colony indicated hydrolysis. Catalase activity was determined by assaying bubble production in a 3% hydrogen peroxide solution. Gram staining was performed by using the modified Hucker method [9]. Each experiment was done in triplicate.

Strain S-27<sup>T</sup> was Gram-stain-positive and had positive catalase activity. It grew at temperatures between 20 and 37°C, with an optimum growth temperature of 25 to 30°C.acid, serine and glycine. The G+C content of their genomic DNA ranges from 53.9 to 60.2 mol%.

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Strain S-27<sup>T</sup> was Gram-stain-positive and had positive catalase activity. It grew at temperatures between 20 and 37°C, with an optimum growth temperature of 25 to 30°C and no growth at 15 or 45°C. It was able to grow within a pH range of pH 3.5 to 8.6; the pH for optimum growth was pH 6.0, and no growth was observed at pH 3.0 or 9.2. It did not require NaCl for growth, and grew with <2% NaCl. It was able to assimilate L-rhamnose and gellan gum and hydrolyse Avicel, carboxymethylcellulose, xylan, starch and casein, but no growth was observed on chitin. This may mean that chitin prevented strain S-27<sup>T</sup> from growing. The results of the

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**Fig. 1.** Scanning electron micrographs of cultures of strain S-27<sup>T</sup>. Bars, (a) 500 µm; (b) 5 µm; (c) 5 µm. Arrow in (c) indicates a spore.
phenotypic examination of strain S-27<sup>T</sup> are given below in the species description and in Table 1.

Biomass for chemotaxonomic studies was obtained by culturing strain S-27<sup>T</sup> in shake flasks of liquid NBRC medium 231 (0.1 % yeast extract, 0.1 % beef extract, 0.2 % NZ-Amine type A and 1.0 % maltose) for 7 days at 28 °C and 100 r.p.m. Cellular fatty acid methyl esters were prepared and analysed by using the protocol of the MIDI Sherlock Microbial Identification System [10] and gas chromatography (GC; model 6890 N; Agilent Technologies) with the Sherlock software (v. 6.2) and the TSBA6 database (MIDI). Isooprenoid quinones were analysed as described by Hamada et al. [11].

The DNA G+C content was determined by the method of Tamaoka and Komagata [12] using HPLC (model LC-10A; Shimadzu) with a Cosmosil 5C18-MS-II column (100<sup>C</sup>4.6 mm internal diameter; Nacalai Tesque). Acetoni-
trile/water containing 0.2 M ammonium phosphate was used as the mobile phase (5 % acetonitrile, 15 min) with UV detection at 270 nm [10].

Cell walls of strains S-27<sup>T</sup> and SK20-1<sup>T</sup> were prepared by using the methods described by Schleifer and Kandler [13]; the amino acids in the cell-wall hydrolysate were identified by TLC [14] and their phenylthiocarbamoyl derivatives were analysed as described by Hamada et al. [11].

Table 1. Characteristics of strain S-27<sup>T</sup>, <i>K. racemifer</i> SOSP1-21<sup>T</sup>, <i>Thermosporothrix hazakensis</i> SK20-1<sup>T</sup> and <i>Thermosporothrix narukonensis</i> F4<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain S-27&lt;sup&gt;T&lt;/sup&gt;</th>
<th>&lt;i&gt;K. racemifer&lt;/i&gt; SOSP1-21&lt;sup&gt;T&lt;/sup&gt;</th>
<th>&lt;i&gt;Thermosporothrix hazakensis&lt;/i&gt; SK20-1&lt;sup&gt;T&lt;/sup&gt;</th>
<th>&lt;i&gt;Thermosporothrix narukonensis&lt;/i&gt; F4&lt;sup&gt;T&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Gram stain</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td>Colony colour on 1/10 R2A agar</td>
<td>Orange</td>
<td>Cream to orange</td>
<td>White (this study)</td>
<td>White (this study)</td>
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<tr>
<td>DNA G+C content (mol %)</td>
<td>55.7</td>
<td>53.9</td>
<td>54.0</td>
<td>52.5</td>
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<tr>
<td>Growth in the presence of:</td>
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<tr>
<td>1 % NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>2 % NaCl</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Temperature for growth (°C)</td>
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<tr>
<td>Range</td>
<td>20–37</td>
<td>17–40</td>
<td>31–58</td>
<td>30–60</td>
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<tr>
<td>Optimum</td>
<td>25–30</td>
<td>28–33</td>
<td>50</td>
<td>50</td>
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<td>pH for growth</td>
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<tr>
<td>Range</td>
<td>3.5–8.6</td>
<td>4.2–7.2</td>
<td>5.4–8.7</td>
<td>4.9–9.5</td>
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<tr>
<td>Optimum</td>
<td>6.0</td>
<td>6.0</td>
<td>7.0</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Avicel</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Xylan</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Utilization of:</td>
<td></td>
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<tr>
<td>α-L-Arabinose</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Xylose</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Glucose</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Fructose</td>
<td>–</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>Sucrose</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Rhamnose</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>+</td>
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<td>Raffinose</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Inositol</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<td>D-Mannitol</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>–</td>
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<td>Major cellular fatty acids</td>
<td>iso-C&lt;sub&gt;17&lt;/sub&gt;:0, C&lt;sub&gt;16&lt;/sub&gt;:1 2-OH</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;:1 2-OH, iso-C&lt;sub&gt;17&lt;/sub&gt;:0</td>
<td>iso-C&lt;sub&gt;17&lt;/sub&gt;:0</td>
<td>iso-C&lt;sub&gt;17&lt;/sub&gt;:0</td>
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<tr>
<td>Cell-wall amino acids</td>
<td>Glu, Gly, Ala, β-Ala, Orn</td>
<td>Glu, Ser, Gly, Ala, Orn</td>
<td>Glu, Ser, Gly, Ala, β-Ala, Orn</td>
<td>Glu, Ser, Gly, Ala, β-Ala, Orn</td>
</tr>
<tr>
<td>Cell-wall sugars</td>
<td>Ara</td>
<td>Ara, Gal</td>
<td>Man</td>
<td>Man</td>
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<tr>
<td>Polar lipids</td>
<td>PI, PG</td>
<td>PI, PG, DPG</td>
<td>PI, PIM, PG, DPG</td>
<td>PI, PG, DPG</td>
</tr>
</tbody>
</table>
Cell-wall sugars were determined as alditol acetate derivatives on an Agilent 5975C GC-MS instrument equipped with a DB-1 (60 m × 0.25 mm × 0.50 µm) capillary column. GC was programmed with a 4 min solvent delay and a flow rate of 1.5 ml min⁻¹, with an initial hold at 160 °C for 2 min, 20 °C min⁻¹ ramp to 200 °C and hold for 5 min, and 20 °C min⁻¹ ramp to 245 °C and hold for 12 min. Peaks were identified from the mass profiles and retention times of standards. Phospholipids of strain S-27ᵀ were extracted and identified by two-dimensional TLC, followed by spraying with appropriate detection reagents according to the method of Tindall [17, 18].

The DNA G+C content of strain S-27ᵀ was 55.7 mol%, as determined by HPLC analysis. The predominant menaquinone in strain S-27ᵀ was MK-9 (H₂). The major fatty acids were iso-C₁₇:₀ (34.0 %) and C₁₆:₁ 2-OH (32.3 %; Table 1). The cellular fatty acid compositions are shown in Table S1 (available in the online Supplementary Material). The polar lipids consisted of phosphatidylglycerol, phosphatidylinositol and two unknown glycolipids (Fig. S1). We previously detected an unknown amino acid in the peptidoglycan amino acids of Thermosporothrix hazakensis SK20-1ᵀ and Thermosporothrix narukonensis F4ᵀ [4]. Here, we detected the same unknown amino acid in strain S-27ᵀ and in strain SK20-1ᵀ and identified it as β-alanine by analysing FDLA derivatives using LC-MS (Fig. S2) on the basis of the comparison with authentic β-alanine. The peptidoglycan amino acids of Thermosporothrix hazakensis SK20-1ᵀ contained d-glutamic acid, L-serine, glycine, L-alanine, D-alanine, L-ornithine and β-alanine in a molar ratio of 1.0 : 1.0 : 1.0 : 1.2 : 1.1 : 0.9 : 1.1. Those of S-27ᵀ contained d-glutamic acid, glycine, L-alanine, D-alanine, L-ornithine and β-alanine in a molar ratio of 1.0 : 1.6 : 1.4 : 0.6 : 0.9 : 1.1 (Fig. S2). The major cell-wall sugar of strain S-27ᵀ was arabinose.

Crude DNA was extracted by heating a single colony in 100 µl 0.05 M NaOH at 95 °C for 15 min. After centrifugation of the mixture at 10 000 g for 5 min, the supernatant was diluted 10-fold and used as a template for PCR. The almost full-length 16S rRNA gene was amplified by PCR with the primers 27F and 1492R [19] with ExTaq DNA polymerase (Takara) on a GeneAmp PCR system 9800 thermal cycler (Applied Biosystems, Life Technologies), under the following conditions: initial denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min; the final extension was extended for 4 min. The products amplified were purified and sequenced commercially by Dragon Genomics Centre (Takara Bio, Shiga, Japan). Sequencing was done with the automatic Applied Biosystems 3730xl DNA Analyzer (Life Technologies) and the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies).

The 16S rRNA gene sequence of strain S-27ᵀ was compared with sequences obtained from GenBank. Multiple alignments of the sequences were performed with CLUSTAL W (v. 1.83) [20], and gaps and unidentified base positions were edited in the BioEdit software [21]. Phylogenetic trees were reconstructed by using the maximum-likelihood method [22] and the neighbour-joining method [23] in MEGA v. 7.0 software [24], with bootstrap values based on 100 and 1000 replications, respectively [25]. Evolutionary distances were computed by using the Kimura two-parameter method [26].

The results of 16S rRNA gene sequencing showed that the closest phylogenetic relative of strain S-27ᵀ with a validly published name was K. racemifer SOSP1-21ᵀ, belonging to the family Kedonobacteraceae, with 89.6 % sequence identity. Subsequently, the results showed that strain S-27ᵀ was similar to Thermosporothrix hazakensis SK20-1ᵀ (88.0 % identity) and Thermosporothrix narukonensis F4ᵀ (88.7 % identity) in the family Thermospororthicaceae. Species of the family Thermogemmatisporaceae of the order Thermogemmatisporales with validly published names were related more distantly, showing 16S rRNA gene sequence identities of no more than 83 % (Thermogemmatispora onikobensis ONI-1ᵀ, 83.0 %; Thermogemmatispora foliorum ONI-5ᵀ, 82.8 %; Thermogemmatispora carboxidivorans PMSᵀ, 82.4 %). Moreover, the clade of the order Kedonobacterales supported by a bootstrap value of 100 % included strain S-27ᵀ, indicating that the strain belonged to the order Kedonobacterales. Strain S-27ᵀ had high 16S rRNA gene sequence identities with those of the unclassified strains SOSP1-79 (97.9 %) and SOSP 1-9 (96.0 %). The three formed a clade supported by a bootstrap value of 100 %. The levels of pairwise identity between members of the clade and the other SOSP strains, including K. racemifer SOSP1-21ᵀ, were around 88.0 to 91.6 %. Cavalletti et al. [1] indicated that the SOSP strains represented at least five families (or higher-rank taxa) consisting of clades ‘GER 1’ (five SOSP strains, including K. racemifer SOSP1-21ᵀ), ‘GER 2’ (SOSP1-1 and 1-165), ‘GER 3’ (SOSP1-79 and 1-9; to which strain S-27ᵀ belongs), SOSP1-63 and SOSP1-142 (Fig. 2). These facts suggest that GER3 (strain S-27ᵀ, SOSP1-79 and SOSP1-9) may be separated at the family level from other strains in the order Kedonobacterales. However, since this order has very few species with validly published names, the difference in phenotypic traits among families is not clear. Therefore, at this time we do not propose GER3 as a new family.

Strain S-27ᵀ can be differentiated from other species of the order Kedonobacterales by the following chemotaxonomic characteristics. Strain S-27ᵀ does not have serine as a peptidoglycan amino acid and does not have diphosphatidylglycerol as a polar lipid (Table 1). Strain S-27ᵀ and species of the family Thermospororthicaceae have β-alanine, although it was not detected in K. racemifer SOSP1-21ᵀ by Cavalletti et al. [1].

The results of morphological, physiological and chemotaxonomic investigations, together with the phylogenetic analysis, reveal that strain S-27ᵀ is clearly distinguishable from K. racemifer and species of the genus Thermosporothrix at the genus level. Therefore, we propose the name Dictyobacter aurantiacus gen. nov., sp. nov., to accommodate strain S-27ᵀ.
DESCRIPTION OF DICTYOBACTER GEN. NOV.

Dictyobacter (Dic.ty.o.bac'ter. Gr. n. dictyon net; N.L. masc. n. bacter rod, bacterium; N.L. masc. n. Dictyobacter net-like bacterium).

Branched-mycelium-forming bacteria. Gram-stain-positive. Mesophilic, aerobic heterotrophs. Contain D-glutamic acid, glycine, l-alanine, d-alanine, β-alanine and l-ornithine as peptidoglycan amino acids. The cell-wall sugar is arabinose. iso-C17:0 and C16:1 2-OH are the major fatty acids, and MK-9 (H2) is the major menaquinone. The
G+C content of the genomic DNA of the type strain of the type species is 55.7 mol%. The type species is *Dictyobacter aurantiacus*.

**DESCRIPTION OF DICTYOBACTER AURANTIACUS SP. NOV.**

*Dictyobacter aurantiacus* (au.ran.ti’a.cus N.L. fem. adj. aur-antiacus orange-coloured).

Displays the following properties in addition to those described for the genus. Produces spores. The non-motile spores are spherical and are 0.8 to 1.0 mm long and wide. Growth occurs at 20 to 37 °C (optimum 25 to 30 °C) and pH 3.5 to 8.6 (optimum pH 6.0). Does not require NaCl; growth is observed with 2% NaCl. Casein, starch, xylan and cellulose are hydrolysed. Catalase-positive. L-Rhamnose is utilized as a sole carbon source.

The type strain is S-27T (=NBRC 109595T=InaCC B312T), isolated from paddy soil in Gunung Salak (Mount Salak), West Java, Indonesia.

**EMENDED DESCRIPTION OF THE GENUS THERMOSPOROTHRIX**

The genus *Thermosporothrix* has been previously described and emended by us [2, 4]. According to that emendation, *Thermosporothrix hazakensis* SK20-1T and *Thermosporothrix narukonensis* F4T have an unidentified amino acid among their peptidoglycan amino acids. In this study, we identified this as β-alanine. Therefore, the description of the genus *Thermosporothrix* is as given by Yabe *et al.* [2, 4] but with the following emendations. The peptidoglycan amino acids include D-glutamic acid, glycine, L-alanine, D-alanine, β-alanine and L-ornithine.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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