Thermotunica guangxiensis gen. nov., sp. nov., isolated from mushroom residue compost

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A novel thermophilic actinomycete, designated AG2-7^T, was isolated from mushroom residue compost in Guangxi University, Nanning, China. The strain grew optimally at 45–60 °C, at pH 7.0 and with 0–3.0 % (w/v) NaCl. Vegetative mycelia were branched and whitish to pale yellow without fragmentation. Aerial mycelium was abundant, whitish and differentiated into long chains of spores, with a membranous structure or tunic partially covering the surface of aerial hyphae. The non-motive spores were oval in shape with a ridged surface. Strain AG-27^T contained meso-diaminopimelic acid as the diagnostic diamino acid, and the whole-cell sugars were galactose and ribose. Major fatty acids were iso-C16 : 0 (27.51 %), iso-C17 : 0 (10.47 %) and anteiso-C17 : 0 (12.01 %). MK-9(H4) was the predominant menaquinone. The polar phospholipids were diphosphatidylglycerol, niphyn-drin-positive glycoporphospholid, phosphatidylinositol, phosphatidylinositol mannoside, phosphatidylethanolamine, phosphatidylethanolamine, an unknown phospholipid and unknown glucosamine-containing phospholipoids. The G+C content of the genomic DNA was 63.6 mol%. 16S rRNA gene sequence analysis showed that the organism belonged to the family Pseudonocardiaceae, suborder Pseudonocordinaeae and showed more than 5 % divergence from other members of the family. Based on the phenotypic and phylogenetic data, strain AG2-7^T represents a novel species of a new genus in the family Pseudonocardiaceae, for which the name Thermotunica guangxiensis gen. nov., sp. nov. is proposed. The type strain of the type species is AG2-7^T (= ATCC BAA-2499^T = CGMCC 4.7099^T).

The family Pseudonocardiaceae was established by Embley et al. (1988), and emended by Stackebrandt et al. (1997) based on 16S rRNA gene sequence analysis, and contained the type genus Pseudonocardia (Henssen, 1957) and the genera Actinopolyspora (Gochnauer et al., 1975), Actinosynnema (Hasegawa et al., 1978), Amycolatopsis (Lechevalier et al., 1986), Kibdelosporangium (Shearer et al., 1986), Kutneria (Stackebrandt et al., 1994), Lentzea (Yassin et al., 1995), Saccharomonospora (Nonomura & Ohara, 1971), Saccharopolyspora (Lacey & Goodfellow, 1975), Saccharothrix (Labeleda et al., 1984), Streptotalloteichus (Tomita et al., 1978) and Thermocrispum (Korn-Wendisch et al., 1995). Subsequently, 14 genera have been added to family, Prauserella (Kim & Goodfellow 1999), Actinoalloteichus (Tamura et al., 2000), Crossiella (Labeleda, 2001), Actinomycetospora (Jiang et al., 2008), Allokutneria (Labeleda & Kroppenstedt, 2008), Goodfellowiella (Labeleda et al., 2008), Sciscionella (Tian et al., 2009), Actinophytoidea (Indananda et al., 2010), Alloactinosynnema (Yuan et al., 2010), Halocchinothrix (Tang et al., 2010), Yuhushiella (Mao et al., 2011) and Labeledae (Lee, 2012). Labeleda & Kroppenstedt (2000) proposed the family Actinosymmetaceae to accommodate the genera of Actinokineospora, Actinosymmet, Lentzea and Saccharothrix. However, Labeleda et al. (2011) considered that not enough phylogenetic or chemotaxonomic evidence was available to maintain the family Actinosymmetaceae, and all genera in this family, including the genus Umezawaia proposed by Labeleda & Kroppenstedt (2007), should be moved back to the family Pseudonocardiaceae. Zhi et al. (2009) proposed another new family, Actinopolysporaceae, for the genus Actinopolyspora. At the time of writing, the family Pseudonocardiaceae contains 27 genera.

During a survey of thermophilic microbial diversity, a mycelium-forming actinomycete strain was isolated and assigned to the family Pseudonocardiaceae based on 16S rRNA gene sequence analysis and chemotaxonomic and morphological properties comparisons. A novel species in a new genus of this family is proposed to accommodate the isolated strain.

Strain AG2-7^T was isolated from mushroom residue compost in Guangxi University, Nanning, south-western China using the dilution plate technique with HV medium.
AG2-7T were observed. Other data for physiological and sporangia or naked sporangium-like structures on strain observed by scanning electron microscopy (Fig. 1 a, c). No aerial hyphae, a membranous structure or tunica was surface and were non-motile (Fig. 1 b, c). On the surface incubation for 3–7 days on HV media at 50°C. Morphological characteristics were observed under a light microscope (80i; Nikon) and a scanning electron microscope (VEGA3 SBU; TESCAN) after incubation for 3–7 days on HV media at 50°C. Growth temperature and resistance to NaCl was tested on modified Bennett’s agar at 4, 16, 28, 37, 40, 45, 50, 55, 60, 65 and 70°C and observed after 7 and 14 days. Tolerance to NaCl between 0 and 15% (at intervals of 1%) was read after 3 and 7 days at 50°C. The pH range and the optimum pH for growth were examined on Sauton’s agar with the pH range between pH 4.0 and 11.0 (in intervals of 1.0 pH unit) using the following buffer systems: for pH 4.0–6.0, 0.1 M citric acid/0.1 M sodium citrate; pH 7.0–9.0, 0.1 M Tris/0.1 M HCl; pH 9.5–10.0, 0.1 M NaHCO3/0.1 M Na2CO3 and for pH 11.0, 0.05 M Na2HPO4/0.1 M NaOH. After 3 and 7 days incubation at 50°C, growth was scored as a positive result. Carbon source utilization for growth was examined as described by Shirling & Gottlieb (1966), ISP4 medium without a carbon source was used, because strain AG2-7T could not grow on ISP9 medium. Acid fastness was tested by using the method of Gordon et al. (1974). Oxidase, catalase and esterase activities, nitrate reduction, and degradation of adenine, guanine, hypoxanthine, xanthine, aesculin, L-tyrosine, xylan, casein, gelatin, starch, allantoin and urea were conducted as described by Williams et al. (1983).

Strain AG2-7T grew well on HV medium, Sauton’s agar and Bennett’s agar, exhibited moderate growth on ISP4, ISP6 and potato agar, poor growth on ISP2, ISP3, Czapek’s media and water agar, and no growth on Gause’s asparagine agar, ISP5 or ISP7 (Table S1, available in the online Supplementary Material). The strain could produce blue-violet diffusible pigment on ISP6 media. Substrate mycelia were whitish to pale yellow and extensively branched but non-fragmenting. Aerial mycelium was white and branched, and abundantly produced on HV medium but scarcely on other media. Long chains of spores were observed on aerial hyphae at maturity. Spores were 1 μm in diameter, oval in shape with a ridged surface and were non-motile (Fig. 1 b, c). On the surface of aerial hyphae, a membranous structure or tunica was observed by scanning electron microscopy (Fig. 1 a, c). No sporangia or naked sporangium-like structures on strain AG2-7T were observed. Other data for physiological and biochemical properties are given in the species description.

Biomass for chemotaxonomic studies was obtained by centrifugation and freeze-dried, following culture in Bennett’s broth for 3 days at 50°C. The cell-wall diamino acid of strain AG2-7T was determined from whole-cell hydrolysates as described by Hasegawa et al. (1983). Whole-organism sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). Polar lipids were extracted and examined by two-dimensional TLC and identified according to the method of Minnikin et al. (1984). Menaquinones were extracted and purified using the method of Collins et al. (1987) and separated by HPLC (Kroppenstedt, 1982). Extraction and analysis of mycolic acids followed the procedure described by Minnikin et al. (1980). Determination of the N-acyl type of muramic acid residues followed the procedure described by Uchida et al. (1999). Cellular fatty acid methyl esters were prepared and analysed by GC according to the instructions of the Microbial Identification System (version 2.11; MIDI), using the AEROBE package including the TSBA (version 3.9), CLIN (version 3.9) and MI7H10 (version 3.8) databases for the identification of fatty acids. The DNA G+C content was determined by thermal denaturation (Mandel & Marmur, 1968). Chromosomal DNA for genomic DNA G+C content analysis was extracted as described by Marmur (1961).

Whole-cell hydrolysates of strain AG2-7T contained meso-diaminopimelic acid as the diagnostic cell-wall peptidoglycan.
and the whole-cell sugars were galactose and ribose as diagnostic sugars (cell-wall chemotype IV; Lechevalier & Lechevalier, 1970). The peptidoglycan was the N-acetylated type. No mycolic acids were detected. The polar lipid profile of strain AG2-7T contained diphasphatidylglycerol, phosphatidylglycerol, ninyhdrin-positive glycolipid, phosphatidylglycol, phosphatidylglycerol, phosphatidylmannoside, phosphatidylethanolamine, phosphatidymethylthanolamine, an unknown phospholipid and an unknown glucosamine-containing phospholipid (Fig. S1). The predominant menaquinone of strain AG2-7T was MK-9(H4). The fatty acid profile consisted of major amounts of iso-branched hexadecanoic acids: iso-C16:0 (27.51 %), anteiso-C17:0 (12.01 %) and iso-C17:0 (10.47 %), and the other components were C17:0 (8.99 %), iso-C18:0 (7.39 %), C16:0 (6.55 %), iso-C15:0 (6.03 %), C18:1ω7c (2.17 %), iso-C13:0 (2.06 %), C18:0 (1.97 %), iso-C14:0 (1.52 %), C17:1ω8c (1.95 %) and C16:1ω7c/C16:1ω6c (3.11 %). The DNA G+C content of strain AG2-7T was 63.6 mol%.

Genomic DNA for PCR amplification was prepared using the method described by Li et al. (2007) and the 16S rRNA gene was amplified using primers 27f (5'-GAGTTTGATCCTGGGCTCAG-3') and 1525r (5'-AGAAGGAGGTGATCCAGCC-3') as described by Rainey et al. (1996). The PCR product was purified with a gel extraction kit (BIOMIGA) and sequenced on an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequence kit (BIOMIGA) and sequenced on an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequence was aligned and compared with available sequences in the GenBank/EMBL/DDBJ database using BLAST searches (Altschul et al., 1997) and analysed using the EzTaxon-e (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012), revealing that the isolate was a member of the suborder Pseudonocardinae. Multiple alignments with sequences from the type species of all recognized genera in the suborder Pseudonocardinae were carried out using CLUSTAL X (Thompson et al., 1997). Phylogenetic trees were reconstructed with representative sequences using neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A neighbour-joining tree was reconstructed by using the MEGA version 5.0 software package (Tamura et al., 2011) and calculated by using distances corrected according to Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed by using 1000 neighbour-joining datasets (Felsenstein, 1985).

The 16S rRNA gene sequence of strain AG2-7T (1446 nt) showed highest similarity to members of genera in the suborder Pseudonocardinae: Kutzneria (93.0–94.9 %), Streptoalloteichus (94.0–94.5 %), Actinokineospora (93.6–94.3 %) and Labedaea (94.3 %). The 16S rRNA gene sequence similarity values between strain AG2-7T and type strains of other members of the suborder Pseudonocardinae were less than 94.0 %. In the neighbour-joining tree (Fig. 2), strain AG2-7T was phylogenetically located in the clade of the suborder Pseudonocardinae. Strain AG2-7T formed a monophyletic clade supported by a high bootstrap value of 81 % and all the treeing algorithms applied. The monophyletic position of strain AG2-7T was between the genus Labedaea and a large cluster including the genera Kutzneria, Streptoalloteichus and Actinokineospora.

The morphological and chemotaxonomic characteristics of strain AG2-7T that distinguish it from closely related genera of the family Pseudonocardinae are shown in Table 1. Strain AG2-7T did not produce sporangium-like structures, which enabled it to be morphologically differentiated from members of the genera Kutzneria and Streptoalloteichus, and the oval spores produced are different from those of the genera Labedaea and Actinokineospora. Furthermore, a membranous structure or tunica was formed on the surface of aerial hyphae, indicating that the new genus differs from other phylogenetically related genera. Strain AG2-7T is a thermophilic actinobacterium that can grow at 65 °C, while no members of the four related genera can grow at that temperature. Moreover, Strain AG2-7T contains ninyhdrin-positive glycolipid and an unknown glucosamine-containing phospholipid which are distinct from all of the closest genera, and in addition, the fatty acid type and DNA G+C content clearly distinguish the novel isolate from the phylogenetically closest genera.

Therefore, on the basis of phenotypic and phylogenetic differentiation, strain AG2-7T is considered to represent a novel species of a new genus in the family Pseudonocardinae, for which the name Thermotunica guangxiensis gen. nov., sp. nov. is proposed.

**Description of Thermotunica gen. nov.**

*Thermotunica* (Ther.mo.to.ni.ca. Gr. adj. thermos warm, hot; L. fem. n. tunica tunic; N.L. fem. n. Thermotunica a thermophilic organism with a tunic).

Thermophilic, aerobic, Gram-reaction-positive, non-acid-fast, non-motile actinomycete. Substrate mycelium is branched and non-fragmented. Aerial mycelium is branched and abundantly produced on HV medium but scarcely on other media, long chains of spores are formed when mature. The non-motile spores are oval in shape with a ridged surface. On the surface of the aerial hyphae, a membranous structure or tunica can be observed by scanning electron microscopy (Fig. 1 a, c), transmission electron microscopy (Fig. S2) and light microscopy (Fig. S3), but sporangium-like structures are not produced. The diagnostic diamino acid in the cell-wall peptidoglycan is meso-diaminopimelic acid. The acyl type of the muramic acid residues is N-acetylated. Whole-cell sugars are galactose and ribose. The predominant menaquinone is MK-9(H4). The polar lipid profile contains diphasphatidylglycerol, ninyhdrin-positive glycolipid, phosphatidylglycol, phosphatidylglycerol, phosphatidylmannoside, phosphatidylethanolamine, phosphatidylmethylthanolamine, unknown phospholipid and unknown glucosamine-containing phospholipid. Phylogenetically, the genus belongs to the family Pseudonocardinae (suborder Pseudonocardinae).

The type species is *Thermotunica guangxiensis*.
**Description of Thermotunica guangxiensis sp. nov.**

*Thermotunica guangxiensis* (guang.xi.en’sis. N.L. fem. adj. guangxiensis pertaining to Guangxi Zhuang Autonomous Region, south-western China, where the type strain was isolated).

Morphological and chemotaxonomic characteristics are as given in the genus description. Grows well on HV medium,
Table 1. Differential morphological and chemotaxonomic characteristics of strain AG2-7T (Thermotunica gen. nov.) and related genera of the suborder Pseudonocardineae

Genera: 1, Thermotunica gen. nov. (strain AG2-7T; data from this study); 2, Kutzenia (Stackebrandt et al., 1994; Suriyachadkun et al., 2013); 3, Streptosphaeroteca (Tamura et al., 2008); 4, Actinokineospora (Tamura et al., 1995; Otojuro et al., 2001; Lisdiyanti et al., 2010; Tang et al., 2012); 5, Labedaea (Lee, 2012). +, Present; −, absent; V, variable.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td>Aerial mycelium</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fragmented mycelia</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<td>Sporangium-like structures</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>−</td>
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<tr>
<td>Tunica</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Temperature range (°C)</td>
<td>37−65</td>
<td>25−37</td>
<td>20−54</td>
<td>14−33</td>
<td>25−42</td>
</tr>
<tr>
<td>Diagnostic sugars*</td>
<td>Gal, Rib</td>
<td>Gal, Rha, Rib†, Glu†, Man†</td>
<td>Gal, Man, Glc†, Rha†</td>
<td>Gal, Ara, Rib†, Glu†, Man†</td>
<td>Glic, Rha, Gal, Rib, Man, Ara, Xyl</td>
</tr>
<tr>
<td>Phospholipids†</td>
<td>DPG, NPG, PE, PME, PE, OH-PE, DPG, PE, OH-PE, DPG, PE, PME†, PG†</td>
<td>PE</td>
<td>PE</td>
<td>PE, OH-PE†, PF, DPG†</td>
<td>PE, OH-PE†, PF, DPG†</td>
</tr>
<tr>
<td>Major menaquinones</td>
<td>MK-9(H4)</td>
<td>MK-9(H4)</td>
<td>MK-9(H6)</td>
<td>MK-10(H6)</td>
<td>MK-9(H4)</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>anteiso-C17:0, isoc16:0, 2-OH-isoc16:0</td>
<td>iso-C16:0, 10-methyl-C16:0, anteiso-C17:0</td>
<td>iso-C15:0, 2-OH-isoc15:0</td>
<td>iso-C15:0, 2-OH-isoc15:0, anteiso-C17:0, isoc15:0, 2-OH-isoc15:0</td>
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</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>63.6</td>
<td>70.3−70.7</td>
<td>71.6</td>
<td>68.9−71.9</td>
<td>64.2</td>
</tr>
</tbody>
</table>

*Ara, Arabinose; Gal, galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.
†Variable depending on species.
‡DPG, Diposphatidylglycerol; PE, phosphatidylethanolamine; OH-PE, hydroxy-phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PDE, phosphatidylglycerol mannoside; PME, phosphatidylmethylmethylethanolamine; NPG, ninyhdrin-positive glycolphospholipid; PC, phosphatidylcholine; GluNu, unknown glucosamine-containing phospholipid; L, unknown lipid; PL, unknown phospholipid.

Sauton’s agar and Bennett’s agar. Grows moderately on ISP4, ISP6 and potato agar, poorly on ISP2, ISP3, Czapek’s solution agar, and does not grow on Sauton’s asparagine agar, ISP5 or ISP7. Substrate mycelia are whitish to pale yellow, extensively branched and non-fragmenting. Blue-violet diffusible pigments are observed on ISP6 media. Aerial mycelium is white and branched, fragmenting. Blue-violet diffusible pigments are observed. 

The type strain, AG2-7T (=ATCC BAA-2499T=CGMCC 4.7099T), was isolated from mushroom residue compost in Guangxi Zhuang Autonomous Region, south-western China. The DNA G+C content of the type strain is 63.6 mol%.

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