Arthrobacter pityocampae sp. nov., isolated from Thaumetopoea pityocampa (Lep., Thaumetopoeidae)

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A bacterium (strain Tp2T) was isolated from a caterpillar of the pine processionary moth, Thaumetopoea pityocampa (Den. & Schiff.) (Lepidoptera: Thaumetopoeidae), a destructive pine forest pest. The bacterium is a Gram-stain-positive, red-pigmented coccus, oxidase-negative, nitrate-reducing, non-motile and non-spore-forming. Strain Tp2T was subjected to a taxonomic study using polyphasic approach that included morphological and biochemical characterizations, 16S rRNA gene sequence analysis, DNA–DNA hybridization, DNA G+C content analysis, comparative fatty acid profiles, and analyses of quinones and polar lipids. The 16S rRNA gene sequence of strain Tp2T revealed that Arthrobacter agilis DSM 20550T was the closest known strain (98 % 16S rRNA gene sequence similarity). DNA–DNA hybridization of A. agilis DSM 20550T and strain Tp2T resulted in a DNA–DNA relatedness value of 11.9 % (20.2 % reciprocal). The DNA base composition of strain Tp2T was 69.5 mol%, which is consistent with the other recognized members of Actinobacteria that have a high G+C content in their genome. The polar lipid pattern of strain Tp2T consisted of diphosphatidylglycerol (major), phosphatidylglycerol and phosphatidylinositol and unknown glycolipids. The cellular fatty acids were anteiso C15 : 0 and anteiso C17 : 0 and the major menaquinone was MK-9(H2). The peptidoglycan type was A3a with an L-Lys–L-Thr–L-Ala3 interpeptide bridge. The above-mentioned characterization qualifies strain Tp2T as genotypically and phenotypically distinct from closely related species of the genus Arthrobacter with validly published names. Strain Tp2T is therefore proposed to represent a novel species of the genus Arthrobacter, described as Arthrobacter pityocampae sp. nov. The type strain is Tp2T (=DSM 21719T =NCCB 100254T).

The genus Arthrobacter was first proposed by Conn and Dimmick (1947). The genus was later described and emended by Koch et al. (1995). At the time of writing, the genus Arthrobacter comprises 82 species with validly published names, isolated from diverse environments (http://www.bacterio.net/a/arthrobacter.html; Euzeby, 2014). Members of the genus Arthrobacter include Gram-stain-positive coryneform bacteria with an aerobic metabolism and little or no acid production from glucose. Members of the genus Arthrobacter contain diagnostic lysine molecules in the peptidoglycan structure and high base composition (Keddie et al., 1986). According to recommended and accepted criteria for delineating bacterial species, strains with DNA–DNA relatedness values below 70 % as measured by hybridization, or strains with 16S rRNA gene sequence dissimilarity above 3 %, are considered to belong to separate species (Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002; Wayne et al., 1987). Based on these criteria and with the support of morphological, physiological, biochemical and genotypic data, we propose strain Tp2T isolated from the Lepidopteran insect Thaumetopoea pityocampa represents a novel species of the genus Arthrobacter.

The pine processionary moth is one of the most harmful pests of pine species in Mediterranean countries. The insect larvae were collected from their nests located on Scots pine (Pinus sylvestris L.) from the Middle Black Sea region of Turkey while screening the bacterial flora of the pest. Strain Tp2T was one of the 14 different isolates (Tp1–Tp14) that were determined as a member of the bacterial flora of the...
larval stage of the pine processionary moth (Ince et al., 2008). Strain Tp2\textsuperscript{T} was clearly distinguished from the other isolates on the basis of morphological, physiological and biochemical characteristics. The isolation procedures were extensively described in previous studies (Ince et al., 2008; Kati et al., 2010).

The taxonomic position of strain Tp2\textsuperscript{T} was further analysed for its allocation to species level by using a polyphasic approach, which provided a detailed characterization of the strain. The strain was isolated as a part of the microbial flora of the pine processionary moth in our earlier studies (Ince et al., 2008; Kati et al., 2009, 2010). In addition, identification of several other important members of the microbial flora of this insect pest was achieved, including viruses and fungi (Ince et al., 2007; Sevim et al., 2010).

The colony and cell morphology of strain Tp2\textsuperscript{T} were examined by light microscopy and stereomicroscopic observations of the single colonies at different time points in culture. Gram staining was performed according to the procedure described by Claus (1992), and confirmed by the KOH string test (Murray et al., 1999). *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as positive and negative controls, respectively. Cell motility was examined by the presence of turbidity throughout tubes of semi-solid medium (Leifson, 1960), and by light microscopy (CX21; Olympus).

For biochemical tests, API 20E strips (bioMérieux) were used according to the manufacturer’s instructions, incubated at 30 °C, and read after 5 days. Catalase activity was assessed by the production of bubbles from 3 % (v/v) H\textsubscript{2}O\textsubscript{2}, and oxidase activity was tested by using the disc test (Fluka/Sigma Aldrich) according to the manufacturer’s instructions. The oxidase test result was compared with the known positive control *Pseudomonas aeruginosa* ATCC 27853 that turns the oxidase disc purple, and with the negative control *Escherichia coli* ATCC 25922.

Different phenotypic, physiological and biochemical characteristics of strain Tp2\textsuperscript{T} are described in the species description and compared with those of the closely related strain *Arthrobacter agilis* DSM 20550\textsuperscript{T} in Table 1.

To investigate the taxonomic position of the strain Tp2\textsuperscript{T}, 16S rRNA gene sequence analysis was performed. Extraction of genomic DNA was performed as described by Sambrook et al. (1989) and PCR amplification of the 16S rRNA gene was conducted as described by Ince et al. (2008). The PCR product was cloned into the pGEM-T vector, and approximately 1400 nt of 16S rRNA gene sequence was determined by Macrogen (Seoul, Korea). A total of 1408 nt of the 16S rRNA gene sequence from strain Tp2\textsuperscript{T} was deposited in the GenBank database. The sequence was aligned and compared to sequences within global databases with verified type species from LPSN (http://www.bacterio.net/arthrobacter.html; Ezube, 2014), the BLAST program (Altschul et al., 1990) and the EZ-Taxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Multiple sequence alignments were performed using ClustalW2 (Larkin et al., 2007) with default settings (EMBL European Bioinformatics Institute, http://www.ebi.ac.uk), edited and further submitted to MEGA software (version 6.0) to perform phylogenetic analysis (Tamura et al., 2013). The optimal evolutionary model tested on the final aligned dataset was defined by MEGA 6; the TN93 + G + I model was determined as the best fitting model (Tamura et al., 2013). The maximum-likelihood tree was reconstructed and distances were calculated using the Tamura–Nei distance model. The tree is shown in Fig. 1 with 1000 bootstrap replicates to estimate branch support.

Evolutionary relationships of closely related members of genus *Arthrobacter* were inferred using three different phylogenetic tree reconstruction algorithms: maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. The relationships among taxa were coherent in all tree-making methods used. The final presented phylogenetic tree was reconstructed using the maximum-likelihood method (Fig. 1). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The phylogenetic tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993). The analysis involved 31 nucleotide sequences including *Mycobacterium tuberculosis* ATCC 27294\textsuperscript{T} as the outgroup. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 1284 positions in the final dataset. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The 16S rRNA gene sequence analyses revealed that strain Tp2\textsuperscript{T} is a member of the genus *Arthrobacter* that forms a distinct clade with *A. agilis* DSM 20550\textsuperscript{T}. Strain Tp2\textsuperscript{T} was most closely related to *A. agilis* DSM 20550\textsuperscript{T}, with a 16S rRNA gene sequence similarity of 98 %.

DNA–DNA hybridization was determined at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), as described by De Ley et al. (1970), with the modifications of Huss et al. (1983). DNA was isolated by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization studies were performed with strain Tp2\textsuperscript{T} and *A. agilis* DSM 20550\textsuperscript{T}. At the DNA–DNA level, the relatedness between *A. agilis* DSM 20550\textsuperscript{T} and strain Tp2\textsuperscript{T} was only 11.9 % (20.2 % reciprocal).

The DNA base composition was determined by the DSMZ using HPLC and the G+C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT)
Table 1. Differential characteristics of strain Tp2T and its five closest phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>pH range for growth (optimum)</td>
<td>6.5–10 (7)</td>
<td>ND (7)*</td>
<td>7–10 (8–9)</td>
<td>5.3–10.5</td>
<td>6–9 (8)</td>
<td>6–9 (7–8)</td>
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<tr>
<td>Colony colour</td>
<td>Red</td>
<td>Rose red</td>
<td>Yellow–orange</td>
<td>Pale yellow</td>
<td>Yellow</td>
<td>Yellow–orange</td>
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<td>Major Fatty acids (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>anteiso-C₁₅:₀</td>
<td>54</td>
<td>65.5</td>
<td>57</td>
<td>35.4</td>
<td>44</td>
<td>51</td>
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<tr>
<td>anteiso-C₁₇:₀</td>
<td>19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>iso-C₁₅:₀</td>
<td>3</td>
<td>13</td>
<td>17</td>
<td>ND</td>
<td>37</td>
<td>29</td>
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<td>iso-C₁₆:₀</td>
<td>9</td>
<td>ND</td>
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<td>iso-C₁₆:₁</td>
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<td>6</td>
<td>ND</td>
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<td>anteiso-C₁₆:₁₀⁻⁷c</td>
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<td>ND</td>
<td>ND</td>
<td>15</td>
<td>ND</td>
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<td>Oxidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Urease</td>
<td>–</td>
<td>–</td>
<td>V</td>
<td>–</td>
<td>–</td>
<td>V</td>
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<td>Nitrate reduction</td>
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<td>–</td>
<td>V</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>V</td>
<td>+</td>
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<tr>
<td>Growth in 5% NaCl (optimum % NaCl for growth)</td>
<td>– (0–3)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Minor menaquinones</td>
<td>ND</td>
<td>MK-9 (H₂)</td>
<td>MK-9 (H₂)</td>
<td>MK-9 (H₂)</td>
<td>MK-9 (H₂)</td>
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<td>DNA G+C content (mol%)</td>
<td>69.5</td>
<td>67–70</td>
<td>64.7</td>
<td>62.7</td>
<td>63.7</td>
<td>63.8</td>
</tr>
<tr>
<td>Origin</td>
<td>Insect</td>
<td>Water, soil, human skin</td>
<td>Deteriorated mural painting</td>
<td>Deep subsurface water</td>
<td>Deteriorated mural painting</td>
<td>Deteriorated mural painting</td>
</tr>
</tbody>
</table>

*Strains of A. agilis and A. pityocampae sp. nov. cultured in this study at pH 7.
according to the method of Mesbah et al. (1989). The genomic DNA G + C content of strain Tp2T was 69.5 mol%, which is similar to A. agilis DSM 20550T which ranges from 67.0 to 69.0 mol% (Table 1). The G + C content of the DNA of strain Tp2T also falls within the range for the genus Arthrobacter (59–70 mol%) as reported by Keddie et al. (1986).

Analyses of respiratory quinones, polar lipids and peptidoglycan structure were performed at the DSMZ. Peptidoglycan structure identification was performed according to published protocols (Schumann, 2011). The predominant menaquinone isoprenolog of strain Tp2T was MK-9(II-H2), and the polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and some unknown glycolipids (Fig. S1, available in the online Supplementary Material). The peptidoglycan type was A3α (A11.28) with an L-Lys–L-Thr–L-Ala3 interpeptide bridge (Table 1).

Fatty acid methyl esters were extracted concurrently from biomass obtained in the same physiological state from both A. agilis DSM 20550T and strain Tp2T and analysed by gas chromatography (Hewlett Packard 5890 II plus) with the Sherlock Microbial Identification System using version 4.10 of the TSBA40 library (Microbial ID). The cellular fatty acid profile of strain Tp2 was characterized by saturated

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequence data, showing the phylogenetic position of strain Tp2T and the type strains of related species of the genus Arthrobacter. The tree was reconstructed and distances were calculated using the Tamura–Nei distance model. Mycobacterium tuberculosis ATCC 27294T was used as an outgroup. Bayesian Information Criterion (BIC) scores for different models were computed using the MEGA 6 program with the default settings. TN93 with a proportional discrete Gamma distribution (+G) and a fraction of invariant sites (+I), was selected as best-fitted model with the lowest BIC scores. The transition/transversion bias for TN93 + G + I model was estimated to be 1.46. The maximum-likelihood tree was reconstructed using MEGA 6 software by applying estimated model. Bootstrap values (percentages) are based on 1000 replicates and are shown for branches with more than 50% support. GenBank accession numbers are given in parentheses. Bar, 0.05 expected changes per site.
branched fatty acids such as anteiso-C15:0 and anteiso-C17:0, accounting for more than 70% of the total cellular fatty acids. Predominant fatty acids were anteiso-C15:0 (54%) and anteiso-C17:0 (19%). Other fatty acids were iso-C15:0 (3.55%) and iso-C16:0 (9.42%). The fatty acid composition of the novel species was in good agreement with the genus description (Keddie et al., 1986). Members of the genus novel Arthrobacter contain major amounts of iso- and anteiso-methyl branched acids (iso-C15:0 and anteiso-C15:0) together with small amounts of straight-chain saturated acids (C16:0); monounsaturated acids are absent or present only in trace amounts.

Strain Tp2T could grow on trypticase soy agar or broth (TSA: 1.5% casein peptone, 0.5% soy peptone, 0.5% NaCl, 1.5% agar, pH 7; TSB: excluding 1.5% agar from TSA). The optimal growth temperature was determined by culturing strain Tp2T at 20°C to 50°C in intervals of 5°C. The optimal growth temperature determined to be 30°C. Salt-tolerance testing was performed using TSB containing 0, 3, 5, 10, 15 and 18% (w/v) NaCl. The pH range and the optimal pH supporting bacterial growth were evaluated with TSB at pH 4.0 to 10.0 in 0.5 pH unit intervals. Salt and the pH tolerance tests were measured after 72 h of incubation at 30°C. Bacterial growth was observed at pH 6–10 (optimum pH 7) and between 0–3% (w/v) NaCl.

**Description of Arthrobacter pityocampae sp. nov.**

*Arthrobacter pityocampae* (pi.ty.o.cam’pae, N.L. fem. gen. n. *pityocampae* of pityocampa, referring to isolation of the type strain from *Thaumetopoea pityocampa* larvae). Cells are Gram-stain-positive, non-motile, non-sporo-forming, red-pigmented cocci (0.82 μm in diameter). Cells are rods during exponential growth and cocci in their stationary phase occurring singly, in pairs, or in tetrads. The red pigment is water-insoluble. Sediment is formed in nutrient broth. Colonies on TSA are small (<1 mm), red, round with entire margins, smooth and bright after 48 h at 30°C. The growth temperature ranges from 25 to 35°C, with optimal growth at 30°C. Growth is not observed at 35°C. The pH range for growth is pH 6.5–10, with optimum growth at pH 7; growth is not observed at this pH range. Growth occurs with 0–3% (w/v) NaCl; there is no growth observed with 5% (w/v) NaCl. Catalase-positive, oxidase-negative and able to reduce nitrate to nitrite. Tests for β-galactosidase, citrate utilization and gelatinase are positive. Tests for lysine decarboxylase, ornithine decarboxylase, H2S production, urease, tryptophan deaminase, arginine dihydrolase, acetoin production and indole production are negative. As the fermentation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose, amygdalin and L-arabinose are negative, the strain is proposed to be asaccharolytic. The peptidoglycan type is A3γ with an L-Lys–L-Thr–L-Ala3 interpeptide bridge. The predominant menaquinone isoprenolog is MK-9(II-H2) and the polar lipids are diphospha-tidylglycerol, phosphatidylglycerol, phosphatidylinositol and some unknown glycolipids. The major cellular fatty acids are anteiso-C15:0 and anteiso-C17:0.

The type strain, Tp2T (=DSM 21719T=NCIB 100254T), was isolated from healthy larvae of the pine processionary moth. The isolate is the first member of the genus *Arthrobacter* isolated from insects, *Thaumetopoea pityocampa*. The DNA G+C content of the type strain is 69.5 mol%.

**Acknowledgements**

This work was supported by The Republic of Turkey Prime Ministry, State Planning Organization (21.111.004.1).

**References**


