Arthrobacter gyeryongensis sp. nov., isolated from soil of a Gynostemma pentaphyllum field

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A Gram-stain-positive, flagellate, rod-shaped, catalase- and oxidase-positive bacterium, designated DCY72T, was isolated from the soil of a Gynostemma pentaphyllum field. Growth occurred at 4–34 °C (optimum 30 °C), at pH 4–10 (optimum pH 7), and with 0–5 % NaCl (w/v). The major menaquinones of strain DCY72T were MK-9(H2) (81.0 %) and MK-10(H2) (12.2 %). The major amino acid present in the cell-wall peptidoglycan was L-lysine. The major fatty acids were anteiso-C15 : 0 and anteiso-C17 : 0. The genomic DNA G+C content was 64.5 mol%. 16S rRNA gene sequence analysis revealed that strain DCY72T belonged to the family Micrococccaceae and was most closely related to Arthrobacter ramosus CCM 1646T (98.2 % similarity). The DNA–DNA relatedness between strain DCY72T and A. ramosus KACC 14391T (98.2 % 16S rRNA gene sequence similarity), Arthrobacter nitroguajacolicus KACC 14581T (97.6 %), Arthrobacter nicotinovorans KACC 20508T (97.3 %) and Arthrobacter aurescens KACC 20528T (97.3 %), was 12.9 % ± 0.3, 25.6 % ± 0.3, 26.6 % ± 0.5 and 23.2 % ± 0.9, respectively. On the basis of the phenotypic characteristics, genotypic analysis and physiological characteristics, strain DCY72T represents a novel species of the genus Arthrobacter, for which the name Arthrobacter gyeryongensis sp. nov. is proposed. The type strain is DCY72T (=KCTC 33072T =JCM 18514T).

The genus Arthrobacter, belonging to the class Actinobacteria, was first proposed by Conn & Dimmick (1947), and the description was emended by Koch et al. (1995). Members of genus Arthrobacter are Gram-stain-positive, their cell-wall peptidoglycan contains the diagnostic diamino acid lysine and they have a high DNA G+C content (59–66 mol%) (Keddie et al., 1986; Jones & Keddie, 1992). At the time of writing, the genus Arthrobacter comprises 68 recognized species (http://www.bacterio.net/a/arthrobacter.html). Members of the genus Arthrobacter have been isolated from a variety of environmental sources, such as soil, air, water, oil brine, plants, biofilms, cyanobacteria mats, sediment, poultry litter, cheese, human clinical specimens and animal specimens. In this taxonomic study, we characterized a novel isolate belonging to the genus Arthrobacter using a polyphasic approach. The phenotypic and genotypic characterizations of the novel strain are described in this report.

Gynostemma pentaphyllum, belonging to the family Cucurbitaceae, is a traditional medicinal herb. A 500 g sample of the rhizosphere soil, without any stones or particles, was carefully collected in clean zip-lock covers and transferred to the laboratory. One gram of soil sample was dissolved in 10 ml saline solution, and serial dilutions were prepared up to 10⁻⁴ using 0.85 % (w/v) saline solution. Subsequently, 100 μl of each diluted sample was plated onto R2A agar (Difco) that was diluted five times. The plates were incubated at 30 °C for 5 days. Single colonies were purified by transfer to new R2A agar plates. One isolate, designated DCY72T, was selected for further characterization in this study. The isolate was routinely cultured on R2A agar at 30 °C and stored at −80 °C as a suspension in 30 % (v/v) glycerol. For the comparative study, Arthrobacter ramosus KACC 14391T, Arthrobacter nitroguajacolicus KACC 14581T, Arthrobacter nicotinovorans KACC 20508T and Arthrobacter aurescens KACC 20528T were obtained from the Korean Agricultural Culture Collection (KACC) as reference type strains. These strains were cultured under the same conditions as strain DCY72T.

The genomic DNA of strain DCY72T was extracted and purified using a commercial genomic DNA isolation kit (Core Bio System). The 16S rRNA gene sequence was amplified from the chromosomal DNA using the universal bacterial primer set 27F and 1492R (Lane, 1991), then the purified PCR products were sequenced by Genotech (Daejeon) (Kim et al., 2005). The almost complete sequence (1412 bp) of the 16S rRNA gene was assembled by using SeqMan software (DNASTA). BLAST searches with the
nearly complete (1412 bp) 16S rRNA gene sequence of strain DCY72T performed in EzTaxon-e server (Kim et al., 2012) revealed that the novel isolate shared the highest sequence similarity with A. ramosus KACC 14391T, while the sequence similarity with type strains of other species of the genus Arthrobacter was 93.0–98.2 %. Strain DCY72T had the highest 16S rRNA gene sequence similarity with A. ramosus KACC 14391T (98.2 %), A. nitrogaucalicolicus KACC 14581T (97.6 %), A. nicotinovorans KACC 20508T (97.3 %) and A. aurescens KACC 20528T (97.3 %). The 16S rRNA gene sequences with similarities of 95.7–98.2 % with strain DCY72T were taken for reconstruction of a phylogenetic tree. A phylogenetic tree was reconstructed using neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods, by using the MEGA4 program (Tamura et al., 2007). Bootstrap analysis with 1000 replications was also conducted in order to evaluate the confidence level of the branch nodes (Felsenstein, 1985). The distances were calculated according to Kimura’s two-parameter method (Kimura, 1983). (Fig. 1).

For the analysis of G+C content, the genomic DNA of strain DCY72T was extracted and purification was done using an Exgene Cell SV mini-kit (Gene All Biotechnology) according to the manufacturer’s instructions, after which 10 μl of solution containing 10 μg of DNA was heated in a boiling water bath for 5 min and then cooled in an iced water bath. The denatured DNA solution was mixed with 10 μl nuclease P1 solution (nuclease P1 20 U ml−1 of 40 mM acetic buffer, pH 5.4) and incubated at 37°C for 1 h. Next, 10 μl glycine buffer (pH 10.0) and 10 μl alkaline phosphatase (40 U ml−1) were added to the sample, and incubated for 1 h at 37°C (Mesbah et al., 1989). Subsequently, the obtained nucleoside mixture was separated using HPLC (model NS-6000A, Futecs, reversed-phase column YMC-Triart C18, 4.6 x 250 mm x 5 μm), using a mixture of 0.2 M (NH4)2HPO4 and acetonitrile (20:1, v/v) for the mobile phase, a flow rate of 1.2 ml min−1 and a wavelength of 270 nm. The DNA G+C content of strain DCY72T was 64.5 mol%, which is within the range reported for members of the genus Arthrobacter (59–66 mol%) (Keddie et al., 1986).

DNA–DNA hybridization experiments were performed in triplicate using the fluorometric microplate method (Ezaki et al., 1989). The DNA–DNA relatedness values between strain DCY72T and A. ramosus KACC 14391T (98.2 %), A. nitrogaucalicolicus KACC 14581T (97.6 %), A. nicotinovorans KACC 20508T (97.3 %) and A. aurescens KACC 20528T (97.3 %) were below the 70 % threshold proposed for species delineation (Wayne et al., 1987), demonstrating that strain DCY72T represents a distinct genomic species.

Colony morphology of strain DCY72T was observed on R2A agar after incubation at 30°C for two days. Cell size, shape and flagella were observed through a phase-contrast microscope (x 1000 magnification, Nikon Optiphoto-2) and by transmission electron microscopy with cells grown in R2A broth at 30°C for 24 h. Suspended cells were placed on carbon and Formvar-coated nickel grids for 30 s; grids were floated on one drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a Carl Zeiss electron microscope (LOE912AB) at 100 kV under standard operating conditions. The presence of motility was determined using the hanging-drop technique (Bernardet et al., 2002). The bioMérieux Gram stain kit was used to evaluate the Gram reaction according to the manufacturer’s instructions. Growth of strain DCY72T was tested on different media: nutrient (NA), tryptasec soy (TSA), R2A, Luria–Bertani (LB), potato-dextrose (PDA) and MacConkey agars (all Difco) at 30°C. The temperature range for growth was assessed in R2A broth at 4, 10, 20, 25, 30, 37 and 40°C. Salt tolerance was tested using R2A broth supplemented with 0–5.0 % (w/v) NaCl (in 0.5 % increments) for 5 days at 30°C. Growth at pH 5.0–10.0 (in 0.5 pH unit intervals) was assessed in R2A broth with the pH adjusted with 10 mM phosphate-citrate buffer (pH 5.0), MES buffer (pH 5.5–6.5), HEPES buffer (pH 7.0–8.0), Tris buffer (pH 8.5–9.0) or NaHCO3/Na2CO3 (pH 9.5–10.0). Catalase activity was evaluated by determining the production of an oxygen bubble in a 3 % (v/v) H2O2 solution. Oxidase activity was tested by determining the oxidation of 1 % N,N,N',N'-tetramethyl-1,4-phenylenediamine reagent (bioMérieux) according to the manufacturer’s instructions. Hydrogen sulfide (H2S) production was evaluated on triple-sugar iron agar. Hydrolysis of the following substrates was tested: gelatin (on a medium containing 12 % gelatin, 0.3 % beef extract and 0.5 % peptone), starch [on R2A agar containing 1 % starch (Difco)], DNase [on DNase agar medium (Scharlau)], casein [on R2A agar supplemented with 2 % skimmed milk (Difco)] (Gowan & Steel, 1974), aesculin [on R2A agar containing 0.3 % aesculin (Sigma) and 0.02 % ferric citrate (Fluka)], Tween 20 and 80 (on R2A agar containing 1 % Tween 20 or 80 and 0.02 % CaCl2). Indole production was analysed using Kovács’s reagent in 1 % tryptone broth. Nitrate reduction was tested in nitrate broth containing 0.2 % KNO3 (Skerman, 1967). Urease activity was evaluated in Christensen’s medium (Christensen, 1946). The commercial system BD GasPak EZ Gas Generating System (Becton Dickinson) was used to test anaerobic growth. Antibiotic susceptibility was tested by using Oxoid antibiotic paper discs on Mueller–Hinton agar, as described by Bauer et al. (1966). The inhibition zone was interpreted according to the manufacturer’s manual. The following antibiotics were tested: penicillin G (10 units), erythromycin (15 μg), cefazolin (30 μg), oleandomycin (15 μg), ceftazidime (30 μg), vancomycin (30 μg), tetracycline (30 μg), novobiocin (30 μg), carbamycin (100 μg), rifampicin (5 μg) and neomycin (30 μg). Carbon source assimilation and enzyme production tests were conducted using API 20 NE, API ID 32 GN and API ZYM strips according to the manufacturer’s instructions (bioMérieux). The API ID 32 GN and API 20NE strips were recorded after 48 h, while API ZYM strips were recorded after 6 h of incubation. All systems were incubated at 30°C. The results of the reactions are described in Table 1.
For menaquinone analysis, cell biomass was grown on R2A media at 30 °C for two days and freeze-dried. Respiratory quinones were extracted and purified according to Collins (1985); purified menaquinones were determined by reverse-phase HPLC (Wu et al., 1989) with the MK-9(H2) from *A. ramosus* KACC 14391T used as a reference. Strain DCY72T was shown to have MK-9(H2) (81.0%) and MK-10(H2) (12.2%) as the major respiratory quinones, while minor quinones were MK-7(H2) (2.4%) and MK-8(H2) (4.4%).

Peptidoglycan was analysed as described by Schleifer & Kandler (1972) by TLC on cellulose sheets. The lysine type was found in the cell wall of species of the genus.
**Table 1.** Differential characteristics of strain DCY72\(^T\) and its closest neighbours in the genus *Arthrobacter*

<table>
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<th>Characteristic</th>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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</table>

Strains: 1. *Arthrobacter gyeryongensis* sp. nov. DCY72\(^T\); 2. A. ramosus KACC 14391\(^T\); 3. A. nitroguajacolicus KACC 14581\(^T\); 4. A. nicotinovorans KACC 20508\(^T\); 5. A. aurescens KACC 20528\(^T\).

For fatty acid analysis, the cell mass of strain DCY72\(^T\) and the four reference strains were grown on TSA agar (Difco) plates after incubation at 28 °C for 1 day. Cellular fatty acids were extracted and prepared according to the protocol of the Sherlock Microbial Identification System (MIDI), and analysed by capillary GLC (6890; Hewlett Packard) using the Microbial Identification software package with the Sherlock system MIDI 6.1 and the Sherlock Aerobic Bacterial Database (TSBA6.1) (Sasser, 1990). The fatty acid analysis was performed in duplicate. The fatty acid profile of strain DCY72\(^T\) showed a large amount of branched-chain-saturated fatty acids; the major fatty acids were anteiso-C\(_{15:0}\) (71.4 %) and anteiso-C\(_{17:0}\) (10.2 %) (Table 2). The four reference strains also showed very similar profiles for major fatty acids. Therefore, the composition of this novel strain is consistent with that of members of the genus *Arthrobacter*, showing a predominance of anteiso-C\(_{15:0}\).

On the basis of the phylogenetic, phenotypic and chemotaxonomic data, strain DCY72\(^T\) represents a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter gyeryongensis* sp. nov. is proposed.

**Description of Arthrobacter gyeryongensis** sp. nov.

*Arthrobacter gyeryongensis* (gyer.yong.en’sis. N.L. masc. adj. *gyeryongensis* of or belonging to the Gyeryong mountain).

Cells are Gram-stain-positive, flagellate, aerobic and rod-shaped (approximately 0.57 µm wide and 1.29 µm long) (Fig. S2). Tests for oxidase and catalase activity are positive. Cells grow on LB, TSA, R2A and NA, but do not grow on MacConkey agar. Growth occurs at 4–34 °C (optimum 30 °C), at pH 4–10 (optimum pH 7), and with 0–5 % (w/v) NaCl. Nitrate is not reduced to nitrite. Cellulose, skimmed milk, gelatin and Tween 20 are hydrolysed, but DNase, starch and Tween 80 are not. Resistant to ceftazidime (30 µg), but sensitive to penicillin G (30 µg), erythromycin (15 µg), cefazolin (30 µg), oleandomycin (15 µg), vancomycin (30 µg), carbamicillin (100 µg), rifampicin (5 µg), neomycin (30 µg), novobiocin (30 µg) and tetracycline (30 µg). In API ZYM tests, positive for alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, reported previously by Minnikin et al. (1984). Polar lipids were separated by 2D-TLC using TLC Kieselgel 60F\(_{254}\) (Merck) plates (10 × 10 cm). Chromatograms were developed in the first dimension with chloroform/methanol/water (65:25:4, by vol.) and in the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) as solvent systems. Total polar lipids were detected by spraying the TLC plates with 5 % molybdate-phosphoric acid followed by charring at 120 °C for 10 min. The polar lipid profile of strain DCY72\(^T\) contained diphasphatidylglycerol and unidentified glycolipids GL1–2 as the major polar lipids with unidentified phospholipids PL1–2 as minor polar lipids. This profile was similar to that of the reference strain, *A. nicotinovorans* KACC 20508\(^T\) (Fig. S1, available in the online Supplementary Material).

*Arthrobacter* and is also the diagnostic diamino acid in the peptidoglycan of strain DCY72\(^T\). The major amino acids of the cell wall were lysine and alanine.

Polar lipids of strain DCY72\(^T\) and *A. nicotinovorans* KACC 20508\(^T\) were extracted from 50 mg dried cells as described previously by Minnikin et al. (1984). Polar lipids were separated by 2D-TLC using TLC Kieselgel 60F\(_{254}\) (Merck) plates (10 × 10 cm). Chromatograms were developed in the first dimension with chloroform/methanol/water (65:25:4, by vol.) and in the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) as solvent systems. Total polar lipids were detected by spraying the TLC plates with 5 % molybdate-phosphoric acid followed by charring at 120 °C for 10 min. The polar lipid profile of strain DCY72\(^T\) contained diphasphatidylglycerol and unidentified glycolipids GL1–2 as the major polar lipids with unidentified phospholipids PL1–2 as minor polar lipids. This profile was similar to that of the reference strain, *A. nicotinovorans* KACC 20508\(^T\) (Fig. S1, available in the online Supplementary Material).
The type strain is DCY72T (KCTC 33072T=JCM 18514T), isolated from the soil of a Gymnostema pentaphyllum field in Gyeryong mountain in Chungcheong province (36°21’ 40” N 127°12’ 40” E), South Korea. The DNA G+C content of the type strain is 64.5 mol% (HPLC).

**Table 2.** Cellular fatty acids profile of strain DCY72T and type strains of other related species of the genus Arthrobacter

<table>
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<th>Fatty acid</th>
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<tr>
<td>Saturated</td>
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<tr>
<td>C14:0</td>
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<td>0.6</td>
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<td>0.8</td>
<td>0.7</td>
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<tr>
<td>C16:0</td>
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<td>1.4</td>
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<tr>
<td>Branched-chain</td>
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<tr>
<td>iso-C14:0</td>
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<tr>
<td>iso-C15:0</td>
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<td>5.2</td>
<td>9.4</td>
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<tr>
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<td>6.9</td>
<td>10.2</td>
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<td>9.1</td>
<td>10.0</td>
<td>11.1</td>
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*Summed feature 3, which contains C16:1ω7c and/or C18:1ω6c, could not be separated by the Microbial Identification System (MIDI).

**Acknowledgements**

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**References**


