Angustibacter speluncae sp. nov., isolated from a lava cave stalactite
Dong Hyo Ko and Soon Dong Lee*

Abstract
Gram-reaction-positive, strictly aerobic, motile coccoid- to rod-shaped actinobacteria, designated strains YC2-20T and YC2-19 were isolated from pieces of stalactites collected at the Yongcheon Cave in Jeju, Republic of Korea. The cells of orange-coloured colonies grew at 20–37 °C, pH 6–10 and in the absence of NaCl. In the neighbour-joining tree based on 16S rRNA gene sequences, the novel isolates formed a distant sublineage at the base of the radiation of the genus Angustibacter. The novel isolates shared identical 16S rRNA gene sequences to each other and revealed a 16S rRNA gene sequence similarity of 95.6 % to the closest relative, Angustibacter aerolatus and <95 % to other members of the family Kineosporiaceae. Albeit with a low similarity of 16S rRNA gene sequences and a distinct phylogenetic position, most of the chemotaxonomic characteristics were in agreement with those of the genus Angustibacter: meso-diaminopimelcic acid as the diagnostic diamino acid in the cell wall; MK-9(H4) as the major menaquione; polar lipids including diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside; and a DNA G+C content of 73.1 mol%. On the basis of the phenotypic and phylogenetic distinctiveness, the novel isolates are considered to represent members of a novel species of the genus Angustibacter, for which the name Angustibacter speluncae sp. nov. is proposed, with type strain YC2-20T (=KCTC 39842T=DSM 103769T).

The genus Angustibacter was proposed by Tamura et al. [1] with the description of Angustibacter luteus as the type species, which was isolated from subarctic forest soil on Rishiri Island, Hokkaido, Japan and belonged to the family Kineosporiaceae [2] along with the genera Kineosporia [3], Kineococcus [4] and Pseudokineococcus [5] and Quadrisphaera [6], based on 16S rRNA gene sequence analysis. Thereafter, two new species have been added to the genus: Angustibacter aerolatus [7] from an air sample collected in Jeju Island, Republic of Korea; and Angustibacter peucedani [8] from a soil sample adhering to a plant (Peucedanum japonicum Thunb.) collected on Mara Island, Jeju, Republic of Korea. Members of the genus were Gram-reaction-positive, aerobic or facultatively anaerobic, coccoid- to rod-shaped and represented chemotaxonomically by the presence of meso-diaminopimelcic acid (DAP) as the diagnostic diamino acid in the cell wall; MK-9(H4) as the major menaquione, polar lipids including diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside, and DNA G+C content of 70.9–73.6 mol% [1, 7, 8]. During an investigation on bacterial diversity in a lava cave, two actinobacterial strains, designated YC2-20T and YC2-19, were isolated from pieces of stalactites and found to be loosely associated to members of the genus Angustibacter based on preliminary analysis of the 16S rRNA gene sequences. In the present study, these Angustibacter-like strains are taxonomically characterized by a polyphasic approach and described as members of a new species, Angustibacter speluncae sp. nov.

Strains YC2-20T and YC2-19 were isolated from pieces of stalactites collected at the Yongcheon Cave in Jeju, Republic of Korea. About 0.5 g of stalactites pieces were crushed with a pestle and suspended in distilled water. The suspension was mixed for 30 min in a tube rotator and allowed to settle. Bacterial isolation was carried out by using standard dilution plating methods on starch-casein agar and the plates were incubated at 30 °C for 1 month. Colonies were streaked on International Streptomycetes Project (ISP) 2 agar [9] two or three times and the pure cultures were maintained in the cell-wall peptidoglycan, MK-9(H4) as the major menaquione, polar lipids including diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside, and DNA G+C content of 70.9–73.6 mol% [1, 7, 8]. During an investigation on bacterial diversity in a lava cave, two actinobacterial strains, designated YC2-20T and YC2-19, were isolated from pieces of stalactites and found to be loosely associated to members of the genus Angustibacter based on preliminary analysis of the 16S rRNA gene sequences. In the present study, these Angustibacter-like strains are taxonomically characterized by a polyphasic approach and described as members of a new species, Angustibacter speluncae sp. nov.

Strains YC2-20T and YC2-19 were isolated from pieces of stalactites collected at the Yongcheon Cave in Jeju, Republic of Korea. About 0.5 g of stalactites pieces were crushed with a pestle and suspended in distilled water. The suspension was mixed for 30 min in a tube rotator and allowed to settle. Bacterial isolation was carried out by using standard dilution plating methods on starch-casein agar and the plates were incubated at 30 °C for 1 month. Colonies were streaked on International Streptomycetes Project (ISP) 2 agar [9] two or three times and the pure cultures were maintained in 20 % (v/v) glycerol solutions at −80 °C and as lyophilized cells. Growth of strains YC2-20T and YC2-19 on various media were examined using ISP 2 agar, trypticase soy agar (TSA; Difco), nutrient agar (NA; Difco) and R2A agar (Difco). As references for fatty acid analysis, Angustibacter
**luteus** KACC 14249\(^T\), **A. aerolatus** KACC 15527\(^T\) and **A. peucedani** KCTC 19628\(^T\) were grown on NA at 30 °C.

Cell morphology was observed by light and transmission electron microscopy, with cells grown on TSA for 5 days at 30 °C. Gram-staining was performed using Gram 2 Kit (bio-Mérieux) according to the manufacturer’s instructions. Cell motility was checked on semisolid agar including trypticase soy broth (TSB; Difco) with the addition of 0.3 % (w/v) Bacto agar (Difco). Flagellation on cell surfaces was observed under a field emission-stereoscan electron microscope with a scanning transmission electron microscope detector (SUPRA 55VP; Zeiss) after staining cells with 2 % uranium acetate. Cell morphology was also observed by scanning electron microscopy (JSM-6700F, Jeol), with cells grown on ISP 2 agar for 7 days at 30 °C. Before fixation with a solution of 3 % glutaraldehyde, cells were attached on cover slips pre-coated with a solution of poly-L-lysine. Colony morphology, size and pigmentation were observed on TSA plates incubated for 7 days at 30 °C. Oxidase and catalase activities were examined as previously described [10]. Anaerobic growth was tested on TSA at 30 °C by using the GasPak EZ Anaerobe Pouch System (BD) according to the manufacturer’s instructions. Enzyme activities, assimilation of carbohydrates and acid production from various substrates were examined using API ZYM, API 20NE and API 50CH strips (bio-Mérieux), respectively, according to the recommendations of the manufacturer, except that API strips were recorded after incubation at 30 °C for 5 days (API 20NE) and for 14 days (API 50CH). Anaerobic conditions for acid production were maintained with supplementation of sterile mineral oils in each well at 3 day intervals. All the API systems were tested twice with independent cultures. The temperature range for growth was examined on TSA at temperatures 4, 10, 20, 30, 37 and 40 °C. The pH range for growth was determined in TSB at pH 4–10 (at intervals of pH 1.0 unit), which was adjusted by using sodium citrate/citric acid (pH 4–5), dihydrogen phosphate/sodium hydroxide (pH 6–8) and carbonate/bicarbonate (pH 9–10) buffers. Salt tolerance was tested in TSB with addition of 0–9 % (w/v) NaCl (at intervals of 1 %). Cells of the novel isolates were Gram-reaction-positive, strictly aerobic, non-spore-forming, motile cocci to rods (Fig. S1, available in the online Supplementary Material). Colonies were circular, convex, orange-coloured with entire margins and 0.5–1.2 mm in diameter after 5 days of incubation. Data for phenotypic features including morphological, physiological and biochemical properties are given in the species description and Table 1.

Genomic DNA isolation, amplification and sequencing of the 16S rRNA genes were carried out as previously described [8]. The **CLUSTALX** program [11] was used for multiple alignments of the 16S rRNA gene sequences and several programs from the **PHYLIP** software package [12] were used for phylogenetic analyses. Evolutionary distances were calculated by using the Jukes and Cantor model [13] and used for reconstruction of a neighbour-joining tree [14], with bootstrap analysis [15] based on 1000 replicates.

DNA-DNA hybridization experiments between strains YC2-20\(^T\) and YC2-19\(^T\) were carried out using a photobiotin-labelled DNA probe and microdilution wells [16].

The nearly complete 16S rRNA gene sequences of strain YC2-20\(^T\) and YC2-19 were determined in this study and found to be identical to each other. The preliminary search of phylogenetic neighbours through **BLAST** of NCBI and EzTaxon-server revealed that the novel isolates were related to members of the family **Kineosporiaceae**. 16S rRNA gene sequence analysis (Fig. 1) showed that strains YC2-20\(^T\) and YC2-19 formed a tight monophyletic clade and associated loosely to members of the genus **Angustibacter**, despite a moderate bootstrap support (54 %). Strains YC2-20\(^T\) and YC2-19 shared a high 16S rRNA gene sequence similarity of 95.3 % to **A. aerolatus** 7402 J-48\(^T\) and of <95 % to other members of the family **Kineosporiaceae**. DNA relatedness between strains YC2-20\(^T\) and YC2-19 was 92.6 % (using a probe DNA of strain YC2-20\(^T\)), revealing that both isolates were members of same species [17].

For analyses of chemotaxonomic characteristics, cells of strains YC2-20\(^T\) and YC2-19 were grown in TSB for 6 days at 30 °C and harvested by centrifugation. The isomer of dianimopimelic acid in the cell-wall peptidoglycan was determined as described by Staneck and Roberts [18]. Respiratory quinones and polar lipids were extracted by using the integrated procedure of Minnikin et al. [19]. The respiratory quinones were analysed by high-performance liquid chromatography (HPLC) [20]. The polar lipids were analysed by two-dimensional thin-layer chromatography (TLC) [21]. Mycolic acids were examined by the method of Minnikin et al. [22]. The DNA G+C contents were determined by HPLC [23]. For determination of cellular fatty acids, strains YC2-20\(^T\) and YC2-19 and the three type strains of the genus **Angustibacter** were grown on NA for 5 days at 30 °C. Fatty acid methyl esters were prepared and analysed according to the instructions of the Sherlock Microbial Identification System (**MIDI**, version 6.1) with the RTSBA6 library for identification of each chromatographic peak.

Despite low 16S rRNA gene sequence similarity, most of chemotaxonomic characteristics of strains YC2-20\(^T\) and YC2-19 were in agreement with those of the genus **Angustibacter** [1, 8]. The diagnostic diaminoc acid in cell-wall peptidoglycan was **meso**-DAP. The major respiratory quinone was menaquinone (MK)–9(H\(_2\)). Small amounts of MK-7(H\(_2\)) and MK-6 were also detected. The polar lipids contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside, an unknown phospholipid and two unknown lipids (Fig. S2). Mycolic acids were not present. The DNA G+C content of strain YC2-20\(^T\) was 73.1 mol%, within the range described in the genus [8]. On the other hand, strains YC2-20\(^T\) and YC2-19 were chemotaxonomically different from members of the other genera of the family **Kineosporiaceae**. Members of the genera **Kineococcus** [4] and **Pseudo-kineococcus** [5] differ from both isolates in that they...
contain MK-9(H$_2$) as the major menaquinone and do not have phosphatidylinositol and phosphatidylinositol mannoside as the polar lipids. The genus *Kineosporia* [3] can be distinguished from the novel strains by the presence of LL-DAP and phosphatidylcholine as the additional diamino acid in the cell wall and the polar lipid, respectively. In contrast to the novel isolates having MK-9(H$_2$), the genus *Quadrisphaera* [6] has MK-8(H$_2$) as the predominant menaquinone.

The cellular fatty acid profiles of the novel isolates and the three type strains of the genus *Angustibacter* are given in Table 2. Strains YC2-20$^T$ and YC2-19 showed similar profiles of cellular fatty acids in that they consisted mainly of saturated, unsaturated and iso-branched fatty acids; the major fatty acids of strain YC2-20$^T$ were C$_{17}:1\omega8c$ (20.1%), C$_{16}:0$ (18.9%), iso-C$_{16}:0$ (11.9%), iso-C$_{15}:0$ (11.1%) and C$_{18}:0$ (8.9%). In our study, performed under the same cultivation and analysis conditions, the three type strains of

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Cell morphology</td>
<td>Cocci, rods</td>
<td>Rods</td>
<td>Cocci, rods</td>
<td>Rods</td>
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<tr>
<td>Cell motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cell size (µm)</td>
<td>0.7–0.9×1.3–1.6</td>
<td>0.9–1.2×1.5–1.8</td>
<td>0.3–0.5×0.3–2.0</td>
<td>0.4–0.8×0.9–1.4</td>
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<tr>
<td>Temperature range for growth</td>
<td>20–37</td>
<td>10–37</td>
<td>5–30</td>
<td>10–37</td>
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<tr>
<td>pH range for growth</td>
<td>6–10</td>
<td>6–8</td>
<td>6–8</td>
<td>6–11</td>
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<tr>
<td>Growth at 3% (w/v) NaCl</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth under anaerobic condition</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Enzyme activity (API ZYM):</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Esterase</td>
<td>+</td>
<td>+</td>
<td>w*</td>
<td>–</td>
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<td>Valine arylamidase</td>
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<td>α-Chymotrypsin</td>
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<td>Acid production from (API 50CH):</td>
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<tr>
<td>d-Arabinose</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>–</td>
<td>+*</td>
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<tr>
<td>Arbutin</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>d-Arabitol</td>
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<tr>
<td>d-Fucose</td>
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<td>Gentiobiose</td>
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<td>w</td>
<td>+</td>
<td>–</td>
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<tr>
<td>d-Lyxose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Melezitose</td>
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<tr>
<td>Methyl α-d-glucoside</td>
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<td>–</td>
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<tr>
<td>d-Ribose</td>
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<td>+</td>
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<tr>
<td>Sorbitol</td>
<td>w</td>
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<td>L-Sorbose</td>
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<td>Trehalose</td>
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<td>–</td>
<td>+*</td>
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<td>Turanose</td>
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<td>Xylitol</td>
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<tr>
<td>D-Xylose</td>
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<td>–</td>
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<tr>
<td>L-Xylose</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<td>DNA G+C (mol%)</td>
<td>73.1</td>
<td>73</td>
<td>70.9</td>
<td>73.6</td>
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</tbody>
</table>

*Different from the results reported by Tamura et al. [1].

Table 1. Differential characteristics between *Angustibacter speluncae* sp. nov. (strains YC2-20$^T$ and YC2-19) and the type strains of the genus *Angustibacter*

species of the genus *Angustibacter* revealed similar profiles with those previously reported [1, 7, 8], albeit with differences in relative proportions and differing from one another by the presence of anteiso-C<sub>15:0</sub> (23.2 %) and iso-C<sub>16:0</sub> (1.6 %) in *A. aerolatus* KACC 15527<sup>T</sup>, of iso-C<sub>16:0</sub> (19.1 %) and iso-C<sub>17:1ω9c</sub> (12.5 %) in *A. luteus* KACC 14249<sup>T</sup> and of anteiso-C<sub>17:0</sub> (12.0 %) and anteiso-C<sub>15:0</sub> (7.4 %) in *A. peucedani* KCTC 19628<sup>T</sup>.

Strains YC2-20<sup>T</sup> and YC2-19 can be clearly differentiated from members of the genus *Angustibacter* by the presence of a large amount of C<sub>17:1ω8c</sub> (Table 2). Furthermore, both isolates differ phenotypically from the three type strains of species of the genus *Angustibacter* in that they possess the activities of trypsin, α-chymotrypsin and α-mannosidase, and produce acid from D-arabinose, melezitose, methyl α-D-glucoside, sorbitol, L-sorbose and xylitol, but do not
isolates and members of the genus *Angustibacter* proposed.

The combination of the phenotypic and phylogenetic dissimilarity supports that strains YC2-20 which the name *Angustibacter speluncae* sp. nov. is additionally contained C16:1ω7c and/or C17:1ω6c as the additional major components is variable depending on the species.

### DESCRIPTION OF *ANGUSTIBACTER SPELUNCAE* SP. NOV.

*Angustibacter speluncae* (spe.lun’cae. L. gen. n. speluncae of a cave, the site where the type strain was isolated).

Cells are Gram-positive, strictly aerobic, non-sporulating, oxidase-negative, catalase-positive, motile cocci (0.54–0.83 μm in diameter) to rods (0.7–0.9×1.3–1.6 μm). Colonies are circular, convex, entire, orange-coloured and 0.5–1.2 mm after incubation on TSA for 7 days at 30 °C. Grow well on TSA, R2A and ISP2 but moderately on NA. Growth occurs at 20–37 °C with an optimum at 30 °C, pH 6–10 with an optimum at pH 6–7 and in the absence of NaCl. Nitrate is not reduced to nitrite. Positive for gelatin hydrolysis and aesculin degradation but negative for glucose fermentation, indole production, arginine dihydrolase and urease. D-Glucose, D-arabinose (weak), D-mannitol, N-acetylgalactosamine, maltose, glucosan and malate (weak) are assimilated but D-mannose, caprate, citrate, adipate and phenylacetate are not (API 20NE). Enzyme activities for esterase, esterase lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase (weak), trypsin (weak), α-chymotrypsin, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase (weak) are positive but alkaline phosphatase, acid phosphatase and lipase, valine arylamidase, cystine arylamidase, β-galacturonidase, α-galactosidase, N-acetyl-β-glucosaminidase and α-fucosidase are negative (API ZYM). Acid is produced from D-arabinose, L-arabinose, D-xylene, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, sorbitol (weak), methyl α-D-glucoside, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-arabitol and D-ribulose but not from glycerol, erythritol, D-rhamnose, L-xylene, adonitol, methyl β-D-xyllose, rhamnose, dulcitol, inositol, D-mannitol, methyl α-D-mannoside, N-acetylglucosamine, amygdalin, arbutin, inulin, D-tagatose, D-fucose, L-fucose, L-arabitol, gluconate and D-xylose (API 50CH). The cell-wall peptidoglycan contains meso-DAP as the diagnostic diamino acid. The predominant menaquinone is MK-9(H4). Mycolic acids are absent. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol mannoside, an unknown phospholipid and two unknown lipids. The major fatty acids are C17:1ω8c, C16:0ω7c, isoc15:0ω7c, anteiso-C15:0ω7c, anteiso-C17:0ω7c, anteiso-C15:0ω8c and iso-C17:0ω8c as the additional major components is variable depending on the species.

### EMENDED DESCRIPTION OF THE GENUS *ANGUSTIBACTER* TAMURA ET AL. 2010

**EMEND KIM ET AL. 2013 EMEND LEE 2013**

The description is as given by Tamura et al. [1], Kim et al. [7] and Lee [8] with the following amendments. The predominant fatty acids (>10% in all species) are C16:0 and iso-C15:0. The presence of C17:1ω8c, iso-C16:0, anteiso-C15:0ω7c and or/and iso-C17:1ω6c as the additional major components is variable depending on the species.
Resources (NIBR) under the Ministry of Environment, Republic of Korea.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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