Spelaeicoccus albus gen. nov., sp. nov., an actinobacterium isolated from a natural cave

Soon Dong Lee

Department of Science Education, Jeju National University, Jeju 690-756, Republic of Korea

A novel Gram-stain-positive, non-endospore-forming, coccoid actinobacterium, designated strain D3-40T, was isolated from the soil of a natural cave and characterized by means of a polyphasic taxonomic analysis. 16S rRNA gene sequence analysis showed that strain D3-40T is a member of the suborder Micrococccineae and forms a distinct branch at the base of a Brevibacteriaceae cluster. Its closest relative is the type strain of Brevibacterium samyangense (95.7 % sequence similarity). The chemotaxonomic characteristics were as follows: the cell-wall peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid; the major menaquinone was MK-9(H2); the polar lipids consisted of phosphatidylglycerol, phosphatidylinositol, an unknown glycolipid and an unknown phospholipid; the major fatty acids were anteiso-C15:0, anteiso-C17:0, iso-C15:0, C16:0 and cyclohexyl-C17:0; mycolic acids were absent. The G+C content of the DNA was 64.3 mol%. On the basis of morphological, chemotaxonomic and phylogenetic data, it is suggested that the organism represents a novel species of a new genus within the family Brevibacteriaceae, for which the name Spelaeicoccus albus gen. nov., sp. nov. is proposed. The type strain of the type species is D3-40T (KCTC 29141T = DSM 26341T).

During a culture-dependent study on the diversity of cave bacteria, strain D3-40T was isolated from the soil of a natural cave in Jeju, Republic of Korea. For bacterial isolation, a soil sample (1 g) was suspended in 9 ml sterile distilled water and mixed in a tube rotator for 30 min. After serial dilution with sterile distilled water, aliquots (100 µl) of each dilution were transferred on to isolation media (soluble starch 1%, casein 0.03%, KNO₃ 0.2%, NaCl 0.2%, CaCO₃ 0.002%, MgSO₄·7H₂O 0.005%, FeSO₄·7H₂O 0.001%, agar 1.8%, pH 7.2) and the agar plates were incubated for 14 days at 30°C. Single colonies were picked up and streaked on International Streptomyces Project (ISP) medium 2 (Shirling & Gottlieb, 1966) several times and the pure culture was maintained in 20% (v/v) glycerol solution at −20°C and −80°C.

Growth was tested on ISP medium 2, nutrient agar (NA; Difco), R2A agar (Difco) and trypticase soy agar (TSA; Difco). Colony morphology and pigmentation was observed visually and recorded after 7 days of growth at 30°C on ISP medium 2. For checking the presence of a rod–coccus cycle during growth, cells from both young (12 h and 24 h) and older (2–7 day) cultures grown in ISP 2 broth were observed using a light microscope (×1000 magnification). Cell morphology and motility was

The family Brevibacteriaceae Breed 1953 emend. Stackebrandt et al. 1997 was emended with the update for a set of the other 13 families, at the time of writing, in the suborder Micrococccineae (Zhi et al., 2009) and currently contains the only and type genus Brevibacterium Breed 1953 emend. Collins et al. 1980. Elevation of this suborder to the order Micrococcales was recently proposed by Busse (2012). This genus is chemotaxonomically characterized by having meso-diaminopimelic acid as the diagnostic diamino acid in cell-wall peptidoglycan, MK-8(H₂) or MK-7(H₂) and MK-8 (H₂) as the major menaquinone(s), anteiso-methyl-branched components (anteiso-C₁₅:0 and anteiso-C₁₇:0) as the predominant fatty acids, no mycolic acids and DNA G+C contents of 55–71 mol% (Goodfellow & Trujillo, 2012; Kim et al., 2013). Strains of species of the genus Brevibacterium have a rod–coccus cell cycle during growth on complex media; many of which exhibit cells arranged at an angle to give V-forms (Goodfellow & Trujillo, 2012). At the time of writing, the genus contains 28 recognized species and its members have been isolated and cultured from dairy products, clinical specimens, poultry, sludge, and terrestrial and aquatic environments (Goodfellow & Trujillo, 2012; Kumar et al., 2013; Kim et al., 2013). During the characterization of bacterial isolates from a natural cave, a novel strain with an uncertain taxonomic affiliation was recovered. In this paper, the results of a polyphasic taxonomic study of the novel strain are reported, with the proposal of the name Spelaeicoccus albus gen. nov., sp. nov.

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequences of strain D3-40T is HF570029.

Three supplementary figures are available with the online version of this paper.
observed using a transmission electron microscope (JEM-1010; JEOL), with cultures grown for 48 h. Before negative staining with 1% phosphotungstic acid, cells were washed with distilled water twice and suspended in distilled water.

Growth at 4, 10, 20, 30, 37 and 42 °C was tested on ISP 2 agar. NaCl tolerance for growth was examined by using NaCl concentrations of 0–9% (w/v) at intervals of 1% (w/v); the recording was done after 7 days of incubation at 30 °C. The pH growth range and optimum was investigated between pH 4.0 and 10.0 (at intervals of 1.0 pH unit) and the agar plates, after inoculation, were placed for 5 days at 30 °C. Catalase and oxidase activities were determined by using previously described methods (Lee, 2006). Some physiological and biochemical properties were tested by using API CORYNE, API 20NE, API ZYM and API 50CH strips (bioMérieux) according to the manufacturer’s instructions. Assimilation tests in API 20NE and fermentation tests in API 50CH strips were observed and recorded after incubation for 4 and 6 days, respectively.

Cells were aerobic, Gram-stain-positive, non-endospore-forming, non-motile cocci (Fig. S1, available in IJSEM Online) that occurred singly, in pairs, in chains or in clusters. A clear rod–coccus cycle was not observed during growth. Colonies were white, opaque, circular and convex with entire margins. The results of other biochemical and physiological characterizations are given in the species description.

For determination of DNA G+C content and phylogenetic analysis, genomic DNA was isolated and purified according to the method of Hopwood et al. (1985). PCR amplification and sequencing of the 16S rRNA gene from strain D3-40T was performed as described by Lee (2006). The phylogenetic neighbours of strain D3-40T were examined through a preliminary BLAST search against GenBank and EMBL databases. The CLUSTAL X program (Thompson et al., 1997) was used for multiple alignments of the 16S rRNA gene sequences from strain D3-40T and related taxa. Phylogenetic relationships were inferred using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) treeing methods. A neighbour-joining tree was drawn from evolutionary distances computed by the method of Jukes & Cantor (1969), with bootstrap analysis (Felsenstein, 1985) of the 1000 replicate datasets.

The 16S rRNA gene sequence comparisons with entries in the GenBank and EMBL databases revealed that strain D3-40T was related to members of the suborder Micrococineae, being particularly related more closely to members of the family Brevibacteriaceae. A neighbour-joining tree showing the position of strain D3-40T within the radiation encompassing members of the family Brevibacteriaceae and representatives of 13 other families of the suborder Micrococineae is given as Fig. 1. Strain D3-40T formed a distinct monophyletic clade at the base of a cluster containing the family Brevibacteriaceae, with support by a high bootstrap value (83%) and from the maximum-parsimony treeing method. The highest 16S rRNA gene sequence similarity (95.7%) was found with Brevibacterium samyangense SST-8T. Levels of 16S rRNA gene sequence similarity between the organism and the other members of the family Brevibacteriaceae ranged from 92.1 to 95.7%, whereas the organism revealed sequence similarities lower than 93.6% to representatives of the 13 other families of the suborder Micrococcineae.

For chemotaxonomic analyses, cell biomass of the organism was obtained from a 5 day culture in ISP 2 broth at 30 °C. Polar lipids were extracted and identified by using two-dimensional TLC (Minnikin et al., 1977). Mycolic acids were analysed according to the method of Minnikin et al. (1980). Respiratory quinones were analysed by using HPLC (Kroppenstedt, 1985). The kind and isomer of diaminopimelic acid in the cell-wall peptidoglycan was determined by using one-dimensional TLC (Staneck & Roberts, 1974). The G+C content of the DNA was determined by HPLC (Mesbah et al., 1989).

The diagnostic diamino acid in cell-wall peptidoglycan was meso-diaminopimelic acid, which has been shown to be present in all brevibacteria examined so far. The polar lipid profile of strain D3-40T contained significant amounts of phosphatidylglycerol, phosphatidylinositol and an unknown glycolipid, with small amount of an unknown phospholipid (Fig. S2). Diphosphatidylglycerol, being present in some strains of species of the genus Brevibacterium (Heyrman et al., 2004; Kämpfer et al., 2010; Kim et al., 2013), and other characteristic phospholipids were not detected. The presence of the major lipids phosphatidylglycerol and an unidentified glycolipid has also been reported for Brevibacterium yomogidense (Tonouchi et al., 2013). Mycolic acids were not present. The DNA G+C content was 64.3 mol%. Analysis of respiratory quinones revealed that strain D3-40T contained menaquinone MK-9(H2), being a distinctive feature that can differentiate strain D3-40T from members of the family Brevibacteriaceae in that its phylogenetically close neighbours, all the type strains of species of the genus Brevibacterium, have MK-8(H2) or MK-7(H2) and MK-8(H2) as the predominant menaquinone(s) (Goodfellow & Trujillo, 2012; Tonouchi et al., 2013; Kim et al., 2013).

For determination of cellular fatty acids, cells of strain D3-40T and B. samyangense SST-8T were grown on ISP medium 2 for 5 days at 30 °C. The preparation and analysis of fatty acid methyl esters (FAMEs) were performed by GC according to the instructions of the Sherlock Microbial Identification System (MIDI), using version 6.1 of the ACTIN6 library. A main peak including two or more fatty acids which could not be separated by GLC using the MIDI system was analysed and identified by GC-MS, as described by Manaia & Moore (2002) with a 30 m RTX-5MS fused silica capillary column (0.25 mm internal diameter and 0.25 μm film thickness; RESTEK).

The cellular fatty acid composition of strain D3-40T and B. samyangense SST-8T is given in Table 1. The cellular fatty acid profile of strain D3-40T was represented by the presence of straight-chain saturated, iso- and anteiso-methyl-branched
fatty acids together with a cycohexyl fatty acid; the major fatty acids were anteiso-C_{15:0} (20.6%), anteiso-C_{17:0} (19.8%), iso-C_{15:0} (18.2%), C_{16:0} (11.9%) and cyclohexyl-C_{17:0} (11.7%). The cyclohexyl-C_{17:0} was confirmed by GC-MS (Fig. S3), albeit being identified as summed feature 7 (C_{18:1ω9t}ω6tω11c) which could not be separated by GLC using the MIDI system. As an authentic sample of cyclohexyl-C_{17:0}, FAMEs of *Humibacter albus* DSM 18994^T* (Vaz-Moreira et al., 2008) were also analysed and compared. On the other hand, *B. samyangense* SST-8^T in this analysis contained significant amounts of straight-chain saturated, iso- and anteiso-methyl-branched fatty acids, having anteiso-C_{17:0} (36.8%) and anteiso-C_{15:0} (32.7%) as the predominant fatty acids, but no unsaturated or cyclohexyl fatty acids were detected; this feature is typical of members of the genus *Brevibacterium* (Goodfellow & Trujillo, 2012). Some strains of species of the genus *Brevibacterium*, such as *Brevibacterium album* (Tang et al., 2008), *Brevibacterium daeugense* (Cui et al., 2013), *Brevibacterium siliguriense* (Kumar et al., 2013) and *Brevibacterium ammoniilyticum* (Kim et al., 2013), have been reported to have iso-methyl-branched (iso-C_{15:0}) or straight-chain saturated (C_{16:0}) fatty acid as additional major components. However, strain D3-40^T can be readily differentiated from members of the genus *Brevibacterium* in having a cycohexyl-C_{17:0} as a major fatty acid.

Affiliation of strain D3-40^T to the family *Brevibacteriaceae* was supported by possession of all the 16S rRNA gene signature nucleotides that have been defined for the family *Brevibacteriaceae* (Zhi et al., 2009). In addition, strain D3-40^T possesses a unique set of signature nucleotides that place it in a novel lineage within the family *Brevibacteriaceae*, such as 293 : 304 (G–U), 289 : 311 (G–U), 381 (C), 379 : 384 (G–G), 445 : 489 (G–U), 501 : 544 (C–G), 612 : 628 (U–A), 613 : 627 (A–U) and 837 : 849 (C–G), with low levels of 16S rRNA gene sequence similarities (<95.7%) to members of the family *Brevibacteriaceae*. Furthermore, the combination of the morphological and chemotaxonomic data (Table 2) clearly excludes strain D3-40^T from the genus *Brevibacterium* and places it in a novel genus within the family *Brevibacteriaceae*.

On the basis of morphological, chemotaxonomic and phylogenetic data presented here, it is suggested that strain D3-40^T represents a new genus and novel species of the family *Brevibacteriaceae*, for which the name *Spelaeicoccus albus* gen. nov., sp. nov. is proposed.

### Description of *Spelaeicoccus albus* gen. nov.

*Spelaeicoccus* [Spe.lae.i.coc’cus. L. neut. n. spelaeum a cave; N.L. masc. n. coccus (from Gr. masc. n. kokkos grain, seed) coccus; N.L. masc. n. *Spelaeicoccus* a coccus from cave]. Cells are Gram-stain-positive, non-endospore-forming, non-motile cocci that occur singly, in pairs, in chains or in clusters and are aerobic. Oxidase-negative and catalase-positive. The diagnostic diaminoc acid in the cell wall is meso-diaminopimelic acid. Mycolic acids are not present. The major menaquinone is MK-9(H2). The major polar lipids are phosphatidylglycerol, phosphatidylinositol and an unknown glycolipid. The cellular fatty acid profile is represented by the presence of iso- and anteiso-methyl-branched, straight-chain saturated and cycohexyl fatty acids. The type species is *Spelaeicoccus albus*. Phylogenetically, the genus belongs to the family *Brevibacteriaceae*, order *Micrococcales*. The G+C content of the DNA of the type strain of the type species is 64.3 mol%.

### Description of *Spelaeicoccus albus* sp. nov.

*Spelaeicoccus albus* (al’bus. L. masc. adj. albus white, referring to the colour of the colonies).

The following characteristics are given in addition to properties described for the genus. Cocci are 0.7–0.8 μm in diameter. Colonies are white, opaque, circular and convex and have entire margins. Temperature range for growth is 10–37 °C, with an optimum temperature of 28–30°C. Growth does not occur at 4 °C or 42 °C. pH range for...
growth is pH 5–9, with an optimum at pH 7. Tolerates 0–6 % NaCl, with optimum growth at 0–3 % NaCl. Leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-galactosidase (weak) and N-acetyl-β-glucosaminidase activities are present, but alkaline phosphatase, esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities are absent (API ZYM). In tests with the API CORYNE strip, nitrate reduction is positive. Pyrazinamidase and pyrrolidonyl arylamidase activities are absent. In tests using API 20NE strips, aesculine degradation shows a weak response but indole production, glucose fermentation, arginine dihydrolase, urease and gelatin hydrolysis are not observed. D-Glucose, D-mannose, D-mannitol (weakly), L-xylose, adonitol, D-mannose, D-mannitol, methyl-D-glucoside, methyl-D-glucosamine, N-acetylglucosamine, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, glucuronate, 2-ketogluconate or 5-ketogluconate (API 50CH).

The type strain is strain D3-40T (=KCTC 29141T=DSM 26341T), which was isolated from soil of a natural cave in Jeju, Republic of Korea. The major fatty acids of the type strain are anteiso-C15:0, anteiso-C17:0, iso-C15:0, C16:0 and cyclohexyl-C17:0. The G+C content of the DNA of the type strain is 64.3 mol%.

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## References


### Table 2. Differential characteristics between strain D3-40T and the species of the genus *Brevibacterium*

<table>
<thead>
<tr>
<th>Characters</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Cell morphology</td>
<td>Cocci</td>
<td>Rod–coccus cycle</td>
</tr>
<tr>
<td>Cell motility</td>
<td>–</td>
<td>v</td>
</tr>
<tr>
<td>Major menaquinone type</td>
<td>MK-9(H2)</td>
<td>‘MK-8(H2)’ or ‘MK-7(H2) and MK-8(H2)’</td>
</tr>
<tr>
<td>Major fatty acid types</td>
<td>S, A, I, Ch</td>
<td>S, A, I</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>64.3</td>
<td>55–71</td>
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