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Arthrobacter subterraneus sp. nov., Isolated from Deep Subsurface Water of the South Coast of Korea

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Abstract Strain CH7^T, a pale yellow-pigmented bacterium and new isolate from deep subsurface water of the South Coast of Korea, was subjected to a polyphasic taxonomic study. CH7^T grew between 5 and 37°C, pH 5.3–10.5, and tolerated up to 13% NaCl. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain CH7^T was associated with the genus *Arthrobacter* and phylogenetically closely related to the type strains *Arthrobacter tumbae* (99.4%) and *Arthrobacter parietis* (99.1%). However, DNA-DNA hybridization experiments revealed 2.1% and 12% between strain CH7^T and *Arthrobacter tumbae* and *Arthrobacter parietis*, respectively. Thus, the phenotypic and phylogenetic differences suggested that CH7^T should be placed in the genus *Arthrobacter* as a novel species, for which the name *Arthrobacter subterraneus* sp. nov. is proposed. In addition, the type strain for the new species is CH7^T (=KCTC 9997^T=DSM 17585^T).

Keywords: *Arthrobacter*, 16S rRNA gene, phylogenetic characteristic

Microorganisms inhabit the entire world in which we live, yet for a comprehensive understanding of microorganisms, it is important to culture, isolate, and characterize new species isolated from environmental niches [2, 3, 19, 20, 33, 34]. The genus *Arthrobacter* was discovered by Conn and Dimmick [5], where the members are Gram-positive, aerobic, catalase-positive, and produce little or no acid from glucose. Most *Arthrobacter* spp. also have a rod-coccus growth cycle, DNA G+C content of 59–66 mol%, branched cellular fatty acids, and L-lysine in the peptidoglycan [12]. The genus *Arthrobacter* has since been divided into two groups based on their peptidoglycan structures and menaquinone composition [28]. The *Arthrobacter* species in group I contain an A3α peptidoglycan variant, in which murein is cross-linked by interpeptide bridges involving monocarboxylic L-amino acids, or glycine, or both, as observed in most species of *Arthrobacter*, including *Arthrobacter globiformis*, the type strain of the genus [30]. The strains in group II possess an A4α peptidoglycan variant, in which the peptidoglycans are cross-linked by bridges containing a dicarboxylic acid, such as l-Lys-Ala-Glu or l-Lys-Glu, as in *Arthrobacter nicotianae*, *Arthrobacter uratoxydans*, *Arthrobacter protophormiae*, *Arthrobacter sulfurous*, *Arthrobacter mysorens*, *Arthrobacter creatinolyticus*, and *Arthrobacter rhombi* [6, 10, 18, 28, 30, 31]. The genus *Arthrobacter* constitutes a predominant group of microorganisms from various environments all over the world. At the time of writing, 52 species of the genus *Arthrobacter* (http://www.bacterio.cict.fr/a/arthrobacter.html) have been recognized and identified as phenotypically heterogeneous. However, *Arthrobacter* species isolated from deep subsurface water have rarely been reported. Accordingly, this study describes the morphological, biochemical, and phylogenetic characteristics of an *Arthrobacter* species isolated from deep subsurface water. Recently, an *Arthrobacter*-like, Gram-positive, and pale-yellow pigmented bacterial strain CH7^T was isolated using a dilution-plating technique from deep subsurface water collected from the South Coast of Korea. The collection area was located in the center of the Pohang basin from the Southeastern Coast of the Korean peninsula, and was the focus of drilling for deep hydrothermal systems to utilize subterranean heat

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energy. The basin has a basement consisting of Cretaceous sedimentary rocks, including tuffs and granites, which are covered with Miocene Yeonil formations, semiconsolidated clastic sedimentary rocks with marine and terrestrial origins. Based on drilling to a depth of 1,000 m, groundwater was pumped from below 500 m with a temperature above 42°C, salinity of about 0.1%, and pH of about 8.0.

Strain CH7^T was routinely grown on a nutrient agar (NA) at 25°C with replating every 3 days. The cell morphology was examined by light microscopy (E600; Nikon) and transmission electron microscopy (TEM). The presence of flagella was investigated by TEM, using cells from exponentially growing cultures. The Gram reaction was determined using a Gram Stain kit (Difco) according to the manufacturer's instructions. For reference strains, the most closely related strains, based on 16S rDNA similarity, *Arthrobacter tumbae* DSM 16406^T, *Arthrobacter parietis* DSM 16404^T, *Arthrobacter tecti* DSM 16407^T, and *Arthrobacter agilis* DSM 20550^T, were obtained from DSMZ, Germany, and grown under the same conditions. For morphological and physiological characterization, strain CH7^T was generally cultivated in a nutrient broth (NB) and incubated by shaking at 25°C. The growth at various NaCl concentrations, temperatures, and pHs were measured in an NB broth, whereas the growth under anaerobic conditions was determined after incubation for 7 days in anaerobic Gaspak jars (BBL) containing an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. The catalase activity was determined by bubble production in a 3% (v/v) H_2O_2 solution, the oxidase activity determined using an oxidase reagent (bioMerieux Inc., Marcy L'Etoile, France), whereas API Staph and API ZYM test strips (bioMerieux Inc., Marcy L'Etoile, France) were used to analyze the biochemical and physiological traits of the bacterial strain, and additional biochemical tests were performed using the methods and media described by Gordon *et al.* [7]. The bacterial strains were grown on NA for 3 days at 25°C to analyze the fatty acid methyl esters (FAMEs), which were extracted and prepared according to the standard protocols provided by the MIDI/Hewlett Packard Microbial Identification System [26]. Using the method of Rosenthal and Dziarski [23], the peptidoglycan was hydrolyzed with 4 M HCl at 120°C for 60 min, and the composition of the major chain determined according to the method of Schleifer and Kandler [28]. The isoprenoid quinones in the CH7^T strain were extracted from 100 mg of freeze-dried cells, according to previously described methods [4], and purified *via* preparative thin-layer chromatography (TLC, silica gel F254; Merck). Additionally, the ubiquinone fraction was analyzed *via* a high-performance liquid chromatograph (HPLC, Hitachi L-5000) equipped with a reverse-phase column (YMC pack ODS-AM; YMC Co.), as described

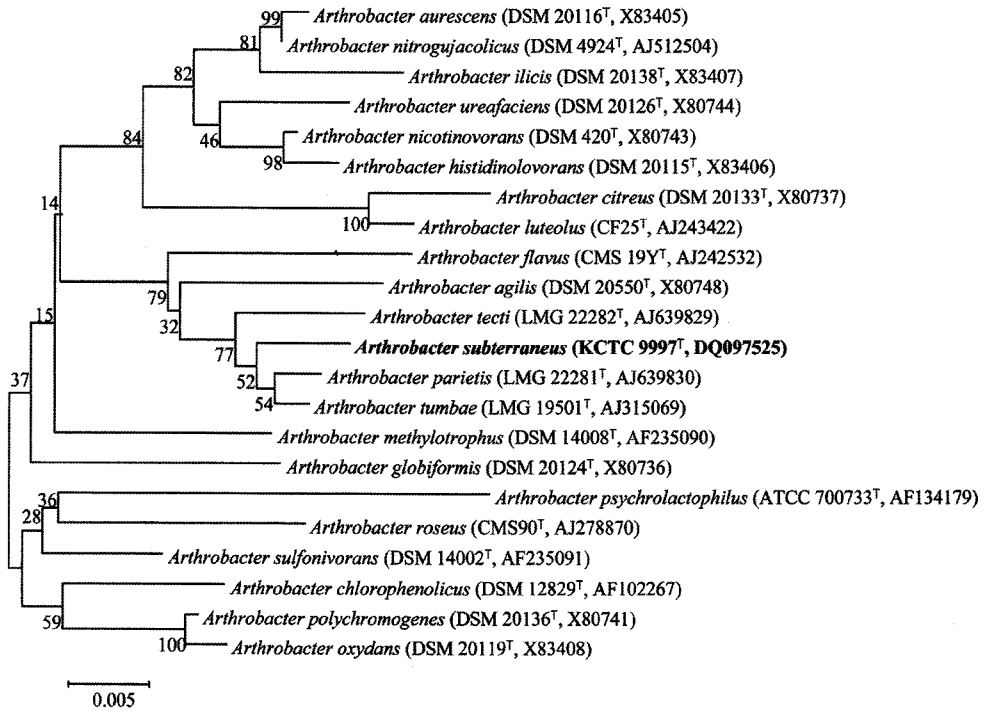

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain CH7^T with respect to other species of genus *Arthrobacter.*

The tree was generated using the neighbor-joining method. The numbers at the nodes indicate the bootstrap values (1,000 replications). Bar, 0.005 accumulated changes per nucleotide.

previously [29]. The cell-wall sugars were prepared and analyzed according to the method described by Komagata and Suzuki [15], and the chromosomal DNA extracted and purified according to the method described by Sambrook *et al.* [25]. The 16S rRNA gene was amplified by a PCR using two universal primers, as previously described [36], and the DNA-DNA hybridization performed fluorometrically by the method of Bae *et al.* [1] using Cy5-labeled genomic DNA probes and genome-spotted microarrays. The 16S rRNA gene sequence of CH7^T was then aligned with 21 reference sequences (Fig. 1) from the RDP database using the multiple sequence alignment program CLUSTAL_X (1.8) [35]. The phylogenetic relationships between representatives from the genus *Arthrobacter* were determined using MEGA version 2.1 software. The distance matrices were determined following the assumptions described by Kimura [13], and these matrices used to elaborate dendrograms using the neighbor-joining method [24]. A bootstrap analysis to investigate the stability of the trees was then performed by obtaining a consensus tree based on 1,000 randomly generated trees.

The isolate did not grow under anaerobic conditions and presented a negative Voges-Proskauer test. The characteristics that differentiated the new isolate from related species are shown in Table 1, and the CH7^T FAMEs were iso-$C_{15:0}$ (12.8%), anteiso-$C_{15:0}$ (35.4%), iso-$C_{16:1}$H (4.1%), iso-$C_{16:0}$ (5.6%), $C_{16:1}\omega7c$/iso-$C_{15:0}$2-OH (4.6%), $C_{16:0}$ (1.6%), iso-$C_{17:1}\omega9c$ (3.7%), anteiso-$C_{17:1}\omega9c$ (15%), iso-$C_{17:0}$ (1.8%), anteiso-$C_{17:0}$ (12.1%), and iso-$C_{15:0}$2-OH/$C_{16:1}\omega7c$ (4.6%).

Although the genus *Arthrobacter* includes a few species that are unpigmented (*A. globiformis*, *A. crystallopoietes*, *A. pascens*, and *A. istidinolovorans*), the majority produces various types of pigment; for example, yellow (*A. aurescens*, *A. ilicis*, *A. citreus*, *A. flavus*, *A. tecti*, *A. koreensis*, *A. psychrophenolicus*, *A. arilaitensis*, *A. bergerei*, *A. nicotianae*, *A. protophormiae*, *A. uratoxydans*, *A. sulfurous*, *A. gangotriensis*, *A. kerguelensis*, and *A. mysorens*), light yellow (*A. monumenti*, *A. castelli*, *A. gandavensis*, and *A. pigmenti*), orange to yellow (*A. parietis* and *A. tumbae*), grey to yellow (*A. ureafaciens*, *A. oxydans*, and *A. siderocapsulatus*), blue to black (*A. atrocyaneus*), and red (*A. agilis* and *A. roseus*) [8, 9, 11, 12, 14, 16, 17, 21,

Table 1. Characteristics that differentiate *Arthrobacter subterraneus* sp. nov. from its five closest phylogenetic relatives.

Species	1	2	3	4	5	6
Colony color	Pale yellow	Yellow-orange	Yellow-orange	Yellow	Rose-red	Yellow
Growth in 5% NaCl	+	+	+	+	-	+
Nitrate reduction	-	v	+	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	+	-
Urease	-	v	v	-	-	-
Alkaline phosphatase	-	-	-	+	NG	NG
Esterase lipase (C_8)	+	+	-	+	NG	NG
Lipase (C_{14})	-	-	-	-	+	-
β-Galactosidase	+	-	+	v	+	+
Cell-wall sugars*	Gal, Glc, Rib	Gal	Gal	Gal, Man	GlcN	Gal, Glc, Rib
Peptidoglycan type	Lys-Thr-Ala_3	Lys-Thr-Ala_3	Lys-Thr-Ala_2	Lys-Thr-Ala_3	Lys-Thr-Ala_3	Lys-Thr-Ala_3
Predominant cellular fatty acids	Anteiso-$C_{15:0}$ (35.4%), anteiso-$C_{17:1}\omega9c$ (15%)	Anteiso-$C_{15:0}$ (57%), iso-$C_{15:0}$ (17%)	Anteiso-$C_{15:0}$ (51%), iso-$C_{15:0}$ (29%),	Anteiso-$C_{15:0}$ (44%), iso-$C_{15:0}$ (37%)	Anteiso-$C_{15:0}$ (65.5%), iso-$C_{15:0}$ (13%)	Anteiso-$C_{15:0}$ (52%), iso-$C_{16:0}$ (17%)
Percentage of menaquinones						
MK-7(H_2)	-	6	-	-		
MK-8(H_2)	1	4	2	-		
MK-9(H_2)	56	54	69	41	Major	Major
MK-10(H_2)	36	25	20	28		
MK-11(H_2)	2	7	4	11		
MK-12(H_2)	-	-	-	2		
MK-9	-	-	-	7		
MK-10	-	-	-	4		
MK-11	-	-	-	1		
DNA G+C content (mol%)	62.7	64.7	63.8	63.7	67–70	62–66

Species: 1, *A. subterraneus* sp. nov.; 2, *A. tumbae*; 3, *A. parietis*; 4, *A. tecti*; 5, *A. agilis*; 6, *A. flavus*. Data from known species of *Arthrobacter* are from Heyrman *et al.* [9], Koch *et al.* [14], and Reddy *et al.* [21]. +, Positive; v, variable; -, negative; w, weak reactions; NG, not given.
*Abbreviations for cell-wall sugars: Gal, galactose; Glc, glucose; GlcN, glucosamine; Man, mannose; Rib, ribose.

22, 27, 32]. The prominent characteristic that differentiated strain CH7^T from other related species was the production of a pale-yellow pigment. Moreover, while exhibiting certain phenotypically recognizable traits, strain CH7^T displayed a strong valine arylamidase activity in contrast to the type strains of phylogenetically closely related species, such as *A. tecti*, *A. tumbae*, *A. castelli*, *A. monumenti*, *A. parietis*, and *A. pigmenti*. Furthermore, the predominant fatty acids for CH7^T were anteiso-$C_{15:0}$ (35.4%) and anteiso-$C_{17:1}\omega9c$ (15%), whereas those for *A. flavus* were anteiso-$C_{15:0}$ (52%) and iso-$C_{16:0}$ (17%), those for *A. parietis* were anteiso-$C_{15:0}$ (51%) and iso-$C_{15:0}$ (29%), those for *A. tumbae* were anteiso-$C_{15:0}$ (57%) and iso-$C_{15:0}$ (17%), and those for *A. tecti* were anteiso-$C_{15:0}$ (44%) and iso-$C_{15:0}$ (37%).

The phylogenetic trees based on the 16S rRNA gene sequences from members of different species of *Micrococcaceae*, including *Arthrobacter* species, placed strain CH7^T within the cluster of *Arthrobacter* species (Fig. 1). CH7^T exhibited the highest 16S rDNA similarity to *Arthrobacter parietis*^{T} (99.1%), *Arthrobacter tumbae*^{T} (99.4%), *Arthrobacter tecti*^{T} (98.5%), and *Arthrobacter agilis*^{T} (98.1%). DNA-DNA homology studies were then performed to determine the genomic relationship between CH7^T and type strains from the closest relatives as regards 16S rDNA similarity. As a result, the DNA-DNA relatedness between strain CH7^T and *A. parietis*^{T}, *A. tumbae*^{T}, *A. tecti*^{T}, and *A. agilis*^{T} was 12%, 2.1%, 4%, and 17%, respectively. Thus, when considering the phenotypic, phylogenetic, and genotypic characteristics of the isolate, it was concluded that CH7^T belonged to the genus *Arthrobacter*. However, based on the phylogenetic and DNA-DNA hybridization data, it is proposed that strain CH7^T should be the type strain for a novel species, *Arthrobacter subterraneus* sp. nov.

Description of *Arthrobacter subterraneus* sp. nov.

Arthrobacter subterraneus (sub.terr.an'e.us. L. masc. adj. *subterraneus* under the earth, indicating the source of isolation).

The cells are Gram-positive, short rods, and cocci (diameter 0.8–1 μm) occurring singly, in pairs, or in clusters. They are nonmotile and do not form endospores. Colonies grown on NA after 48 h are small (<1 mm), pale yellow, round with entire margins, of a low convexity, opaque, and smooth. No growth in an anaerobic chamber on NA. Optimum temperature for growth is 20–30°C. Weak growth at 37°C, and no growth at 45°C. Growth is apparent at 4°C after 1 week of incubation. Growth occurs on media with 13% NaCl, yet not with 15% NaCl. Growth occurs in the pH range 5.3 to 10.5. Catalase-positive and oxidase-negative. Using the API Staph system, positive reactions are observed for fermentation with D-mannitol, xylitol, D-melibiose, and raffinose, whereas negative reactions are obtained for fermentation with D-glucose, D-fructose, D-mannose, maltose, lactose, D-trehalose, potassium nitrate, β-naphthyl-acid phosphate, sodium pyruvate, xylose, sucrose, α-methyl-D-glucoside, *N*-acetyl-glucosamine, arginine, and urea. Using the API ZYM system, activity is detected with esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, and α-glucosidase, whereas no activity is detected with alkaline phosphatase, lipase (C_{14}), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The predominant fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:1}\omega9c$ (approximately 35% and 15%, respectively). The cell-wall peptidoglycan type is A3α, with a Lys-Thr-Ala_3 interpeptide bridge. The major menaquinone is MK-9(H_2). The cell-wall sugars are galactose, glucose, and ribose. The type strain, CH7^T (=KCTC 9997^T, =DSM 17585^T), was isolated from deep subsurface water in Pohang, Gyeongbuk Province, Korea.

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