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# *Methylobacterium dankookense* sp. nov., Isolated from Drinking Water

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**A pink-pigmented bacterium, designated SW08-7$^T$ was isolated from the drinking water of a water purifier. Cells were Gram-negative, rod-shaped, strictly aerobic, and non-spore-forming. It grew optimally at 25°C, pH 6~7. Phylogenetic analysis based on 16S rRNA gene sequence showed that strain SW08-7$^T$ belongs to the genus *Methylobacterium*. The highest 16S rRNA gene sequence similarities were found to *Methylobacterium mesophilicum* JCM 2829$^T$ (96.9%), *Methylobacterium brachiatum* B0021$^T$ (96.9%), *Methylobacterium phyllosphaerae* CBMB27$^T$ (96.6%), *Methylobacterium radiotolerans* JCM 2831$^T$ (96.6%), and *Methylobacterium hispanicum* GP34$^T$ (96.5%). DNA-DNA hybridization experiment revealed low-level (28.5%) of DNA-DNA relatedness between strain SW08-7$^T$ and *Methylobacterium hispanicum*. The genomic DNA G+C content was 68.9 mol% and the major isoprenoid quinone was Q-10. The major cellular fatty acid of strain SW08-7$^T$ was $C_{18:1}$ $\omega$7*c* (79.8±2.1%). Results of phylogenetic, phenotypic, and biochemical analyses revealed that strain SW08-7$^T$ could be classified as representing a novel species of genus *Methylobacterium*, for which the name *Methylobacterium dankookense* sp. nov. is proposed. The type strain is SW08-7$^T$ (=KCTC 22512$^T$ =DSM 22415$^T$).**

***Keywords*:** drinking water, alphaproteobacteria, *Methylobacterium*

The genus *Methylobacterium* was first proposed by Patt *et al*. (1976). The genus was composed of a variety of pink-pigmented, facultatively methylotrophic (PPFM) bacteria which are capable of growth on one-carbon compounds such as formate, formaldehyde, and methanol as the sole source of carbon and energy as well as on a wide range of multi-carbon growth substrates (Green, 1992). Members of the genus *Methylobacterium* have been isolated from soil, dust, freshwater, lake sediments, leaf surfaces, nodules, rice grain, air, and hospital environments (Green and Bousfield, 1983). The type species *Methylobacterium organophilum*, is not able to grow on methane. In contrast, *M. populi*, a recently described species isolated from poplar tissues, can utilize methane as the sole source of carbon and energy (Van Aken *et al*., 2004). *M. nodulans* is a legume root nodule-dwelling bacterium that is a nitrogen-fixer (Jourand *et al*., 2004) and *M. phyllosphaerae* was isolated from the phyllosphere of rice (Madhaiyan *et al*., 2009). Recently, five novel species were isolated from drinking-water system (Gallego *et al*., 2005a, 2005b, 2006) and another five novel species were isolated from freshwater (Kato *et al*., 2008). In this paper, the taxonomic position of a novel *Methylobacterium* strain, SW08-7$^T$, isolated from drinking water in Korea, is reported.

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## Materials and Methods

### Bacterial strain

Drinking water samples were plated on Plate Count Agar (PCA; Difco) and R2A agar medium (Difco, USA). Strain SW08-7$^T$ was isolated on PCA medium after incubation at 25°C for 7 days. The isolate was routinely cultured on PCA medium and maintained as a glycerol suspension (20%, w/v) at -70°C. Reference strain, *Methylobacterium phyllosphaerae* KACC 11716$^T$ and *Methylobacterium hispanicum* KACC 11432$^T$ were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. Strain SW08-7$^T$ was deposited to KCTC (Korean Collection for Type Cultures) as KCTC 22512$^T$, and DSMZ (German Collection of Microorganisms and Cell Cultures) as DSM 22415$^T$.

### Morphological and physiological characteristics

Air-dried smears from cultures were stained using a commercial Gram staining kit (Sigma-Aldrich, USA) to determine the Gram reaction. Cell morphology was ascertained by light microscopy using a CHT microscope (Olympus, Japan) at ×1000 magnification, and by scanning electron microscopy following recovery of cells grown for 7 days at 25°C on Tryptic Soy Agar (TSA; Difco). Preparation for electron microscopy involved fixation of cells in 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol, and substituted by isoamyl acetate. Then they were dried at the critical point in $CO_2$. Finally the samples were sputtered with gold in a sputter coater (SC502, Polaron) and ob-

served using the scanning electron microscope, HITACHI S4300N (HITACHI, Japan) installed in KRIBB (Korea Research Institute of Bioscience and Biotechnology). Motility test was carried out on TSA medium contained 0.5% of agar. Growth was ascertained at temperature of 4, 10, 15, 20, 25, 30, 37, and 45°C in Tryptic Soy Broth (TSB; Difco) and pH of 3~10. The influence of salinity was tested using modified TSB supplemented with 0, 0.2, 0.5, 1, 1.5, 2, 2.5, and 3% (w/v) NaCl. Substrate utilization and enzyme activity tests were conducted by using the API 20NE, API ID 32GN, and API ZYM galleries according to the instructions of the manufacturer (bioMérieux, France). Aerobic growth was tested in a thioglycollate medium (Difco) at 25°C for 7 days. The utilization of various electron acceptors was examined on TSB media containing yeast extract (0.1%) as electron donor. Electron acceptors were added in the form of autoclaved stock solutions. The following electron acceptors were used at the final concentration of 10 mM: $KNO_3$, Fe(III)-citrate, sulfate, and $NaHCO_3$. The medium (10 ml) was dispensed in 50 ml serum bottles and bubbled solution and aerated the headspace with filtered $N_2$ gas to achieve the anaerobic condition.

### DNA G+C content and quinone composition

For measurement of the G+C content of chromosomal DNA, the genomic DNA of strain SW08-7$^T$ was extracted and purified as described by Moore and Dowhan (1995) and enzymatically degraded in nucleosides. The DNA G+C content was determined as described by Mesbah *et al*. (1989), using reversed-phase high-pressure liquid chromatography (HPLC, Supelco). Quinone analyses were accomplished using high performance thin layer chromatography (HPTLC) and HPLC (Hiraishi *et al*., 1996).

### Cellular fatty acid profiles

Fatty acid profiles of strain SW08-7$^T$ and type strains were determined on the identical conditions. Cells grown on TSA for 7 days at 25°C were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analyzed by gas chromatography (model 6890; Hewlett Packard, USA) using the Microbial Identification software package. Triplicate experiments were performed.

### Determination of 16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted using the InstaGene™ Matrix (Bio-Rad, USA). PCR amplification of 16S rRNA gene was accomplished using the 27F-1492R universal primer and GeneAmp PCR system 9700 (Applied Biosystems, USA). The phylogenetic position of strain SW08-7$^T$ was determined from their 16S rRNA gene sequences. The sequences obtained were aligned with those of representative members of selected genera belong to the genus *Methylobacterium* using Bioedit sequence alignment editor. Phylogenetic analysis was performed using the software package Mega (Molecular Evolutionary Genetics Analysis) version 4.1 (Tamura *et al*., 2007). Distance calculation (distance options according to the Kimura two-parameter, Jukes-Cantor and Taiima-Nei model) and clustering with the neighbour-joining and maximum evolution were performed, and bootstrap values were estimated based on 1,000 replications. Similarity of 16S rRNA gene was compared using the EzTaxon server version 2.1 (http://www.EzTaxon.org).

### DNA-DNA hybridization

The taxonomic relationship between strain SW08-7$^T$ and the type strain of *M. hispanicum* was further examined using DNA-DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Seldin and Dubnau, 1985) according to the method described by Baik *et al*. (2006).

## Results and Discussion

### Morphological and physiological characteristics

Strain SW08-7$^T$ was Gram-negative, motile, and non-spore-forming. The isolate was catalase positive and positive for oxidase reaction with N,N,N,N-tetramethyl-p-phenylenediamine. Cells were rod (0.8~0.9×1.2~1.6 μm) and occurred singly, in pairs or in rosettes. The colonies grown on PCA agar for 7 days were circular, pink color and 0.4~2.0 mm

**Table 1.** Comparison of the characteristics of strain SW08-7$^T$ with members of other *Methylobacterium* species
Strains: 1, *M. dankookense* sp. nov. SW08-7$^T$; 2, *M. hispanicum* GP34$^T$ (data from Kato *et al*., 2008); 3, *M. phyllosphaerae* CBMB27$^T$ (data from Madhaiyan *et al*., 2009). +, positive; -, negative; w, week; -, no data.

| Characteristics | 1 | 2 | 3 |
|---|---|---|---|
| Colony pigmentation | Pink to red | Pink | Pink to red |
| Cell length (μm) | 1.2~1.6 | 2.0~2.5 | 1.8~2.7 |
| Cell width (μm) | 0.8~0.9 | 1.0~1.5 | 0.63~0.64 |
| Diameter of colony (mm) | 0.4~2.0 | 1.0~2.0 | 0.2~0.8 |
| Range (optimum) | | | |
| Templature (°C) | 10~30 (25) | 15~30 (28) | 20~30 (28) |
| pH | 5.0~8.0 (6.0~7.0) | - | 5.0~9.0 (6.8) |
| NaCl tolerance (%) | 0~1.5 (2.0) | - | 0~2.0 (-) |
| Composition of ubiquinones | | | |
| Ubiquinone Q-9 | 10.1 | 2.8 | - |
| Ubiquinone Q-10 | 89.9 | 97.2 | - |
| DNA G+C content (mol%) | 68.9 | 69.9 | 66.8 |

**Table 2.** Differential biochemical characteristics between the novel strains and their nearest phylogenetic neighbours
Strains: 1, *M. dankookense* sp. nov. SW08-7$^T$; 2, *M. hispanicum* KACC 11432$^T$; 3, *M. phyllosphaerae* KACC 11716$^T$. +, positive; -, negative; w, weak.

| Characteristics | 1 | 2 | 3 |
|---|---|---|---|
| Nitrate reduction | + | - | - |
| Enzyme activity | | | |
| Crystine arylamidase | + | - | - |
| Trypsin | + | - | - |
| Urease | + | + | w |
| Utilization of | | | |
| Itaconic acid | + | + | - |
| Suberic acid | - | + | + |
| Sodium malonate | + | + | - |
| Sodium acetate | + | + | - |
| L-Alanine | + | - | - |
| L-Arabinose | - | - | + |
| Propionic acid | + | - | - |

in diameter. Growth occurred at 10~30°C but not at 4 or 37°C and pH 5.0~8.0, with a pH optimum of 6.0~7.0. Growth occurred in the absence of NaCl and in the presence of 0~1.5% (w/v) NaCl, but not in the presence of 2% (w/v) NaCl (Table 1). The isolate grew on the surface of thioglycollate medium. There was no growth on TSB media with yeast extract as the electron donor and $KNO_3$, Fe(III)-citrate, sulfate, or $NaHCO_3$ as the electron acceptor. Biochemical characteristics that differentiate strain SW08-7$^T$ from other members of the genus *Methylobacterium* were listed in Table 2.

**Table 3.** Fatty acid composition of strain SW08-7$^T$and related species of genus *Methylobacterium*
Strains: 1, *M. dankookense* sp. nov. SW08-7$^T$; 2, *M. hispanicum* KACC 11432$^T$; 3, *M.* phyllosphaerae KACC 11716$^T$. -, trace (<1%; fatty acids that account for <1% of the total are not shown).

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| $C_{16:0}$ | 3.1±0.5 | 1.7±0.1 | 3.0±0.2 |
| $C_{18:0}$ | 5.8±1.7 | 2.9±0.1 | 3.4±0.1 |
| $C_{18:0}$ 3-OH | 3.4±1.0 | - | - |
| $C_{18:1}$ $\omega7c$ | 79.8±2.1 | 82.9.1±0.7 | 87.4±0.2 |
| Summed feature 2[a] | 3.3±1.6 | 2.3±0.2 | 1.3±0.4 |
| Summed feature 3[a] | 3.0±0.5 | 8.5±0.4 | 3.9±0.2 |

[a] Summed features represent groups two or three fatty acids that could not be separated by GLC with MIDI system. Summed feature 2 contains iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH. Summed feature 3 contains iso-$C_{16:1}$ $\omega7c$ and/or $C_{15:0}$ 2-OH.

## DNA G+C content and quinone composition

The DNA G+C content of strain SW08-7$^T$ was 68.9 mol%, which is slightly different values reported for *Methylobacterium phyllosphaerae* (66.8 mol%) (Madhaiyan *et al*., 2009), *Methylobacterium hispanicum* (69.9 mol%) (Kato *et al*., 2008). However, the value still lies in the range expected for members of the same genus and the G+C content range of genus *Methylobacterium* should be extended taking into account our result. SW08-7$^T$ contained the characteristic chemical markers of the genus *Methylobacterium*, that is ubiquinone Q-10 and Q-9 (Table 1).

## Cellular fatty acid profiles

Our isolate showed cellular fatty acid profiles with large

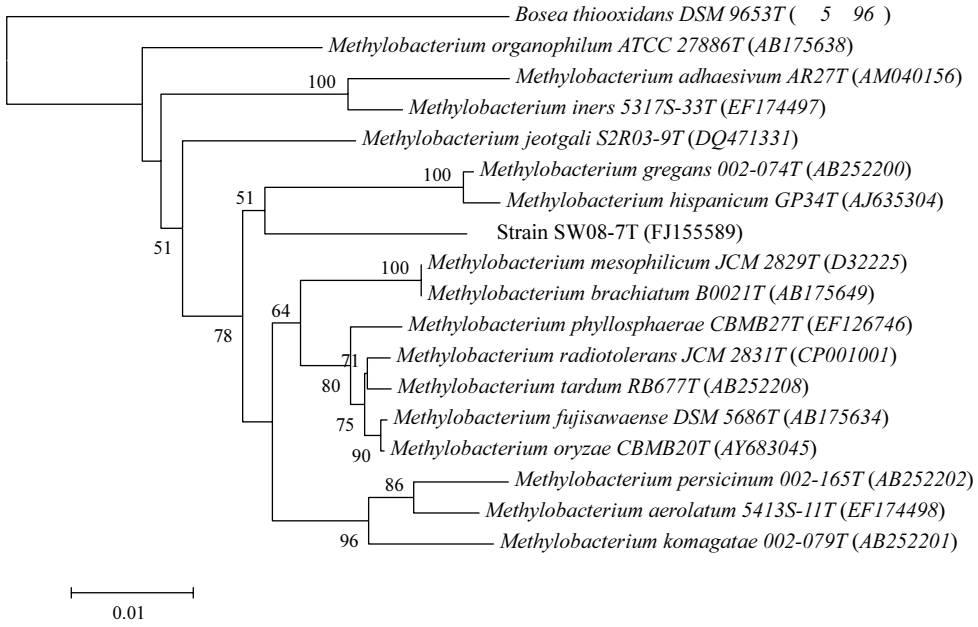


**Fig. 1.** Phylogenetic position of strain SW08-7$^T$ within the genus *Methylobacterium*, based on 16S rRNA gene sequences. Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining were performed by using the software package Mega (Molecular Evolutionary Genetics Analysis) version 4.1 (Tamura *et al*., 2007). Bootstrap percentages (> 50%) based on 1,000 replications are given at branch points. Bar, 0.01 nucleotide substitutions per nucleotide position.

amounts of the saturated and unsaturated fatty acids $C_{16:0}$ (3.1±0.5%), $C_{18:0}$ (5.8±1.7%), $C_{18:0}$ 3-OH (3.4±1.0%), $C_{18:1}$ *ω7c* (79.8±2.4%), summed feature 2 (3.3±1.6%; iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH), and summed feature 3 (3.0±0.5%; iso-$C_{16:1}$ *ω7c* and/or $C_{15:0}$ 2-OH). Strain SW08-7$^T$ has a fatty acid profile similar to those of the type strains of *Methylobacterium* species, but it has a larger proportion of $C_{18:0}$ 3-OH and a smaller proportion of $C_{18:1}$ *ω7c* (Table 3).

**Phylogenetic analysis**

The sequence of the 16S rRNA gene of strain SW08-7$^T$ (1,424 bp) was obtained. Strain SW08-7$^T$ showed the highest 16S rRNA gene sequence similarity with *M. mesophilicum* JCM 2829$^T$ (96.9%) and *M. brachiatum* B0021$^T$ (96.9%), followed by *M. phyllosphaerae* CBMB27$^T$ (96.6%), *M. radiotolerans* JCM 2831$^T$ (96.6%), and *M. hispanicum* GP34$^T$ (96.5%). A neighbour-joining tree showed that strain SW08-7$^T$ was placed in the genus *Methylobacterium*, most closely related to *M. hispanicum* and *M. gregans* (Fig. 1).

**DNA-DNA hybridization**

DNA-DNA hybridization was conducted to ascertain the degree of genomic relatedness between strain SW08-7$^T$ and *M. hispanicum*. DNA relatedness of strain SW08-7$^T$ with the type strain of *M. hispanicum* GP34$^T$ was 28.5%.

**Taxonomic conclusions**

All characteristics determined for strain SW08-7$^T$ are in accordance with those of the genus *Methylobacterium*. Strain SW08-7$^T$ clearly forms an independent line with the genus *Methylobacterium* in the phylogenetic tree, with low 16S rRNA gene sequence similarities with other type species (<97%). This close relationship was supported by chemotaxonomic characteristics, i.e. the dominance of $C_{18:1}$ *ω7c* fatty acid, the presence of ubiquinone-10, and the DNA G+C content (68.9 mol%). However, genomic relatedness between strain SW08-7$^T$ and *M. hispanicum* was only in the range of 28.5%, and a number of biochemical characteristics (Table 2) readily distinguished the test strain from other *Methylobacterium* species. Therefore, on placed in the genus *Methylobacterium* novel species, for which the name *Methylobacterium dankookense* sp. nov. is proposed.

**Description of *Methylobacterium dankookense* sp. nov.**

*Methylobacterium dankookense* (dan.koo.ken'se. NL. neut. adj. *dankookense* named after Dankook University, where taxonomic studies on this species were performed).

Cells are Gram-negative, rod-shaped (0.8~0.9 μm wide and 1.2~1.6 μm long), motile, and non-spore-forming. After 7 days incubation at 25°C on PCA, colonies are circular, pink to red color, and 0.4~2.0 mm in diameter. The isolate grows on TSB at 10~30°C but not at 4 or 37°C. The pH range for growth is 5.0~8.0. No growth is observed in the presence of 2% or higher NaCl concentration. Anaerobic growth does not occur. Positive for catalase and also for oxidase reaction with N,N,N,N-tetramethyl-p-phenylenediamine. Nitrate is reduced, but nitrite is not reduced. The type strain shows positive reaction for urease activity. Negative for production of indole, arginine dihydrolase, and protease activities (gelatin hydrolysis). The following compounds are assimilated as sole carbon sources: potassium gluconate, adipic acid, malic acid, trisodium citrate, itaconic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, propionic acid, and 3-hydroxybutyric acid. The following compounds are not assimilated as sole carbon sources: D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, capric acid, phenylacetic acid, L-rhamnose, D-ribose, inositol, D-saccharose, suberic acid, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, L-serine, salicin, D-melibiose, L-fucose, D-sorbitol, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 3-hydroxybenzoic acid, and L-proline. Enzymic activity is detected for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, acid phospatase, and naphtol-AS-BI-phosphohydrolase. No activity is detected for lipase (C14), α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Simons' citrate test is positive. Starch and casein are hydrolysed. The DNA G+C content is 68.9 mol%. The major respiratory quinone is Q-10, and major fatty acid is $C_{18:1}$ *ω7c* (79.8±2.1%). The GenBank accession no. for 16S rRNA gene sequence is FJ155589. The type strain, SW08-7$^T$ (=KCTC 22512$^T$ =DSM 22415$^T$), was isolated from drinking water of water purifier in Cheonan, Republic of Korea.

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