

## *Nocardioides soli* sp. nov., a bacterium isolated from a mountain soil

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**Abstract** Strain SR-1<sup>T</sup>, a Gram-positive, strictly-aerobic, short-rod shaped, non-motile bacterium, was isolated from a mountain soil collected in Seoul Women's University in South Korea. Growth occurred between 15 and 37 °C (optimum, 30 °C), at pH 6.0–9.0 (optimum, pH 7.0) and with 0–2 % NaCl. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SR-1<sup>T</sup> belongs to the genus *Nocardioides* and is closely related to *Nocardioides simplex* KCTC 9106<sup>T</sup> (96.8 %), *Nocardioides caeni* MN8<sup>T</sup> (96.7 %), *Nocardioides aromaticivorans*

H-1<sup>T</sup> (96.6 %), and *Nocardioides kongjuensis* A2-4<sup>T</sup> (96.6 %). Chemotaxonomic data revealed that strain SR-1<sup>T</sup> possesses MK-8(H<sub>4</sub>) as predominant menaquinone, LL-2,6-diaminopimelic acid as the diagnostic diamino acid, phosphatidylglycerol and diphosphatidylglycerol as predominant polar lipids and iso-C<sub>16:0</sub>, 10-methyl-C<sub>18:0</sub>, and C<sub>18:1 ω9c</sub> are major fatty acids. The DNA G+C content of the strain SR-1<sup>T</sup> was 72.4 mol%. Based on polyphasic evidence, strain SR-1<sup>T</sup> (= KEMC 9004-134<sup>T</sup> = JCM 19684<sup>T</sup>) should be classified as the type strain of a novel *Nocardioides* species, for which the name *Nocardioides soli* sp. nov. is proposed.

The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SWU8<sup>T</sup> (= KEMC 9004-134<sup>T</sup> = JCM 19684<sup>T</sup>) is KF955611.

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### Introduction

The genus *Nocardioides* was first described by Prauser (1976) with *Nocardioides albus* as the type species, belonging to the family *Nocardioideaceae*. At the time of writing, the genus *Nocardioides* comprised 66 species with validly published names (<http://www.bacterio.net/nocardioides.html>). Members of the genus *Nocardioides* are Gram-positive, aerobic, rod- or cocci shaped, motile or non-motile, have L,L-2,6-diaminopimelic acid (LL-DAP) and glycine in the cell wall peptidoglycan.

In a series of studies, we attempted to isolate microorganisms from various soils; we have isolated a strain designated as SR-1<sup>T</sup> from a mountain soil, in Seoul Women's University, South Korea. Strain SR-1<sup>T</sup> showed Gram-staining-positive and pale yellow colored colonies on nutrient agar (NA, Difco). In this study, strain SR-1<sup>T</sup> was characterized by a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences, and chemotaxonomic and physiological properties.

## Materials and methods

### Isolation of bacterial strain and culture conditions

Strain SR-1<sup>T</sup> was isolated from soil collected in a mountain in Seoul Women's University, South Korea. One gram of the soil was immersed in 50 ml normal saline (0.9 % NaCl) solution, vortexed, serially diluted and 100 µl of the aliquot was inoculated on ten times diluted nutrient agar (NA, Difco) plate. The purified colony was tentatively identified using partial 16S rRNA gene sequences, using the EzTaxon-e (<http://eztaxon-e.ezbiocloud.net>) (Kim et al. 2012). Strain SR-1<sup>T</sup> was routinely cultured on NA at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C. The reference type strains, *Nocardioides simplex* KCTC 9106<sup>T</sup>, *Nocardioides caeni* KCTC 19600<sup>T</sup>, *Nocardioides aromaticivorans* KACC 20613<sup>T</sup>, and *Nocardioides kongjuensis* KCTC 19054<sup>T</sup> were obtained from the Korean Agricultural Culture Collection (KACC) and the Korean Collection for Type Cultures (KCTC). All the strains were maintained and cultivated on NA (Difco) at pH 7.0 aerobically, unless otherwise mentioned.

### Phenotypic and biochemical characteristics

Cell morphology of strain SR-1<sup>T</sup> was determined by both phase contrast microscopy and transmission electron microscopy. Gram-stained (Doetsch 1981) bacterial cells were examined on a BX51 (Olympus, Japan) phase contrast microscope (1,000×). To examine the motility, the hanging drop technique was used after the cells had grown for 2 days at 30 °C on NA agar. Growth and colony morphology were examined on different media such as trypticase soy agar (TSA, Difco), R2A agar (Difco), and Luria–

Bertani agar (LB, Difco). Growth under anaerobic conditions was tested by culturing the organisms on NA, and TSA plates in GasPak jars (BBL) at 30 °C. Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, and 42 °C) was assessed on NA (Difco) for 3 days. Growth at various pH levels (from pH 4 to pH 11 at 1 increment) was assessed in nutrient broth (NB, Difco) at 30 °C. The pH of the medium was maintained using three buffers (final concentration of 50 mM): acetate buffer (for pH 4.0–5.0); phosphate buffer (for pH 6.0–8.0) and tris buffer (for pH 9.0–11.0). NaCl tolerance was tested on NB (Difco) at 30 °C that had been supplemented with 0–10 % (w/v) NaCl (1 % intervals). Catalase activity was determined by bubble production with 3 % (v/v) H<sub>2</sub>O<sub>2</sub>, and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine (Cappuccino and Sherman 2002). Hydrolysis of casein, starch and tween-80 (Atlas 1993) was also investigated; reactions were read after 5 days. The utilization of different substrates as sole carbon-source and some enzyme activities were tested by using the API 20NE, API 32GN and API ZYM galleries according to the instructions of the manufacturer (bioMérieux).

### 16S rRNA sequencing and phylogenetic analysis

Genomic DNA of the strain SR-1<sup>T</sup> was extracted with a commercial genomic DNA extraction kit (Solgent, Korea) and the 16S rRNA gene was amplified using the 9F and 1492R universal bacterial primer set (Weisburg et al. 1991) and sequenced by Genotech (Daejeon, Korea) using the 9F, 518F, 785F, and 1492R universal bacterial primer set. The nearly full sequences of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc., Madison, WI, USA) and then compared by use of the EzTaxon-e server. The 16S rRNA sequences of related taxa obtained from GenBank were edited with the BioEdit program (Hall 1999). Multiple alignments were performed with the CLUSTAL\_X program (Thompson et al. 1997) and a phylogenetic tree was constructed using the MEGA5 program (Tamura et al. 2011). Pairwise distances for the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) were calculated according to the Kimura two-parameter model (Kimura 1983). A bootstrap analysis with 1,000 replicates was conducted (Felsenstein, 1985). The min-mini heuristic method (Fitch 1971) with a search factor of

**Table 1** Some selective phenotypic characteristics of strain SR-1<sup>T</sup> and type strains of phylogenetically related *Nocardioides* species

Characteristic	1	2	3	4	5
Habitat	Soil	Soil	Domestic wastewater	River water, Sediment, Soil	Soil
Motility	–	+	–	–	–
Morphology	Shor-rod	Rods, Cocci	Rods, Cocci	Rods	Rods, Cocci
Size (µm)					
Length	1.0–1.2	1.5–6.0	0.7–2.5	1.0–2.0	0.8–3.0
Width	0.5–0.7	1.0–1.2	0.3–0.7	0.5–0.7	0.4–0.7
Colony color	Pale yellow	Pale yellow	Greyish yellow	Milky white	Yellowish white
Growth at					
37 °C	+	+	–	+	+
42 °C	–	–	–	+	+
Oxidase	+	+	+	–	+
Enzyme activity (API ZYM)					
Alkaline phosphatase	–	+	+	+	+
Cystine arylamidase	+	+	–	+	–
Esterase (C4)	+	–	–	–	+
α-Fucosidase	+	–	–	–	–
Trypsin	+	–	–	+	+
Urease	+	–	+	+	–
Valine arylamidase	+	+	w	+	–
Assimilation (API 20NE)					
3-Hydroxybenzoate	+	+	+	–	+
2-Ketogluconate (α)	–	+	+	+	–
Acetate	+	+	–	+	–
Adipate	–	–	w	–	+
Citrate	+	+	–	–	–
Itaconate	+	+	–	+	–
D,L-Lactate	+	–	–	–	–
Phenyl acetate	+	+	–	–	–
Propionate	+	+	+	–	–
<i>n</i> -Valerate	+	+	+	+	–
L-Arabinose	+	–	–	–	–
D-Mannose	+	–	+	w	w
L-Rhamnose	–	–	+	+	–
D-Ribose	+	–	–	–	–
D-Sucrose	–	–	–	–	–
D-Mannitol	+	–	+	–	+
L-Alanine	+	+	+	+	–
L-Histidine	+	+	–	–	–
L-Proline	–	+	+	–	+
L-Serine	+	+	+	–	+
<i>N</i> -acetyl-D-glucosamine	+	–	+	+	–
Glycogen	+	+	–	+	–
Hydrolysis of					
Tween 80	+	+	+	–	+
Starch	+	+	+	–	–

**Table 1** continued

Characteristic	1	2	3	4	5
Casein	–	–	+	+	+
Esculin	+	+	+	+	–
G+C content	72.4	71.7	71.5	72.2	72.1

All species are positive for Gram staining, for esterase lipase (C8) and utilization of D-glucose. All species are negative for lipase (c14),  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -glucuronidase, N-acetyl- $\beta$ -glucosaminidase and  $\alpha$ -mannosidase activities

Strains: 1 Strain SR-1<sup>T</sup>, 2 *N. simplex* KCTC 9106<sup>T</sup>, 3 *N. caeni* KCTC 19600<sup>T</sup>, 4 *N. aromaticivorans* KACC 20613<sup>T</sup>, 5 *N. kongjuensis* KCTC 19054<sup>T</sup>. All the data were obtained in this study

+ positive reaction, – negative reaction, w weak reaction

one was applied in a maximum-parsimony (MP) analysis (MEGA 5 Program).

#### Chemotaxonomic characteristics

To analyse quinones, cell wall di-amino acid, and polar lipids strain SR-1<sup>T</sup> was cultured in NB, collected at late-exponential growth phase and freeze dried. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane quinone solution was then purified using silica Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC, as described previously (Hiraishi et al. 1996). The amino acids of cell wall composition were analyzed using cellulose TLC sheets, with alanine, aspartic acid, meso-diaminopimelic acid (meso-DAP), LL-DAP, glutamic acid and glycine being used as standard amino acids (Schleifer and Kandler 1972).

Polar lipids were extracted according to the procedures described by Minnikin et al. (1977; 1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata and Suzuki 1987; Schleifer and Kandler 1972). For TLC development, first mobile phase was chloroform/methanol/water (65:25:4, v/v/v) and second mobile phase was chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Total lipid profile was detected by spraying with molybdophosphoric acid solution (Sigma-Aldrich; St. Louis, Mo) followed by heating at 150 °C; aminolipids by spraying with 0.2 % (w/v) ninhydrin solution followed by heating at 105 °C for 10 min; glycolipids with 0.5 % 1-naphthol in methanol/water (1:1, v/v) and sulfuric acid/ethanol (1:1, v/v) followed by heating at 120 °C for 5–10 min;

phospholipids by spraying with Zinzadze reagent; and phosphatidylcholine by spraying with Dragendorff reagent (Sigma-Aldrich; St. Louis, Mo).

In order to perform fatty acid methyl ester (FAME) analysis, strain SR-1<sup>T</sup>, and reference strains were grown on TSA for 48 h at 30 °C. Two loopful of bacterial mass were collected from the fourth quadrant of the quadrant streaked plate, and subjected to saponification, methylation and extraction using the methods of Kuykendall et al. (1988). The FAME mixtures were separated using the Sherlock Microbial Identification System (TSBA, Version 6.0; MIDI), and then analysed by gas chromatography (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser 1990).

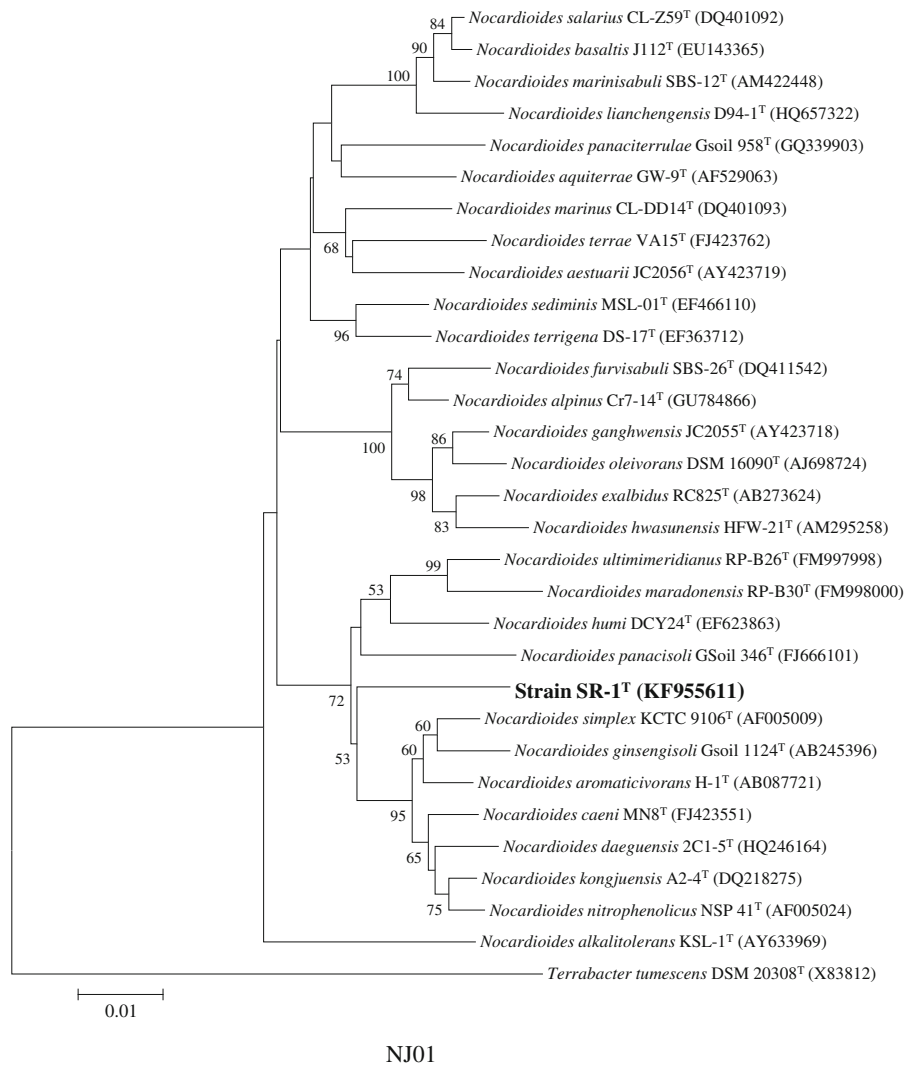
#### DNA base content

For the determination of the DNA G+C content, genomic DNA was extracted as mentioned above and treated with nuclease P1 and alkaline phosphatase to be enzymatically degraded into nucleosides. The mixture was analyzed using HPLC as described previously (Mesbah et al. 1989; Tamaoka and Komagata 1984).

## Results

#### Morphological and physiological characteristics

Strain SR-1<sup>T</sup> showed pale yellow color, circular (colony size 0.3–1.0 mm), convex with entire margins on NA agar plate after 2 days. Cells are Gram-positive, non-motile and short-rod-shaped. The physiological characteristics of strain SR-1<sup>T</sup> are summarized in the



**Fig. 1** A neighbor-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequences of strain SR-1<sup>T</sup> and representatives of related taxa. Numbers at the nodes indicate the bootstrap

values (greater than 50 %) expressed as percentage of 1,000 replicates. Bar represents 0.01 substitutions per nucleotide position

species description and a comparison of selective characteristics with those of closely related *Nocardioidea* species is shown in Table 1.

**Phylogenetic analysis**

The 16S rRNA gene sequence of strain SR-1<sup>T</sup> was compiled with SeqMan software (DNASTAR Inc.) and showed a continuous stretch of 1,448 nucleotides. According to the 16S rRNA gene sequence, strain SR-1<sup>T</sup> was determined to belong to the phylum *Actinobacteria*. The highest degrees of sequence similarities

were found to be with *N. simplex* KCTC 9106<sup>T</sup> (96.8 %), *N. caeni* MN8<sup>T</sup> (96.7 %), *N. aromaticivorans* H-1<sup>T</sup> (96.6 %), and *N. kongjuensis* A2-4<sup>T</sup> (96.6 %). The other members of *Nocardioidea* species showed less than 96.5 % of the 16S rRNA gene similarity. In the phylogenetic tree (Fig. 1), strain SR-1<sup>T</sup> clearly belonged to the genus *Nocardioidea* lineage. The Min-mini heuristic method with maximum-parsimony analysis (Supplementary Fig S1) and maximum-likelihood analysis also clearly showed that strain SR-1<sup>T</sup> belonged to the *Nocardioidea* lineage.

**Table 2** Fatty acid compositions of strain SR-1<sup>T</sup> and closely related *Nocardiooides* species

Fatty acids	1	2	3	4	5
<b>Saturated</b>					
16:0	2.9	1.5	4.2	3.1	5.9
17:0	1.8	ND	1.5	2.5	2.9
18:0	2.4	2.4	1.4	ND	ND
<b>Hydroxy</b>					
15:0 3OH	2.6	ND	ND	2.4	3.5
<b>Branched</b>					
14:0 iso	1.3	1.2	1.2	3.4	1.9
15:0 iso	3.8	1.9	2.5	2.5	4.1
16:0 10-methyl	5.5	5.1	ND	ND	ND
16:0 iso	19.6	36.1	42.3	48.2	50.1
17:0 10-methyl	3.4	5.8	ND	3.2	2.8
17:0 anteiso	1.2	ND	1.1	ND	1.8
17:0 iso	2.1	3.1	5.1	2.5	4.8
18:0 10-methyl (TBSA)	17.1	11.1	2.6	1.5	2.5
18:0 iso	8.4	tr	1.6	5.6	2.2
<b>Unsaturated</b>					
16:1 iso H	2.5	2.6	ND	ND	ND
17:1 $\omega$ 6c	4.5	14.3	8.3	7.2	4.1
17:1 $\omega$ 8c	3.9	2.1	7.5	4.6	2.5
18:1 $\omega$ 9c	13.2	9.2	17.1	10.8	8.4
Summed Feature3 (16:1 $\omega$ 6c/16:1 $\omega$ 7c)	3.8	3.1	2.1	2.1	1.8

Only the fatty acids with an amount of more than 1 % were shown. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system

Strains: 1 Strain SR-1<sup>T</sup>, 2 *N. simplex* KCTC 9106<sup>T</sup>, 3 *N. caeni* KCTC 19600<sup>T</sup>, 4 *N. aromaticivorans* KACC 20613<sup>T</sup>, 5 *N. kongjuensis* KCTC 19054<sup>T</sup>. All the data were obtained in this study

tr less than 1 %, ND not detected

### Chemotaxonomic characteristics

Chemotaxonomic properties of strain SR-1<sup>T</sup> confirmed the phylogenetic affiliation to a member of genus *Nocardiooides* (Tamura and Yokota 1994; Yoon et al. 1997; Lawson et al. 2000; Urzì et al. 2000). Strain SR-1<sup>T</sup> contained predominant amounts of MK-8(H<sub>4</sub>) menaquinone. Cell wall peptidoglycan of strain SR-1<sup>T</sup> contained LL-DAP as the diagnostic diamino acids. Strain SR-1<sup>T</sup> exhibited similar polar lipid profile with those of other *Nocardiooides* species (Dastager et al. 2008; O'Donnell et al. 1982), containing a

predominance of phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and an unknown phospholipid.

Strain SR-1<sup>T</sup> contained iso-C<sub>16:0</sub> (19.6 %), 10-methyl-C<sub>18:0</sub> (17.1 %) and C<sub>18:1</sub>  $\omega$ 9c (13.2 %) as the predominant fatty acids which are also common in the closely related *Nocardiooides* species. Moderate amounts of iso-C<sub>18:0</sub> (8.4 %), 10-methyl-C<sub>16:0</sub> (5.5 %), C<sub>17:1</sub>  $\omega$ 6c (4.5 %), C<sub>17:1</sub>  $\omega$ 8c (3.9 %), and iso-C<sub>15:0</sub> (3.8 %) are also present. The major fatty acids of strain SR-1<sup>T</sup> is similar with other members of the genus *Nocardiooides* and the complete fatty acid profile of strain SR-1<sup>T</sup> is shown in Table 2 and compared with other *Nocardiooides*. The DNA G+C content of the strain SR-1<sup>T</sup> was 72.4 mol%, and it is within the range of the genus *Nocardiooides*.

### Taxonomic conclusion

The results of chemotaxonomic analysis (presence of MK-8(H<sub>4</sub>) as menaquinone; major fatty acids 10-methyl-C<sub>18:0</sub>, C<sub>18:1</sub>  $\omega$ 9c, and iso-C<sub>16:0</sub>; PG, DPG as major polar lipid; cell wall peptidoglycan LL-DAP as main di-amino acid) supports the recognition of strain SR-1<sup>T</sup> as a member of the genus *Nocardiooides*. Strain SR-1<sup>T</sup> can be distinguished from closely related species for ability to utilize D,L-lactate, L-arabinose and D-ribose as sole carbon sources. Strain SR-1<sup>T</sup> is non-motile, but the closest species *N. simplex* is motile. The physiological and chemotaxonomic characteristics which differentiate strain SR-1<sup>T</sup> from closely related members (Tables 1 and 2) novel species with the genus *Nocardiooides*, for which the name *Nocardiooides soli* sp. nov. is proposed.

### Description of *Nocardiooides soli* sp. nov

*Nocardiooides soli* (so'li. L. gen. n. soli of soil, the isolation source of the type strain).

Cells are Gram-positive, aerobic, non-motile, and short-rod-shaped, about 0.5–0.7  $\mu$ m in width and 1.–1.2  $\mu$ m in length. Colonies grown on NA (Difco) for 2 days are smooth, small and pale yellow in color. Growth occurs at 1–37 °C (optimum about 30 °C) but no growth observed below 10 or above 42 °C. Growth occurs at pH 6.–9.0 with optimum growth at pH 7. Cells tolerate up to 2 % NaCl (w/v) with optimum growth with no NaCl. Oxidase and catalase positive. Hydrolysis of tween 80, and starch is positive but not casein. Growth observed on R2A, LB and TSA.

Negative in tests for nitrate reduction, indole production, glucose acidification, arginine dihydro-lase, and  $\beta$ -galactosidase; positive for urease, gelatinase, and esculin hydrolysis in API 20NE test. The following carbon sources are utilized in the API 20NE and API 32GN tests: acetate, *N*-acetyl-D-glucosamine, adipate, L-alanine, L-arabinose, gluconate, D-glucose, glycogen, L-histidine, 3-hydroxybenzoate, itaconate, D,L-lactate, D-mannose, phenyl acetate, propionate, D-ribose, L-serine and *n*-valerate. Utilization of the following substrates is negative: caprate, citrate, L-fucose,  $\beta$ -galactosidase (PNPG), 4-hydroxybenzoate, D,L-3-hydroxybutyrate, 2-ketogluconate ( $\alpha$ ), 5-ketogluconate, L-malate, malonate, D-maltose, D-mannitol, D-melibiose, myo-Inositol, L-proline, L-rhamnose, salicin, D-sorbitol, suberate, and D-sucrose.

In assays with API ZYM, acid phosphatase, cystine arylamidase, esterase (C4), esterase (C8),  $\alpha$ -fucosidase,  $\alpha$ -glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase are present, but *N*-acetyl- $\beta$ -glucosaminidase, alkaline phosphatase,  $\alpha$ -chymotrypsin,  $\beta$ -galactosidase (ONPG),  $\alpha$ -galactosidase,  $\beta$ -glucosidase,  $\beta$ -glucuronidase, lipase (C14), and  $\alpha$ -mannosidase are absent. Chemotaxonomic characteristics were the following. The predominant menaquinone is MK-8(H<sub>4</sub>) and major polar lipids are PG, DPG. The major cellular fatty acids are iso-C<sub>16:0</sub>, 10-methyl-C<sub>18:0</sub>, and C<sub>18:1</sub>  $\omega$ 9c. The DNA G+C content of the type strain is 72.4 mol%.

The type strain, SR-1<sup>T</sup> (= KEMC 9004-134<sup>T</sup> = JCM 19684<sup>T</sup>), was isolated from a mountain soil collected in Seoul Women's University at South Korea.

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