

Novosphingobium humi sp. nov., isolated from soil of a military shooting range

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Abstract

A Gram-stain-negative, strictly aerobic bacterium, designated R1-4^T, was isolated from soil from a military shooting range in the Republic of Korea. Cells were non-motile short rods, oxidase-positive and catalase-negative. Growth of R1-4^T was observed at 15–45 °C (optimum, 30 °C) and pH 6.0–9.0 (optimum, pH 7.0). R1-4^T contained summed feature 8 (comprising C_{18:1}ω7c/C_{18:1}ω6c), summed feature 3 (comprising C_{16:1}ω7c/C_{16:1}ω6c), cyclo-C_{19:0}ω8c and C_{16:0} as the major fatty acids and ubiquinone-10 as the sole isoprenoid quinone. Phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, sphingoglycolipid, phosphatidylcholine, an unknown glycolipid and four unknown lipids were detected as polar lipids. The major polyamine was spermidine. The G+C content of the genomic DNA was 64.4 mol%. The results of phylogenetic analysis based on 16S rRNA gene sequences indicated that R1-4^T formed a tight phylogenetic lineage with *Novosphingobium sediminicola* HU1-AH51^T within the genus *Novosphingobium*. R1-4^T was most closely related to *N. sediminicola* HU1-AH51^T with a 98.8% 16S rRNA gene sequence similarity. The DNA–DNA relatedness between R1-4^T and the type strain of *N. sediminicola* was 37.8±4.2%. On the basis of phenotypic, chemotaxonomic and molecular properties, it is clear that R1-4^T represents a novel species of the genus *Novosphingobium*, for which the name *Novosphingobium humi* sp. nov. is proposed. The type strain is R1-4^T (=KACC 19094^T=JCM 31879^T).

The genus *Novosphingobium*, belonging to the family *Sphingomonadaceae*, order *Sphingomonadales*, class *Alphaproteobacteria*, phylum ‘*Proteobacteria*’, was first proposed as a result of the reclassification of *Sphingomonas aromaticivorans*, *Sphingomonas capsulata*, *Sphingomonas rosa*, *Sphingomonas stygia*, *Sphingomonas subarctica* and *Sphingomonas subterranea* [1]. The genus *Novosphingobium* comprises Gram-stain-negative, strictly aerobic, catalase-positive, motile or non-motile rods that form yellow or whitish-brown colonies [1]. In addition, members of the genus *Novosphingobium* contain ubiquinone-10 (Q-10) as the major respiratory quinone and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, sphingoglycolipid and phosphatidylcholine as the major polar lipids and produces spermidine as the major polyamine [1–7]. Species of the genus *Novosphingobium* with diverse physiological and functional properties have been isolated from diverse habitats, such as rice roots, waste-water treatment plants, humic-matter-rich bog lake, estuarine sediment and deep

sea [7–11], indicating that members of the genus *Novosphingobium* play important roles in environmental habitats. In this study, a presumably novel strain of the genus *Novosphingobium*, designated strain R1-4^T, was isolated from soil of a shooting range and its taxonomic properties were characterized using a polyphasic approach.

R1-4^T was isolated from soil from a military shooting range in Jangseong (35° 30′ 01″N 126° 71′ 23″ E), Republic of Korea, by an enrichment culture using hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). For the enrichment of RDX-metabolizing bacteria, 1 g soil sample obtained from the shooting range was added to 5 ml enrichment medium (2.78 g KH₂PO₄, 2.78 g Na₂HPO₄, 5 mg succinate and 15 mg RDX l⁻¹) and the culture medium was incubated at 30 °C under aerobic conditions. The enrichment medium was subcultured into fresh medium five times every two weeks. The final enrichment culture was serially diluted in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) and aliquots of

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Abbreviations: DDH, DNA–DNA hybridization; LB, Luria–Bertani; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; Q-10, ubiquinone-10; RDP, Ribosomal Database Project; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TSA, tryptic soy agar.

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The 16S rRNA gene sequence of strain R1-4^T has been deposited in GenBank under the accession number KY658458.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

each serial dilution were spread on R2A agar (BD) and incubated at 30 °C for 3 days. The 16S rRNA genes from colonies grown on R2A agar were PCR-amplified using the universal primers F1 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R13 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), as described previously [12]. The PCR amplicons were sequenced using the universal primers 340F (5'-CCT ACG GGA GGC AGC AG-3'), 518R (5'-ATT ACC GCG GCT GCT GG-3') and 805F (5'-GAT TAG ATA CCC TGG TAG TC-3') to obtain almost complete 16S rRNA gene sequences. The resulting 16S rRNA gene sequences were compared with those of type strains of species with validly published names using the Nucleotide Similarity Search program in the EzTaxon-e server [13]. From the 16S rRNA gene sequence analysis, a putative novel strain representing a member of the genus *Novosphingobium*, designated strain R1-4^T, was selected for further phenotypic and phylogenetic analyses. R1-4^T was routinely cultured aerobically on R2A agar at 30 °C for 3 days. R1-4^T was stored in R2A broth containing 15 % (v/v) glycerol at -80 °C for long-term preservation. *Novosphingobium sediminicola* KCTC 22311^T, *Novosphingobium lotistagni* KACC 18541^T, *Novosphingobium oryzae* JCM 30537^T and *Novosphingobium capsulatum* KACC 12316^T were used as reference strains for the comparisons of phenotypic properties and fatty acid analysis.

The 16S rRNA gene sequences of R1-4^T (1457 nucleotides) and closely related type strains were aligned using the fast secondary-structure aware Infernal aligner available in the Ribosomal Database Project (RDP) [14]. Phylogenetic trees based on the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms were reconstructed using the PHYLIP software (ver. 3.695; [15]), and their tree topologies were evaluated through bootstrap analyses based on a 1000-resampled dataset. A phylogenetic tree with bootstrap values based on the maximum-likelihood (ML) algorithm was also reconstructed using RAxML-HPG BlackBox (version 8.2.4) of the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; [16]). An additional taxonomic analysis was performed using the RDP naïve Bayesian rRNA Classifier tool (<http://rdp.cme.msu.edu/classifier>) [17]. DNA–DNA relatedness between R1-4^T and the type strain of *N. sediminicola* (KCTC 22311^T) was evaluated by DNA–DNA hybridization (DDH) using a genome-probing microarray method, as described by Chang *et al.* [18]. The DDH experiments were confirmed by reciprocally interchanging probe and target DNA.

The NJ-algorithm-based phylogenetic tree indicated that R1-4^T formed a tight phylogenetic lineage with *Novosphingobium sediminicola* HU1-AH51^T within the genus *Novosphingobium* with a high bootstrap value (99 %) (Fig. 1). Phylogenetic trees reconstructed using the ML and MP algorithms also supported the hypothesis that R1-4^T formed a phylogenetic lineage with *N. sediminicola* HU1-AH51^T within the genus *Novosphingobium* (Fig. 1). The taxonomic analysis using the RDP Classifier tool also indicated that

R1-4^T represented a novel member of the genus *Novosphingobium* at a 90 % confidence threshold. Comparative analysis based on the 16S rRNA gene sequences revealed that R1-4^T was most closely related to *N. sediminicola* HU1-AH51^T with 98.8 % similarity. The 16S rRNA gene sequence similarities with other type strains of species of the genus *Novosphingobium* with validly published names were less than 97.7 %. Because it has been suggested that 98.65–98.7 % of 16S rRNA gene sequence similarity between two strains equates to a 70 % DNA–DNA relatedness, the gold standard for species delineation [19–22], DDH experiments between R1-4^T and the type strain of *N. sediminicola* were performed. The DNA–DNA relatedness between R1-4^T and the type strain of *N. sediminicola* was 37.8±4.2 % (reciprocal 39.1±2.8 %), which was clearly below the 70 % threshold generally accepted for species delineation [19]. These results indicate that R1-4^T represents a novel species of the genus *Novosphingobium*.

Growth of R1-4^T on R2A agar, laboratory prepared Luria–Bertani (LB) agar, nutrient agar (BD) and tryptic soy agar (TSA; BD) was tested at 30 °C for 3 days. Growth of R1-4^T at different temperatures (10–50 °C at 5 °C intervals) and pH values (5.0–10.0 at 0.5 pH-unit intervals) was evaluated in R2A broth for 3 days. The R2A broth with pH below 7.0 and pH 8.0–10.0 was prepared using Na₂HPO₄/NaH₂PO₄ and Tris/HCl buffers, respectively, as described previously [23]. The pH values were checked again after sterilization (121 °C for 15 min) and adjusted if necessary. Growth of R1-4^T at different NaCl concentrations [0–5 % (w/v) at 1 % intervals] was evaluated in R2A broth prepared in the laboratory according to the BD formula. Gram staining was investigated using the bioMérieux Gram stain kit according to the manufacturer's instructions. Oxidase activity was evaluated by the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was tested by the production of oxygen bubbles in 3 % (v/v) aqueous H₂O₂ [24]. Cell morphology and motility were investigated using phase-contrast microscopy (AXIO Scope A1; Zeiss) and transmission electron microscopy (JEM-1010; JEOL) with cells from a culture grown to exponential phase on R2A agar at 30 °C. Anaerobic growth was assessed on R2A agar under anaerobic conditions (with 4–10 % CO₂) using the GasPak Plus system (BBL) at 30 °C for 21 days. The following phenotypic properties of R1-4^T and the four reference strains were investigated under the same conditions in parallel. Hydrolysis of casein, starch, aesculin, tyrosine, urea, gelatin, Tween 20 and Tween 80 was tested on R2A agar, according to the methods described previously [24, 25]. Nitrate reduction was assessed according to the method described by Lányi [25]. Additional enzymatic activities, biochemical features and oxidation of various carbon sources were evaluated using API ZYM, API 20NE (bioMérieux) and GN2 MicroPlate (Biolog), respectively, according to the manufacturer's instructions, except that the test strains were incubated at their optimal growth temperatures.

R1-4^T grew well on R2A agar and nutrient agar, but grew very slowly on TSA and LB agar. Cells of R1-4^T were

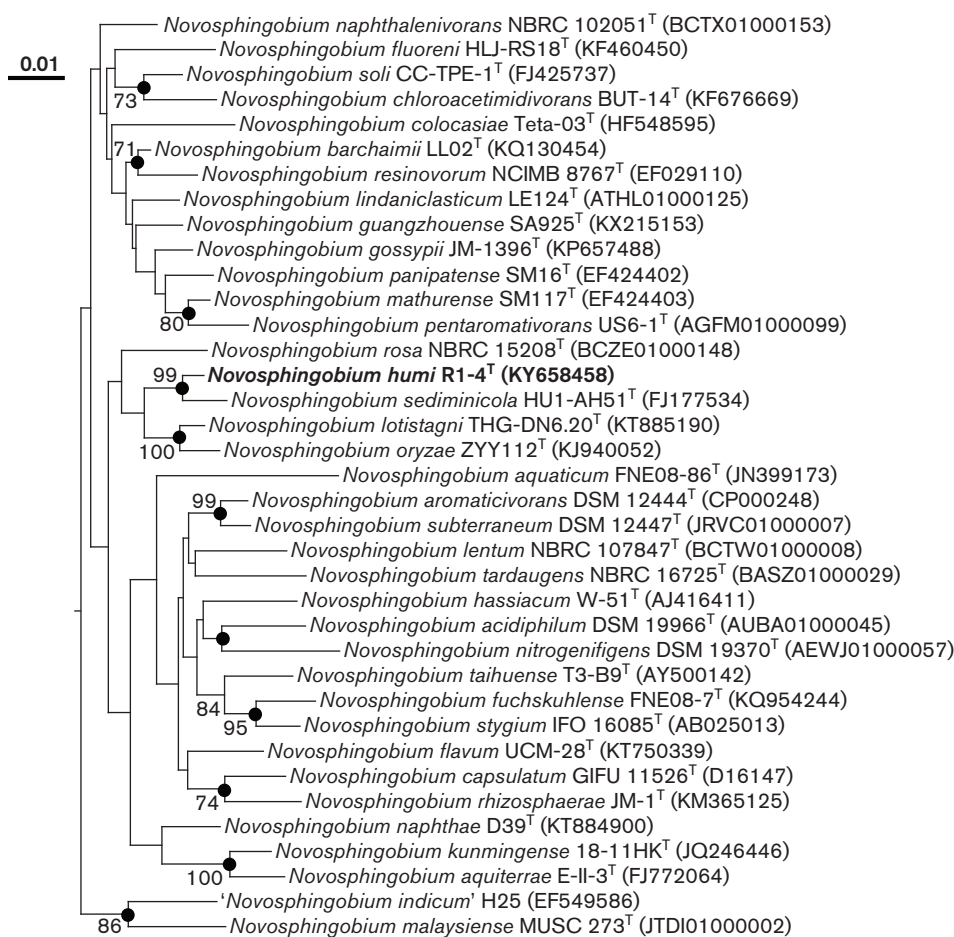


Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of R1-4^T and strains of members of the genus *Novosphingobium*. Bootstrap values are shown at nodes as percentages of 1000 replicates, for values over 70 %. Filled circles (•) indicate that the corresponding nodes were also recovered in the trees reconstructed using the ML and MP algorithms. *Zymomonas mobilis* ATCC 10988^T (AF281031) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

Gram-stain-negative and non-motile short rods (0.8–0.9 µm in diameter and 1.0–1.2 µm in length) (Fig. S1, available in the online Supplementary Material). R1-4^T grew well in R2A broth, but did not grow in R2A broth supplemented with 1 % NaCl. Anaerobic growth of R1-4^T was not observed after 21 days of incubation at 30 °C. Although some phenotypic properties of R1-4^T, including hydrolysis of tyrosine, aesculin and Tween 20 and enzyme activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-glucosidase, were identical with those of the four reference strains of species of the genus *Novosphingobium*, many other phenotypic properties, such as catalase and oxidase activities, nitrate reduction to nitrite and hydrolysis of Tween 80, differentiated R1-4^T from other closely related species of the genus *Novosphingobium* (Table 1). Physiological and biochemical characteristics of R1-4^T are additionally described in the species description and compared with those of the closely

related species of the genus *Novosphingobium* in Tables 1 and S1.

The DNA G+C content of R1-4^T was determined by the fluorometric method [26] using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). Isoprenoid quinones were extracted, according to the method of Minnikin *et al.* [27] and analyzed using a model LC-20A HPLC system (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250×64.6 mm, Kromasil; Akzo Nobel), as described by Komagata and Suzuki [28]. For the cellular fatty acid analysis, R1-4^T and the four reference strains were cultivated in R2A broth at 30 °C and microbial cells were harvested at the same growth stage (exponential phase, OD₆₀₀ = 0.8). The cellular fatty acids of microbial cells were saponified and methylated using the standard MIDI protocol. The fatty acid methyl esters were analyzed by gas chromatography (model 6890; Hewlett Packard) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B; [29]). The polar lipids of R1-4^T were

Table 1. Comparisons of phenotypic characteristics of R1-4^T and the type strains of closely related species of the genus *Novosphingobium*

Strains: 1, R1-4^T (this study); 2, *N. sediminicola* KCTC 22311^T [5]; 3, *N. lotistagni* KACC 18541^T [6]; 4, *N. oryzae* JCM 30537^T [7]; 5, *N. capsulatum* KACC 12316^T [1, 32]. All strains were positive for the following characteristics (determined under the same conditions during this study): hydrolysis of tyrosine, aesculin and Tween 20 and activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and β -glucosidase. All strains were negative for the following characteristics: Gram-staining, hydrolysis of starch, casein, urea and gelatin, reduction of nitrate to nitrogen, indole production, fermentation of glucose, activities of arginine dihydrolase, trypsin, α -chymotrypsin, α -mannosidase and α -fucosidase and assimilation of D-mannitol, capric acid, adipic acid, trisodium citrate and phenylacetic acid. +, Positive; –, negative.

Characteristic	1	2	3	4	5
Ranges for growth*:					
Temperature (°C)	15–45	15–35	10–40	20–40	10–35
pH	6.0–9.0	5.5–9.5	5.0–9.0	5.0–8.0	6.0–8.0
NaCl (% w/v)	0	0–1.0	0–1.0	0–1.0	0–2.0
Catalase*	–	+	+	+	+
Oxidase*	+	+	+	+	–
Reduction of nitrate to nitrite*	+	+	+	–	+
Hydrolysis* of:					
Tween 80	–	–	+	–	+
Activity (API ZYM)* of:					
Lipase (C14), α -galactosidase	–	–	–	+	–
Valine arylamidase, cystine arylamidase	–	+	–	+	+
Acid phosphatase	+	+	–	+	+
β -Glucuronidase	+	–	–	+	+
α -Glucosidase	–	+	–	+	–
N-Acetyl- β -glucosaminidase	–	–	–	+	+
Assimilation of (API 20NE)*:					
D-Glucose and maltose	+	+	+	–	+
L-Arabinose	–	+	+	+	+
D-Mannose, potassium gluconate	–	–	–	–	+
N-Acetyl-glucosamine	–	+	–	+	+
Malic acid	+	–	–	–	+
Polar lipids†	PE, PG, DPG, PC, SGL, GL, L1–4	PE, PDE, PG, PC, SGL, GL1–2, L1–2	PE, PG, DPG, PC, SGL, APL, L	PE, PG, DPG, SGL, PL, AL	PE, PME, PG, DPG, PDE, PC, SGL, PL1–2, APL, GL
DNA G+C content (mol%)	64.4	62.6	63.1	60.8	63.1

*These analyses were conducted under the same conditions in this study.

†PE, phosphatidylethanolamine; PME, phosphatidylmonomethylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PDE, phosphatidylmethylethanolamine; PC, phosphatidylcholine; SGL, sphingoglycolipid; AL, unknown aminolipid; PL, unknown phospholipid; APL, unknown amino-phospholipid; GL, unknown glycolipid; L, unknown lipid

analyzed by TLC using cells harvested during the exponential growth phase, according to the procedure described by Minnikin et al. [30]. The following reagents were used to detect different polar lipids: 10 % ethanolic molybdophosphoric acid (for total polar lipids), ninhydrin (for aminolipids), Dittmer–Lester reagent (for phospholipids), α -naphthol (for glycolipids) and Dragendorff reagent (for choline). Polyamines were extracted and analyzed according to the methods described by Busse et al. [31].

The genomic DNA G+C content of R1-4^T was approximately 64.4 mol%, which was in the range of the DNA

G+C contents reported previously for species of the genus *Novosphingobium* [3]. The only isoprenoid quinone detected from R1-4^T was ubiquinone-10 (Q-10). The major cellular fatty acids (>5 % of the total fatty acids) of R1-4^T were summed feature 8 (comprising C_{18:1} ω 7c/C_{18:1} ω 6c), summed feature 3 (comprising C_{16:1} ω 7c/C_{16:1} ω 6c), cyclo-C_{19:0} ω 8c and C_{16:0}, which was in good agreement with those of species of the genus *Novosphingobium* (Table 2). However, there were some differences in their respective proportions or in the presence/absence of some fatty acid components. The major polar lipids of R1-4^T were diphosphatidylglycerol,

Table 2. Cellular fatty acid compositions (percentages) of R1-4^T and the type strains of closely related species of the genus *Novosphingobium*

Strains: 1, R1-4^T; 2, *N. sediminicola* KCTC 22311^T; 3, *N. lotistagni* KACC 18541^T; 4, *N. oryzae* JCM 30537^T; 5, *N. capsulatum* KACC 12316^T. All data were from this study. Data are expressed as percentages of the total fatty acids. Major components (>5.0%) are highlighted in bold type; –, not detected.

Fatty acid	1	2	3	4	5
Saturated:					
C _{14:0}	–	–	1.2	0.2	–
C _{16:0}	9.4	17.6	12.6	18.3	19.0
C _{17:0}	–	–	1.1	–	–
C _{18:0}	–	–	0.8	0.4	–
cyclo-C _{17:0}	–	–	–	5.4	–
Unsaturated:					
C _{14:1} ω5c	–	1.0	–	0.2	–
C _{15:1} ω6c	–	–	0.6	–	–
C _{16:1} ω5c	–	1.0	0.9	0.4	–
C _{17:1} ω6c	–	–	4.1	–	–
C _{17:1} ω8c	–	–	0.7	–	–
C _{18:1} ω9c	–	–	0.9	–	–
cyclo-C _{19:0} ω8c	13.2	1.6	–	4.6	–
Hydroxy:					
C _{14:0} 2-OH	4.2	4.3	12.5	10.7	10.7
C _{15:0} 2-OH	–	–	1.8	–	–
Summed feature*:					
3	35.4	39.4	34.4	15.5	6.1
8	37.8	35.1	28.4	44.3	64.2

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3, C_{16:1}ω7c and/or C_{16:1}ω6c; Summed feature 8, C_{18:1}ω7c and/or C_{18:1}ω6c.

phosphatidylethanolamine, phosphatidylglycerol and sphingoglycolipid. Phosphatidylcholine, an unknown glycolipid and four unknown lipids were also detected as the minor polar lipids (Fig. S2). The polar lipids profile of R1-4^T was in good accordance with those of members of the genus *Novosphingobium* [1–7]. However, there were some differences in minor components, such as unknown aminophospholipids, aminolipids, phospholipids and glycolipids (Table 1). Spermidine was identified as the major polyamine and spermine was also detected as a minor component, which was in good agreement with the previous description as a characteristic trait of the genus *Novosphingobium* that spermidine was the predominant polyamine [1]. Spermine has also been also detected as a minor component in *N. sediminicola* HU1-AH51^T, the most closely related type strain [5]. In conclusion, the physiological and chemotaxonomic features and the phylogenetic inference of R1-4^T support the proposition that R1-4^T represents a novel species of the genus *Novosphingobium*, for which the name *Novosphingobium humi* sp. nov. is proposed.

DESCRIPTION OF *NOVOSPHINGOBIUM HUMI* SP. NOV.

Novosphingobium humi (hu'mi. L. gen. n. *humi* of earth, soil, from where the type strain was isolated).

Cells are Gram-stain-negative, strictly aerobic, non-motile short rods. Colonies on R2A agar are whitish-yellow and circular. Growth occurs at 15–45 °C (optimum, 30 °C) and pH 6.0–9.0 (optimum, pH 7.0). Oxidase is positive but catalase is negative. Indole production and fermentation of glucose do not occur. Nitrate is reduced to nitrite but does not produce nitrogen gas. Hydrolysis of tyrosine, aesculin and Tween 20 occurs, but not of starch, casein, Tween 80, gelatin and urea. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase and β-glucosidase activities are positive, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and arginine dihydrolase activities are negative. Assimilation of D-glucose, maltose and malic acid is positive, but assimilation of L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid is negative. Major cellular fatty acids are summed feature 8 (comprising C_{18:1}ω7c/C_{18:1}ω6c), summed feature 3 (comprising C_{16:1}ω7c/C_{16:1}ω6c), cyclo-C_{19:0}ω8c and C_{16:0}. The sole isoprenoid quinone is Q-10. Phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, sphingoglycolipid, phosphatidylcholine, an unknown glycolipid and four unknown lipids are detected as polar lipids. The major polyamine is spermidine and spermine is also detected as a minor polyamine.

The type strain is R1-4^T (=KACC 19094^T=JCM 31879^T), isolated from soil from a military shooting range in the Republic of Korea. The DNA G+C content is 64.4 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interests.

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