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Microvirga terrae sp. nov., Isolated from Soil

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Abstract

A Gram-stain-negative and strictly aerobic bacterium, strain R24^T, was isolated from soil in South Korea. Cells were non-motile short rods showing catalase- and oxidase-positive activities. Growth was observed at 15–40 °C (optimum, 25–30 °C) and pH 6.0–10.0 (optimum, 8.0–9.0), and in the presence of 0–3.0% NaCl (optimum, 0%). Strain R24^T contained ubiquinone-10 as the sole respiratory quinone, $C_{16:0}$, $C_{18:0}$, and summed feature 8 (comprising $C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$) as the major fatty acids, and phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylcholine as the major polar lipids. The DNA G+C content calculated from the whole genome sequence was 64.4%. Strain R24^T was most closely related to *Microvirga aerilata* 5420S-16^T with a 98.6% 16S rRNA gene sequence similarity. Average nucleotide identity and digital DNA–DNA hybridization values between strain R24^T and all *Microvirga* species were less than 82.5 and 23.8%, respectively. Phylogenetic analyses based on the 16S rRNA gene and whole genome sequences revealed that strain R24^T formed a phyletic lineage within the genus *Microvirga*. Based on its phenotypic, chemotaxonomic, and molecular characteristics, strain R24^T represents a novel species of the genus *Microvirga*, for which the name *Microvirga terrae* sp. nov. is proposed. The type strain is R24^T (=KACC 21784^T=JCM 34259^T).

Abbreviations

Q-10	Ubiquinone-10
NJ	Neighbor-joining
MP	Maximum-parsimony
ML	Maximum-likelihood
ANI	Average nucleotide identity
DDH	DNA-DNA hybridization
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
DPG	Diphosphatidylglycerol
PC	Phosphatidylcholine

Introduction

The genus *Microvirga* as a genus of the family *Methylobacteriaceae* in the class *Alphaproteobacteria* was first proposed by Kanso and Patel [1] with *Microvirga subterranea* as the type species, which was a moderate thermophile isolated from a deep subsurface thermal aquifer. However, members of the genus *Microvirga* are broadly distributed and thus have been isolated from various other environmental habitats, including air [2], soil [3–8], hot spring [9, 10], plants [11–14], human stool [15], and skin [16]. At the time of writing, the genus *Microvirga* includes 21 valid and 11 not yet validated species (https://lpsn.dsmz.de/genus/microvirga). As part of the Korean government's domestic microbial resource collection project, we have isolated and characterized bacteria from various environmental samples. During such a process, a putative novel *Microvirga* species was isolated from humus soil, and in this study, we taxonomically characterized it using a polyphasic approach.

The GenBank accession numbers for the 16S rRNA gene and genome sequences of strain R24^T are MT233327 and CP102845–8, respectively.

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Materials and Methods

Bacterial Isolation and Cultivation

Strain R24^T was isolated from a humus soil sample collected near the roots of pine trees in Yeongwol-gun of Gangwon-do province (37°07′46.3"N 128°31′58.5"E) in South Korea. For the isolation, the collected soil sample was resuspended and serially diluted in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.2), and aliquots of each serial dilutions were spread on R2A agar (BD, USA) and incubated aerobically at 30 °C for 3 days. Different colonies grown on R2A agar were randomly selected and their 16S rRNA genes 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') [17]. PCR amplicons were double-digested with *Hae*III and *Hha*I and representative PCR amplicons indicating discrete fragment patterns were partially sequenced using the universal primer 340F (5'-CCT ACG GGA GGC AGC AG-3'), as described previously [18]. The resulting 16S rRNA gene sequences were compared with those of all reported validated and invalidated type strains using the EzBioCloud server (http://www.ezbiocloud.net/identify) [19]. From the analysis, a putative novel strain belonging to the genus *Microvirga*, designated as strain R24^T, was selected. The isolate was routinely cultured on R2A agar for 3 days at 30 °C and preserved at –80 °C in R2A broth containing 15% (v/v) glycerol for a long-term preservation. The type strains, *Microvirga lupini* KACC 16864^T, *Microvirga aerilata* KACC 12744^T, *Microvirga zambiensis* KACC 16865^T, and *Microvirga makkahensis* KCTC 23863^T, and *M. subterranea* KACC 12828^T, were used as reference strains for the comparison of phenotypic properties and fatty acid compositions.

Phylogenetic and Genotypic Analysis

The 16S rRNA gene amplicon of strain R24^T amplified by F1 and R13 primers were further sequenced using the universal primers 518R (5′-ATT ACC GCG GCT GCT GG-3′) and 805F (5′-GAT TAG ATA CCC TGG TAG TC-3′) [18] at Macrogen (Korea) and the sequences obtained by the primers 340F, 518R, and 805F were assembled to get an almost complete 16S rRNA gene sequence (1409 nucleotides). Sequence similarities of 16S rRNA genes between strain R24^T and closely related type strains were calculated using the EzBioCloud server (http://www.ezbiocloud.net/identify) [19]. The 16S rRNA gene sequences of strain R24^T and closely related type strains were aligned using Infernal (version 1.1.4) with the covariance model of Rfam family RF00177 [20]. Phylogenetic trees based on the neighbour-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) algorithms with bootstrap values (1000 replications) were constructed in MEGA11 software [21].

For the whole genome sequencing, the genomic DNA of strain R24^T was extracted according to the procedure of the phenol–chloroform extraction and ethanol precipitation method [22] and sequenced using a combination of Oxford Nanopore MinION platform at the laboratory and an Illumina Hiseq X instrument with 151 bp paired-end reads at Macrogen (South Korea). The sequencing reads derived from the Nanopore and Illumina sequencing were de novo-assembled using the Unicycler program (version 0.4.7) with hybrid assembly option, and multiple rounds of polishing were performed with Pilon 1.23 in the Unicycler pipeline to correct small sequence errors [23]. The quality of the assembled strain R24^T genome was checked based on the genome completeness and contamination values using the CheckM software [24]. The whole genome sequence of strain R24^T was deposited in GenBank, and ORF finding and functional annotation were conducted by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [25]. Automated carbohydrate-active enzyme annotation web server, dbCAN meta server (http://bcb.unl.edu/dbCAN2/blast.php) [26], was employed to identify genes associated with Carbohydrate-Active enZYmes (CAZy) from the genome. A phylogenomic tree based on the concatenated nucleotide sequences of 81 housekeeping core genes was reconstructed with bootstrap values (1,000 replications) using the up-to-date bacterial core genes 2 (UBCG2) pipeline (http://leb.snu.ac.kr/ubcg2) [27] and visualized using the MEGA 11 program. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (DDH) values between strain R24^T and most closely related *Microvirga* strains were calculated using the Orthologous ANI Tool online (www.ezbiocloud.net/sw/oat) [28] and Genome-to-Genome Distance Calculator (GGDC) version 2.1 (https://ggdc.dsmz.de/ggdc.php#) with formula 2 [29], respectively.

Phenotypic, Physiological and Biochemical Characteristics

The growth of strain R24^T was examined on R2A agar, tryptic soy agar (BD, USA), nutrient agar (BD, USA), marine agar (BD, USA), and Luria–Bertani agar (BD, USA) for 2 days at 30 °C. The growth at different temperatures (10, 15, 20, 25, 30, 37, 40, and 45 °C) and pH values (5.0–11.0 at 1.0 pH unit intervals) was evaluated for 2 days on R2A agar and in R2A broth, respectively. R2A broths with pH 5.0, pH 6.0–7.0, pH 8.0–9.0, and pH 10.0–11.0 were prepared using Na-citrate, Na_2HPO_4/NaH_2PO_4, Tris–HCl, and

Na_2CO_3/$NaHCO_3$ buffers, respectively [30]. After autoclaving (for 15 min at 121 °C), the pH values of R2A broths were adjusted again if necessary. Salt tolerance was tested in R2A broth supplemented with different NaCl concentrations (0–4% at 0.5% intervals, w/v). Anaerobic growth of strain R24^T was assessed on R2A agar under anaerobic conditions using the GasPak Plus system (BBL, USA) at 30 °C for 21 days. The following biochemical analyses and physiological tests of strain R24^T were conducted using cells grown on R2A agar for 2 days at 30 °C. Gram staining was performed using the Gram stain kit (bioMérieux, France), according to the manufacturer's instructions. Cell morphology was investigated using phase-contrast microscopy (Carl Zeiss, Germany) and transmission electron microscopy (JEM–1010; JEOL, Japan). Oxidase activity was evaluated by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck, USA), and catalase activity was tested by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution (Junsei, Japan) [30]. The following properties of strain R24^T and four reference strains were investigated in parallel under the same conditions: hydrolysis of tyrosine, casein, esculin, starch, Tween 20, and Tween 80 was tested on R2A agar following the methods described by Smibert and Krieg [31] and Lányí [32] and additional biochemical features and enzymatic activities were tested using the API 20NE and API ZYM kits (bioMérieux, France), respectively, according to the manufacturers' instructions.

Chemotaxonomic Characteristics

Isoprenoid quinones of strain R24^T were extracted according to the procedure described by Minnikin et al. [33] and analyzed using an LC-20A HPLC system (Shimadzu, Japan) equipped with a diode array detector (SPD-M20A; Shimadzu, Japan) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel, Japan). For the analysis of cellular fatty acids, strain R24^T and four reference strains were cultivated in R2A broth at their optimal temperatures, and their microbial cells were harvested at the same growth stage (middle exponential phase; optical density = 0.6–0.8 at 600 nm). Cellular fatty acids of the harvested cells were saponified, methylated, and extracted, according to the procedure of the standard MIDI protocol. Extracted fatty acid methyl esters were analyzed using a gas chromatograph (Hewlett Packard 6890, USA) and identified using the RTSBA6 database of the Microbial Identification System (Sherlock ver. 6.0B) [34]. Polar lipids of strain R24^T were extracted from cells harvested during the exponential growth phase and analyzed by two-dimensional thin-layer chromatography (TLC), according to the procedure described by Minnikin et al. [35]. The following reagents were used to identify different polar lipids: 10% ethanolic molybdophosphoric acid (for total polar lipids), ninhydrin (for aminolipids), Dittmer-Lester reagent (for phospholipids), and α-naphthol (for glycolipids). Phosphatidylethanolamine (PE) phosphatidylglycerol (PG), phosphatidylcholine (PC), and diphosphatidylglycerol (DPG) were confirmed using standard polar lipid compounds purchased from Sigma-Aldrich (USA).

Results and Discussion

Molecular Phylogenetic Analysis

The phylogenetic analysis of 16S rRNA gene sequences based on the NJ algorithm revealed that strain R24^T formed a phylogenetic lineage distinct from *M. aerilata* 5420S-16^T and *M. zambiensis* WSM3693^T within the genus *Microvirga* (Fig. 1). Phylogenetic trees based on the ML and MP algorithms also indicated that strain R24^T formed a distinct phylogenetic lineage within the genus *Microvirga* (Fig. S1, available in the online version of this article). The comparative analysis of 16S rRNA gene sequences of strain R24^T with validly named type strains showed that the strain was most closely related to *M. aerilata* 5420S-16^T, *M. zambiensis* WSM3693^T, *M. makkahensis* SV1470^T, and *M. lupini* Lut6^T with 98.6, 98.2, 98.2, and 98.1% sequence similarities, respectively. In addition, strain R24^T was most closely related to '*Microvirga tunisiensis*' LmiM8 (99.2% 16S rRNA gene sequence similarity) among all *Microvirga* species, including invalidly named species. In conclusion, the phylogenetic and comparative analyses based on 16S rRNA

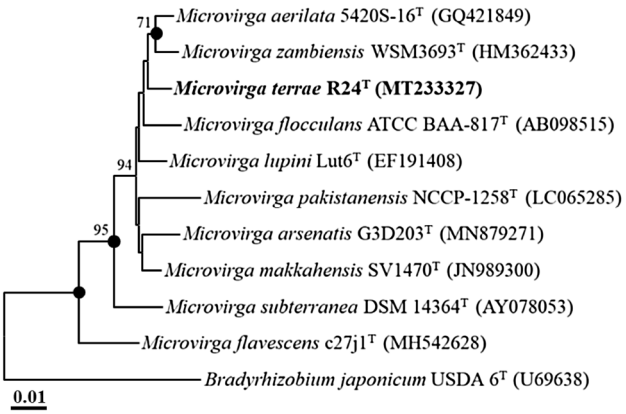

Fig. 1 A neighbor-joining tree showing the phylogenetic relationships between strain R24^T and closely related taxa in the genus *Microvirga*, based on 16S rRNA gene sequences. Numbers on nodes correspond to bootstrap values for branches (1000 replicates); only values over 70% are shown. Filled circles (●) indicate the corresponding nodes that were also recovered in trees constructed using the maximum-likelihood and maximum-parsimony algorithms. *Bradyrhizobium japonicum* USDA 6^T (U69638) was used as outgroup. Scale bar, 0.01 substitutions per nucleotide

gene sequences clearly suggest that strain R24^T represents a member of the genus *Microvirga*.

The de novo assembly of strain R24^T generated a complete genome consisting of a circular chromosome (5,295,221 bp) and three circular plasmids (556,397, 238,488, and 39,726 bp). The genome completeness and contamination rates were 99.1 and 1.1%, respectively, which clearly satisfied the criteria (≥ 90 and ≤ 10%, respectively) for the consideration as a high-quality genome [24]. The genome harbored 5718 total genes, and among them, 5505 protein coding genes, 4 rRNA gene operons (5S, 16S, and 23S rRNA), and 63 tRNA genes for the synthesis of 20 amino acids were predicted. The G+C content calculated from the whole genome sequence including chromosome and plasmids was 64.4%, which is within the range (61.1 to 65.1%) of *Microvirga* species [6]. Phylogenomic analysis based on concatenated 81 housekeeping gene sequences also showed that strain R24^T formed a phylogenetic lineage with *M. lupini* Lut6^T within the genus *Microvirga* (Fig. 2). The ANI and digital DDH values between strain R24^T and closely related type strains, *M. lupini* Lut6^T, *M. aerilata* 5420S-16^T, *M. zambiensis* WSM3693^T, and *M. makkahensis* KCTC 23863^T, and were 85.2, 82.5, 83.1, and 79.7% and 30.6, 23.8, 27.4, and 26.6%, respectively, which were clearly lower than the prokaryotic species delineation thresholds (ANI, < 95%, digital DDH, < 70%) [36, 37]. In addition, the ANI and digital DDH values between strain R24^T and '*M. tunisiensis*' LmiM8, which was the most closely related invalid species, were 85.1 and 30.0%, respectively. These results suggest that strain R24^T represents a novel species distinct from the members of the genus *Microvirga*.

Members of the family *Methylobacteriaceae*, including the genus *Microvirga*, have been reported as methylotrophs to have abilities to utilize one-carbon compounds except for

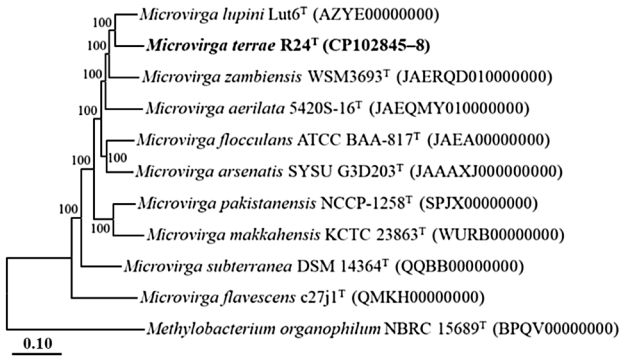

Fig. 2 A phylogenomic tree showing the phylogenetic relationships between strain R24^T and closely related taxa in the genus *Microvirga*, based on the concatenated 81 housekeeping core gene sequences. Numbers on nodes correspond to bootstrap values for branches (1000 replicates); only values over 70% are shown. *Methylobacterium organophilum* NBRC 15689^T (BPQV00000000) was used as outgroup. Scale bar, 0.10 substitutions per nucleotide

methane, such as methanol, methylamine, formaldehyde, and formic acid, as a sole energy and carbon source [38] and to fix nitrogen [39]. Bioinformatic analysis of the strain R24^T genome showed that strain R24^T harbors one PQQ-dependent methanol dehydrogenase gene cluster (Locus_tag: HPT29_23395– HPT29_23400) and six NAD-dependent alcohol dehydrogenase genes (HPT29_03515, 04235, 10425, 10935, 25040, and 27900) responsible for oxidizing methanol to formaldehyde on the periplasm and in the cytosol, respectively. In addition, the strain also harbors nitrogen fixation gene cluster (*fixGHLSJ*; HPT29_13405–13420, 13435–13440) responsible for fixing nitrogen to ammonia neighboring to the high-affinity cytochrome cbb3-type oxidase gene cluster (*ccoNOQP*; HPT29_13385–13400), which may generate ATP necessary for nitrogen fixation by nitrogen fixation gene cluster. These genomic features suggest that strain R24^T may confer benefits to plants through symbiotic relationships in the rhizosphere of plants. Carbohydrate active enzyme (CAZy) that are associated with carbohydrate metabolisms are classified into six major categories: auxiliary activities (AAs), glycosyltransferases (GTs), carbohydrate esterases (CEs), polysaccharide lyases (PLs), glycoside hydrolases (GHs), and carbohydrate-binding modules (CBMs). Bacteria living in rhizospheres can have an ability to decompose plant materials using CAZy. Therefore, the genome-wide distribution of genes encoding CAZys were analyzed in the genome of strain R24^T. A total of 139 putative CAZy-encoding genes were identified in the genome. The major carbohydrate degrading CAZy classes harboring more than 5 CAZy genes were GH1 (6), GH109 (9), GT2 (31), GT4 (12), and GT51 (5). These results suggest that strain R24^T may have an ability to decompose plant materials in the rhizosphere of plants.

Phenotypic, Physiological and Biochemical Characteristics

Strain R24^T grew well on R2A agar and showed slow growth on tryptic soy agar, nutrient agar, and Luria–Bertani agar but did not grow on marine agar. Cells of strain R24^T were short rods with approximately 0.8–1.6 μm in width and 1.5–2.6 μm in length (Fig. S2). Anaerobic growth was not observed after 21 days of incubation on R2A agar at 30 °C. The phenotypic, physiological, and biochemical properties of strain R24^T were compared with those of closely related *Microvirga* species in Table 1.

Chemotaxonomic Characteristics

Q-10 was identified in of strain R24^T as the sole respiratory quinone, which were in common with those of other species of the genus *Microvirga* [1, 6]. Strain R24^T contained $C_{16:0}$, $C_{18:0}$, and summed feature 8 (comprising $C_{18:1}$

Table 1 Comparison of the phenotype characteristics of strain R24^T and closely related type strains of the genus *Microvirga*

Characteristic	1	2	3	4	5	6
Colony color	Pink	Cream	Pink	Cream	Pink	Pink
Isolation source	Soil	Root nodule	Air	Plant root	Soil	Water
Flagella motility	−	−	−	+	+	+
Nitrate reductiona	−	−	−	+	−	+
Growth range of						
Temperature (°C)	15–40	10–43	10–35	15–38	20–45	25–45
pH	6.0–10.0	5.5–9.5	7.0–10.0	7.0–8.5	6.0–9.0	6.0–9.0
NaCl (%, w/v)	0–3.0	0–1.5	0–3.0	0–0.5	0–2.0	0–1.0
Oxidase activity	+	+	+	−	+	−
Hydrolysisa of:						
Tween 20	−	−	−	−	+	−
Starch	+	−	+	−	−	−
Enzyme activity (API ZYM)a of						
Arginine dihydrolase, urease	+	−	+	+	+	+
Esterase lipase (C8)	+	+	−	+	+	−
Lipase (C14)	−	+	−	+	−	−
Leucine arylamidase	−	−	+	−	−	−
α-Glucosidase, *α*-mannosidase, *α*-fucosidase	−	−	+	−	−	−
Trypsin	+	−	−	−	−	+
α-Chymotrypsin	+	−	−	−	+	+
α-Galactosidase	+	+	−	−	−	−
β-Glucosidase	+	−	−	−	−	−
Assimilation (API 20NE)a of:						
D-Maltose, capric acid, malic acid, phenylacetic acid	−	+	−	−	−	−
D-Glucose	+	+	–	–	+	–
D-Mannose	–	+	–	–	+	–
D-Mannitol	+	–	–	+	–	–
N-Acetyl-glucosamine	–	+	–	+	+	+
DNA G+C content (%)[b]	64.4	60.3	62.3	62.7	63.1	65.1

Taxa: 1, strain R24^T (this study); 2, *M. lupini* KACC 16864^T [11]; 3, *M. aerilata* KACC 12744^T [2]; 4, *M. zambiensis* KACC 16865 ^{T} [11]; 5, *M. makkahensis* KCTC 23863^T [3]; 6, *M. subterranea* KACC 12828^T [1]. All strains are positive for the following characteristics: activity* of catalase, alkaline phosphatase, esterase (C4), acid phosphatase, and naphthol-AS-BI-phosphohydrolase, and assimilation* of L-arabinose. All strains are negative for the following characteristics: indole production*, glucose fermentation*, hydrolysis* of casein, Tween 80, tyrosine, and esculin, activity* of valine arylamidase, cystine arylamidase, *β*-galactosidase, *β*-glucuronidase, and *N*-acetyl-*β*-glucosaminidase, and assimilation* of gluconate, adipic acid, and citric acid. Symbols:+, positive; –, negative

[a] These analyses were conducted under the same conditions in this study

[b] The DNA G+C contents were calculated based on their genome sequences in this study

*ω*7*c* and/or $C_{18:1}$ *ω*6*c*) as the major fatty acids (> 10% of the total fatty acids) (Table S1). The overall fatty acid profile of strain R24^T was similar to those of closely related type strains of the genus *Microvirga*, although there were some differences in the respective proportions of some fatty acid components, such as $C_{18:0}$ and $C_{19:0}$ cyclo *ω*8*c* [1–3, 6, 11]. PG, PE, PC, and DPG were identified as the major polar lipids (Fig. S3, available in the online version of this article).

Taxonomic Conclusion

The phylogenetic, chemotaxonomic, and physiological features suggest that strain R24^T represents a novel species of the genus *Microvirga*, for which the name *Microvirga terrae* sp. nov. is proposed.

Description of *Microvirga terrae* sp. nov.

Microvirga terrae (ter'rae L. gen. fem. n. *terrae*, of the soil).

Colonies on R2A agar are pink, circular, convex, and entire. Cells are Gram-stain-negative, strictly aerobic, and non-motile short rods. Catalase- and oxidase-positive. Growth occurs at 15–40 °C (optimum, 25–30 °C) and pH 6.0–10.0 (optimum, 8.0–9.0) and in the presence of 0–3.0% NaCl (optimum, 0%). Does not produce indole. Hydrolyzes starch, but not gelatin, casein, Tween 80, Tween 20, esculin, and tyrosine. Nitrate is not reduced to nitrite. Positive for the activity of arginine dihydrolase, urease, alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, trypsin, α-chymotrypsin, α-galactosidase, and β-glucosidase and the assimilation of L-arabinose, D-glucose, and D-mannitol. Other properties in the API ZYM and 20NE kits are negative. Q-10 is detected as the sole isoprenoid quinone. Major cellular fatty acids are $C_{16:0}$, $C_{18:0}$, and summed feature 8 (comprising $C_{18:1}$ $\omega 7c$ and/or $C_{18:1}$ $\omega 6c$). DPG, PG, PE, and PC are identified as the major polar lipids. The DNA G+C content of the type strain is 64.4%.

The type strain is R24^T (=KACC 21784^T=JCM 34259^T), isolated from soil in South Korea.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-022-03154-3.

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Author Contributions COJ: conceived the ideas and supervised all works. KHK and JHB: collected the samples, isolated the strain and performed initial cultivation, storage and deposition. SEJ and LH: analyzed the phenotypic, biochemical and genomic properties. KHK: sequenced and analysed the genome. KHK, JHB, and COJ: wrote the manuscript and the manuscript has been reviewed and edited by all authors.

Declarations

Conflict of interest The authors declare no competing financial conflict of interests.

Ethical Approval The authors have declared that no ethical issues exist.

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