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ORIGINAL PAPER

Microvirga splendida sp. nov., bacteria isolated from soil

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Abstract Two bacterial strains, BT325^T and BT690, were isolated from soil samples collected in Korea. Both strains were Gram stain-negative, short rod-shaped, and formed light-pink colored colonies. The 16S rRNA sequence similarity of strains BT325^T and BT690 shared a sequence similarity of 99.7%. Both strains shared the highest 16S rRNA gene similarity of 98.6% with *Microvirga arabica* SV2184P^T, followed by *Microvirga ossetica* V5/3 M T (98.5% and 98.2%, respectively), *Microvirga soli* R491^T (98.3% and 98.2%, respectively), *Microvirga aerilata* (98.2% and 98.08%, respectively), *Microvirga makkahensis* (98.08% and 97.8%, respectively). Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain BT325^T and BT690 were positioned in a distinct lineage within the family *Methylobacteriaceae* (order *Rhizobiales*, class *Alphaproteobacteria*). The genome size of strain BT325^T was 5,200,315 bp and the genomic DNA G+C content was 64.3 mol%. The sole respiratory quinone of strain BT325^T was Q-10 and the predominant cellular fatty acids were summed feature 3 ($C_{16:1}$ $\omega7c$/$C_{16:1}$ $\omega6c$) and summed feature 8 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine. Polyphasic taxonomic analysis of biochemical, chemotaxonomic, and phylogenetic analyses suggested that strains BT325^T represents a novel bacterial species within the genus *Microvirga*, for which the name *Microvirga splendida* is proposed. The type strain of *Microvirga splendida* is BT325^T (= KCTC 72406^T = NBRC 114847^T).

Keywords Novel species · *Microvirga* · *Methylobacteriaceae* · Taxonomy

Abbreviations

R2A	Reasoner's 2A
KACC	Korean agricultural culture collection
NA	Nutrient agar
LBA	Laked Blood Agar
TSA	Tryptic Soy Agar

The GenBank accession numbers for the 16S rRNA gene sequences of strains BT325^T and BT690 are MT795758 and MW463443, respectively. The whole-genome sequences of strain BT325^T have been deposited into DDBJ/EMBL/GenBank under the accession number JAELXT000000000.

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MAC	MacConkey
PGAP	Prokaryotic genome annotation pipeline
ANI	Average nucleotide identity
DDH	In silico DNA–DNA hybridization
NJ	Neighbor-joining
ML	Maximum–likelihood
MP	Maximum–parsimony
HPLC	High performance lipid chromatography
TLC	Thin layer chromatography
FAME	Fatty acid methyl esters
MIS	Microbial identification system
CDS	Coding genes
DPG	Diphosphatidylglycerol
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
L	Unidentified polar lipid
AL	Unidentified aminolipid

Introduction

The genus *Microvirga* is a member of the family *Methylobacteriaceae* in the class *Alphaproteobacteria.* Genus *Microvirga* was first reported with *Microvirga subterranea* as type species (Kanso and Patel 2003). At the time of writing the manuscript (August 2021), the genus *Microvirga* consists of 18 validated species (https://lpsn.dsmz.de/genus/microvirga). Members of the genus *Microvirga* have been isolated from various environments such as soil (Li et al. 2020; Zhang et al. 2019), root nodule (Safronova et al. 2017; Msaddak et al. 2019) and air (Weon et al. 2010). The genus *Microvirga* species have ubiquinone 10 (Q-10) as the major quinone and $C_{18:1\ w7c}$ as the major fatty acid. Many strains of the genus *Microvirga* have an important role in symbiotic nitrogen-fixing such as *M. zambiensis, M. lupini, M. lotononidis, M. guangxiensis,* and *M. vignae* (Ardley et al. 2012; Zilli et al. 2015).

In this study, we report polyphasic taxonomic analysis of a novel strains BT325^T and BT690 those isolated from soil samples collected in Uijeongbu and Wonju city, Korea and propose the name *Microvirga splendida* sp. nov.

Materials and methods

Organism and culture conditions

Strains BT325^T and BT690 were isolated from the soil samples collected in Uijeongbu and Wonju city, respectively (37°45′37.7"N, 127°04′31.4″ E/37°19′33.6″ N, 127°55′16.3″ E). Briefly, soil samples were serially diluted in distilled water and spreaded onto Reasoner's 2A (R2A, Difco) agar. The colonies were selected after 3 days of incubation at 25 °C. They were sub-cultured three times under the same conditions to obtain a purified colony. Strains BT325^T and BT690 were preserved at − 80 °C in 20% (v/v) glycerol with R2A broth until use. The reference strains *Microvirga arabica* KCTC 23864 ^{T}, *M. soli* KACC 18969 ^{T}, *M. aerilata* KACC 12744 ^{T}, and *M. zambiensis* KACC 16865 ^{T} were obtained from Korean Agricultural Culture Collection (KACC). Reference strains were cultured under the same conditions for the comparative experiment.

Morphology, physiology and biochemical analysis

Cell morphology was examined by transmission electron microscopy (JEOL, JEM1010) using BT325^T and BT690 colonies cultured 25 °C and 3–5 days. The effects of temperature and media of the strains were determined by incubating cultures in R2A at 20, 25, 28, 37, 40, 45 °C, and on R2A agar, Nutrient agar (NA), Laked Blood Agar (LBA), Tryptic Soy Agar (TSA), MacConkey (MAC) agar, respectively. Gram-staining was done according to manufacturer's instructions (bioMérieux). Bacterial growth was tested in R2A broth at 25 °C, with various pH values (5 to 9, 1 pH intervals) and various NaCl concentrations (0–5% [w/v], 1% intervals). Catalase activity was tested by observing the bubble production after application of 3% (v/v) hydrogen peroxide solution (Cappuccino and Sherman 2002). Oxidase activity was assessed using 1% (w/v) tetramethyl- *p*-phenylene diamine (Smibert and Krieg 1981). API 20NE and API ZYM (bioMérieux) tests were carried out according to the manufacturer's instructions (bioMérieux).

Genomic DNA extraction and genome sequencing

The genomic DNAs of a type strain (strain BT325^T) and other reference strains (*Microvirga arabica* KCTC 23864 ^{T}, *M. soli* KACC 18969 ^{T}, *M. aerilata* KACC 12744 ^{T}, and *M. zambiensis* KACC 16865 ^{T}) were extracted using a genomic DNA extraction kit (Solgent, Korea). Each DNA concentration was measured using a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). The sequencing library was generated with the Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA) and genome sequencing was done using iSeq 100 (150 bp paired end). Obtained genome sequences were assembled using SPAdes 3.13.0 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). The genome sequences were annotated using the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016) and deposited in GenBank (www.ncbi.nlm.nih.gov/) database. The sequence similarities between the strain BT325^T and closely related *Microvirga* species were analyzed using the average nucleotide identity (ANI) and in silico DNA–DNA hybridization (DDH) as described previously (Lee et al. 2016; Meier–Kolthoff et al. 2013). The genomic DNA G + C content of the strains was calculated from their genome sequences.

Phylogenetic analysis

The 16S rRNA genes of strains BT325^T and BT690 were amplified using universal bacterial primer set (Weisburg et al. 1991). Each amplified 16S rRNA gene was sequenced with universal primers (337F, 518R, 785F and 926R) by Macrogen (Korea). To determine the taxonomic positions of strains BT325^T and BT690, 16S rRNA gene sequences of closely related taxa were obtained from EzBioCloud (http://ezbiocloud.net). The phylogenetic tree was conducted by the neighbor-joining (NJ) algorithm (Saitou and Nei 1987), maximum–likelihood (ML, Felsenstein 1981), and maximum–parsimony (MP, Fitch 1971) methods as performed in the program MEGA X (Kumar et al. 2018). The evolutionary distances were calculated using the Kimura 2 parameter model (Kimura 1983). The bootstrap values were determined based on 1,000 replications (Felsenstein, 1985). The genome sequences of the strains and closely–related species were obtained from EZBioCloud and listed in Table S1. Finally, a whole-genome sequence based phylogenetic tree was reconstructed using the UBCG set pipeline (www.ezbiocloud.net/tools/ubcg) (Na et al. 2018) using default settings.

Chemotaxonomic characteristics

Isoprenoid quinones of strain BT325^T were extracted using Sep-Pak Vac cartridges (Waters, USA) and analyzed by high performance lipid chromatography (HPLC) as described previously (Hiraishi et al. 1996) after cells were grown on R2A agar (Difco) for 3 days at 25 °C. Polar lipids were extracted (Minnikin et al. 1984) and analyzed using two-dimensional thin layer chromatography (TLC). They were identified by spraying detecting reagents (Komagata and Suzuki 1988). The cellular fatty acids were purified by saponification, methylation and extraction procedures (Sasser 1990). The fatty acid methyl esters (FAME) were identified using the Sherlock Microbial Identification System V6.01 (MIS, data base TSBA6, MIDI Inc., Newark, DE, USA).

Result and discussion

Morphology, physiology and biochemical analysis

Strain BT325^T formed circular, glistening, convex, and light-pink colonies on R2A agar at 25 °C. Besides, colonies of strain BT690 were circular, convex, and light-pink colored. Cells of strains BT325^T and BT690 were Gram negative and short rod shaped (Fig. 1). Bacterial growth occurred on R2A agar, NA, LBA and TSA while no growth was observed on MCA agar. The other physiological and biochemical characteristics of strain BT325^T is presented in the description. The difference of phenotypic properties between the strains BT325^T and BT690 and closely related species in the genus *Microvirga* are listed in Table 1.

Phylogenetic analysis and genome sequencing

The 16S rRNA gene sequence similarity between the strains BT325^T and BT690 was 99.7%, indicating that they represent an identical species. Based on 16S rRNA gene sequence similarity, strains BT325^T

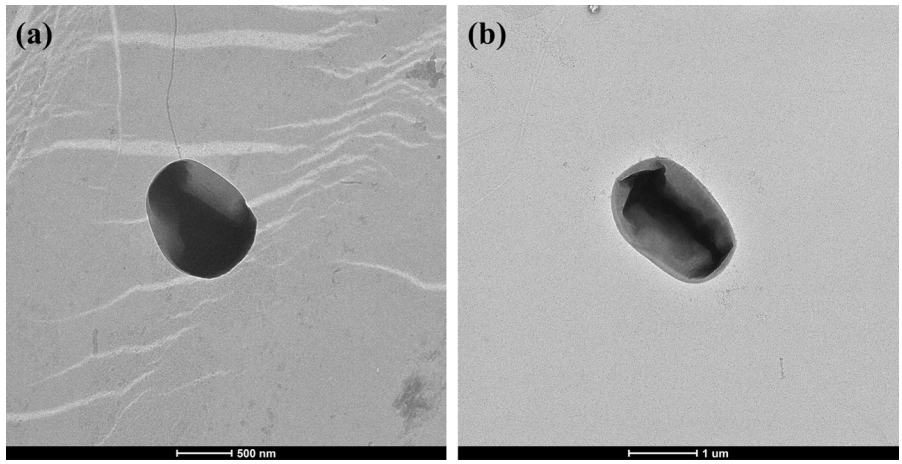

Fig. 1 Transmission electron micrographs of strains BT325^T and BT690

Table 1 Differential characteristics of *Microvirga splendida* sp. nov. and closely related species

Taxa: 1, strain BT325^T; 2, strain BT690; 3, *M. arabica* SV2184P^T; 4, *M. ossetica* V5/3M^T

Data of strains BT325^T and BT690 were obtained in this study. Those of *M. arabica* SV2184P^T and *M. ossetica* V5/3M^T were obtained from previous papers (Veyisoglu et al. 2016; Safronova et al. 2017)

+, positive; −, negative; w, weak positive; ND, no data available

All strains were positive for pH 8–9, but negative for protease (gelatin hydrolysis), D-Mannitol, and D-Maltose

Number of strains	1	2	3	4
Isolation source	Soil	Soil	Root nodule	Root nodule
Colony color	Light pink	Light pink	Pale	Transparent
Catalase	+	+	−	+
pH range	5–9	5–9	6–9	6–9
Growth at pH 5	+	+	−	−
NaCl % range	0–3	0–3	0–1	0–4
Nitrate reduction	+	+	−	+
Production of acid from glucose	−	−	+	+
Enzyme activity				
Alkaline phosphatase	−	−	+	ND
Cystine arylamidase	−	−	+	ND
Trypsin	−	w	−	ND
Urease	−	−	+	+
Valine arylamidase	−	w	+	ND
Assimilation				
L-Malate	−	−	ND	−
L-Arabinose	w	−	+	ND
D-Glucose	w	−	+	+
D-Mannose	−	−	+	+

and BT690 revealed high sequence similarities with the genus *Microvirga*. The strain BT325^T was closely related to *Microvirga arabica* SV2184P^T (98.6%, 16S rRNA gene sequence similarity), *M. ossetica* V5/3 M T (98.5%), *M. soli* (98.3%), *M. aerilata* (98.2%), *M. makkahensis* (98.0%), *M. flocculans* (97.9%), *M. zambiensis* (97.9%), *M. guangxiensis* (97.7%), *M. vignae* (97.7%), *M. lupini* (97.7%), *M. indica* (97.5%), *M. pakistanensis* (97.1%), and *M. subterranean* (97.0%). Levels of sequence similarity with other *Microvirga* species were less than 96.8%. The 16S rRNA gene sequence analysis and phylogenetic tree analysis clearly showed that the strains BT325^T and BT690 belonged to the genus *Microvirga* and represented a novel species (Fig. 2).

The genome sequences of closely related species were obtained from EZBioCloud and listed in Table S1. The draft genome of strain BT325^T contained 62 contigs and an N50 length of 144,833 bp. The genome of strain BT325^T consisted of 4,806

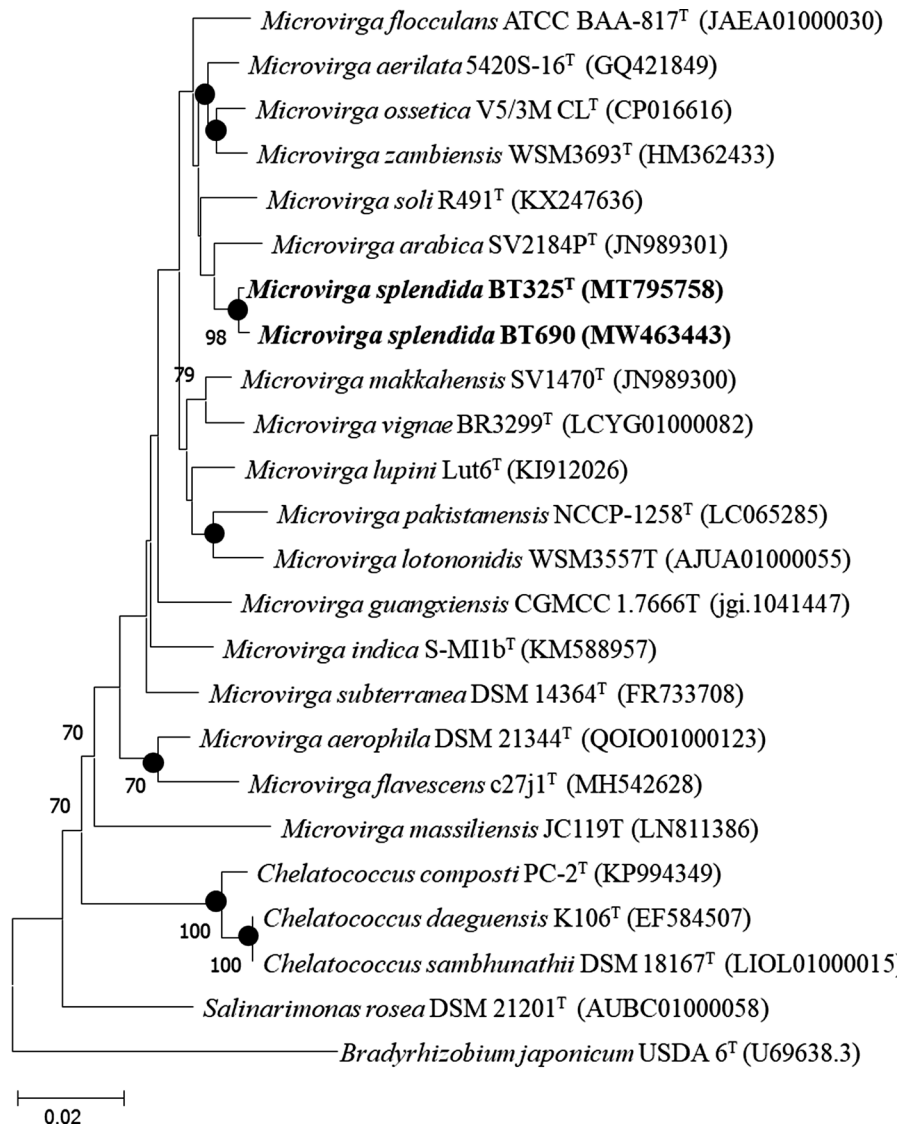

Fig. 2 Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strains BT325^T and BT690 with closely related validly published species. Bootstrap values (> 70%) based on neighbor-joining methods are shown at the branch nodes. The circles indicate that the corresponding branches were also recovered in the maximum-parsimony and maximum-likelihood trees. Bar, 0.02 substitutions per nucleotide position. *Bradyrhizobium japonicum* USDA 6 ^{T} was used as outgroup

coding genes (CDS), 45 tRNA genes, and 4 ncRNA genes. The draft genome size of strain BT325^T was 5,200,315 bp. The G + C content of genomic DNA of strain BT325^T was 64.3 mol%. The ANI and in silico DDH values between strain BT325^T and other closely related species were in the range of 79.3–87.7% and 12.0–18.9%, respectively (Table S2). These values were significantly lower than the accepted threshold values for delineating prokaryotic species using ANI (94–96%) and in silico DDH (70%) (Meier–Kolthoff et al. 2013; Konstantinidis and Tiedje 2005). The genome-based phylogenetic analysis showed (Fig S1) showed that strains BT325^T was most closely association with *Microvirga flocculans* ATCC-BAA-817 ^{T} and *Microvirga flocculans* DSM15743^T. Genome properties of the strain BT325^T based on RAST annotations are detailed in Table S3. The phylogenetic analysis results clearly showed that strains BT325^T and BT690 are a novel species within the genus *Microvirga*.

Chemotaxonomic characterization

The total cellular fatty acids of strain BT325^T and its closely related 2 species were shown in Table S4. The predominant fatty acids of strain BT325^T were

summed feature 8 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$) (76.5%) and summed feature 3 ($C_{16:1}$ $\omega7c$/$C_{16:1}$ $\omega6c$) (11.0%) The fatty acids profile of strain BT325^T was similar to those of closely related type strains, *Microvirga arabica* SV2184P^T and *Microvirga Ossetica* V5/3M^T. However, strain BT325^T differs from reference strains especially in its small amount of $C_{16:0}$, $C_{18:0}$, and summed feature 2 ($C_{14:0}$ 3OH/$C_{16:1}$ iso I).

The polar lipids of strain BT325^T were found to be diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC), an unidentified polar lipid (L), and an unidentified aminolipid (AL) (Fig. S2). Strain BT325^T differed from its closest species by the absence of an unidentified Phosphatidylmonomethylethanolamine (PME). Phosphatidylmonomethylethanolamine was also not produced by some members of the genus *Microvirga* such as *M. makkahensis*, *M. flavescens*, and *M. antarctica* (Veyisoglu et al. 2016; Zhang et al. 2019; Zhu et al. 2021). The polar lipids of strain BT325^T were similar to those of *Microvirga* species. The major respiratory quinone of strain BT325^T was Q-10 which is a common quinone within the species of the genus *Microvirga.*

Description of Microvirga *splendida* sp. nov.

Microvirga splendida (splen'di.da. L. fem. adj. splendida)

The cells are short rod-shaped and Gram-stain-negative. Colonies on R2A agar are circular, convex and colored after 72 h of growth at 25 °C. Cell sizes are approximately 0.8–1.2 µm wide and 1.1–1.5 µm in length. The strain is oxidase and catalase positive. Growth occurs at 18–37 °C (optimal temperature of 30 °C) and pH 5.0–9.0 (optimal pH of 7.0). Cells were grown well on R2A and NA agar; weak on LBA and TSA and but not on Macconkey agar. In API 20NE test, strain BT325^T was positive for nitrate reduction to NO_2; weakly positive for β-galactosidase, D-glucose, and L-arabinose but negative for indole production on tryptophan, glucose fermentation, arginine dihydrolase, urease, hydrolysis of esculin and gelatin, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. In API ZYM test, strain BT325^T was positive for esterase (C4), esterase lipase (C8), and leucine arylamidase, while negative for alkaline phosphatase, lipase (C14), valline arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The major respiratory quinone is Q-10. The dominant cellular fatty acids are summed feature 3 ($C_{16:1}$ $\omega7c$/$C_{16:1}$ $\omega6c$) and summed feature 8 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$). The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC).

The type strain for *Microvirga splendida*, strain BT325^T (= KCTC 72406^T = NBRC 114847^T), was isolated from soil in Korea. The GenBank accession number for the 16S rRNA gene sequence of strains BT325^T and BT690 is MT795758 and MW463443, respectively. The genome sequence of strain BT325^T has been deposited in GenBank/DDBJ/EMBL under the accession number JAELXT000000000.

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Author's contribution All authors equally contributed in this work.

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Declarations

Conflict of interest All authors certify that there is no conflict of interest.

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