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Paraburkholderia lacunae sp. nov., isolated from soil near an artificial pond[§]

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A Gram-stain-negative, strictly aerobic bacterial strain, designated strain S27^T, was isolated from soil near an artificial pond in South Korea. Cells were non-motile short rods showing oxidase- and catalase-positive activities. Growth of strain S27^T was observed at 20–40°C (optimum, 30°C), pH 5.0–7.0 (optimum, pH 6.0), and 0–0.5% (w/v) NaCl (optimum, 0%). Ubiquinone-8 was detected as the sole respiratory quinone and the major fatty acids were $C_{16:0}$, cyclo-$C_{17:0}$, and cyclo-$C_{19:0}$ ω8c. The G + C content of the genomic DNA was 62.4 mol%. Phosphatidylglycerol, phosphatidylethanolamine, and an unidentified aminophospholipid were detected as the major polar lipids. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S27^T formed a clearly distinct phyletic lineage from closely related *Paraburkholderia* species within the genus *Paraburkholderia*. Strain S27^T was most closely related to *Paraburkholderia rhynchosiae* WSM3937^T, *Paraburkholderia ginsengiterrae* DCY85^T, *Paraburkholderia fungorum* NBRC 102489^T, and *Paraburkholderia graminis* C4D1M^T with 98.8%, 98.4%, 98.4%, and 97.7% 16S rRNA gene sequence similarities, respectively. The DNA-DNA relatedness level between strain S27^T and the type strain of *P. rhynchosiae* was 36.8 ± 2.6%. On the basis of phenotypic, chemotaxonomic and molecular properties, strain S27^T represents a novel species of the genus *Paraburkholderia*, for which the name *Paraburkholderia lacunae* sp. nov. is proposed. The type strain is S27^T (KACC 19714 ^{T} = JCM 32721^T).

Keywords: *Paraburkholderia lacunae*, taxonomy, new taxa, soil

Introduction

Since the genus *Burkholderia* was first proposed by Yabuuchi *et al.* (1992) as a member of the family *Burkholderiaceae*, with *Burkholderia cepacia* as the type species, more than 100 *Burkholderia* species have been reported as the members of the genus *Burkholderia*. However, the members of the genus *Burkholderia* did not form a monophyletic lineage and they were split into three genera *Burkholderia*, *Paraburkholderia*, and *Caballeronia* based on their 16S rRNA gene sequences and conserved sequence indels (CSIs) (Sawana *et al.,* 2014; Dobritsa and Samadpour, 2016; Dobritsa *et al.,* 2017). It has been known that the majority of *Burkholderia* species cause infections in humans, while most *Paraburkholderia* and *Caballeronia* members are not commonly associated with human infections (Sawana *et al.,* 2014; Dobritsa and Samadpour, 2016; Dobritsa *et al.,* 2017). Members of the genus *Paraburkholderia* have been isolated from diverse environments and are also metabolically diverse. Some *Paraburkholderia* species have been confirmed to have nodulation and nitrogen-fixing ability in symbiosis with legumes (Bournaud *et al.,* 2017; Choi and Im, 2018; De Meyer *et al.*, 2018; Oh and Lim, 2018), while some have compound- or pollutant-degrading ability (Gao *et al.,* 2016, 2018; Lee and Jeon, 2018), suggesting that they may play important ecological and environmental roles. At the time of writing, the genus *Paraburkholderia* includes 74 species with validly published names (http://www.bacterio.net/paraburkholderia.html). In this study, we isolated an additional novel strain belonging to the genus *Paraburkholderia* from soil near an artificial pond and described its taxonomic assignment using a polyphasic approach.

Materials and Methods

Isolation of bacterial strain and culture conditions

Strain S27^T was isolated from soil near an artificial pond (37°30′15.0″N, 126°57′24.9″E) in Seoul, South Korea. In brief, a pond-side soil sample was serially diluted in 0.9% (w/v) saline, spread on R2A agar (BD), and incubated at 25°C for 5 days. The 16S rRNA genes of 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′) and double-digested with *Hae*III and *Hha*I, as described previously (Kim *et al.,* 2008). Representative PCR products showing distinct fragment patterns were partially sequenced using the primer 340F (5′-CCT ACG GGA GGC AGC AG-3′). The resulting 16S rRNA gene sequences were compared with those of all validated type strains using the Nucleotide Similarity Search

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and the genome sequences of strain S27^T are MG745917 and QHKS00000000, respectively.

program in the EzBioCloud server (http://www.ezbiocloud.net/identify/) (Yoon *et al.,* 2017) and eventually a putative novel strain belonging to the genus *Paraburkholderia*, designated strain S27^T, was selected for further phenotypic and phylogenetic analysis. Strain S27^T was routinely cultured aerobically on R2A agar at 25°C for 3 days. Strain S27^T was preserved at -80°C in R2A broth (BD) containing a final concentration of 15% (v/v) glycerol. Strain S27^T has been deposited in the Korean Agricultural Culture Collection (KACC 19714^T) and Japan Collection of Microorganism (JCM 32721^T).

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene amplicon of strain S27^T that was PCR-amplified using the F1 and R13 primers was sequenced using the primers 518R (5′-ATT ACC GCG GCT GCT GG-3′) and 805F (5′-GAT TAG ATA CCC TGG TAG TC-3′) at Macrogen and an almost complete 16S rRNA gene sequence (1,458 nucleotides) was obtained through the assembly of 518R, 805R, and 340F sequencing information. The 16S rRNA gene sequence similarity values between strain S27^T and closely related type strains were calculated using the EzTaxon-e server (http://www.ezbiocloud.net/). The 16S rRNA gene sequences of strain S27^T and closely related type strains were aligned using the fast secondary-structure aware infernal aligner available in the Ribosomal Database Project (Nawrocki and Eddy, 2007). Phylogenetic trees based on the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) algorithms with bootstrap values (1,000 replications) were constructed using the MEGA7 software (Kumar *et al.,* 2016).

DNA-DNA hybridization and genomics analysis

For DNA–DNA hybridization (DDH) between strain S27^T and the type strain of *P. rhynchosiae* (LMG 27174^T) and whole genome sequencing of strain S27^T, genomic DNA was extracted using a phenol-chloroform extraction and ethanol precipitation method (Sambrook and Russell, 2001). DNA-DNA relatedness between strain S27^T and the type strain of *P. rhynchosiae* was evaluated by DDH using a genome-probing microarray method, as described by Chang *et al.* (2008). The DDH experiments were confirmed through reciprocally interchanging DDH between probe and target DNA.

The whole genome of strain S27^T was sequenced with 101 bp paired-end sequencing reads by an Illumina Hiseq 2500 instrument at Macrogen. The resulting sequence reads were *de novo* assembled by using SOAPdenovo2 (Luo *et al.,* 2012). The DNA G + C content of strain S27^T was calculated based on its draft genome sequence.

Phenotypic and biochemical characteristics

The growth of strain S27^T was tested at 30°C for 3 days on R2A agar, marine agar (BD), LB agar (BD), nutrient agar (BD), and tryptic soy agar (BD). The growth of strain S27^T was assessed on R2A agar at different temperatures (4, 10, 15, 20, 25, 30, 37, 40, and 45°C) and pH values (3.5–10.0 at 1.0 pH unit intervals) was evaluated in R2A broth for 3 days. R2A broth media below pH 7.0, pH 7.5–9.0, and pH 9.5–10.0 were prepared using Na_2HPO_4/NaH_2PO_4, Tris-HCl and $Na_2CO_3/NaHCO_3$ buffers, respectively (Gomori, 1955). After sterilization (121°C for 15 min), the pH values were readjusted if necessary. The growth of strain S27^T at different NaCl concentrations (0–1.0% at 0.5% intervals and 2.0–7.0% at 1.0% intervals) was tested in R2A broth. The anaerobic growth of strain S27^T was assessed on R2A agar and R2A agar supplemented with sodium nitrate (10 mM), sodium nitrite (5 mM), dimethyl sulfoxide (10 mM), or disodium fumarate (10 mM) under the anaerobic (with 4–10% CO_2) condition using the GasPak Plus system (BD BBL) at 30°C for 21 days. Nitrate reduction was assessed in R2A broth according to the method described previously (Lányi, 1987). The following physiological and biochemical tests were conducted using cells grown on R2A agar for 3 days at 30°C. Cell morphology and motility was investigated using phase-contrast microscopy (Carl Zeiss Scope.A1) and transmission electron microscopy (JEM-1010; JEOL). 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 H_2O_2 (Smibert and Krieg, 1994). The following properties of strain S27^T and the reference strains were investigated under the same conditions in parallel. Hydrolysis of casein, starch, esculin, tyrosine, Tween 20 and Tween 80 was tested on R2A agar, according to the methods described previously (Gomori, 1955; Smibert and Krieg, 1994). Additional enzymatic activities and biochemical features were evaluated using API ZYM and API 20NE (bioMérieux), respectively. The manufacturer's protocols were applied for both assessments, except that cells resuspended in 0.9% saline were used as the inocula and the test strains were incubated at their optimal growth temperatures.

Chemotaxonomic analysis

The isoprenoid quinones of strain S27^T were extracted according to the method of Choi *et al.* (2018) and analyzed using HPLC (LC-20A; Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described by Komagata and Suzuki (1987). Strain S27^T and the reference strains were cultivated in R2A broth at their respective optimal temperatures and their microbial cells were harvested at the same growth stage (exponential phase, OD_{600} = 0.6–0.8) for the cellular fatty acid analysis. The cellular fatty acids of the microbial cells were saponified, methylated, and extracted using the standard MIDI protocol. The fatty acid methyl esters were analyzed by a gas chromatography (Hewlett Packard 6890) and identified by using the RTSBA6 database of the Microbial Identification System (Sherlock ver. 6.0B) (Sasser, 1990). The polar lipids of strain S27^T were analyzed by two-dimensional thin-layer chromatography (TLC) using cells harvested during the exponential growth phase according to the procedure described by Minnikin *et al.* (1977). The following reagents were used to detect different polar lipids: 10% ethanolic molybdophosphoric acid (for total polar lipids), ninhydrin (for aminolipids), and Dittmer-Lester reagent (for phospholipids).

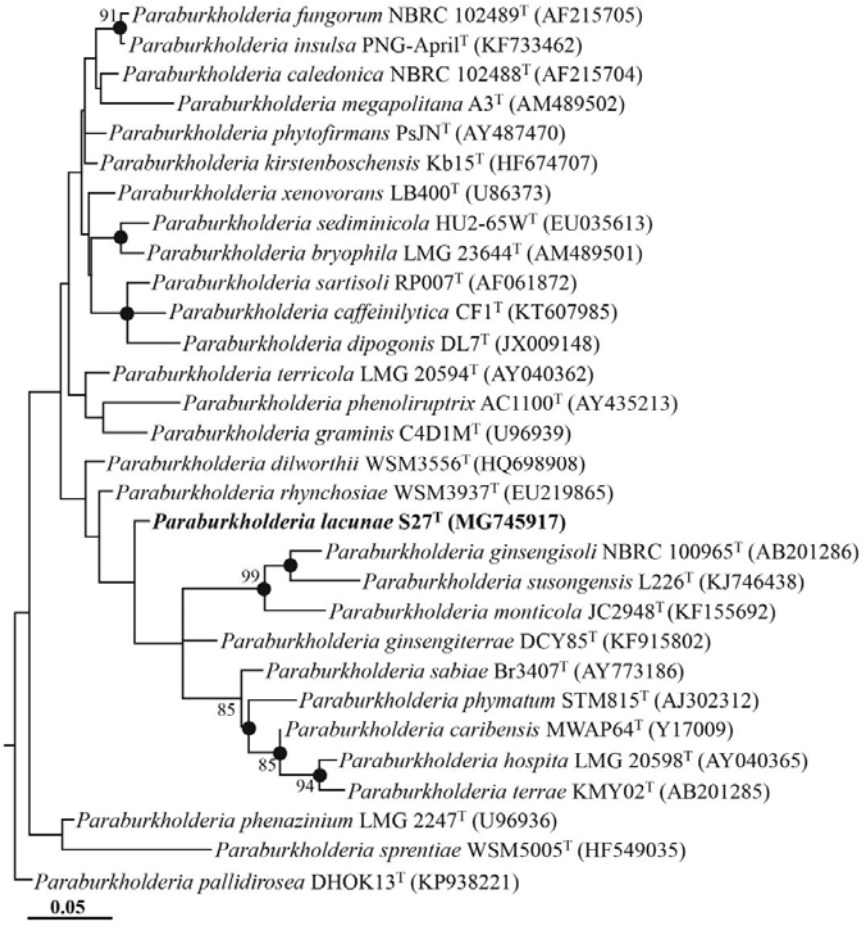

Fig. 1. A maximum-likelihood tree showing phylogenetic relationships between strain S27^T and closely related taxa of the genus *Paraburkholderia*, based on 16S rRNA gene sequences. Filled circles (●) on the nodes indicate that the relationships were also recovered by the neighbor-joining and maximum-parsimony algorithms. Bootstrap values are shown on nodes as percentages of 1,000 replicates for values over 70%. *Cupriavidus taiwanensis* LMG 19424^T (AF300324) was used as an outgroup (not shown). The scale bar equals 0.05 changes per nucleotide position.

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and the genome sequences of strain S27^T are MG-745917 and QHKS00000000, respectively. The accession numbers of the reference strains closely related to strain S27^T are indicated in Fig. 1.

Results and Discussion

Phylogenetic analysis

Comparative analysis based on the 16S rRNA gene sequences revealed that strain S27^T was most closely related to *Paraburkholderia rhynchosiae* WSM3937^T, *Paraburkholderia ginsengiterrae* DCY85^T, *Paraburkholderia fungorum* NBRC 102489^T, and *Paraburkholderia graminis* C4D1M^T with 98.8%, 98.4%, 98.4%, and 97.7% sequence similarities, respectively. The phylogenetic analysis using the ML algorithm revealed that strain S27^T formed a clearly distinct phyletic lineage from closely related *Paraburkholderia* species within the genus *Paraburkholderia* (Fig. 1). The phylogenetic trees based on the NJ and MP algorithms also showed that strain S27^T formed a distinct phyletic lineage from closely related *Paraburkholderia* species within the genus *Paraburkholderia* (Supplementary data Fig. S1). *P. rhynchosiae* LMG 27174^T, *P. ginsengiterrae* KCTC 42054^T, *P. fungorum* LMG 16225^T, and *P. graminis* DSM 17151^T were used as reference strains for the comparisons of phenotypic properties and fatty acid compositions.

Phenotypic characterization

Strain S27^T grew well on R2A agar and nutrient agar, but did not grow on marine agar, LB agar, and tryptic soy agar. Cells of strain S27^T were Gram-stain-negative, non-motile short rods (0.9–1.1 μm in width and 1.5–1.8 μm in length) (Supplementary data Fig. S2). Anaerobic growth was not observed after 21 days of incubation at 30°C. The phenotypic characteristics of strain S27^T are presented in Table 1 and in the species description. Many properties of strain S27^T such as rod-shape, catalase activity and hydrolysis of casein and starch are in common with those of the reference strains of the genus *Paraburkholderia*, whereas other phenotypic properties such as oxidase activity, nitrate reduction to nitrite, hydrolysis of Tween 80, Tween 20, and esculin allowed the differentiation of strain S27^T from other *Paraburkholderia* species (Table 1).

Chemotaxonomic characteristics

The major fatty acids (> 10% of the total fatty acids) of strain S27^T were $C_{16:0}$, cyclo-$C_{17:0}$ and cyclo-$C_{19:0}$ $\omega 8c$, which are consistent with those in the reference strains of the genus

Table 1. Comparison of the phenotypic characteristics of strain S27^T and closely related type strains of the genus *Paraburkholderia*
Taxa: 1, strain S27^T (this study); 2, *P. rhynchosiae* LMG 27174^T (De Meyer *et al.*, 2013); 3, *P. ginsengiterrae* KCTC 42054^T (Farh *et al.*, 2015); 4, *P. fungorum* LMG 16225^T (Coenye *et al.*, 2001); 5, *P. graminis* DSM 17151^T (Viallard *et al.*, 1998). All strains are positive for the following characteristics: catalase activity and tyrosine hydrolysis*, activity* of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, assimilation* of potassium gluconate, trisodium citrate, and phenylacetic acid. All strains are negative for the following characteristics: hydrolysis* of casein and starch, activity* of valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase and assimilation* of D-maltose. Symbols: +, positive; –, negative; NA, not available.

Characteristic	1	2	3	4	5
Source	Soil	Root nodule	Rhizosphere	Fungus	Rhizosphere
Morphology	Short rod	Rod	Rod	Straight rod	Curved rod
Growth at:					
Temperature (optimum,°C)	20–40 (30)	10–37 (28)	20–37 (30)	NA (30)	NA (28)
NaCl range (%, w/v)	0–0.5	0–10	0–1.0	0.5	NA
Oxidase	+	+	–	+	+
Nitrate reduction*	+	+	–	–	+
Hydrolysis* of:					
Tween 80	+	–	+	+	–
Tween 20	–	–	+	+	+
Esculin	+	+	–	–	–
Enzyme activity (API ZYM)* of:					
Lipase (C14)	–	–	+	–	–
β-Glucosidase	–	+	–	–	–
Assimilation (API 20NE)* of:					
Capric acid	–	+	–	+	+
Adipic acid	–	–	–	+	+
Malic acid	+	–	+	+	+
D-Glucose, L-Arabinose, D-Mannose, D-Mannitol, *N*-Acetyl-glucosamine	+	+	+	–	+
DNA G + C content (mol%)†	62.4†	61.2	66.0	62.0	63.0

* All data were obtained from this study.
† The DNA G + C content of strain S27^T was calculated from its genome, while others were determined by HPLC.

Paraburkholderia (Table 2). Although the overall fatty acid profile of strain S27^T was similar to those of closely related *Paraburkholderia* type strains, there were some differences in the respective compositions of some fatty acid components. For example, cyclo-$C_{17:0}$ was identified as a major fatty acid from strain S27^T, but it was detected in just trace amounts (< 0.5%) from the type strains of *P. ginsengiterrae*, *P. fungorum*, and *P. graminis*. Whereas $C_{17:1}$ *ω5c* was detected in trace amounts in strain S27^T, but it was one of major fatty acid components in the type strains of *P. ginsengiterrae*, *P. fungorum*, and *P. graminis*. The major polar lipids of strain S27^T were phosphatidylglycerol, phosphatidylethanolamine, and an unidentified aminophospholipid. Four unidentified lipids were also detected as minor polar lipids (Supplementary data Fig. S3).

Molecular characteristics

It has been suggested that 98.65–98.7% 16S rRNA gene sequence similarity between two strains equates to 70% DNA-DNA relatedness, the gold standard for species delineation (Stackebrandt and Ebders, 2006; Kim *et al.,* 2014). Therefore, a DDH experiment was performed between strain S27^T and *P. rhynchosiae*, which showed a 98.8% 16S rRNA gene sequence similarity. The DDH relatedness value between strain S27^T and the *P. rhynchosiae* type strain LMG 27174^T was 36.8 ± 2.6%, which was clearly lower than the 70% threshold that has been generally accepted for species delineation (Chun *et al.,* 2018). In conclusion, the phylogenetic analyses and DDH experiment clearly suggest that strain S27^T represents a novel species of the genus *Paraburkholderia*.

De novo assembly of the genome sequencing data of strain S27^T resulted in 96 contigs, an N50 of 266,119 bp, and average genome coverage of 493 ×. The draft genome size of strain S27^T was 8,405,011 bp of total length. A total of 7,770 genes were predicted, and among them, 7,712 genes protein-coding genes and 58 RNA genes were identified. The genomic DNA G + C content of strain S27^T was 62.4 mol%, which was in the range of those of *Paraburkholderia* members (Table 1) (Viallard *et al.,* 1998; Coenye *et al.,* 2001; De Meyer *et al.,* 2013; Farh *et al.,* 2015). The draft genome sequence of strain S27^T was deposited to GenBank with the accession number QHKS00000000.

Taxonomic conclusion

The results of physiological characterization, the 16S rRNA gene and whole genome sequence comparison, and chemotaxonomic tests indicated that the strain S27^T was a member of the genus *Paraburkholderia.* However, based on the phylogenetic distances from he established *Paraburkholderia* species, and the combination of phenotypic characteristics (Table 1), it can be observed that the strain S27^T is not affiliated with any recognized species of the genus *Paraburkholderia.* In conclusion, the phylogenetic, physiological, and chemotaxonomic features clearly show that strain S27^T re-

Table 2. **Cellular fatty acid compositions (%) of strain S27^T and closely related taxa of the genus *Paraburkholderia***
Taxa: 1, strain S27^T; 2, *P. rhynchosiae* LMG 27174^T; 3, *P. ginsengiterrae* KCTC 42054^T; 4, *P. fungorum* LMG 16225^T; 5, *P. graminis* DSM 17151^T. All fatty acid data were obtained from this study. Data are expressed as percentages of the total fatty acids and fatty acids amounting to less than 0.5% in all strains are not shown. Major components (> 10.0%) are highlighted in bold. tr, trace amount (< 0.5%); –, not detected.

Fatty acid	1	2	3	4	5
Saturated:					
$C_{12:0}$	3.1	tr	3.7	–	–
$C_{14:0}$	1.5	4.7	1.1	5.2	5.4
$C_{16:0}$	**30.6**	**15.7**	**20.4**	**17.5**	**24.7**
$C_{18:0}$	1.1	0.6	1.1	1.1	1.3
$C_{19:0}$	1.5	0.8	1.1	0.7	1.1
iso-$C_{17:0}$	0.8	–	0.5	–	0.5
Unsaturated:					
$C_{17:1}$ *ω5c*	tr	tr	**23.9**	**19.8**	**26.6**
$C_{17:1}$ *ω7c*	0.7	tr	tr	tr	tr
$C_{20:2}$ *ω6,9c*	0.6	tr	tr	tr	tr
$C_{18:1}$ *ω7c* 11-methyl	1.0	tr	tr	tr	tr
Cyclic:					
cyclo-$C_{17:0}$	**17.7**	**21.0**	tr	tr	tr
cyclo-$C_{19:0}$ *ω8c*	**17.9**	**17.8**	**15.3**	**21.1**	**16.5**
Hydroxy					
$C_{16:0}$ 2-OH	3.7	4.1	4.1	3.0	2.8
$C_{16:0}$ 3-OH	4.1	4.4	4.5	4.0	4.4
$C_{16:1}$ 2-OH	1.3	4.8	1.4	2.0	tr
$C_{18:1}$ 2-OH	1.8	1.9	1.6	2.1	2.1
Summed feature*:					
2	5.1	6.6	7.5	7.2	tr
3	1.3	4.0	2.2	2.7	2.3
5	1.6	0.8	0.9	0.7	1.5
8	3.0	**10.4**	8.8	**10.0**	8.7

* Summed features represent groups of two fatty acids that cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 2, one or more of $C_{12:0}$ aldehyde and/or an unknown fatty acid with an equivalent chain-length of 10.9525; summed feature 3, $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; summed feature 5, $C_{18:0}$ ante/$C_{18:2}$ *ω6,9c*; summed feature 8, $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*.

presents a novel species of the genus *Paraburkholderia*, for which the name *Paraburkholderia lacunae* sp. nov. is proposed.

Description of *Paraburkholderia lacunae* sp. nov.

Paraburkholderia lacunae (la.cu'nae. L. fem. gen. n. *lacunae* of a pond; referring to the isolation of the type strain isolated from soil near a pond). Cells are Gram-stain-negative, strictly aerobic, non-motile short rods (0.9–1.1 μm in width and 1.5–1.8 μm in length). Catalase- and oxidase-positive. Growth occurs at 20–40°C (optimum, 30°C), pH 5.0–7.0 (optimum, pH 6.0), and 0–0.5% (w/v) NaCl (optimum, 0%). Hydrolyzes Tween 80, esculin, and tyrosine, but not casein, Tween 20, or starch. Nitrate is reduced to nitrite. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities are positive, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase activities are negative. Assimilation of D-glucose,carabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, malic acid, trisodium citrate, and phenylacetic acid is positive, but assimilation of D-maltose, capric acid, and adipic acid is negative. Q-8 is detected as the sole isoprenoid quinone. The major polar lipids consist of phosphatidylglycerol, phosphatidylethanolamine, and an unidentified aminophospholipid. The major fatty acids are $C_{16:0}$, cyclo-$C_{19:0}$ *ω8c*, and cyclo-$C_{17:0}$. The DNA G + C content of the type strain is 62.4 mol%. The type strain is S27^T (KACC 19714^T = JCM 32721^T), isolated from soil near an artificial pond in Seoul, South Korea.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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