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NOTE

Rummeliibacillus suwonensis sp. nov., Isolated from Soil Collected in a Mountain Area of South Korea[§]

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A Gram-positive, facultatively aerobic, rod-shaped, non-motile, terminal spore-forming bacterium, designated strain G20^T, was isolated from soil collected in a mountain region of Suwon, South Korea. On the basis of 16S rRNA gene sequence similarity, this strain was shown to be related to *Rummeliibacillus pycnus* NBRC 101231^T (97.4%) and *Rummeliibacillus stabekisii* KSC-SF6g^T (95.7%). DNA-DNA hybridization studies showed 42% and 50% similarity of strain G20^T with *R. pycnus* NBRC 101231^T *and R. stabekisii* KSC-SF6g^T, respectively. The DNA G+C content of G20^T was 37.8 mol%, the major cellular fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$, and the predominant menaquinones were MK-7 and MK-8. On the basis of phylogenetic, chemotaxonomic, and phenotypic characteristics, we propose this strain to be a novel species and the third member of genus *Rummeliibacillus*. We suggest the name *Rummeliibacillus suwonensis* sp. nov. The type strain is G20^T (KACC 17316^T =KEMB 9005-003^T =JCM 19065^T).

Keywords: *Rummeliibacillus suwonensis*, soil, spore-forming

The genus *Rummeliibacillus* belongs to the *Bacillaceae* (family), *Bacillales* (order), *Bacilli* (class), and *Firmicutes* (phylum). To date, two species have been identified: *Rummeliibacillus stabekisii* and *Rummeliibacillus pycnus* (Vaishampayan *et al*., 2009). The latter species was reclassified from *Bacillus pycnus* at the time when the novel *Rummeliibacillus* genus was proposed, because the polyphasic taxonomic properties of *B. pycnus* were most closely affiliated with the novel genus (Nakamura *et al*., 2002; Vaishampayan *et al*., 2009).

Strain G20^T was isolated from soil collected in a forest region of Suwon, South Korea. The sampling site of soil was geographically located at 37° 14′ 35.9″ N and 127° 4′ 57.2″ E. Soil pH, salinity and soil texture were 4.2, 0.02% and loamy sand (79.8% sand, 11.1% silt, and 9.0% silt), respectively. Exchangeable Ca^{2+}, Mg^{2+}, K^+, and Na^+ were 61.2, 11.4, 17.7, and 10.4 mg/kg, respectively.

The morphology of cells grown for 3 days at 30°C on nutrient broth was observed by phase-contrast microscopy using an Olympus BX 51 microscope (Olympus, Japan) at a magnification of ×1000. Motility was tested with cells grown on 0.4% agar-containing trypticase soy agar (TSA; Oxoid, UK). Sporulation was induced by growth in modified Schaeffer's medium [KCl 0.1%, $MgCl_2$ 0.01%, $Ca(NO_3)_2$ 1.0 mM, $MnCl_2$ 0.01 mM, $FeSO_4$ 1.0 μM, and nutrient broth 8 g/L] (Kempf *et al*., 2005), and spore morphology was examined by phase-contrast microscopy, as described above, after 1 week at 30°C. Gram reactions were carried out according to the established Gram procedure (Doetsch, 1981). Oxidase activity was evaluated by oxidation of 1% *ρ*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production after application of a 3% (v/v) hydrogen peroxide solution. Growth on different media was investigated with TSA, Luria-Bertani agar (LB; Oxoid), nutrient agar (NA; Oxoid), and R2A agar (MB Cell, USA). The API 20NE, API ID32GN, API ZYM, and API 50CHB microtest systems (bioMérieux, France) were used to determine carbon source utilization and enzyme activities according to the manufacturer's instructions. Growth at various temperatures (10, 15, 20, 25, 30, 37, 40, 45, 50, and 55°C) was assessed on TSA for 5 days. Growth at pH values between 0 and 12 (in 0.5 unit increments) was assessed on TSB at 30°C. To examine the growth pattern according to O_2 level, traditional deep tube culture method was applied by using 0.7% agar medium (Johnson and Case, 2007). Cells of strain G20^T were Gram-positive, facultatively aerobic, non-motile, and rod-shaped. After 3 days incubation at 30°C on TSA plates, the colonies were 0.5–1.5 mm in diameter, circular, viscous, raised, entire, and yellowish. The strain grew well on TSA, LB, NA, and R2A. Spore formation of strain G20^T was confirmed and terminal cylindrical spores were observed in unswollen sporangia.

The 16S rRNA gene was amplified by PCR using 27F and 1492R primers, as previously described (Frank *et al*., 2008). Sequencing was performed on an Applied Biosystems 3730XL DNA analyzer using a Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystems, USA). The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G20^T is KC677630. A nearly complete se-

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quence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc., USA). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited with the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with bootstrap values based on 1,000 replications (Felsenstein, 1985). The phylogenetic tree was constructed by using the 30 closest type strains and one outgroup (*Bacillus subtilis* IAM 12118^T). The analysis showed that strain G20^T was most closely related to the genus *Rummeliibacillus* (Fig. 1).

Polar lipids were extracted using a previously published procedure (Minnikin *et al*., 1984) and identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). The polar lipid profile of G20^T consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unknown phospholipids, and an unknown polar lipid (Supplementary data Fig. S1).

Isoprenoidquinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC, evaporated under vacuum conditions, and re-extracted in n-hexane:water (1:1, v/v). The crude n-hexane quinone solution was purified and then analyzed by HPLC, as previously described (Collins and Jones, 1981). For fatty acid methyl ester analysis, cells were grown for 3 days on TSA at 30°C. Fatty acid methyl esters were saponified, methylated, extracted, and analyzed according

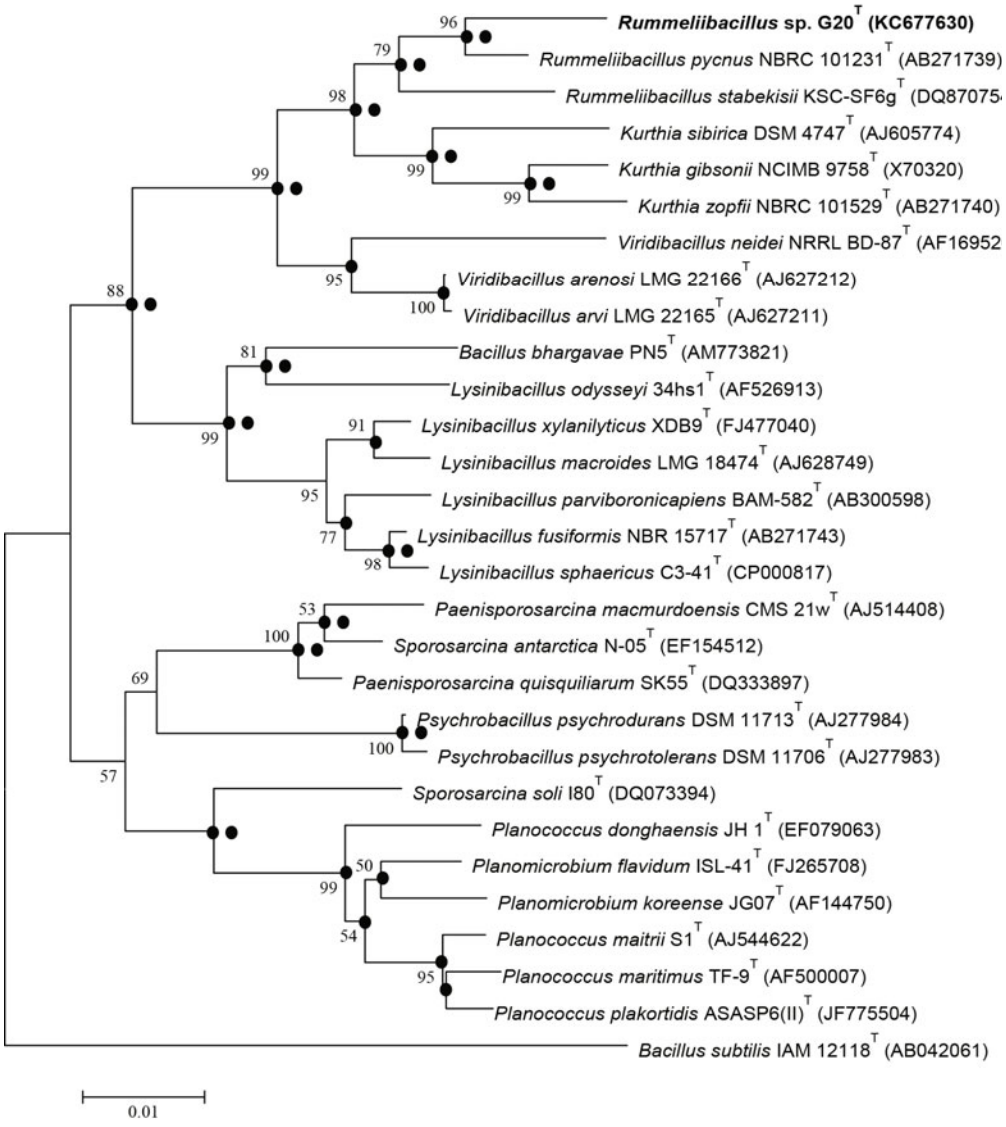

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain G20^T and related taxa. Bootstrap percentages were based on 1,000 replications and are shown at the branch points. A closed circle indicates the common node checked by either the maximum-parsimony method or the maximum-likelihood method. Two closed circles reveal the common node recovered from both the maximum-parsimony method and the maximum-likelihood method.

to the procedures of the Sherlock Microbial Identification System (TSBA6, Version 6.0; MIDI, Inc., USA) (Sasser, 1990). Fatty acids were analyzed by GC (HP 6890 series, Hewlett Packard, USA).

For the determination of the DNA G+C content, genomic DNA was extracted and enzymatically degraded into nucleosides. The nucleosides were analyzed by HPLC as described previously (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989). The predominant respiratory quinones of strain G20^T were MK-7 and MK-8, whereas in the closest type strains, *R. pycnus* NBRC 101231^T and *R. stabekisii* KSC-SF6g^T, the main quinone was MK-7 (Vaishampayan *et al*., 2009). The fatty acid profile of strain G20^T consisted of iso-$C_{15:0}$ (55.2%), anteiso-$C_{15:0}$ (22.7%), iso-$C_{14:0}$ (8.7%), iso-$C_{16:0}$ (4.9%), $C_{16:1}$ *ω7c* alcohol (2.2%), and anteiso-$C_{17:0}$ (1.7%). The major fatty acids (>10%) of strain G20^T, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ (22.7%), were quantitatively different from those of other *Rummeliibacillus* species. $C_{16:0}$ was slightly (<1%) detected in strain G20^T, but it was somewhat (>1%) detected in other *Rummeliibacillus* species (Table 2). The five most abundant fatty acids were the same for strain G20^T and *R. pycnus* NBRC 101231^T, which was the species most related to strain G20^T according to 16S rRNA gene sequence similarity. The G+C content of G20^T was 37.8 mol%.

DNA-DNA hybridization experiments were performed by the fluorometric method developed by Ezaki *et al.* (1989), using photobiotin-labeled DNA probes and microdilution wells. Hybridization was performed at 50.2°C in 50% (v/v) formamide and 0.3 M NaCl using five replicates per sample. The highest and lowest values obtained for each sample

Table 1. Differential phenotypic and chemotaxonomic properties of strain G20^T and its related species in genus *Rummeliibacillus*. Strains: 1, *R. suwonensis* G20^T; 2, *R. pycnus* NBRC 101231^T; 3, *R. stabekisii* KSC-SF6g^T. The data for strain G20^{T}and its related taxa were obtained in this study, and some data (major quinones and DNA G+C content) for other strains are taken from Nakamura *et al.* (2002) and Vaishampayan*et al.* (2009). +, positive; -, negative; w, weakly positive. All strains are negative for enzyme activity of lipase (C14), valinearylamidase, cystinearylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucuronidase, α-glucosidase, β-glucosidase, *N*-Acetyl-β-glucosaminidase, α-mannosidase,α-fucosidase, urease and argininedihydrolase. All strains are negative for assimilation of d-melibiose, l-fucose, d-sorbitol, propionic acid, capric acid, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, l-proline, l-rhamnose, d-ribose, inositol, d-saccharose, d-maltose, itaconic acid, suberic acid, sodium malonate, lactic acid, l-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, l-serine, adipic acid and phenylacetic acid. All strains are negative for fermentation of d-glucose.

Characteristics	1	2	3
Motility	-	+	+
Optimum growth temp. (°C)	37–45	25–45	25–45
Anaerobic growth	w	-	-
Growth in 7% NaCl	-	-	+
Reduction (NO_3→NO_2)	+	-	-
Indole production	+	+	-
Gelatin hydrolysis	+	-	+
Assimilation of:			
Salicin	-	+	-
Valeric acid	+	+	-
Trisodium citrate	-	+	-
L-Histidine	-	+	-
Sodium acetate	-	+	-
Glycogen	+	+	-
D-Glucose	w	+	-
L-Arabinose	-	w	-
D-Mannose	-	w	-
D-Mannitol	-	w	-
N-Acetylglucosamine	-	w	-
Potassium gluconate	-	+	-
Malic acid	w	w	-
Enzyme activity:			
Alkaline phosphatase	+	w	-
Esterase (C4)	-	+	+
Esterase lipase (C8)	-	+	+
Leucinearylamidase	-	+	+
α-Chymotrypsin	-	+	+
Acid phosphatase	+	-	w
Acid production from D-glucose	-	+	-
Major quinones	MK-7, MK-8	MK-7	MK-7
Major polar lipids[a]	DPG, PE, PG, 2PL, UL	DPG, PE, PG, APL, 2PL, AL	DPG, PE, PG, APL, 2PL, AL
Major fatty acids (>10%)	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{14:0}$
DNA G+C content (mol%)	37.8	35.0	34.3

[a] Abbreviations: AL, unknown aminolipid; APL, aminophospholipid; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown phospholipid; UL, unknown polar lipid

Table 2. Cellular fatty acid composition of strain G20^T and its related species in genus *Rummeliibacillus*. All data were obtained in this study and are expressed as percentages. Strains: 1, *R. suwonensis* G20^T; 2, *R. pycnus* NBRC 101231^T; 3, *R. stabekisii* KSC-SF6g^T. –, less than 1.0% or not detected.

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	–	–	10.9
$C_{16:0}$	–	1.7	8.0
$C_{18:0}$	–	–	1.9
Branched saturated			
iso-$C_{14:0}$	8.7	9.3	3.0
iso-$C_{15:0}$	55.2	64.8	27.2
iso-$C_{16:0}$	4.9	5.3	2.7
iso-$C_{17:0}$	–	1.1	–
anteiso-$C_{15:0}$	22.7	11.7	35.6
anteiso-$C_{17:0}$	1.7	–	7.7
Monosaturated			
$C_{16:1}$ $\omega 11c$	–	–	1.4
$C_{16:1}$ $\omega 7c$ alcohol	2.2	2.5	1.7

were excluded, and the hybridization values were calculated with the remaining three values. Strain G20^T DNA and salmon sperm DNA were used as the positive and negative controls, respectively. The DNA relatedness of strain G20^T to *R. pycnus* NBRC 101231^T and *R. stabekisii* KSC-SF6g^T was 42% and 50%, respectively, indicating the same genus but different species (70% > value ≥ 25%) (Madigan *et al*., 2012).

The phenotypic, chemotaxonomic, and phylogenetic characteristics classify strain G20^T as a member of the genus *Rummeliibacillus*. However, some other characteristics, including phenotypic properties and DNA-DNA relatedness, indicate that strain G20^T differs from other members of the genus *Rummeliibacillus* and is a unique species. The phenotypic and chemotaxonomic characteristics that were uniquebetween strain G20^{T}and its closest relatives were non-motile, facultative growth, positive nitrate reduction, different major quinone and polar lipid profiles and DNA G+C content (Table 1). The other properties that distinguished strain G20^T from each member of the same genus are revealed in Table 1. Therefore, we propose strain G20^T to be a novel species and the third member of the genus *Rummeliibacillus*, and suggest the name *Rummeliibacillus suwonensis* sp. nov. (KACC 17316^T =KEMB 9005-003^T =JCM 19065^T).

Description of *Rummeliibacillus suwonensis* sp. nov.

Rummeliibacillus suwonensis (su.won.en'sis. N.L. masc. adj. suwonensis referring to the city of Suwon, South Korea, where the type strain was isolated).

Cells are Gram-positive rods 0.5–0.7 μm wide and 3–8 μm long. After 2 days growth on TSA, colonies are 0.5–1.5 mm in diameter, circular, viscous, raised, entire, and yellowish. Cells can grow at 20–50°C (optimum, 37–45°C), at pH 5.5–10 (optimum, pH 6.5–8.0), and at NaCl concentrations of 0–5% (optimum, 0–1.5%). Growth occurs on LB, NA, R2A, and TSA (best). The strain is catalase positive and oxidase positive, reduces nitrate to nitrite, and produces indole. Valeric acid, glycogen, D-glucose, and malic acid are assimilated (API 20NE and API ID 32GN). Esterase (C4), acid phosphatase, naphthol-AS-BI-phosphohydrolase, and gelatinase (protease) are present (API ZYM). Acid is not produced from all substrates (API 50CHB). The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unknown phospholipids, and an unknown polar lipid. The predominant respiratory quinones are MK-7 and MK-8. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The DNA G+C content is 37.8 mol%. The type strain G20^T (KACC 17316^T =KEMB 9005-003^T =JCM 19065^T) was isolated from forest soil collected in Suwon (city), Gyeonggi Province, South Korea.

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