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Paenibacillus agri sp. nov., isolated from soil

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Abstract

A strict aerobic bacterium, strain JW14^T was isolated from soil in the Republic of Korea. Cells were Gram-stain-positive, non-endospore-forming and motile rods showing catalase-positive and oxidase-negative activities. Growth of strain JW14^T was observed at 20–37 °C (optimum, 30 °C), pH 6.0–10.0 (optimum, pH 7.0) and in the presence of 0–2.0% NaCl (optimum, 0%). Strain JW14^T contained menaquinone-7 as the sole isoprenoid quinone, anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$ as the major fatty acids (>10.0%), and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified aminophospholipids and an unidentified lipid as the major polar lipids. The cell-wall peptidoglycan of strain JW14^T contained *meso*-diaminopimelic acid. The DNA G+C content of strain JW14^T calculated from the whole genome sequence was 48.1 mol%. Strain JW14^T was most closely related to *Paenibacillus graminis* DSM 15220^T with 97.4% 16S rRNA gene sequence similarity. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain JW14^T formed a distinct phyletic lineage from closely related type strains within the genus *Paenibacillus*. Based on the results of phenotypic, chemotaxonomic and molecular analyses, strain JW14^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus agri* sp. nov. is proposed. The type strain is JW14^T (=KACC 21840^T=JCM 34279^T).

The genus *Paenibacillus* as a member of the family *Paenibacillaceae* was first proposed by Ash *et al.* in 1994 [1] with *Paenibacillus polymyxa* as the type species and the genus description was subsequently emended [2, 3]. At the time of writing, 254 validly published species (www.bacterio.net/paenibacillus.html) isolated from diverse environmental habitats, including soil [4–6], plant rhizosphere [7, 8], plants [9, 10], water [11], sediment [12], foods [13] and clinical samples [14] belong to this genus. Typically, members of the genus *Paenibacillus* are rod-shaped, aerobic or facultative anaerobic and endospore-forming bacteria, having anteiso-$C_{15:0}$ as the major cellular fatty acid and menaquinone (MK)-7 as the major menaquinone although some members produce MK-8 [1, 2, 15]. The genus *Paenibacillus* has been reported to produce various extracellular enzymes, such as chitosanase, glucanase, amylase, proteases, antimicrobial substances and phytohormones [16–18]. Additionally, it has been reported that some bacterial species in this genus have nitrogen-fixing abilities [19]. In this study, we isolated a putative novel species of the genus *Paenibacillus*, designated strain JW14^T, from soil and taxonomically characterized the strain using a polyphasic approach.

ISOLATION AND ECOLOGY

Strain JW14^T was isolated from a soil sample collected from Gongju in Chung-nam Province, Republic of Korea (36° 33′ 27.4″ N 126° 57′ 00.8″ E). For the isolation of a novel strain, collected soil samples were resuspended and serially diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and aliquots of each serial dilutions were spread on Reasoner's 2A (R2A) agar (BD) 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′-AGAGTTTGATCMTGGCTCAG-3′) and R13 (5′-TACGGYTACCTTGTTACGACTT-3′). Amplified PCR products 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

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; LB, Luria–Bertani; MA, marine agar; MK, menaquinone; MK, menaquinone; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; R2A, Reasoner's 2A; TSA, tryptic soy agar.
The GenBank accession numbers for the 16S rRNA gene and genome sequences of strain JW14^T are MT233330 and JABWCS000000000, respectively.
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One supplementary table and four supplementary figures are available with the online version of this article.

(5′-CCTACGGGAGGCAGCAG-3′), as described previously [20]. The resulting 16S rRNA gene sequences were compared with those of all reported validated type strains using the Nucleotide Similarity Search program in the EzBioCloud server (www.ezbiocloud.net/identify) [21] and strain JW14^T was selected as a putative novel species of the genus *Paenibacillus* for further taxonomic characterizations. Strain JW14^T was routinely cultured on R2A agar and/or in R2A broth for 2–3 days at 30 °C and stored at –80 °C in R2A broth containing 15 % (v/v) glycerol for long-term preservation. *Paenibacillus graminis* KACC 11529^T, *Paenibacillus jilunlii* KACC 16679^T and *Paenibacillus polymyxa* KACC 10485 ^{T} were used as reference strains of strain JW14^T for the comparison of phenotypic properties and fatty acid compositions.

16S rRNA GENE SEQUENCE PHYLOGENY

The 16S rRNA gene of strain JW14^T amplified by F1 and R13 primers was further sequenced using 518R (5′-ATTACCGCGGCTGCTGG-3′) and 805F (5′-GATTAGATACCCTGGTAGTC-3′) universal primers at Macrogen (Seoul, Republic of Korea) and the sequences obtained by the primers 340F, 518R and 805F were assembled to produce an almost-complete 16S rRNA gene sequence (1482 nucleotides). The similarities of 16S rRNA gene sequences between strain JW14^T and closely related type strains were calculated using the EzBioCloud server (www.ezbiocloud.net/identify) [21]. The 16S rRNA gene sequences of strain JW14^T and closely related type strains were aligned using the fast secondary-structure aware infernal aligner available in the Ribosomal Database Project [22]. Phylogenetic trees based on the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms with bootstrap values (1000 replications) were reconstructed using MEGA7 software [23].

Comparative analysis of 16S rRNA gene sequences using the EzBioCloud server showed that strain JW14^T was closely related to *P. graminis* DSM 15220^T, *Paenibacillus helianthi* P26E^T, *Paenibacillus riograndensis* SBR5^T and *P. jilunlii* Be17^T with 97.4, 97.2, 97.2 and 97.1% 16S rRNA gene sequence similarities, respectively. All phylogenetic analyses based on NJ, ML and MP algorithms revealed that strain JW14^T formed a separate phylogenetic lineage from the closely related type strains within the genus *Paenibacillus* (Figs 1 and S1, available in the online version of this article). In conclusion, the phylogenetic analyses based on 16S rRNA gene sequences clearly suggested that strain JW14^T could represent a novel species of the genus *Paenibacillus*.

GENOME FEATURES

The genomic DNA of strain JW14^T was extracted using the Wizard Genomic DNA purification Kit (Promega) and sequenced using an Illumina Hiseq X instrument at Macrogen (Seoul, Repulic of Korea) with 151 bp paired-end reads. The resulting sequencing reads of strain JW14^T were *de novo*-assembled using SOAPdenovo2 program [24]. The whole genome sequence of strain JW14^T was deposited in GenBank and was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [25]. The DNA G+C content of strain JW14^T was calculated from the whole genomic sequence. Average nucleotide identity (ANI) and digital DNA–DNA hybridization (DDH) values between strain JW14^T and closely related *Paenibacillus* type strains, *P. graminis* (CP009287), *P. jilunlii* (LIPY01000000) and *P. helianthi* (LVWI00000000), were calculated using the Orthologous Average Nucleotide Identity Tool software available in the EzBioCloud server (www.ezbiocloud.net/sw/oat) [26] and the Genome-to-Genome Distance Calculator version 2.1 (http://ggdc.dsmz.de/distcalc2.php) [27], respectively.

The *de novo* assembly of the genome sequencing data of strain JW14^T resulted in 221 contigs with an N50 value of 177.2 kb and an average genome coverage of 248.6×. The draft genome of strain JW14^T was 6375712 bp in size and had 5899 total genes, 5614 protein-coding genes and 89 tRNA genes. Seven complete rRNA gene operons were predicted from the genome and the G+C content of strain JW14^T was 48.1 mol%. The 16S rRNA gene sequence of strain JW14^T obtained by the above PCR approach was identical to that from its genome. The draft genome sequence of strain JW14^T was deposited to GenBank with the accession number JABWCS000000000. ANI and digital DDH values between strain JW14^T and the type strains of *P. graminis*, *P. jilunlii* and *P. helianthi* were 75.2, 75.6 and 85.0% and 21.1, 21.2 and 20.7%, respectively, which are clearly lower than the criteria for the prokaryotic species delineation thresholds (ANI, ~95 %; digital DDH, 70%) [28, 29], which suggests that strain JW14^T represents a new species of the genus *Paenibacillus*.

PHYSIOLOGY AND CHEMOTAXONOMY

Growth of strain JW14^T was examined for 2 days at 30 °C on R2A agar, tryptic soy agar (TSA; BD), nutrient agar (NA; BD), marine agar (MA; BD) and laboratory-prepared Luria–Bertani (LB) agar. Growth of strain JW14^T at different temperatures (4, 10, 15, 20, 25, 30, 37, 40 and 45 °C) and pH values (3.0–11.0 at 1.0 pH unit intervals) was evaluated on R2A agar and in R2A broth, respectively, for 2 days. R2A broths at pH 3.0–5.0, 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 sterilization using autoclave (for 15 min at 121 °C), the pH values of the R2A broths were adjusted again if necessary. Growth of strain JW14^T at different NaCl concentrations (0–5% at 1.0% intervals, w/v) was tested in R2A broth prepared in the laboratory. The following biochemical tests and physiological analyses of strain JW14^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), according to the manufacturer's instructions. Cell morphology of strain JW14^T was investigated using phase-contrast microscopy (Carl Zeiss) and transmission electron microscopy (JEM-1010, JEOL). The endospore-forming ability of strain JW14^T was checked by culturing the strain in R2A broth or R2A broth supplemented with $MnCl_2$ (5 mg l^{-1}) for a week and

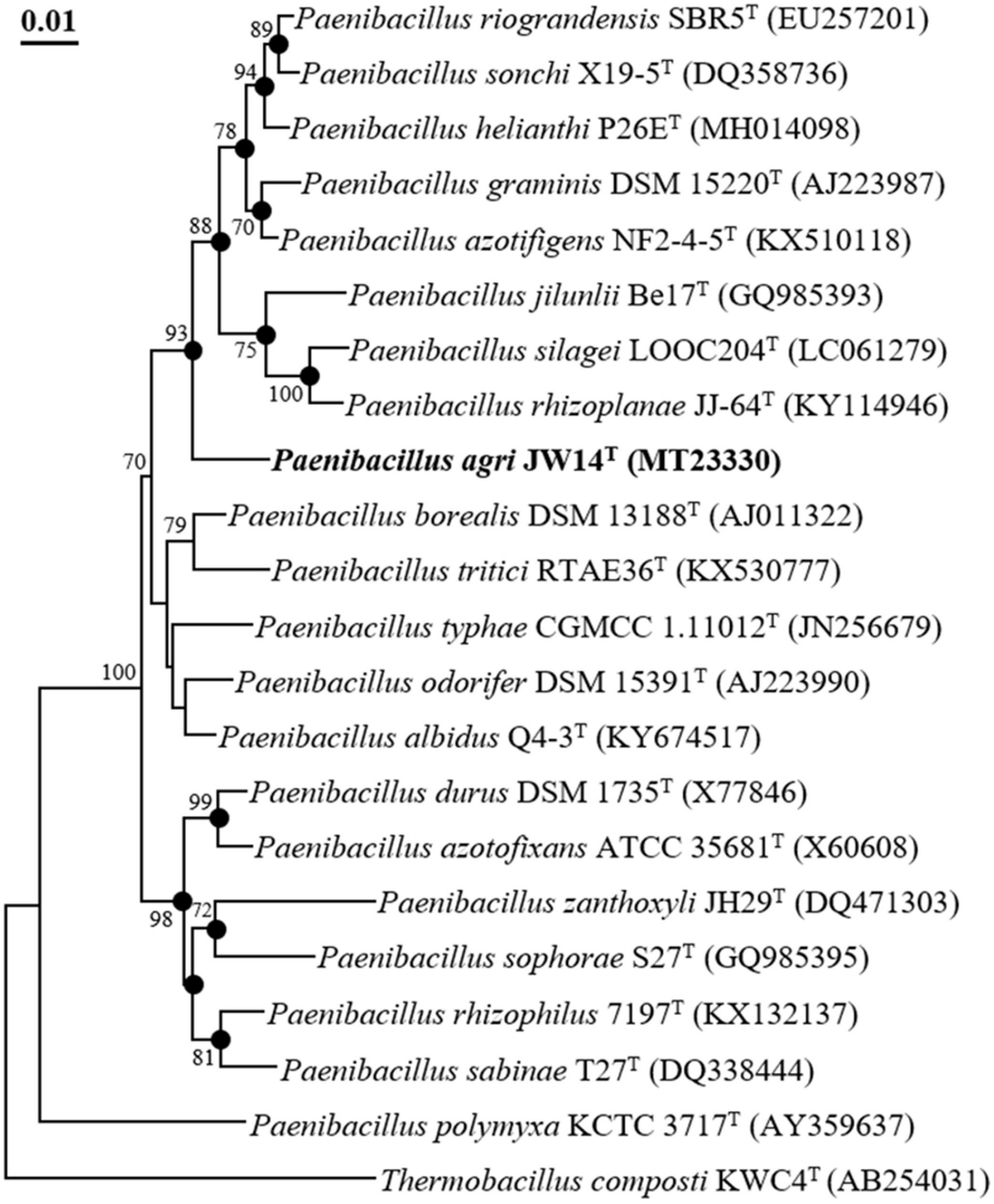

Fig. 1. A neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain JW14^T and their closely related taxa. Bootstrap values above 70% are shown on nodes in percentages of 1000 replicates. Filled circles (●) indicate the corresponding nodes that were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Thermobacillus composti* KWC4^T (AB254031) was used as an outgroup. Scale bar, 0.01 changes per nucleotide position.

observing endospores using a microscopy (Carl Zeiss) after malachite green spore-staining, as described previously [31, 32]. The endospore-forming ability of strain JW14^T was confirmed by spreading on R2A agar and colony-counting after heat treatment (80 °C, 30 min) of $MnCl_2$-treated cells (7 days cultured) and cells exponentially grown in R2A broth. *P. graminis* KACC 11529^T was used as a positive control of the endospore formation [4]. Oxidase activity was evaluated by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was tested by production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution (Junsei) [31]. Anaerobic growth was assessed on

R2A agar under anaerobic conditions using the GasPak Plus system (BBL) at 30 °C for 21 days. The following properties of strain JW14^T and reference strains were investigated in parallel under the same conditions. Hydrolysis of tyrosine, casein, aesculin, starch, Tween 20 and Tween 80 was tested on R2A agar following the methods described by Lányí [33] and Smibert and Krieg [31]. Additional biochemical features and enzymatic activities were tested using the API 20NE, API 50 CH and API ZYM systems (bioMérieux), respectively, according to the manufacturer' instructions.

Strain JW14^T grew well on R2A agar, TSA and NA, but did not grow on MA and LB agar. Growth occurred at 20–37 °C (optimum, 30 °C), pH 6.0–10.0 (optimum, 7.0) and in the presence of 0–2.0% NaCl (optimum, 0%). Cells of strain JW14^T were Gram-stain-positive, motile rods with peritrichous flagella, approximately 0.3–1.0 µm wide and 1.2–3.0 µm long (Fig. S2). Endospore-formation of strain JW14^T was not observed by malachite green spore-staining and the heat treatment test, meaning that strain JW14^T did not form an endospore inside cells under the test conditions, suggesting that strain JW14^T may not form an endospore and this non-endospore-forming property of strain JW14^T was different from the endospore-forming property of closely related *Paenibacillus* strains [1, 4, 7]. Colonies were white, circular and convex with smooth surface. Anaerobic growth was not observed after 21 days of incubation on R2A agar at 30 °C. Nitrate reduction to nitrite, hydrolysis of Tween 80, activity of esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *α*-glucosidase, *α*-fucosidase and *α*-galactosidase, assimilation of 4-nitrophenyl-*β*-D-galactopyranoside and acid production from glycerol, D-xylose, methyl *β*-D-xylopyranoside, galactose, glucose, fructose, mannose, mannitol, sorbitol, methyl *α*-D-mannopyranoside, methyl *α*-D-glucoside, *N*-acetyl glucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, turanose, erythritol, D-arabinose, ribose, L-xylose, sorbose, gentiobiose, L-fucose, 5-keto-gluconate, adonitol, dulcitol, inositol, D-tagatose, D-lyxose and xylitol were in common with other reference strains, while some other properties such as ananerobic growth clearly differentiated strain JW14^T from the most closely related type strains in the genus *Paenibacillus* species (Tables 1 and S1).

Isoprenoid quinone was extracted according to the method of Minnikin *et al.* [34] and analysed using a model LC-20A HPLC system (Shimadzu) equipped with a diode array detector (SPD-M20A, Shimadzu) and a reversed-phase column (250×4.6 mm; Kromasil, Akzo Nobel). For cellular fatty acid analysis, strain JW14^T and the reference strains were cultivated in R2A broth at their optimal temperatures and microbial cells were harvested at the same growth stage (exponential phase, OD_{600}=0.6–0.8). Cellular fatty acids of microbial cells were saponified, methylated and extracted using the standard MIDI protocol. Fatty acid methyl esters were analysed by gas chromatography (Hewlett Packard 6890) and identified by using the RTSBA6 database of the Microbial Identification System (Sherlock version 6.0B) [35]. The cell-wall peptidoglycan of strain JW14^T was analysed according to the procedure described by Schleifer and Kandler [5, 36]. *P. jilunlii* KACC 16679^T [7] was used as a positive control and the presence of *meso*-diaminopimelic acid was confirmed using *meso*-diaminopimelic acid purchased from Sigma-Aldrich. Polar lipids of strain JW14^T were extracted from cells harvested during the exponential growth phase and analysed by two-dimensional thin-layer chromatography, according to the procedure described by Minnikin *et al.* [37]. 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).

The only respiratory quinone identified in strain JW14^T was MK-7, which was in agreement with those of other species of the genus *Paenibacillus* [1–3]. Anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$ were identified as major cellular fatty acids (>10% of the total fatty acids). The overall fatty acid profile of strain JW14^T was similar to those of the closely related reference type strains, but there were some differences in some components (Table 2). The cell-wall analysis showed that the cell walls of strain JW14^T contained *meso*-diaminopimelic acid as a diagnostic diamino acid (Fig. S3), which was in agreement with other *Paenibacillus* species [7, 18]. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified aminolipids and one unidentified phospholipid were detected as major polar lipids (Fig. S4), which was in common with other closely related members of the genus *Paenibacillus* [4, 5, 7]. In conclusion, phylogenetic analysis and physiological and chemotaxonomic features support that strain JW14^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus agri* sp. nov. is proposed.

DESCRIPTION OF *PAENIBACILLUS AGRI* SP. NOV.

Paenibacillus agri (a′gri. L. gen. n. *agri* of a field).

Cells are Gram-stain-positive, strictly aerobic, non-endospore-forming and motile rods (width, 0.3–1.0 µm; length, 1.2–3.0 µm) by means of peritrichous flagella. Catalase-positive and oxidase-negative. Colonies on R2A agar are white, circular and convex with smooth surfaces. Growth occurs at 20–37 °C (optimum, 30 °C), pH 6.0–10.0 (optimum, 7.0) and in the presence of 0–2.0% NaCl (optimum, 0%). Nitrate is reduced to nitrite. Hydrolysis of Tween 80 is positive, but starch, tyrosin, casein, aesculin, Tween 20 and gelatin are negative. Negative for indole production and glucose fermentation. Positive for esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *α*-glucosidase, *α*-fucosidase and *α*-galactosidase, but negative for oxidase, arginine dihydrolase, urease, alkaline phosphatase, valine arylamidase, crystine arylamidase, trypsin, *α*-mannosidase, *N*-acetyl-*β*-glucosaminidase, *β*-glucuronidase, lipase (C14), leucine arylamidase, *α*-chymotrypsin and *β*-glucosidase.

Table 1. Comparison of phenotype characteristics of strain JW14^T and closely related taxa of the genus *Paenibacillus*

Strain: 1, JW14^T (this study); 2, *P. graminis* KACC 11529^T [4]; 3. *P. jilunlii* KACC 16679^T [5, 7] 4, *P. polymyxa* KACC 10485^T [1, 9]. All strains are positive for the following characteristics: nitrate reduction to nitrite*, Tween 80 hydrolysis*, activity* of esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-galactosidase and assimilation of 4-nitrophenyl-*β*-D-galactopyranoside. All strains are negative for the following characteristics: indole production*, hydrolysis* of gelatin, starch and tyrosine, activity* of arginine dihydrolase, urease, oxidase, alkaline phosphatase, valine arylamidase, crystine arylamidase, trypsin, α-mannosidase, *N*-acetyl-*β*-glucosaminidase, *β*-glucuronidase and lipase (C14), glucose fermentation* and assimilation* of citrate, phenylacetate, caprate and adipate. Symbols: +, positive; –, negative; v, variable.

Characteristic	1	2	3	4
Isolation source	Soil	Soil and plant roots	Rhizosphere of *Begonia semperflorens*	Decomposing plants and soil
Gram stain	+	+	+	v
Endospore formation	–	+	+	+
Anaerobic growth	–	+	+	+
Range for growth:				
Temperature (°C)	20–37	5–40	15–50	10–40
pH	6–10	4–9	4–9	6.0–8.0
NaCl (%, w/v)	0–2	0–3	0–3	0–3
Catalase activity*	+	+	–	+
Hydrolysis of:*				
Casein, aesculin, Tween 20	–	–	–	+
Enzyme activity (API ZYM) of:*				
Leucine arylamidase	–	+	–	–
α-Chymotrypsin	–	–	–	+
β-Galactosidase	+	+	–	+
α-Glucosidase	+	+	+	–
β-Glucosidase	–	+	+	+
α-Fucosidase	+	+	–	–
Assimilation (API 20NE) of:*				
D-Glucose, maltose	+	+	–	+
Mannitol, malate	–	–	+	+
Gluconate, arabinose	–	–	–	+
N-Acetyl glucosamine	+	–	–	–
Mannose	–	+	+	+
DNA G+C content (mol%)†	48.1	50.6	50.9	44.9

*These analyses were conducted under the same conditions in this study.
†The DNA G+C contents of *P. graminis* (CP009287), *P. jilunlii* (LIPY01000000) and *P. polymyxa* (CP049783-4) were calculated based on their genome sequences in this study.

Assimilation of D-glucose, maltose, *N*-acetyl glucosamine and 4-nitrophenyl-*β*-D-galactopyranoside is positive, but assimilation of citrate, phenylacetate, caprate, adipate, mannitol, malate, gluconate, arabinose and mannose is negative. Acid production from glycerol, D-xylose, methyl *β*-D-xylopyranoside, galactose, glucose, fructose, mannose, mannitol, sorbitol, methyl *α*-D-mannopyranoside, methyl *α*-D-glucoside, *N*-acetyl glucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, turanose, erythritol, D-arabinose, ribose, L-xylose, sorbose, gentiobiose, L-fucose, 5-keto-gluconate, adonitol, dulcitol, inositol, D-tagatose, D-lyxose and xylitol is positive, but acid production from gluconate, 2-keto-gluconate, L-arabinose, rhamnose, D-arabitol, L-arabitol and D-fucose is negative. Only MK-7 is detected as the isoprenoid quinone. Major cellular fatty acids are anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$ (>10 %). The cell-wall peptidoglycan contains

Table 2. Comparative view of cellular fatty acid compositions (%) between strain JW14^T and closely related type strains of the genus *Paenibacillus*

Strain: 1, JW14^T; 2, *P. graminis* KACC 11529^T; 3. *P. jilunlii* KACC 16679^T; 4. *P. polymyxa* KACC 10485^T. All data are from this study. Data are expressed as percentages of the total fatty acids and fatty acids amounting to less than 1.0% in all strains are not shown. Major components (>10.0%) are highlighted in bold; TR, trace amount (<1.0 %); –, not detected.

Fatty acid	1	2	3	4
Saturated:				
$C_{14:0}$	1.8	2.2	4.4	1.3
$C_{16:0}$	**20.0**	**14.2**	**17.8**	6.8
Unsaturated:				
$C_{15:1}$ $\omega5c$	–	TR	TR	1.1
$C_{16:1}$ $\omega11c$	TR	1.9	–	1.2
$C_{18:3}$ $\omega6c$ (6, 9, 12)	2.2	2.0	2.0	4.8
$C_{16:1}$ $\omega7c$ alcohol	–	TR	–	1.0
Branched:				
iso-$C_{14:0}$	5.0	3.6	2.3	1.5
iso-$C_{15:0}$	2.1	4.3	4.9	2.0
iso-$C_{16:0}$	**15.8**	**8.2**	5.2	**18.4**
iso-$C_{17:0}$	1.4	1.3	3.3	3.5
iso-$C_{19:0}$	1.4	–	1.4	2.0
anteiso-$C_{15:0}$	**41.4**	**52.7**	**43.5**	**33.3**
anteiso-$C_{17:0}$	5.5	4.6	5.9	**16.8**
anteiso-$C_{17:1}$ $\omega9c$	–	–	2.0	–
Hydroxy:				
iso-$C_{17:0}$ 3-OH	TR	TR	–	1.3

meso-diaminopimelic acid. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified aminolipids and one unidentified phospholipid are detected as major polar lipids.

The type strain is JW14^T (=KACC 21840^T=JCM 34279^T), isolated from soil sampled in the Republic of Korea. The DNA G+C content of the type strain is 48.1 mol% (genome).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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