Paenibacillus wooponensis sp. nov., isolated from wetland freshwater

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A rod-shaped, endospore-forming, Gram-reaction-positive bacterium, designated strain WPCB018^T, was isolated from a fresh water sample collected from Woopo wetland, Korea. The isolate was identified as a member of the genus *Paenibacillus* on the basis of phenotypic characteristics and phylogenetic inference based on 16S rRNA gene sequence analysis. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and unknown aminophospholipids. The diamino acid found in the cell-wall peptidoglycan was meso-diaminopimelic acid. The predominant menaquinone was MK-7. The major cellular fatty acids were anteiso-C_{15:0} (32.2 %), C_{16:0} (20.1 %) and C_{18:0} (18.1 %). The DNA G + C content was 56.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain WPCB018^T belongs to a cluster comprising species of the genus *Paenibacillus*, its closest neighbours being *Paenibacillus humicus* PC-147^T (97.5 %) and *Paenibacillus pasadenensis* SAFN-007^T (96.2 %). Genomic DNA–DNA hybridizations performed with strain WPCB018^T and type strains of the species *P. humicus*, *P. pinihumi*, *P. phyllosphaerae*, *P. pasadenensis* and *P. tarimensis* showed relatedness values of only 10, 17, 18, 19 and 20 %, respectively. On the basis of phenotypic, molecular and genetic evidence, strain WPCB018^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus wooponensis* sp. nov. is proposed. The type strain of the novel species is WPCB018^T (=KCTC 13280^T =JCM 16350^T).

The genus *Paenibacillus* was proposed by Ash *et al.* (1993) to accommodate the members of ‘group 3’ within the genus *Bacillus*. At the time of writing, there were 112 species of the genus *Paenibacillus* with validly published names (<http://www.bacterio.cict.fr/p/paenibacillus.html>). Members of the genus *Paenibacillus* possess the general characteristics of the genus *Bacillus*, being aerobic or facultatively anaerobic, endospore-forming and rod-shaped. Members of the genus *Paenibacillus* have physiologically diverse characteristics and some species are known to secrete a variety of extracellular enzymes and produce antibiotic substances (Chung *et al.*, 2000; Seo *et al.*, 1999). Species of the genus *Paenibacillus* have been isolated from a wide range of sources including soil, water, plant rhizospheres, plant materials, food, fodder, faeces and diseased insect larvae (Alexander & Priest, 1989; Kanzawa *et al.*, 1995; Montefusco *et al.*, 1993; Nakamura, 1984; Seldin *et al.*, 1984; Shida *et al.*, 1997; Yoon *et al.*, 1998).

Recently, two species of the genus *Paenibacillus*, *P. humicus* and *P. pasadenensis*, were distinguished from other closely related species by having a high DNA G + C content (>58 mol%), together forming a distinct lineage within the genus *Paenibacillus* (Vaz-Moreira *et al.*, 2007).

During the course of a study to estimate the bacterial diversity of Woopo wetland (Baik *et al.*, 2008), one bacterial strain, designated WPCB018^T, was identified as a member of the genus *Paenibacillus*. The wetland is located at the eastern part of the Nakdong River and is represented by a large oxbow lake as well as small ponds and marshes. Surface water is persistent, even in the dry season, and extensive flooding occurs in surrounding areas during the rainy season (<http://ramsar.wetlands.org>). Strain WPCB018^T was isolated in a previous study (Baik *et al.*, 2008) from a surface water sample taken from the Woopo freshwater wetland in July 2000, where the water temperature was 27 °C and pH was 7.4. Isolation was achieved by using PYGV (Staley, 1968) and R2A agar (Becton Dickinson), incubated at 25 °C for 7 days. The isolate was routinely cultured on R2A agar and preserved at –80 °C as a suspension in distilled water containing 20 % (w/v) glycerol.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpmB* gene sequences of strain WPCB018^T are EU939687 and HQ596203, respectively.

Three supplementary figures and one supplementary table are available with the online version of this paper.

DNA preparation, PCR amplification and sequencing of the 16S rRNA gene and partial RNA polymerase β -subunit (*rpoB*) gene were carried out as described previously (Chun & Goodfellow, 1995; Dahllöf *et al.*, 2000). Sequence similarities were determined from pairwise 16S rRNA gene sequence comparisons using the EzTaxon server (www.eztaxon.org/; Chun *et al.*, 2007). Sequences were aligned by using CLUSTAL_X (Thompson *et al.*, 1997), and the alignment was refined using PHYDIT (Chun, 1995). A total of 1460 nt in unambiguously aligned positions were used for tree reconstruction. Phylogenetic analyses were performed by using the computer packages PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred using neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) algorithms. Distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The robustness of the topology in the neighbour-joining phylogenetic tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Multiple alignments of partial *rpoB* gene sequences (374 nt) were determined using CLUSTAL_X (Thompson *et al.*, 1997) and sequence similarities were calculated using PHYDIT (Chun, 1995). The phylogenetic analysis based on *rpoB* gene sequences was performed using the methods described above.

Strain WPCB018^T showed highest 16S rRNA gene sequence similarities to *Paenibacillus humicus* PC-147^T (97.5%) and *P. pasadenensis* SAFN-007^T (96.2%) followed by *P. pinihumi* S23^T (95.9%), and displayed <95.9% to other members of the genus *Paenibacillus*. The neighbour-joining tree (Fig. 1a) shows that strain WPCB018^T formed a distinct branch within the clade comprising *P. humicus* PC-147^T, *P. pasadenensis* SAFN-007^T, *P. pinihumi* S23^T and *P. tarimensis* DSM 10249^T, as was seen in other trees (Supplementary Fig. S1, available in IJSEM Online). Partial *rpoB* gene sequence similarity values of strain WPCB018^T with *P. pinihumi* S23^T, *P. pasadenensis* SAFN-007^T and *P. humicus* PC-147^T were 86.4, 85.3 and 85.0%, respectively. Although the phylogenetic position of strain WPCB018^T based on 16S rRNA and *rpoB* gene sequences (Fig. 1b) was slightly different, it was clear that strain WPCB018^T could be differentiated from its nearest neighbours.

DNA–DNA hybridizations were performed by using the membrane filter technique with the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals) following the method of Lee *et al.* (2003), except that the hybridization temperature used was 55 °C. DNA–DNA relatedness tests were performed between strain WPCB018^T and the type strains of closely related species of the genus *Paenibacillus*.

Levels of DNA–DNA relatedness between strain WPCB018^T and strains *P. humicus* PC-147^T, *P. pinihumi* S23^T, *P. phyllosphaerae* PALXIL04^T, *P. pasadenensis* SAFN-007^T and *P. tarimensis* SA-7-6^T were only 10, 17, 18, 19 and 20%,

respectively (Supplementary Table S1). Thus, levels of genetic relatedness according to DNA–DNA hybridization experiments were >70%, the recommended threshold value for the delineation of bacterial species (Wayne *et al.*, 1987), suggesting that strain WPCB018^T represents a novel species, distinct from its closest relatives.

Growth on various standard bacteriological media was tested by using nutrient agar (NA; Becton Dickinson), tryptic soy agar (TSA; Becton Dickinson) and R2A agar according to the manufacturer's instructions. Cells of strain WPCB018^T were grown on TSA at 30 °C for 2 days and used for physiological and biochemical tests. Cell morphology was observed by using light microscopy (BX50; Olympus) and scanning electron microscopy (S-4800; Hitachi). Endospore formation was observed by using phase-contrast microscopy (TMS-F; Nikon). Flagellation was determined by using transmission electron microscopy (CM-20; Philips) with cells cultured for 24h in tryptic soy broth (TSB; Becton Dickinson). For this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram-reaction test was performed on cells grown on TSA at 25 °C for 1–16 days by using the bioMérieux Gram-stain kit according to the manufacturer's instructions and the Ryu non-staining KOH method (Powers, 1995). Growth at 0–10% (w/v) NaCl (increments of 1%) was investigated in TSB prepared according to the formula of the Becton Dickinson medium except that no NaCl was used initially. Growth at pH 4–10 was determined using TSB medium containing either 100 mM acetate buffer (for pH 4–5), 100 mM phosphate buffer (pH 6–8) or 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10) (Breznak & Costilow, 2007; Yumoto *et al.*, 2004). The optimal temperature and temperature range for growth was tested on TSA at 4–50 °C. Anaerobic growth was tested on TSA for up to 10 days in a jar containing an AnaeroPack-Anaero (Mitsubishi Gas Chemical), which works as oxygen absorber and CO₂ generator. Catalase activity was determined from bubble production in a 3% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by using a bioMérieux oxidase reagent. Acid production from sugars was tested as described by Yamaguchi & Yokoe (2000). Nitrate reduction was tested on TSB containing 0.2% (w/v) KNO₃ (Skerman, 1967). Hydrogen sulfide production was determined on Kligler iron agar (Becton Dickinson). Hydrolysis of CM-cellulose (1%, w/v), casein (2% skim milk, w/v), egg yolk (10%, w/v), starch (0.2%, w/v), Tween 20 (1%, w/v), Tween 80 (1%, w/v), L-tyrosine (0.5%, w/v) and xylan (1%, w/v), β -galactosidase activity, indole production and the Voges–Proskauer reaction were tested as described by Smibert & Krieg (1994). DNase activity was determined with DNase test agar (Becton Dickinson). Other biochemical and enzymic activity tests were performed using API 20 NE, API 50 CHB and API ZYM kits (bioMérieux) and the GP2 MicroPlate system (Biolog) prepared according to the manufacturer's instructions. Antibiotic resistance was determined using the disc diffusion method with commercial

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA (a) and partial *rpoB* (b) gene sequences showing relationships between strain WPCB018^T and related members of the genus *Paenibacillus*. Percentage bootstrap values >50% (based on 1000 resamplings) are given at branch points. Asterisks indicate that the corresponding nodes (groupings) were also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bars, 0.01 (a) and 0.05 (b) substitutions per nucleotide position.

antibiotic-impregnated discs (BBL Becton Dickinson). After 5 days of incubation at 30 °C on TSA, the results were interpreted according to the guidelines of the CLSI (2009).

Cells of strain WPCB018^T were Gram-reaction-positive, strictly aerobic, motile and catalase- and oxidase-positive. Vegetative cells were rod-shaped, 0.7–1.0 × 2.0–5.0 µm in size and formed peritrichous flagella on TSB medium (Supplementary Fig. S2). Ellipsoidal spores were formed in swollen sporangia and were in a subterminal position in cells. Colonies of WPCB018^T were circular, smooth, opaque, convex, entire and cream-coloured when grown on TSA plates. The diameter of the colonies was 2 mm after 2 days of growth at 30 °C on TSA. Strain WPCB018^T grew at 10–37 °C (optimum 30–35 °C) on TSA but not at 40 °C. Strain WPCB018^T grew in TSB media containing

0–2% (w/v) NaCl but not at 3% (w/v) NaCl or above. The pH range for growth was pH 5–9 (optimum pH 6–8). Detailed results of physiological and biochemical analyses are given in Table 1 and in the species description. There were several phenotypic characteristics that readily separated strain WPCB018^T from phylogenetically related species; for example, strain WPCB018^T was exclusively positive for DNA hydrolysis and acid phosphatase activity, but negative for starch hydrolysis.

Cellular fatty acids of strain WPCB018^T grown on TSA for 2 days at 30 °C were prepared (in duplicate) and analysed as methyl esters by GLC according to the instructions of the Microbial Identification System (MIDI). Polar lipids were analysed by using standard procedures (Minnikin *et al.*, 1984). Extracted lipids were separated by two-

Table 1. Differential characteristics of strain WPCB018^T and the type strains of related species of the genus *Paenibacillus*

Strains: 1, WPCB018^T; 2, *P. humicus* PC-147^T; 3, *P. pasadenensis* SAFN-007^T; 4, *P. pinihumi* S23^T; 5, *P. tarimensis* SA-7-6^T. All data are from this study unless otherwise indicated. Data in the parentheses were different from published data. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5
Catalase	+	+	+	–	+
Oxidase	+	+	+	+	–
Hydrolysis of:					
Casein	+	–	+	(+)	w
DNA	+	–	w	–	–
Starch	–	+	+	+	(+)
Tween 20	+	+	+	–	+
Tween 80	–	+	–	–	–
Acid production from:					
D-Fructose	+	–	+	–	–
D-Galactose	+	–	+	–	+
D-Glucose	+	–	(+)	–	+
D-Mannitol	–	(+)	(+)	–	+
D-Mannose	+	–	+	–	+
Melezitose	+	–	+	–	w
Raffinose	+	–	+	(+)	+
Salicin	–	–	+	–	+
D-Xylose	+	–	+	(+)	+
Enzyme activities (API ZYM):					
Acid phosphatase	+	–	–	–	–
Esterase (C4)	+	+	+	–	+
β-Galactosidase	+	+	+	+	–
α-Glucosidase	–	+	+	–	–
β-Glucosidase	+	+	–	–	+
DNA G+C content (mol%)*	56.0	58.0 ^a	63.0	49.5 ^b	53.7 ^c

*Data from: *a*, Vaz-Moreira *et al.* (2007); *b*, Kim *et al.* (2009); *c*, Wang *et al.* (2008).

dimensional TLC and identified by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The isomer type of the diamino acid of the cell wall was analysed according to the method of Komagata & Suzuki (1987). The respiratory quinones were extracted from 300 mg freeze-dried cells, purified according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described previously (Kroppenstedt, 1985). The DNA G+C content was calculated using the formula described by Mandel *et al.* (1970). The DNA sample was prepared in duplicate and determined by the thermal denaturation method of Marmur & Doty (1962).

The major fatty acids of strain WPCB018^T comprised branched saturated fatty acid anteiso-C_{15:0} (32.2%) and straight-chain saturated fatty acids C_{16:0} (20.1%) and C_{18:0} (18.1%). The fatty acid profile of strain WPCB018^T was similar to those of related species of the genus *Paenibacillus*, but the respective proportions of predominant fatty acids

differed significantly from other species (Table 2); strain WPCB018^T contained larger amounts of C_{16:0} and C_{18:0}, but smaller amounts of anteiso-C_{15:0} in strain WPCB018^T.

The polar lipid pattern of strain WPCB018^T was characterized by the presence of diphosphatidylglycerol, phosphatidylethanolamine, unknown aminolipids and unknown aminophospholipids, similar to the profiles of related species of the genus *Paenibacillus* (Supplementary Fig. S3). The diamino acid in the cell-wall peptidoglycan of strain WPCB018^T was *meso*-diaminopimelic acid. The predominant isoprenoid quinone was menaquinone with seven isoprene units (MK-7). The DNA G+C content of strain WPCB018^T was 56.0 mol%. This high DNA G+C molar ratio supported its position in a distinct phylogenetic lineage with *P. humicus* and *P. pasadenensis* as shown in Fig. 1(a) (Vaz-Moreira *et al.*, 2007).

On the basis of the phylogenetic, genomic and phenotypic data, strain WPCB018^T represents a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus wooponensis* sp. nov. is proposed.

Table 2. Cellular fatty acid profiles (%) of strain WPCB018^T and the type strains of related species of the genus *Paenibacillus*

Strains: 1, WPCB018^T; 2, *P. humicus* PC-147^T; 3, *P. pasadenensis* SAFN-007^T; 4, *P. pinihumi* S23^T; 5, *P. tarimensis* SA-7-6^T. Cells of all strains were harvested after cultivation at 30 °C on TSA medium for 2 days. –, Not detected. All data were obtained in this study.

Fatty acid	1	2	3	4	5
Straight-chain saturated					
C _{12:0}	0.6	–	–	0.9	–
C _{14:0}	2.6	2.0	1.5	2.1	1.8
C _{15:0}	4.6	1.2	2.0	2.1	4.0
C _{16:0}	20.9	12.7	12.5	15.7	10.5
C _{17:0}	1.4	0.5	0.8	0.6	0.7
C _{18:0}	18.1	7.9	9.7	7.3	6.0
Branched saturated					
iso-C _{14:0}	0.8	2.3	1.4	2.1	0.8
iso-C _{15:0}	4.6	5.3	3.3	7.9	4.8
iso-C _{16:0}	5.2	11.3	12.9	10.5	3.8
iso-C _{17:0}	0.8	1.4	1.4	2.1	1.4
anteiso-C _{15:0}	32.2	46.8	43.0	42.5	50.7
anteiso-C _{17:0}	3.0	6.6	9.0	3.2	13.0
Monounsaturated					
C _{16:1ω7c} alcohol	–	–	0.9	–	–
C _{16:1ω11c}	–	–	0.4	–	1.0
C _{18:1ω9c}	2.8	0.9	1.2	1.4	0.7
C _{18:1ω7c}	3.1	1.1	–	1.2	–
Summed feature 4*	–	–	–	–	1.0

*Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained iso-C_{17:1} I and/or iso-C_{17:1} B.

Description of *Paenibacillus wooponensis* sp. nov.

Paenibacillus wooponensis (woo.po.nen'sis. N.L. masc. adj. *wooponensis* of or belonging to Woopo wetland, Republic of Korea, the geographical origin of the type strain of the species).

Cells are Gram-reaction-positive, strictly aerobic, motile rods ($0.7\text{--}1.0 \times 2.0\text{--}5.0\ \mu\text{m}$) with peritrichous flagella. Catalase- and oxidase-positive. Produces ellipsoidal endospores that lie in a subterminal position in cells and usually cause the sporangia to swell. Colonies on TSA are circular, opaque, cream-coloured and convex with entire margins and are usually 2 mm in diameter within 48 h of growth at 30 °C. Growth occurs in the presence of 0–2 % NaCl, at pH 5–9 (optimum pH 6–8) and at 10–37 °C (optimum 30–35 °C). Hydrogen sulfide is not produced. DNA, casein and Tween 20 are hydrolysed but CM-cellulose, egg yolk, gelatin, Tween 80, L-tyrosine and xylan are not. In the API 20 NE system, positive for aesculin hydrolysis (β -glucosidase), β -galactosidase and assimilation of D-glucose and maltose but negative for nitrate reduction, fermentation of D-glucose, indole production, production of arginine dihydrolase and urease and assimilation of N-acetylglucosamine, adipic acid, L-arabinose, capric acid, D-mannitol, D-mannose, malate, phenylacetic acid and trisodium citrate. In the API 50 CHB system, acids are produced from amygdalin, cellobiose, dulcitol (weakly), aesculin, D-fructose, D-galactose, gentiobiose, D-glucose, lactose, maltose, D-mannose, melezitose, melibiose, methyl- α -D-glucopyranoside (weakly), methyl- α -D-mannopyranoside, raffinose, L-rhamnose, sucrose, trehalose, turanose and D-xylose; acids are not produced from other substrates. In the API ZYM gallery, acid phosphatase, leucine arylamidase (weakly), esterase (C4), esterase lipase (C8), α - and β -galactosidase and β -glucosidase activities are present; but alkaline phosphatase, N-acetyl- β -glucosaminidase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α -glucosidase, β -glucuronidase, lipase (C14), α -mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are absent. In the Biolog GP2 Microplate system the following substrates are assimilated: amygdalin, cellobiose, dextrin, D-galactose, gentiobiose, D-gluconic acid, α -D-glucose, maltose, maltotriose, melibiose, melezitose, methyl- β -D-galactoside, raffinose, palatinose, trehalose, stachyose, sucrose and turanose; other substrates are not assimilated. Cells are sensitive to (μg per disc, unless otherwise indicated): amikacin (30), ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin (10 IU), polymyxin B (300 IU), streptomycin (10) tetracycline (30) and vancomycin (30). The major fatty acids are anteiso- $\text{C}_{15:0}$ (32.2 %), $\text{C}_{16:0}$ (20.1 %) and $\text{C}_{18:0}$ (18.1 %). The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and unknown aminophospholipids. The diamino acid in the cell-wall peptidoglycan is meso-diaminopimelic acid. The predominant isoprenoid quinone is MK-7.

The type strain, WPCB018^T (=KCTC 13280^T =JCM 16350^T), was isolated from freshwater collected from Woopo wetland. The DNA G + C content of the type strain is 56.0 mol%.

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