Paenibacillus rigui sp. nov., isolated from a freshwater wetland

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A rod-shaped, endospore-forming, Gram-reaction-variable bacterial strain, designated WPCB173^T, was isolated from freshwater collected from the Woopo wetland, Republic of Korea. Based on its phenotypic characteristics and phylogenetic position inferred from 16S rRNA gene sequence analysis, the isolate was identified as being a member of the genus *Paenibacillus*. Major polar lipids present in strain WPCB173^T included phosphatidylethanolamine and several unidentified phospholipids. The diamino acid found in the cell-wall peptidoglycan was meso-diaminopimelic acid. The predominant menaquinone was MK-7. The major cellular fatty acid was anteiso-C_{15:0} (65.2%). The DNA G + C content was 48.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain WPCB173^T clustered with species of the genus *Paenibacillus*, its closest neighbours being *Paenibacillus chinjuensis* WN9^T (96.7%) and *Paenibacillus soli* DCY03^T (96.4%). DNA–DNA hybridization of strain WPCB173^T with *P. soli* DCY03^T and *P. chinjuensis* WN9^T showed relatedness values of only 10 and 19%, respectively. On the basis of the phenotypic and phylogenetic evidence, strain WPCB173^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus rigui* sp. nov. is proposed. The type strain of the novel species is WPCB173^T (=KCTC 13282^T =JCM 16352^T).

The genus *Paenibacillus* was proposed by Ash *et al.* (1993) to accommodate the members of 'group 3' within the genus *Bacillus*. Since then, the transfer of species of the genus *Bacillus* to this genus and descriptions of novel species have, to date, resulted in 120 species with validly published names being assigned the genus *Paenibacillus* (<http://www.bacterio.cict.fr/p/paenibacillus.html>). Members of the genus *Paenibacillus* share the general characteristics of the genus *Bacillus* of being aerobic or facultatively anaerobic, endospore-forming and rod-shaped. Members of both genera have physiologically diverse characteristics and some species are known to secrete a variety of extracellular enzymes, such as polysaccharide-hydrolysing enzymes, or to produce polysaccharides (Claus & Berkeley, 1986; Priest, 1977; 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, 1987; Seldin *et al.*, 1984; Shida *et al.*, 1997; Yoon *et al.*, 1998).

The Woopo wetland (35° 33' N, 128° 25' E), located to the east of the Nakdong river, Republic of Korea, was created about 140 million years ago, is surrounded by low-level mountains and has a levee that was built to protect agricultural areas. The wetland types are classified as 'O', permanent freshwater lakes over 8 ha, including large oxbow lakes; and 'Tp', permanent freshwater marshes, pools or ponds below 8 ha, marshes and swamps on inorganic soils with emergent vegetation and that are waterlogged for most of the growing season (<http://www.wetlands.org>). The bacterial diversity of the wetland was estimated using surface water samples (at 27 °C and pH 7.4) taken in July 2000. The bacterial community was composed of members of four phyla; *Firmicutes* (49.8%), *Actinobacteria* (32%), *Proteobacteria* (13.8%) and *Bacteroidetes* (4.4%) (Baik *et al.*, 2008). In the present study, we describe one bacterial strain that has been identified as a member of the genus *Paenibacillus*.

Strain WPCB173^T was isolated using PYGV (Staley, 1968) and R2A agar (Becton Dickinson) at 25 °C for 7 days. The isolate was routinely cultured on R2A agar and preserved at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WPCB173^T is EU939688.

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

–80 °C as a suspension in distilled water containing 20 % (w/v) glycerol.

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences of the novel isolate and related taxa were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and the alignment was refined using PHYDIT (Chun, 1995). A total of 1407 unambiguously aligned nucleotide positions were used for tree reconstruction. Phylogenetic analysis was performed using the computer packages PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms. Distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes and Cantor (1969). The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

Strain WPCB173^T showed the highest 16S rRNA gene sequence similarity to *Paenibacillus chinjuensis* WN9^T (96.7 %) followed by *Paenibacillus soli* DCY03^T (96.4 %) and showed <95.7 % similarity to other members of the genus *Paenibacillus*. The neighbour-joining tree (Fig. 1 and Supplementary Fig. S3, available in IJSEM Online) showed that strain WPCB173^T formed a distinct branch within the clade comprising *P. chinjuensis* WN9^T and *P. soli* DCY03^T, which was also shown in the Fitch–Margoliash tree (data not shown). On the basis of 16S rRNA gene sequence similarity data and the results of the phylogenetic analysis, it is clear that strain WPCB173^T represents a novel genomic species within the genus *Paenibacillus* (Wayne *et al.*, 1987) as 16S rRNA gene sequence similarities between strain WPCB173^T and species of the genus *Paenibacillus* were

below the level of similarity typically observed at the species level (97 %; Stackebrandt & Goebel, 1994).

Growth on various standard bacteriological media was tested by using nutrient agar (NA; Becton Dickinson), tryptic soy agar (TSA; Becton Dickinson) and R2A agar. Cells of strain WPCB173^T grown on TSA at 30 °C for 2 days were used for the physiological and biochemical tests. Morphology of the cells was observed using light microscopy (BX50; Olympus) and scanning electron microscopy (S-4800; Hitachi). Endospore formation was observed using phase-contrast microscopy (TMS-F; Nikon). Motility was examined by observing cells grown in wet mounts using a phase-contrast microscope. Flagellation was determined using a transmission electron microscope (CM-20; Philips) with cells cultured for 48 h 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 manufacturer's instructions except that different amounts of NaCl were used. Growth at pH 4–11 (increments of 1 pH unit) was determined in TSB that was adjusted by the addition of HCl and NaOH. The optimum and range of temperatures for growth were tested on TSA at 4–50 °C. Anaerobic growth was tested on TSA in a jar containing an AnaeroPack-Anaero (Mitsubishi Gas Chemical), which works as oxygen absorber and CO₂ generator for up to 10 days. Catalase activity was determined from bubble production in a 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by using bioMérieux oxidase reagent. 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 carboxymethyl 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

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain WPCB173^T and some related members of the genus *Paenibacillus*. Percentage bootstrap values >50 % (based on 1000 resamplings) are given at branch points. Solid circles indicate that the corresponding nodes (groupings) were also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bar, 0.02 substitutions per nucleotide position.

(1 %, w/v), L-tyrosine (0.5 %, w/v) and xylan (1 %, w/v) was 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 a GP2 MicroPlate (Biolog) prepared according to the manufacturer's instructions. Antibiotic resistance was determined after 5 days of incubation at 30 °C on TSA using the disc diffusion method with commercial antibiotic-impregnated discs (BBL, Becton Dickinson), the results of which were interpreted according to the guidelines set forth by the CLSI (2003).

Cells of strain WPCB173^T stained Gram-reaction-variable (Supplementary Fig. S1a). The KOH test was negative as was reported previously (Powers, 1995) for Gram-negative bacteria. Vegetative cells were rod-shaped and 0.4–0.6 × 2.0–5.0 µm. Young cells were peritrichously flagellated. Ellipsoidal spores were formed in swollen sporangia (Supplementary Fig. S1b). Strain WPCB173^T was positive for nitrate reduction, β-galactosidase activity and assimilation of gentiobiose, α-D-glucose, maltose, malic acid, D-mannitol, melibiose, potassium gluconate and pyruvic acid, whereas its closest relatives, *P. chinjuensis* WN9^T and *P. soli* DCY03^T, were negative for these characteristics. Strain WPCB173^T was negative for growth in 2 % NaCl (w/v) and at 42 °C, hydrolysis of Tweens 40 and 80 and naphthol-AS-B1-phosphohydrolase activity, while *P. chinjuensis* WN9^T and *P. soli* DCY03^T were positive for these characteristics. Detailed results of physiological and biochemical tests are given in Table 1 and in the species description.

Cellular fatty acids of cells of strain WPCB173^T grown on TSA for 2 days at 30 °C were prepared (in duplicate) and analysed by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). Polar lipids were analysed by using standard procedures (Minnikin *et al.*, 1984). Extracted lipids were separated by two-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). Respiratory quinones were extracted from 300 mg samples of 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 the G+C content was determined using the thermal denaturation method of Marmur & Doty (1962). Taxonomic relationships between strain WPCB173^T and *P. soli* DCY03^T and *P. chinjuensis* WN9^T were examined further using DNA–DNA hybridization, which was performed using the membrane filter technique with a DIG High Prime DNA Labelling and Detection Starter kit II (Roche) according to the method described in detail by Lee *et al.* (2003) with the modification that the hybridization temperature used was 55 °C.

Table 1. Differential characteristics of strain WPCB173^T and type strains of related species of the genus *Paenibacillus*

Taxa: 1, WPCB173^T; 2, *P. chinjuensis* WN9^T; 3, *P. soli* DCY03^T. +, Positive; –, negative; w, weakly positive.

Characteristic	1*	2	3
Anaerobic growth	–	+	–
Motility	+	+	–
Oxidase	+	+	–
Nitrate reduction	+	–	–
β-Galactosidase*	+	–	–
Growth at/in:			
2 % (w/v) NaCl	–	–	+
42 °C	–	+	+
pH 5–6	+	+	–
Hydrolysis of*:			
DNA	–	+	–
Tween 80	–	+	+
Assimilation of (API 20 NE)*:			
L-Arabinose	+	–	+
Malic acid	+	–	–
Maltose	+	+	+
D-Mannitol	+	–	–
D-Mannose	+	+	–
Postassium gluconate	+	–	–
Enzyme activities (API ZYM)*:			
Alkaline phosphatase	–	+	–
Esterase (C4)	w	–	–
α-Glucosidase	+	–	–
Leucine arylamidase	+	–	+
Naphthol-AS-B1-phosphohydrolase	–	+	+
Utilization of (Biolog GP2)*:			
L-Alanyl glycine	–	+	–
Gentiobiose	+	–	–
α-D-Glucose	+	–	–
Maltose	+	+	–
Melibiose	+	–	–
Monomethyl succinate	+	+	–
Palatinose	+	+	–
Pyruvic acid	+	–	–
Trehalose	+	+	–
Turanose	+	+	–
Tween 40	–	–	+
DNA G+C content (mol %)	48.3	53	56.6

*Data from this study. Other data from Yoon *et al.* (2002) and Park *et al.* (2007).

The major fatty acids of strain WPCB173^T included the branched saturated fatty acids anteiso-C_{15:0} (65.2 %), iso-C_{15:0} (9.3 %), iso-C_{16:0} (5.4 %) and anteiso-C_{17:0} (3.1 %) and the straight-chain saturated fatty acids C_{15:0} (5.7 %) and C_{16:0} (3.6 %). The fatty acid profile of strain WPCB173^T was similar to that of related species of the genus *Paenibacillus* (Table 2). The polar lipids patterns were characterized by the presence of phosphatidylethanolamine, phosphatidylglycerol and several unidentified phospholipids (Supplementary Fig. S2). The diamino acid

in the cell-wall peptidoglycan of strain WPCB173^T was *meso*-diaminopimelic acid. The predominant isoprenoid quinone was menaquinone with seven isoprene units (MK-7). The DNA G+C content of strain WPCB173^T was 48.3 mol%. Genomic DNA–DNA hybridization of strain WPCB173^T with *P. soli* DCY03^T and *P. chinjuensis* WN9^T showed relatedness values of only 10 and 19%, respectively. The levels of genetic relatedness according to DNA–DNA hybridization experiments were less than 70%, the recommended threshold for the delineation of bacterial species (Wayne *et al.*, 1987), which lead to the conclusion that strain WPCB173^T represents a novel species.

Based on the phylogenetic, genomic and phenotypic evidence, strain WPCB173^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus rigui* sp. nov. is proposed.

Description of *Paenibacillus rigui* sp. nov.

Paenibacillus rigui (ri'gu.i. L. gen. n. *rigui* of a well-watered place, referring to where the type strain was isolated).

Cells of strain WPCB173^T are Gram-reaction-variable, motile, rod-shaped, 0.4–0.6 × 2–5 µm and occur singularly or in chains. Produces ellipsoidal endospores that lie in the subterminal position and usually cause the sporangia to

swell. Colonies grown on TSA plates for 2 days at 30 °C are circular, ~1 mm in diameter, opaque, cream, convex and have entire margins. Does not grow under anaerobic conditions. Grows at 15–37 °C (optimum 30 °C), pH 5–8 (optimum pH 6–7) and in up to 1 % (w/v) NaCl. Positive for nitrate reduction. Hydrogen sulfide is not produced. Catalase- and oxidase-positive. Starch and Tween 20 are hydrolysed but carboxymethyl cellulose, casein, DNA, egg yolk, gelatin, Tween 80, L-tyrosine and xylan are not. In the API 20NE system, positive for nitrate reduction, fermentation of D-glucose, aesculin hydrolysis (β-glucosidase), β-galactosidase activity and assimilation of D-glucose, L-arabinose, maltose, D-mannitol, D-mannose and malic acid, but negative for indole production, arginine dihydrolase, gelatin hydrolysis and urease activities and assimilation of N-acetylglucosamine, capric acid, adipic acid, trisodium citrate and phenylacetic acid. In the API ZYM system, α-galactosidase, β-galactosidase, α-glucosidase and leucine arylamidase activities are present and acid phosphatase, esterase (C4) and esterase lipase (C8) are weakly active. In the Biolog GP2 Microplate (Biolog) system, the following substrates are assimilated: dextrin, gentiobiose, α-D-glucose, maltose, maltotriose, melibiose, palatinose, sucrose, trehalose, turanose, monomethyl succinate and pyruvic acid; the following substrates are weakly assimilated: glycogen, L-arabinose, D-galactose, D-mannitol, methyl-α-D-glucoside, raffinose, D-ribose, D-sorbitol, D-xylose, acetic acid and methyl pyruvate; 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). Other physiological and biochemical characteristics are given in Table 1. The major fatty acid is anteiso-C_{15:0}. The major polar lipids are phosphatidylethanolamine and unidentified phospholipids. The diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid. The predominant isoprenoid quinone is MK-7.

The type strain, WPCB173^T (=KCTC 13282^T =JCM 16352^T), was isolated from freshwater collected from the Woopo wetland, Republic of Korea. The DNA G+C content of the type strain is 48.3 mol%.

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Table 2. Cellular fatty acid profiles (%) of strain WPCB173^T and type strains of related species of the genus *Paenibacillus*

Taxa: 1, WPCB173^T; 2, *P. chinjuensis* WN9^T; 3, *P. soli* DCY03^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
Straight-chain saturated			
C _{13:0}	0.3	—	—
C _{14:0}	0.9	0.6	2.0
C _{15:0}	5.7	3.1	9.2
C _{16:0}	3.6	6.5	8.7
C _{17:0}	0.6	—	0.4
C _{18:0}	0.7	0.5	0.5
Hydroxy			
C _{13:0} 2-OH	—	0.6	—
Branched saturated			
iso-C _{13:0}	0.2	—	—
iso-C _{14:0}	2.9	1.6	2.8
iso-C _{15:0}	9.3	3.4	11.1
iso-C _{16:0}	5.4	8.2	6.5
iso-C _{17:0}	2.0	1.6	1.5
anteiso-C _{13:0}	0.2	—	—
anteiso-C _{15:0}	65.2	66.7	53.8
anteiso-C _{17:0}	3.1	7.1	2.4
Summed feature 3*	—	—	1.2

*Summed features represent groups of two fatty acids that could not be separated by GLC in the MIDI system. Summed feature 3 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

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