Bacillus rigui sp. nov., isolated from wetland fresh water

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Two Gram-stain-positive strains, WPCB074^T and WPCB165, were isolated from fresh water collected from the Woopo wetland (Republic of Korea). Both strains were strictly aerobic, motile, endospore-forming rods. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains WPCB074^T and WPCB165 belonged to the genus *Bacillus* and that strain WPCB074^T was most closely related to *Bacillus solisalsi* YC1^T (98.4 % sequence similarity), *B. barbaricus* V2-BIII-A2^T (97.7 %), *B. macauensis* ZFHKF-1^T (96.9 %), *B. arsenicus* Con a/3^T (96.4 %) and *B. gelatini* LMG 21880^T (95.1 %). The 16S rRNA gene sequences of strains WPCB074^T and WPCB165 differed at one position (99.9 % similarity), suggesting that these two strains constitute a single species. DNA–DNA relatedness between strain WPCB074^T and the type strains of *B. solisalsi*, *B. barbaricus*, *B. macauensis*, *B. arsenicus* and *B. gelatini* were 26, 17, 20, 14 and 7 %, respectively. Strain WPCB074^T was characterized by having cell-wall peptidoglycan based on meso-diaminopimelic acid, MK-7 as the predominant menaquinone and iso-C_{15:0} and anteiso-C_{15:0} as the major fatty acids. The DNA G+C content of strain WPCB074^T was 41.9 mol%. On the basis of phenotypic properties, phylogeny and genomic distinctiveness, strain WPCB074^T represents a novel species of the genus *Bacillus* for which the name *Bacillus rigui* sp. nov. is proposed. The type strain is WPCB074^T (=KCTC 13278^T =JCM 16348^T).

Members of the genus *Bacillus* are widely distributed in nature and have physiologically diverse characteristics (Claus & Berkeley, 1986). For a long time, most aerobic, endospore-forming rods were assigned to the genus *Bacillus* (Claus & Berkeley, 1986). The results of genomic analyses proved that the genus *Bacillus* comprised heterogeneous taxa (Priest *et al.*, 1981; Ash *et al.*, 1991; Slepecky & Hemphill, 1992; Stackebrandt & Liesack, 1993; Nakamura, 1996). In particular, 16S rRNA gene sequence analyses have revealed the presence of several phylogenetically distinct lineages within the genus *Bacillus*. Consequently, some phylogenetic groups have been established as the following new genera: *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Alkalibacillus* (Jeon

et al., 2005), *Aneurinibacillus* (Shida *et al.*, 1996), *Brevibacillus* (Shida *et al.*, 1996), *Marinibacillus* (Yoon *et al.*, 2001b), *Paenibacillus* (Ash *et al.*, 1993), *Pullulanibacillus* (Hatayama *et al.*, 2006), *Salibacillus* (Wainø *et al.*, 1999), *Sporolactobacillus* (Hatayama *et al.*, 2006), *Ureibacillus* (Fortina *et al.*, 2001), *Viridibacillus* (Albert *et al.*, 2007) and *Virgibacillus* (Heyndrickx *et al.*, 1998). Also, several species of the genus *Bacillus* have been reclassified or transferred to the following genera: *Geobacillus* (Nazina *et al.*, 2001), *Gracilibacillus* (Wainø *et al.*, 1999), *Lysinibacillus* (Ahmed *et al.*, 2007), *Rummeliibacillus* (Vaishampayan *et al.*, 2009), *Salimicrobium* (Yoon *et al.*, 2007) and *Sporosarcina* (Yoon *et al.*, 2001a). Therefore, classification and identification of Gram-positive, endospore-forming rods should be performed by using a polyphasic taxonomic approach that integrates phylogenetic analysis based on 16S rRNA gene sequences, genomic relatedness and an extensive range of phenotypic characteristics (Shida *et al.*, 1997a, b; Yoon *et al.*, 1998; Tcherpakov *et al.*, 1999). In the course of our study on wetland microbial diversity, two Gram-stain-positive,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WPCB074^T and WPCB165 are EU939689 and EU939690.

DNA–DNA hybridization data for WPCB064^T and WPCB165 and related strains are available as supplementary material with the online version of this paper.

endospore-forming bacterial strains, designated strains WPCB074^T and WPCB165, were isolated from a fresh-water sample and were the subject of a taxonomic investigation.

Strains WPCB074^T and WPCB165 were isolated from a fresh-water sample collected from the wetland of Woopo (35° 33' N 128° 25' E) located in the Republic of Korea using the standard dilution plating technique. Isolation was achieved using PYGV (Staley, 1968) and R2A agar (Oxoid) at 25 °C for 7 days. The isolates were routinely cultured on R2A agar and preserved at -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). 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). The novel sequences were aligned with related sequences by using CLUSTAL_X (Thompson *et al.*, 1997); the alignment was refined using PHYDIT (Chun, 1995). Phylogenetic analysis was performed by 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 & Cantor (1969). The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. The 16S rRNA gene sequences of strains WPCB074^T and WPCB165 were continuous stretches of 1453 and 1463 nt, respectively. 16S rRNA gene sequence similarity between the two strains was 99.9%; hence, the two strains should be assigned to a single taxon (Fig. 1). Based on 16S rRNA gene sequence similarity data, it was found that the closest relatives of strain WPCB074^T were *Bacillus solisalsi* YC1^T (98.4 %), *Bacillus barbaricus* V2-BIII-A2^T (97.8 %), *Bacillus macauensis* ZFHKF-1^T (96.9 %), *Bacillus arsenicus* Con a/3^T (96.4 %) and *Bacillus gelatini* LMG 21880^T (95.1 %).

DNA–DNA hybridization was performed by the membrane filter technique using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals) according to the method described in detail by Lee *et al.* (2003), with the modification that the hybridization temperature was 45 °C. DNA–DNA relatedness tests were performed between strains WPCB074^T and WPCB165 and the type strains of some phylogenetically related *Bacillus* species. Strains WPCB074^T and WPCB165 exhibited two independent levels of DNA–DNA relatedness of 96 and 94 %. Levels of DNA–DNA relatedness between strain WPCB074^T and the related type strains *B. solisalsi* KCTC 13181^T, *B. barbaricus* KACC 12101^T, *B. macauensis*

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strains WPCB074^T and WPCB165 and some related members of the genus *Bacillus*. Bootstrap values (>50%; 1000 resamplings) are given at branch points and solid circles indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bar, 0.01 nucleotide substitutions per position.

KACC 12177^T, *B. arsenicus* KACC 12116^T and *B. gelatini* KACC 12197^T were 26, 17, 20, 14 and 7 %, respectively (Supplementary Table S1; available in IJSEM Online). Thus, levels of genetic relatedness according to DNA–DNA hybridization experiments were less than 70 %, which leads to the conclusion that strains WPCB074^T and WPCB165 represent a novel and distinct species (Wayne *et al.*, 1987).

Cells of strains WPCB074^T and WPCB165 grown on tryptic soy agar (TSA; Becton Dickinson) at 30 °C for 2 days were used for physiological and biochemical tests. Morphology of the cells was observed using a differential interference microscope (BX50; Olympus) and a scanning electron microscope (S-4800; Hitachi). Endospore formation was observed by phase-contrast microscopy. Motility was examined by observing cells grown in wet mounts using a phase-contrast microscope (TMS-F; Nikon). Growth at various NaCl concentrations (0–10 %, w/v, in increments of 1.0 %) was investigated in tryptic soy broth (TSB; Becton Dickinson) prepared according to the formula of the Becton Dickinson medium except that no NaCl was used. The pH range for growth was determined in TSA that was adjusted to pH 4–11 (in increments of 1 pH unit) by the addition of HCl and NaOH. The optimal temperature and temperature range for growth were tested on TSA at 4–50 °C. Anaerobic growth was tested on TSA in a jar containing an AnaeroPak (Mitsubishi Gas Chemical), which works as an oxygen absorber and CO₂ generator, for up to 10 days. Catalase and oxidase activities were

determined using 3 % (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956), respectively. Nitrate reduction was tested in TSB containing 0.2 % KNO₃ (Skerman, 1967). H₂S production was determined on Kligler iron agar (Becton Dickinson). Hydrolysis of CM-cellulose (1 %, w/v), casein (2 % skimmed 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) was tested as described by Smibert & Krieg (1994). DNase activity was determined on DNase test agar (Becton Dickinson). Other biochemical tests and enzymic activities were assessed using API 20NE, API 50CHB, API ZYM kits (bioMérieux) and the GP2 MicroPlate (Biolog) prepared according to the manufacturers' instructions. Antibiotic resistance was determined by the disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 5 days of incubation at 30 °C on TSA, results were interpreted according to the guidelines set forth by the CLSI (2003).

Strains WPCB074^T and WPCB165 had similar characteristics with respect to their cellular and colonial morphologies. Cells of both strains were Gram-stain-positive,

motile rods (0.5 µm wide and 3.0–6.0 µm long) and occurred singly or in chains. They produced ellipsoidal endospores that lay in a subterminal position and usually caused the sporangia to swell. Colonies grown on TSA plates for 2 days at 30 °C were circular, opaque, cream and convex with entire margins and approximately 3–4 mm in diameter. They did not grow under anaerobic conditions. Both strains grew between 10 and 45 °C, with optimum growth at 20–37 °C. The strains grew at pH 6–9; optimal growth was observed between pH 7 and 8. Strains WPCB074^T and WPCB165 grew in 0–9 % (w/v) NaCl, with optimal growth at 1–2 % (w/v) NaCl. From tests done using the API 20NE system (bioMérieux), both strains gave positive results for oxidase, β-galactosidase, gelatin hydrolysis, acid production from glucose and assimilation of D-glucose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, malic acid and trisodium citrate; negative results were observed for reduction of nitrate to nitrite and to nitrogen, indole production, arginine dihydrolase, urease and assimilation of L-arabinose, capric acid, adipic acid and phenylacetic acid. From tests done using the API 50CHB system (bioMérieux), both strains were positive for acid production from aesculin,

Table 1. Phenotypic characteristics that differentiate strains WPCB074^T and WPCB165 from their phylogenetic neighbours in the genus *Bacillus*

Strains: 1, WPCB074^T; 2, WPCB165; 3, *B. arsenicus* KACC 12116^T; 4, *B. barbaricus* KACC 12101^T; 5, *B. gelatini* KACC 12197^T; 6, *B. macauensis* KACC 12177^T; 7, *B. solisalsi* KCTC 13181^T. Except where indicated, data for reference strains were taken from Zhang *et al.* (2006), De Clerck *et al.* (2004), Liu *et al.* (2009), Shivaji *et al.* (2005) and Taübel *et al.* (2003).

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	–	–	–	+	–	+	+
Motility	+	+	+	–	+	+	+
Oxidase	+	+	+	–	–	+	+
Growth at/in:							
15 °C	+	+	–	–	–	–	+
47 °C	–	–	–	–	+	–	+
5 % (w/v) NaCl*	+	+	–	–	+	–	+
10 % (w/v) NaCl*	–	–	–	–	+	–	+
Hydrolysis of:*							
Aesculin	+	+	–	–	+	–	+
Gelatin	+	+	+	–	+	+	–
Tween 20	+	+	–	–	–	–	+
Tyrosine	–	–	–	–	–	–	+
Utilization of carbon sources (Biolog)*							
Cellobiose	–	–	–	–	+	–	–
D-Fructose	–	+	–	–	–	–	+
α-D-Glucose	–	–	+	–	–	+	+
Inulin	–	–	–	–	–	+	+
D-Mannitol	+	+	–	–	–	–	+
D-Mannose	–	–	–	–	+	–	–
Methyl pyruvate	–	–	–	+	–	–	–
D-Ribose	+	–	+	–	–	+	–
D-Xylose	+	–	–	–	–	–	–
DNA G + C content (mol%)	41.9	41.4	35.0	42.0*	41.5	40.8	41.8

*Data from this study.

D-fructose, D-glucose, glycogen, maltose, D-mannose, starch, sucrose and trehalose, but negative for the other substrates tested. Neither strain produced H₂S or hydrolysed CM-cellulose, egg yolk, Tween 80, L-tyrosine or xylan, but casein, DNA, starch and Tween 20 were hydrolysed by both strains. The detailed results of physiological and biochemical analyses are given in Table 1 and the species description. It is evident from Table 1 that there are several phenotypic characters that readily separate strains WPCB074^T and WPCB165 from phylogenetically related species.

Cells of strains WPCB074^T and WPCB165 and reference strains grown on TSA for 1 day at 30 °C were prepared (in duplicate) and their fatty acid methyl esters were 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). 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 freeze-dried cells, purified according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described previously (Kroppenstedt, 1985). The G+C content was calculated using the formula described by Mandel *et al.* (1970). The DNA sample was prepared in duplicate and G+C content was determined by the thermal denaturation method of Marmur & Doty (1962). The cellular fatty acid profiles of strains WPCB074^T and WPCB165 revealed the presence of C₁₄–C₁₈ fatty acids and included mainly iso- and anteiso-branched components. In both strains, iso-C_{15:0} and anteiso-C_{15:0} were the major fatty acids. A comparison of cellular fatty acid compositions of strains WPCB074^T and WPCB165 and type strains of some related species of the genus *Bacillus* is given in Table 2. The major polar lipids present in both strains were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine; minor components were unknown aminolipids, aminophospholipids and a phospholipid. Glycolipids were not detected. The diamino acid in the cell wall peptidoglycan of strains WPCB074^T and WPCB165 was *meso*-diaminopimelic acid. The predominant isoprenoid quinone was unsaturated menaquinone with seven isoprene units (MK-7). The DNA G+C contents of strains WPCB074^T and WPCB165 were 41.9 and 41.4 mol%, respectively.

Phylogenetic, genomic, chemotaxonomic and phenotypic data indicate clearly that strains WPCB074^T and WPCB165 represent a novel genomic species within the genus *Bacillus*, for which the name *Bacillus rigui* sp. nov. is proposed.

Description of *Bacillus rigui* sp. nov.

Bacillus rigui (ri'gu.i. L. gen. n. *rigui* of a well-watered place).

Strictly aerobic. Colonies on TSA are circular, opaque, convex, cream coloured and approximately 3–4 mm in

Table 2. Cellular fatty acid profiles (%) of strains WPCB074^T and WPCB165 and related species of the genus *Bacillus*

Strains: 1, WPCB074^T; 2, WPCB165; 3, *B. arsenicus* KACC 12116^T; 4, *B. barbaricus* KACC 12101^T; 5, *B. gelatini* KACC 12197^T; 6, *B. macauensis* KACC 12177^T; 7, *B. solisalsi* KCTC 13181^T. All data were obtained in this study; cells of all strains were harvested after cultivation at 30 °C on TSA medium for 1 day. –, Not detected.

Fatty acid	1	2	3	4	5	6	7
Straight-chain saturated							
C _{14:0}	0.8	0.8	0.8	1.1	1.3	1.2	1.3
C _{16:0}	2.3	2.0	6.9	5.7	4.7	1.4	7.2
C _{18:0}	–	–	4.4	3.1	1.8	–	3.2
Branched							
iso-C _{14:0}	4.1	4.6	2.1	5.9	1.1	4.0	6.6
iso-C _{15:0}	27.3	30.2	28.3	35.6	44.4	34.5	11.7
iso-C _{15:1} at 12–13	–	–	1.2	1.8	–	–	–
iso-C _{16:0}	2.7	2.1	5.3	5.5	4.6	5.1	5.9
iso-C _{17:0}	1.9	1.6	5.4	2.2	6.4	1.0	1.2
iso-C _{17:1} ω10c	0.8	–	3.2	1.5	1.6	–	–
anteiso-C _{15:0}	49.8	49.3	16.6	20.3	16.4	46.4	55.1
anteiso-C _{17:0}	5.0	3.9	5.3	1.6	13.2	6.5	6.7
anteiso-C _{17:1} A	–	–	1.6	1.3	–	–	–
C _{16:0} N-alcohol	–	–	0.9	1.5	–	–	–
Monounsaturated							
C _{16:1} ω7c	1.7	1.7	5.2	4.6	1.0	–	–
C _{16:1} ω11c	1.5	1.4	4.7	2.7	1.5	–	–
Summed features*							
3	–	–	1.8	1.8	–	–	–
4	1.6	1.4	3.0	1.9	1.7	–	–
Unknown 14.263	0.6	1.0	–	–	–	–	–

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

diameter after 2 days at 30 °C (pH 7). Cells are motile and 0.5 × 3.0–6.0 µm in size. Ellipsoidal endospores are formed in a subterminal position and usually cause sporangia to swell. Growth occurs in 0–9% (w/v) NaCl (optimum 1–2%), at pH 6–9 (optimum pH 7–8) and at 10–45 °C (optimum 20–37 °C). Oxidase- and catalase-positive. In the API ZYM gallery, alkaline phosphatase, α-chymotrypsin, esterase (C4), β-galactosidase, α-glucosidase and leucine arylamidase activities are present; *N*-acetyl-β-glucosaminidase, acid phosphatase, cystine arylamidase, esterase lipase (C8), α-fucosidase, α-galactosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are absent. The following carbon sources are utilized (positive with the Biolog GP2 system): adenosine, dextrin, glycerol, D- and L-α-glycerol phosphate, α-ketovaleric acid, monomethyl succinate, Tween 40 and D-xylose. The remaining substrates of the Biolog GP2 system are not utilized. 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 acids are iso-C_{15:0} and anteiso-C_{15:0}. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major respiratory quinone is MK-7.

The type strain is WPCB074^T (=KCTC 13278^T =JCM 16348^T), isolated from fresh water of the Woopo wetland, Republic of Korea. The DNA G+C content of the type strain is 41.9 mol%. Strain WPCB165 (=KCTC 13279 =JCM 16349), isolated from the same source, is a reference strain.

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