Mucilaginibacter rigui sp. nov., isolated from wetland freshwater, and emended description of the genus *Mucilaginibacter*

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A non-motile, rod-shaped bacterium, designated strain WPCB133^T, was isolated from freshwater collected from the Woopo wetland (Republic of Korea). Cells were Gram-reaction-negative, aerobic and catalase- and oxidase-positive. The major fatty acids were iso-C_{15:0} and anteiso-C_{15:0}. The strain contained MK-7 as the major isoprenoid quinone. The DNA G + C content was 47 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain WPCB133^T forms an independent lineage within the genus *Mucilaginibacter*. Strain WPCB133^T was distantly related to *Mucilaginibacter kameinonensis* SCK^T (94.7 % sequence similarity), *Mucilaginibacter paludis* TPT56^T (94.5 %) and *Mucilaginibacter gracilis* TPT18^T (94.4 %). Phenotypic characteristics distinguished strain WPCB133^T from members of the genus *Mucilaginibacter*. On the basis of evidence presented in this study, strain WPCB133^T represents a novel species of the genus *Mucilaginibacter*, for which the name *Mucilaginibacter rigui* sp. nov. is proposed. The type strain is WPCB133^T (=KCTC 12534^T =NBRC 101115^T). An emended description of the genus *Mucilaginibacter* is also proposed.

Members of the phylum *Bacteroidetes* are widely distributed in aquatic and terrestrial habitats and have a key role in degrading various biopolymers (Floyd *et al.*, 2005). The genus *Mucilaginibacter*, a member of the family *Sphingobacteriaceae* (Steyn *et al.*, 1998; Pankratov *et al.*, 2007), phylum *Bacteroidetes*, was proposed recently to group Gram-reaction-negative, non-spore-forming and non-motile rods producing large amounts of extracellular polymeric substances (Pankratov *et al.*, 2007). Cells of some species undergo a cyclic shape change during culture development. At the time of writing, the genus comprises only three species, *Mucilaginibacter paludis* and *Mucilaginibacter gracilis* isolated from acidic peat (Pankratov *et al.*, 2007) and *Mucilaginibacter kameinonensis* isolated from garden soil (Urai *et al.*, 2008). In the course of our study on wetland microbial diversity, a rod-shaped bacterial strain, designated WPCB133^T, was isolated from a freshwater sample and subjected to a polyphasic taxonomic investigation.

Strain WPCB133^T was isolated from a freshwater 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 agar (Staley, 1968) 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 % glycerol (w/v).

Preparation of bacterial DNA and PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chun & Goodfellow, (1995). The resultant sequence of strain WPCB133^T (1426 nt) was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available for all sequences (positions 33–1380; *Escherichia coli* numbering system) using the neighbour-joining (Saitou & Nei, 1987) and the Fitch–Margoliash (Fitch & Margoliash, 1967) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (available at <http://chunlab.snu.ac.kr/jphydit/>) and PAUP 4.0 (Swofford, 1998), as described by Chun *et al.*, (2000).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WPCB133^T is EU747841.

Scanning and transmission electron micrographs of cells of strain WPCB133^T are available with the online version of this paper.

Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that the isolate was closely related to the genus *Mucilaginibacter*. The newly determined sequence was then aligned manually against representatives of the phylum *Bacteroidetes*. Strain WPCB133^T showed the highest 16S rRNA gene sequence similarity to *M. kameinonensis* SCK^T (94.7 % sequence similarity), *M. paludis* TPT56^T (94.5 %) and *M. gracilis* TPT18^T (94.4 %). Sequence similarity with other members of the family *Sphingobacteriaceae* was <92 %. The neighbour-joining tree (Fig. 1) showed that strain WPCB133^T formed a monophyletic clade with the members of the genus *Mucilaginibacter* with 100 % bootstrap support. The topology of the Fitch–Margoliash tree was essentially the same (data not shown).

As 16S rRNA gene sequence similarities between strain WPCB133^T and described species of the genus *Mucilaginibacter* were below the level indicative of relatedness at the species level (97 %; Stackebrandt & Goebel, 1994), no DNA–DNA hybridization test was needed to define a novel species.

Growth was tested on nutrient (NA, Difco), tryptic soy (TSA, Difco), plate count (PCA, Difco), MM1 (Pankratov *et al.*, 2007) and R2A (Difco) agars. Cells of strain WPCB133^T grown on R2A agar at 25 °C for 3 days were used for physiological and biochemical tests and results were recorded for 10 days unless otherwise indicated. Cell morphology was observed by phase-contrast (TMS-F, Nikon), scanning (S-4800, Hitachi) and transmission electron (CM-20, Philips) microscopy using cells grown for 2 days at 25 °C on R2A. Cell life cycle was examined using cultures grown both on MM1 agar and in MM1 broth for up to 2 weeks. Motility was examined by observing cells in wet mounts using phase-contrast microscopy (Axioskop 40, Zeiss). The pH range for growth was determined on R2A agar. Medium sterilized by autoclaving was adjusted to pH 4–11 (using increments of 1 pH unit) by addition of HCl or NaOH. Growth temperature (4, 10, 15, 20, 25, 30, 37, 40 and 42 °C) and

NaCl tolerance [0, 1, 2, 3, 5 and 10 % (w/v)] were tested on R2A agar. Anaerobic growth on R2A agar was tested using the AnaeroPack-Anaero (Mitsubishi Gas Chemical). Catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide and Kovacs' reagent (Kovacs, 1956), respectively. Hydrolysis of cellulose (filter paper) and chitin was tested by overlaying the respective substrates on R2A. Hydrolysis of CM-cellulose was assessed on R2A agar supplemented with 0.5 % (w/v) CM-cellulose (Sigma). Plates were flooded with 0.2 % aqueous Congo red dye solution followed by removal of extra dye and flooding the plate with 1 M NaCl solution. A clear zone around bacterial growth indicated positive hydrolysis. For assessing hydrolysis of alginate (0.5 % sodium alginate, w/v), casein (2 % skim milk, w/v), chitin (1 % colloidal chitin, w/v), pectin (0.5 %, w/v), starch (0.2 % soluble starch, w/v), L-tyrosine (0.5 %, w/v), Tween 20 (1 %, v/v), Tween 80 (1 %, v/v) and xylan (1 %, w/v), R2A agar was supplemented with each substrate at concentrations recommended by Smibert & Krieg (1994). The presence of flexirubin-type pigments was tested using the KOH test as described by Bernardet *et al.* (2002). Acid production from sugars was examined by using phenol red broth base (Difco) containing 0.5 % of each sugar. Other physiological and biochemical tests were performed using API 20E, API 20NE and API 50CH kits (bioMérieux) and the GN2 MicroPlate (Biolog). Enzymic activities were tested using the API ZYM kit (bioMérieux) following the manufacturer's instructions. Antibiotic resistance of strain WPCB133^T and reference strains was determined by using the disc diffusion method with commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 5 days of incubation at 25 °C on R2A, the results were interpreted according to the guidelines set forth by the CLSI (2003).

The detailed results of morphological, physiological and biochemical analyses are given in Table 1 and the species description. Table 1 shows the phenotypic characteristics that differentiate strain WPCB133^T from *M. kameinonensis*, *M. paludis* and *M. gracilis*.

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing relationships between strain WPCB133^T, members of the genus *Mucilaginibacter* and representative members of the family *Sphingobacteriaceae* and of the phylum *Bacteroidetes*. Percentages at nodes are levels of bootstrap support (>50 %) based on neighbour-joining analyses of 1000 resampled datasets. The sequence of *Hymenobacter rigui* KCTC 12533^T (DQ089669) was used as an outgroup. Bar, 0.02 nucleotide substitutions per position.

Table 1. Phenotypic characteristics that differentiate strain WPCB133^T from other species of the genus *Mucilaginibacter*

Strains: 1, *Mucilaginibacter rigui* WPCB133^T; 2, *M. paludis* TPT56^T; 3, *M. gracilis* TPT18^T (data in columns 2–3 from Pankratov *et al.*, 2007); 4, *M. kameinonensis* SCK^T (Urai *et al.*, 2008). +, Positive; –, negative; w, weakly positive; NA, data not available. All strains are positive for acid production from cellobiose, D-galactose, D-glucose, lactose, maltose, sucrose and trehalose and for catalase activity. All strains are negative for assimilation of citric acid and malic acid, Gram staining, motility and H₂S and indole production.

Characteristic	1	2	3	4
Cell arrangement	Pair, single	Single, pair, short chain	Single, pair, short chain	Irregular
Cyclic cell shape change	–	+	+	NA
Ranges for growth				
Temperature (°C)	4–37	2–33	2–33	5–30
pH	5–10	4.2–8.2	4.2–8.2	4–9
Flexirubin pigments	–	–	–	+
Oxidase activity	+	+	+	–
Hydrolysis of aesculin	+	–	–	+
Acid production from:				
Arabinose	–	–	+	+
Arbutin	w	–	–	–
D-Fructose	–	+	+	+
Inulin	–	–	–	+
D-Mannose	+	+	–	+
Melezitose	–	–	–	+
Melibiose	+	–	–	+
Raffinose	–	–	+	+
Salicin	w	–	+	+
Trehalose	+	–	+	+
D-Xylose	–	+	+	+
Assimilation of:				
N-Acetyl-D-glucosamine	+	+	–	w
D-Galacturonic acid	–	+	–	–
D-Gluconic acid	–	+	–	–
D-Mannose	+	+	–	+
Melibiose	+	–	–	+
Raffinose	+	–	+	+
myo-Inositol	–	+	–	–
Susceptibility to:				
Chloramphenicol (30 µg)	+	+	+	–
Nalidixic acid (30 µg)	+	+	+	–
Erythromycin (15 µg)	–	–	+	–
DNA G + C content (mol%)	47	46.1	42.4	43.7
Quinone(s)	MK-7	MK-7, MK-6	MK-7, MK-6	MK-7

*Data from this study.

Cell biomass of strains WPCB133^T and *M. kameinonensis* SCK^T for cellular fatty acid analysis was obtained from batch cultures grown in MM1 broth at 24 °C for 1 week. Analysis was performed by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). Isoprenoid quinone analysis was performed by reversed phase TLC according to Collins (1985). The G + C content of the DNA was determined by using the thermal denaturation method of Marmur & Doty (1962).

The cellular fatty acid profile of strain WPCB133^T is described in Table 2. Significant differences in the proportions of the predominant fatty acids (anteiso-C_{15:0} and summed feature

3) were found between strain WPCB133^T and the type strains of related species. Quinone analysis revealed a spot that corresponded to menaquinone-7 (MK-7), but menaquinone-6 (MK-6) was not detected. MK-7 predominates in all members of the family *Sphingobacteriaceae*, but some of them also contain minor amounts of MK-6 (Pankratov *et al.*, 2007; Urai *et al.*, 2008). The DNA G + C content of strain WPCB133^T was 47 mol%, a value slightly above the range reported for other species of the genus *Mucilaginibacter* (Pankratov *et al.*, 2007; Urai *et al.*, 2008).

A number of phenotypic characteristics clearly distinguished strain WPCB133^T from other species of the genus

Table 2. Cellular fatty acid composition (%) of strain WPCB133^T and the type strains of species of the genus *Mucilaginibacter*

Strains: 1, *Mucilaginibacter rigui* WPCB133^T; 2, *M. paludis* TPT56^T; 3, *M. gracilis* TPT18^T (data in columns 2–3 from Pankratov *et al.*, 2007). 4, *M. kameinonensis* SCK^T (data in columns 1 and 4 from this study). –, Not detected; tr, trace amount (<1 %); ECL, equivalent chain-length. For fatty acid analysis, all strains studied were grown in MM1 broth at 24 °C for 1 week. Fatty acids amounting to <1 % of the total fatty acids in all strains are omitted.

Fatty acid	1	2	3	4
Straight-chain				
C _{14:0}	tr	tr	tr	2.4
C _{15:0}	—	3.2	4.5	1.7
C _{16:0}	tr	3.1	tr	25.2
C _{17:0}	—	tr	tr	1.3
C _{18:0}	—	tr	—	19.5
Branched				
iso-C _{13:0}	tr	—	—	—
iso-C _{14:0}	3.3	—	—	—
iso-C _{15:0}	34.1	15.7	32.2	10.0
iso-C _{15:1} F	1.9	—	—	—
iso-C _{16:0}	1.3	1.5	tr	—
iso-C _{17:0}	1.1	tr	tr	—
iso-C _{17:1} ω9c	3.4	tr	2.7	—
anteiso-C _{15:0}	26.7	21.1	5.8	—
anteiso-C _{17:0}	—	4.5	—	—
Unsaturated				
C _{14:1} ω5c	—	—	—	tr
C _{15:1} ω6c	—	tr	1.2	—
C _{16:1} ω5c	—	5.8	2.6	1.3
C _{17:1} ω8c	—	tr	1.5	—
C _{18:1} ω5c	1.4	—	—	—
C _{18:1} ω7c	—	tr	—	2.9
C _{18:1} ω9c	—	tr	—	2.5
Hydroxylated				
C _{15:0} 2-OH	2.1	tr	tr	—
C _{16:0} 3-OH	—	tr	tr	2.3
C _{17:0} 2-OH	1.1	tr	tr	—
iso-C _{15:0} 3-OH	5.9	1.8	3.2	—
iso-C _{16:0} 3-OH	2.6	tr	tr	—
iso-C _{17:0} 3-OH	5.4	8.9	13.1	6.3
Unknown				
ECL 14.263	2.2	—	—	—
Summed feature*				
1	1.7	—	—	—
3	tr	24.7	21.5	24.2
4	1.7	—	—	—

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 contained iso-C_{15:1} H and/or iso-C_{15:1} I and/or C_{13:0} 3-OH; 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.

Mucilaginibacter (Table 1). Therefore, strain WPCB133^T should be classified as a novel species within the genus *Mucilaginibacter*, for which the name *Mucilaginibacter rigui* sp. nov. is proposed. On the basis of new data obtained by Urai *et al.* (2008) and in this study, an emended description of the genus *Mucilaginibacter* is also proposed.

Emended description of the genus *Mucilaginibacter* Pankratov *et al.* 2007 emend. Urai *et al.* 2008

The description of the genus *Mucilaginibacter* is as given by Pankratov *et al.* (2007) and emended by Urai *et al.* (2008) with the following modifications. Oxidase activity, hydrolysis of aesculin, pectin, starch and xylan, production of flexirubin-type pigments and cyclic cell shape change are species-dependent. Show strictly aerobic or facultatively anaerobic metabolism. The predominant quinone is MK-7, but some species contain MK-6 as well. The major fatty acids are straight- and branched-saturated fatty acids, but some species contain summed feature 3 (C_{16:1} ω7c and/or iso-C_{15:0} 2-OH) as well. The DNA G+C content varies between 42.4 and 47 mol%.

Description of *Mucilaginibacter rigui* sp. nov.

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

Cells are Gram-reaction-negative, non-endospore-forming, non-motile, aerobic rods, 0.3–0.4 µm in diameter and 1.0–1.7 µm long, occurring singly or in pairs (Supplementary Fig. S1 available in IJSEM Online). No cyclic cell shape change is noticed during culture. Colonies on R2A agar are circular, convex with entire margins, mucoid, translucent, pale pink and approximately 3.0 mm in diameter after 3 days at 25 °C (pH 7). Good growth occurs on R2A, MM1, PCA and TSA, but only weak growth occurs on NA. Large amounts of extracellular polymeric substances are produced. Growth occurs at pH 5–10 (optimum, pH 6–7) and at 4–37 °C (optimum, 25 °C). NaCl inhibits growth at concentrations above 1 % (w/v). Flexirubin-type pigments are absent. Oxidase- and catalase-positive. Positive for hydrolysis of Tween 20, but negative for hydrolysis of alginate, casein, cellulose, chitin, CM-cellulose, pectin, starch, Tween 80, L-tyrosine and xylan. Acid is produced from galactose, lactose, maltose, mannose and sucrose, but not from cellobiose, fructose, glucose, rhamnose, xylan and xylose. Positive reactions (API 20E and 20NE systems) for acetoin production, aesculin hydrolysis, β-galactosidase activity and assimilation of *N*-acetylglucosamine, glucose, mannose and maltose. Negative reactions for arginine dihydrolase, gelatinase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease activities, utilization of citrate, production of indole and H₂S, nitrate reduction, oxidation of amygdalin, arabinose, inositol, mannitol, melibiose, rhamnose, sorbitol and sucrose, fermentation of glucose, and assimilation of adipate, arabinose, caprate, potassium gluconate, malate, mannitol,

phenylacetate and trisodium citrate. In the API 50CH system, acid is produced from *N*-acetylglucosamine, aesculin, amygdalin, arbutin, cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, melibiose, salicin, sucrose and trehalose; acid is not produced from D-adonitol, D- or L-arabinose, D- or L-arabitol, dulcitol, erythritol, D-fructose, D- or L-fucose, gentiobiose, glycerol, glycogen, inositol, inulin, D-lyxose, D-mannitol, melezitose, methyl β -D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, raffinose, L-rhamnose, D-ribose, D-sorbitol, L-sorbose, starch, D-tagatose, turanose, xylitol or D- or L-xylose. In the API ZYM gallery, *N*-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, leucine arylamidase, α -mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present; but α -chymotrypsin, cystine arylamidase, α -fucosidase, β -glucuronidase, lipase (C14) and trypsin activities are absent. Positive for Biolog GN2 MicroPlate substrates *N*-acetyl-D-glucosamine, cellobiose, dextrin, gentiobiose, α -D-glucose, lactose, lactulose, maltose, D-mannose, melibiose, methyl β -D-glucoside, raffinose, sucrose, trehalose and turanose, but negative for acetic acid, *N*-acetyl-D-galactosamine, *cis*-aconitic acid, adonitol, L-alaninamide, D- and L-alanine, L-alanyl glycine, γ -aminobutyric acid, 2-aminoethanol, L-arabinose, D-arabitol, L-asparagine, L-aspartic acid, bromosuccinic acid, 2,3-butanediol, D- and L-carnitine, citric acid, α -cyclodextrin, D-erythritol, formic acid, D-fructose, L-fucose, D-galactonic acid lactone, D-galactose, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, glucose 1-phosphate, glucose 6-phosphate, glucuronamide, D-glucuronic acid, L-glutamic acid, glycerol, D- and L- α -glycerol phosphate, glycogen, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, α -, β - and γ -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, L-hydroxyproline, inosine, *myo*-inositol, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, D- and L-lactic acid, L-leucine, malonic acid, D-mannitol, L-ornithine, L-phenylalanine, phenylethylamine, L-proline, propionic acid, D-psicose, putrescine, L-pyrogutamic acid, pyruvic acid methyl ester, quinic acid, L-rhamnose, D-saccharic acid, sebacic acid, D- and L-serine, D-sorbitol, succinamic acid, succinic acid, succinic acid monomethyl ester, L-threonine, thymidine, Tweens 40 and 80, uridine, urocanic acid and xylitol. Sensitive to the following antibiotics (μ g per disc, unless otherwise indicated): chloramphenicol (30), ciprofloxacin (5), nalidixic acid (30), rifampicin (5) and tetracycline (30). Resistant to the following antibiotics (μ g per disc, unless otherwise indicated): amikacin (30), ampicillin (10), aztreonam (30), cephalothin (30), cefepime (30), cefmetazole (30), cefotaxime (30), ceftazidime (30), ceftriaxone (30), erythromycin (15), gentamicin (10), imipenem (10), isepamicin (30), kanamycin (30), lincomycin (2), moxalactam (30), nitrofurantoin (300), oxacillin (1), penicillin (10 IU), piperacillin (100), streptomycin (100), teicoplanin

(30), ticarcillin/clavulanic acid (75/10), tobramycin (10) and vancomycin (30). The major fatty acids (>25%) are iso-C_{15:0} and anteiso-C_{15:0}. The complete fatty acid composition is given in Table 2. The DNA G+C content of the type strain is 47 mol%. The only respiratory quinone detected is menaquinone 7 (MK-7).

The type strain is WPCB133^T (=KCTC 12534^T =NBRC 101115^T), isolated from freshwater of Woopo wetland, Republic of Korea.

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