Microbacterium paludicola sp. nov., a novel xylanolytic bacterium isolated from swamp forest

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A xylanolytic bacterium, US15^T, was isolated from swamp forest soil in Ulsan, Korea. The cells of the novel strain were Gram-positive, non-motile, short-rod-shaped and showed chemotaxonomic properties that were consistent with its classification in the genus *Microbacterium*. Chemotaxonomic results showed MK-12 and MK-11 as major menaquinones, predominating iso- and anteiso-branched cellular fatty acids, glucose, galactose and mannose as cell-wall sugars, peptidoglycan-type B2 β with glycolyl residues and a DNA G + C content of 66.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain US15^T was closely related to *Microbacterium arborescens* IFO 3750^T, *Microbacterium imperiale* IFO 12610^T and *Microbacterium ulmi* LMG 20991^T (96.9, 96.8 and 96.2% similarities, respectively), and formed a separate lineage within the genus *Microbacterium*. Combined genotypic and phenotypic data showed that strain US15^T (= DSM 16915^T = KCTC 19080^T) merits recognition as the type strain of a novel species within the genus *Microbacterium*, for which the name *Microbacterium paludicola* sp. nov. is proposed.

Micro-organisms are primarily responsible for xylan degradation in nature. The complex chemical structure of xylan has been described as a linear polymer of repeating xylopyranosyl groups substituted at various carbon positions with different sugars or acidic compounds. For the complete and efficient enzymic hydrolysis of the polymer, an array of enzymes with diverse specificity that the microbial cells produce is needed (Biely, 1985; Wong *et al.*, 1988; Uffen, 1997). Current knowledge of microbial action on xylan has already led to suggestions about new technologies that are ready for development in both agriculture and industry (Kuhad & Singh, 1993).

During the course of our studies to screen micro-organisms that were able to degrade xylan from various environmental sources, a yellow-pigmented xylanolytic bacterium, strain US15^T, was isolated from swamp forest soil and subjected to polyphasic taxonomic investigation. The results from phenotypic and genotypic characterizations suggested that the strain belongs to the genus *Microbacterium*, which was first described by Orla-Jensen (1919); its description was emended by Collins *et al.* (1983) and more recently it has been emended by Takeuchi & Hatano (1998) to unite the genera *Microbacterium* and *Aureobacterium*. At the time of

writing, the genus *Microbacterium* consisted of 39 species with validly published names (type species is *Microbacterium lacticum*) that were isolated from various environmental sources including soil, plants, insects, air, dairy products, sewage, steep liquor and human clinical specimens.

A soil sample from swamp forest in Ulsan was collected and diluted serially in 0.85% NaCl solution. Aliquots of each serial dilution were spread onto R2A agar (Difco) supplemented with insoluble chromogenic xylan (25 g l⁻¹; Ten *et al.*, 2004) and incubated at 28 °C for 7 days. A colony surrounded by a conspicuous clearing zone, designated US15^T, was isolated and subcultivated onto nutrient agar (Difco) at 28 °C for 48 h for further analyses.

The Gram reaction was determined as described by Gerhardt *et al.* (1994). Cell morphology and motility were observed under a phase-contrast microscope (Optiphot; Nikon) at $\times 1000$ magnification, with cells grown for 1–7 days on nutrient agar. Oxidase activity was tested using 1% tetramethyl-*p*-phenylenediamine (Tarrand & Groschel, 1982) and catalase activity was tested using 3% H₂O₂. Growth was investigated on R2A agar, trypticase soy agar (TSA; BBL) and nutrient agar at temperatures ranging from 5 to 45 °C, at pH ranging from 4 to 9, in different salt concentrations (1, 2, 4 and 6.5%) and on MacConkey agar (Difco). Hydrolysis of casein and starch was tested on casein agar and starch agar (Difco). A H₂S production test was performed on triple-sugar-iron agar (BBL). Carbon-source utilization tests, acid-production tests and additional

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physiological tests were performed using API 20NE, API 32GN, API 50CH and API ZYM galleries according to the manufacturer's instructions (bioMérieux).

For the analysis of fatty acids, strain US15^T was cultivated on TSA at 28 °C for 4 days. *Microbacterium arborescens* DSM 20754^T (=IFO 3750^T), *Microbacterium imperiale* DSM 20530^T (=IFO 12610^T) and *Microbacterium ulmi* LMG 20991^T were used as reference strains under the same conditions.

Fatty acid methyl esters were prepared and analysed as described previously (Klatte *et al.*, 1994) using the standard Microbial Identification system (MIDI) for automated gas chromatography analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted and purified as described previously (Tindall, 1990), and dried preparations were dissolved in 200 µl 2-propanol; 1–10 µl samples were separated by HPLC without further purification. Purified cell-wall preparations were obtained as described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional TLC on cellulose plates by using solvent systems described by Schleifer & Kandler (1972). Cell-wall sugars were analysed according to the procedures of Stanek & Roberts (1974). Murein acyl type was determined by the colorimetric method of Uchida *et al.* (1999). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1977).

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Rainey *et al.* (1996). The 16S rRNA gene sequence was aligned with published sequences retrieved from EMBL by using CLUSTAL X (Thompson *et al.*, 1997) and was edited using BIOEDIT (Hall, 1999). A phylogenetic tree was constructed on the basis of the neighbour-joining method (Saitou & Nei, 1987); distances were estimated by the method of Jukes & Cantor (1969) using MEGA version 2.1 (Kumar *et al.*, 2001). The DNA G+C content was determined by HPLC after hydrolysis, as described by Tamaoka & Komagata (1984), and non-methylated λ DNA (Sigma) was used as a standard. The resultant neighbour-joining tree topology was evaluated by the bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets.

Strain US15^T formed visible colonies (1–2 mm in diameter) on nutrient agar at 28 °C within 48 h. Good growth occurred at temperatures ranging from 15 to 37 °C, but no growth was observed at 5 °C or at temperatures above 45 °C within 14 days. Growth was observed at pH 6–9. The colonies were lemon-yellow, translucent and circular with entire edges. Cells were Gram-positive, non-motile, non-spore-forming, short rods (0.8–1.0 × 1.0–2.5 µm in size). Strain US15^T differed significantly from *M. arborescens*, *M. imperiale* and *M. ulmi*, its closest phylogenetic neighbours, in terms of acid production, carbon-source utilization and

substrate hydrolysis profiles as well as colony colour. Physiological and biochemical characteristics are summarized in Table 1 and the species description.

The major menaquinones of strain US15^T were MK-12 (49.2 % of total quinones) and MK-11 (44.2 %); a small amount of MK-13 (6.7 %) was also present. The predominant fatty acids were 15:0-anteiso (63.9 %), 17:0-anteiso (14.4 %) and 16:0-iso (11.7 %). The cell-wall peptidoglycan was found to be the B2β type, (L-homoserine)-D-Glu→Gly→D-Orn, with glycolyl residues. The cell-wall sugars were glucose, galactose, mannose, rhamnose and fucose. The polar lipids were composed of diphosphatidylglycerol, phosphatidylglycerol and unknown polar lipids including glycolipid and phospholipid. Strain US15^T showed a different peptidoglycan type from *M. arborescens* and *M. imperiale*, and a different menaquinone profile from *M. ulmi*. Detailed chemotaxonomic characteristics are summarized in Table 2.

To determine the phylogenetic position of strain US15^T, the almost complete 16S rRNA gene sequence (1452 nt) was determined. Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain US15^T was closely related to *M. arborescens* IFO 3750^T, *M. imperiale* IFO 12610^T and *M. ulmi* LMG 20991^T (96.9, 96.8 and 96.2 % similarities, respectively); sequence similarities between US15^T and all *Microbacterium* species with validly published names ranged between 93.7 and 96.9 %. In the phylogenetic tree, strain US15^T formed a separate lineage within the genus *Microbacterium* (Fig. 1), which also supported the placement of strain US15^T in a novel species.

On the basis of phenotypic and genotypic data, strain US15^T merits recognition as the type strain of a novel species within the genus *Microbacterium*, for which the name *Microbacterium paludicola* sp. nov. is proposed.

Description of *Microbacterium paludicola* sp. nov.

Microbacterium paludicola (pa.lu.di'co.la. L. n. *palus* -udis swamp; L. suff. -cola from L. masc. or fem. n. *incola* inhabitant; N.L. masc. or fem. n. *paludicola* inhabitant of a swamp).

Cells are non-motile, non-spore-forming, short rods (0.8–1.0 × 1.0–2.5 µm in size). Gram-positive, oxidase-positive and catalase-positive. Good growth occurs on R2A agar, TSA and nutrient agar at 15–37 °C, but no growth is observed at 5 °C, temperatures above 45 °C or on MacConkey agar. Optimal growth temperature range is 25–30 °C and optimal pH range is 6–8. Growth occurs in the presence of 1, 2, 4 and 6.5 % NaCl. Colonies are lemon-yellow, translucent and circular with entire edges. Indole and H₂S are not produced. Nitrate is not reduced to nitrite. Xylan, starch and aesculin are hydrolysed, but gelatin, casein and cellulose are not hydrolysed. Arginine dihydrolase and urease are produced, but ornithine decarboxylase and lysine

Table 1. Physiological and biochemical characteristics that differentiate strain US15^T from its closest phylogenetic neighbours

Strains: 1, *Microbacterium paludicola* sp. nov. US15^T; 2, *Microbacterium arborescens* DSM 20754^T; 3, *Microbacterium imperiale* DSM 20530^T; 4, *Microbacterium ulmi* LMG 20991^T. +, Positive reaction; –, negative reaction. Acid production from D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, aesculin, salicin, cellobiose, maltose and trehalose; utilization of mannitol, D-glucose, rhamnose and maltose; and hydrolysis of L-leucyl 2-naphthylamide, 2-naphthyl phosphate (pH 5.4) and naphthol-AS-BI-phosphate are positive for all strains tested. Acid production from glycerol, ribose, L-xylose, adonitol, sorbose, dulcitol, sorbitol, inulin, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 2-ketogluconate; utilization of L-fucose, D-sorbitol, caprate, 3-hydroxybutyrate, 4-hydroxybutyrate, L-proline, D-ribose, inositol, itaconate, suberate, malonate, L-alanine, 3-hydroxybenzoate and L-serine; and hydrolysis of 2-naphthyl phosphate (pH 8.5), N-glutaryl-phenylamine 2-naphthylamide, naphthol-AS-BI-β-D-glucuronide and 2-naphthyl α-L-fucopyranoside are negative for all strains tested.

Characteristic	1	2	3	4
Colour of colonies	Lemon-yellow	Dirty orange	Red-orange	White
Arginine dihydrolase	+	–	–	–
Urease	+	+	–	–
Acid production from:				
Gluconate	–	+	+	–
N-Acetylglucosamine	–	+	+	–
Glycerol	+	+	–	–
Glycogen	+	–	–	–
Lactose	–	+	+	+
Melezitose	+	+	–	–
Methyl β-D-xyloside	–	+	–	+
Utilization of:				
Acetate	–	+	+	–
Adipate	+	–	+	–
Malate	–	+	+	–
Propionate	+	–	–	+
Valerate	+	–	+	–
N-Acetylglucosamine	–	+	+	–
Glycogen	+	–	–	–
Mannose	+	–	–	+
Hydrolysis of:				
2-Naphthyl butyrate	+	+	–	–
2-Naphthyl caprylate	+	+	–	–
2-Naphthyl myristate	+	–	–	–
6-Bromo-2-naphthyl α-D-galactopyranoside	–	+	+	–
2-Naphthyl β-D-galactopyranoside	–	+	+	+
2-Naphthyl α-D-glucopyranoside	+	+	–	–
6-Bromo-2-naphthyl β-D-glucopyranoside	–	+	+	–
1-Naphthyl N-acetyl-β-D-glucosaminide	–	+	+	+

*Data from API ZYM tests. The intensity of the colour was measured on a scale from 0 to 5, and interpreted as negative for values ranging between 0 and 1 and positive for values ranging between 2 and 5 (Mudarris *et al.*, 1994).

decarboxylase are not produced. Methyl-red and Voges-Proskauer tests are negative. Acid is produced from glycerol, L-arabinose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, methyl α-D-mannoside, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, starch, glycogen and D-turanose, but not from erythritol,

D-arabinose, ribose, L-xylose, adonitol, methyl β-D-xylose, sorbose, dulcitol, inositol, sorbitol, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, lactose, melibiose, inulin, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The following

Table 2. Chemotaxonomic characteristics of strain US15^T and its closest phylogenetic neighbours

Strains: 1, *M. paludicola* sp. nov. US15^T; 2, *M. arborescens* DSM 20754^T; 3, *M. imperiale* DSM 20530^T; 4, *M. ulmi* LMG 20991^T. Gal, Galactose; Glc, glucose; Fuc, fucose; Man, mannose; Rha, rhamnose; 6dTal, 6-deoxytalose; Xyl, xylose; Lys, L-lysine; Orn, D-ornithine. ND, Not detected.

Characteristic	1	2	3	4
DNA G + C content (mol%)	66.5	70.9	71.3	69.0
Peptidoglycan type	B2β	B1β	B1β	B2β
Cell-wall sugars	Glc, Gal, Man, Rha*, Fuc*	6dTal, Man, Gal	Rha, Man, Gal	Gal, Fuc, Xyl, Rha
Cell-wall diamino acid	Orn	Lys	Lys	Orn
Major menaquinones	11, 12	11, 12	11, 12	12, 13
Fatty acid composition (%)†				
13:0-anteiso	2.3	ND	ND	ND
14:0-iso	3.3	1.7	1.0	1.2
15:0-iso	1.3	ND	ND	12.2
15:0-anteiso	63.9	47.7	50.8	42.0
16:0-iso	11.7	21.3	14.0	12.5
16:0	2.9	2.0	5.9	4.8
17:0-iso	ND	ND	ND	3.0
17:0-anteiso	14.4	27.3	28.2	24.4

*Traces.
†Fatty acids are listed using standard abbreviations (no. carbon atoms: no. double bonds).

compounds are utilized as sole carbon sources: mannose, gluconate, adipate, mannitol, D-glucose, salicin, L-arabinose, propionate, valerate, 2-ketogluconate, rhamnose, D-sucrose, maltose, DL-lactate, 5-ketogluconate and glycogen. The following carbon sources are not utilized: malate, phenyl acetate, D-melibiose, L-fucose, D-sorbitol, caprate,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method (Saitou & Nei, 1987) showing the position of strain US15^T among species of the genus *Microbacterium*. Numbers at branching points refer to bootstrap values (1000 resamplings). Bar, 1 substitution per 100 nt positions.

citrate, histidine, 3-hydroxybutyrate, 4-hydroxybenzoate, L-proline, *N*-acetylglucosamine, D-ribose, inositol, itaconate, suberate, malonate, acetate, L-alanine, glycogen, 3-hydroxybenzoate and L-serine. According to the results from the API ZYM test, 2-naphthyl butyrate, 2-naphthyl caprylate, 2-naphthyl myristate, L-leucyl 2-naphthylamide, L-valyl 2-naphthylamide, 2-naphthyl phosphate (pH 5.4), naphthol-AS-BI-phosphate, 2-naphthyl α-D-glucopyranoside and 6-bromo-2-naphthyl α-D-mannopyranoside are hydrolysed, but 2-naphthyl phosphate (pH 8.5), L-cystyl 2-naphthylamide, *N*-benzoyl-DL-arginine 2-naphthylamide, *N*-glutaryl-phenylalanine 2-naphthylamide, 6-bromo-2-naphthyl α-D-galactopyranoside, 2-naphthyl β-D-galactopyranoside, naphthol-AS-BI-β-D-glucuronide, 6-bromo-2-naphthyl β-D-glucopyranoside, 1-naphthyl *N*-acetyl-β-D-glucosaminide and 2-naphthyl α-L-fucopyranoside are not hydrolysed. The major menaquinones are MK-12 (49.2 % of total quinones) and MK-11 (44.2 %); a small amount of MK-13 (6.7 %) is also present. The predominant fatty acids are 15:0-anteiso (63.9 %), 17:0-anteiso (14.4 %) and 16:0-iso (11.7 %). The cell-wall peptidoglycan is of the B2β type, (L-homoserine)-D-Glu→Gly→D-Orn, with glycolyl residues. The cell-wall sugars are glucose, galactose, mannose, rhamnose and fucose. The polar lipids are composed of diphosphatidylglycerol, phosphatidylglycerol and unknown polar lipids including glycolipid and phospholipid. The G + C content of the DNA is 66.5 mol%.

The type strain, US15^T (=DSM 16915^T=KCTC 19080^T), was isolated from swamp forest soil in Ulsan, Korea.

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