Microbacterium ulmi sp. nov., a xylanolytic, phosphate-solubilizing bacterium isolated from sawdust of *Ulmus nigra*

Raúl Rivas, Martha E. Trujillo, Manuel Sánchez, Pedro F. Mateos, Eustoquio Martínez-Molina and Encarna Velázquez

Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, Universidad de Salamanca, 37007 Salamanca, Spain

Correspondence
Martha E. Trujillo
mett@usal.es

A xylanolytic and phosphate-solubilizing bacterium isolated from sawdust of *Ulmus nigra* in Salamanca was characterized by a polyphasic approach. The novel strain, designated XIL02^T, was Gram-positive, aerobic, catalase- and oxidase-negative, rod-shaped and non-motile. Phylogenetically and chemotaxonomically, it was related to members of the genus *Microbacterium*. According to 16S rRNA gene sequence analysis, it is closely related to *Microbacterium arborescens* and *Microbacterium imperiale*; however, DNA–DNA hybridization showed reassociation values less than 70 % with the type strains of these species. In chemotaxonomic analyses, the major menaquinones detected were MK-12, MK-13 and MK-11 and the major fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$; the peptidoglycan was of the type B2β. The G+C content determined was 69 mol%. Based on the present data, it is proposed that strain XIL02^T (=LMG 20991^T=CECT 5976^T) be classified as the type strain of a novel *Microbacterium* species, for which the name *Microbacterium ulmi* sp. nov. is proposed.

Xylan is a heterogeneous polymer composed of 1,4-linked D-xylosyl residues that is present in plant cell walls. Microbial degradation of xylan requires the action of several enzymes such as β-1,4-xylanases, arabinofuranosidases, β-glucuronidases and β-xylosidases, which are produced by a wide range of micro-organisms, many of them belonging to the high-G+C Gram-positive bacteria (Beg *et al.*, 2000; Busch & Stutzenberger, 1997; Chamberlain & Crawford, 2000; Rivas *et al.*, 2003; Ruiz-Arribas *et al.*, 1995; Mayorga-Reyes *et al.*, 2002; Wang *et al.*, 1998).

A study was undertaken to investigate the bacterial diversity in decayed trees of *Ulmus nigra* with the aim of isolating xylanolytic strains. A series of coryneform bacteria were isolated from various samples; one of these strains produced significant xylan hydrolysis activity and was chosen for further study. Chemotaxonomic, morphological, physiological and genetic characterization of this strain suggested that it belongs to the emended genus *Microbacterium* (Takeuchi & Hatano, 1998), which accommodated 33 species with validly published names at the time of writing. Members of the genus *Microbacterium* contain the unusual type-B peptidoglycan, and strains representing these species have been isolated from various sources (Zlamala *et al.*, 2002). The data presented in this paper indicate that this strain represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium ulmi* sp. nov. is proposed.

A sample of sawdust from a decayed tree of *U. nigra* was collected under aseptic conditions and 1 g was suspended in 100 ml sterile water and stirred for 30 min. From this suspension, 100 µl was spread on XED medium (0·7 % xylan, 0·3 % yeast extract and 2·5 % agar) and incubated at 28 °C. A bacterial strain that produced a conspicuous clearing zone around the area of growth was isolated after 10 days incubation and a pure culture was maintained as a glycerol suspension (25 %, v/v) at −80 °C.

Isolate XIL02^T was observed by phase-contrast microscopy using 48-h-old cultures grown on nutrient broth to check for cell shape and motility. Cells were also Gram-stained as described by Doetsch (1981).

DNA extraction, PCR amplification of the 16S rRNA gene and sequencing of the PCR products were performed as described previously (Rivas *et al.*, 2003). An almost complete 16S rRNA gene sequence was obtained and compared with those from the public databases. Sequences were aligned using the CLUSTAL X software (Thompson *et al.*,

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of isolate XIL02^T is AY062021.

A 16S rDNA-based neighbour-joining tree including all species of *Microbacterium* is available as supplementary material in IJSEM Online.

1997). An evolutionary-distance matrix was calculated using the algorithm of Jukes & Cantor (1969). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and its topology was compared to that of a tree obtained using the maximum-parsimony method (Fitch, 1971). Bootstrap analyses were based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses.

Strain XIL02^T was cultivated in TSB (Becton Dickinson) for 4 days at 28 °C in a rotary shaker (90 r.p.m.) for cell wall and menaquinone analyses. The same medium amended with 1·5 % agar was used to cultivate the strain for fatty acid composition analysis. Menaquinone and cellular fatty acid compositions were determined as described by Zimmermann *et al.* (1998). Determination of the peptidoglycan type was carried out as described by Schleifer (1985) and Schleifer & Kandler (1972). Sugar analyses were performed according to described procedures (Staneck & Roberts, 1974).

The ability to solubilize phosphate was detected in YED-P plates (3 g yeast extract, 7 g glucose, 15 g agar and 3 g dibasic calcium phosphate l^{-1}) as described previously (Peix *et al.*, 2001). Amylase, catalase and oxidase activities were detected as described by Rivas *et al.* (2003). Casein hydrolysis activity was detected on skimmed milk agar. Cellulases were detected after 7 days incubation on plates containing 0·5 % carboxymethylcellulose as the carbon source, 0·3 % yeast extract and 1·5 % agar. Plates were stained with a 1 % aqueous Congo red solution. Other physiological and biochemical tests were done using API 20NE and API 50CH strips (bioMérieux) following the manufacturer's instructions.

DNA for base composition analysis was prepared according to Chun & Goodfellow (1995). The G+C content of DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968).

DNA–DNA relatedness was tested [in 2× SSC plus 10 % (v/v) DMSO at 68 °C] between strain XIL02^T and strains *Microbacterium arborescens* DSM 20754^T and *Microbacterium imperiale* DSM 20530^T. DNA was isolated using the procedure of Cashion *et al.* (1977) and DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modification of Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were calculated using the TRANSFER.BAS program (Jahnke, 1992).

Isolate XIL02^T was a Gram-positive, aerobic, non-motile, non-spore-forming and rod-shaped organism. Colonies on nutrient agar or XED medium showed a typical coryneform morphology. They were convex, smooth, white, opaque and 1–3 mm in diameter within 7 days at 28 °C.

An almost complete 16S rRNA gene sequence was obtained for isolate XIL02^T and this indicated that the organism was phylogenetically related to members of the genus

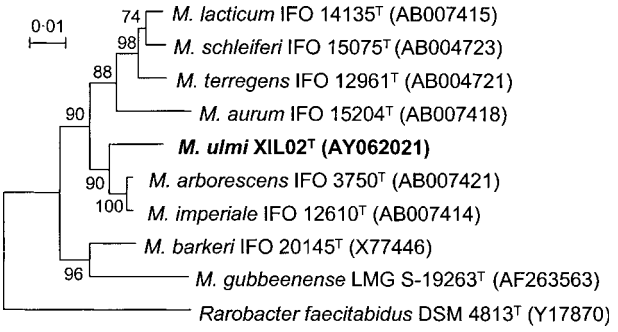

Fig. 1. Phylogenetic dendrogram based on comparison of the 16S rRNA gene sequence of *Microbacterium ulmi* sp. nov. XIL02^T within the genus *Microbacterium*. The significance of branches is indicated by bootstrap percentages based on 1000 resamplings. Bar, 1 substitution per 100 nt.

Microbacterium within the family *Microbacteriaceae*. Fig. 1 shows the relationship of strain XIL02^T with its nearest phylogenetic relatives based on the neighbour-joining method. The closest relatives were *M. imperiale* (97·8 % similarity) and *M. arborescens* (97·4 %). Similar results were obtained using the maximum-parsimony method (data not shown). A fuller phylogenetic tree that includes all *Microbacterium* species with validly published names can be found as supplementary material in IJSEM Online.

Strain XIL02^T showed high xylanase and amylase activity; moderate cellulase production was also observed. Other phenotypic properties can be found in the species description and in Table 1.

The ability of isolate XIL02^T to solubilize phosphate in the form of dibasic calcium phosphate was detected after 7 days. Phosphate-solubilizing bacteria can play an important role in plant nutrition through an increase in phosphorus uptake by plants (Antoun *et al.*, 1998; Chabot *et al.*, 1998; Rodríguez & Fraga, 1999), and their use as plant-growth-promoting rhizobacteria may be an important contribution to biofertilization of agricultural crops. Most strains studied for this purpose belong to the α-proteobacteria, especially members of the genera *Mesorhizobium* and *Rhizobium* (Peix *et al.*, 2001). During a screening programme for phosphate-solubilizing strains, we isolated strain XIL02^T; to our knowledge, this is the first report of a *Microbacterium* strain that solubilizes inorganic phosphate. It is worth mentioning that a laboratory bacterial contaminant characterized and identified as *Friedmanniella spumicola* (BUTO1) also produced significant phosphate-clearing zones when it was incubated on a medium that contained dibasic calcium phosphate as the only phosphorus source (M. E. Trujillo, unpublished data). These data suggest that the biodiversity of phosphate-solubilizing bacteria is still poorly understood and that isolation of these micro-organisms is not restricted to the soil. On the other hand, Gram-positive bacteria with high

Table 1. Characteristics that differentiate *Microbacterium ulmi* sp. nov. XIL02^T from its nearest phylogenetic neighbours

+, Positive; −, negative; V, variable; ND, not determined; W, weak. Abbreviations: ai, anteiso-branched; i, iso-branched; Fuc, fucose; Gal, galactose; Man, mannose; Rha, rhamnose; 6dTal, 6-deoxytalose; Xyl, xylose; Lys, lysine; Orn, ornithine. Data from Behrendt *et al.* (2001), Matsuyama *et al.* (1999) and this study. Cells of all three species are short rods.

Characteristic	Strain XIL02^T	*M. imperiale*	*M. arborescens*
Colony colour	White	Red–orange	Dirty orange
Motility*	−	+	+
Catalase	W	+	+
Hydrogen sulfide	−	V	+
Chemotaxonomic characteristics:			
Peptidoglycan type	B2β	B1β	B1β
Cell-wall sugars	Gal, Fuc, Xyl, Rha	Rha, Man, Gal	6dTal, Man, Gal
Cell-wall diamino acid	Orn	Lys	Lys
Major fatty acids	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{16:0}$
Major menaquinones	MK-12, MK-13, MK-11, M-14, MK-10	MK-11, MK-12	MK-11, MK-12
Growth on sole carbon sources:			
L-Arabinose	−	+	+
Malate†	−	+	+
N-Acetylglucosamine	−	W	+
Citrate	−	−	W
Phenylacetate	+	−	−
Hydrolysis of:			
Starch	+	+	−
Gelatin	−	−	+

*67 % of the *Microbacterium* species described are non-motile.

†50 % of *Microbacterium* strains positive, 33 % negative, while the remaining 17 % either present a weak reaction or different results have been reported.

G+C content have been observed as dominant members of phosphate-removing activated-sludge communities (Bond *et al.*, 1995; Maszenan *et al.*, 1999) and are believed to play a major role in phosphorus turnover. It cannot be concluded whether phosphate removal is linked to the solubilization of rock phosphate; however, a better knowledge of these processes will improve our understanding of the diversity and interactions of these micro-organisms.

The presence of the D-diamino acid D-ornithine in the cell wall indicated that strain XIL02^T contained the unusual peptidoglycan type B2 (Schleifer & Kandler, 1972), which has been reported for 67 % of strains that currently belong to the genus *Microbacterium* (Behrendt *et al.*, 2001). Other species, including *M. arborescens* and *M. imperiale*, contain L-lysine as the cell-wall diamino acid (Behrendt *et al.*, 2001; Takeuchi & Hatano, 1998). The cell-wall sugars detected for strain XIL02^T were galactose, fucose, xylose and rhamnose, while mannose was not found. Galactose and rhamnose have been found in cell walls of many other *Microbacterium* species, while fucose has only been reported for *Microbacterium aurantiacum* and *Microbacterium aurum* and xylose has been detected in *Microbacterium chocolatum* and *Microbacterium laevaniformans*. The cellular fatty acid pattern of strain XIL02^T was composed of iso- and anteiso-branched fatty acids. The main fatty acids detected were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. These results are in accordance with those reported for the genus *Microbacterium*. The major isoprenoid quinones detected for this strain were MK-12 and MK-13, with minor amounts of MK-11 and MK-14 and traces of MK-10. This pattern has been reported for other *Microbacterium* species, including *Microbacterium arabinogalactonolyticum*, *Microbacterium esteraromaticum*, *Microbacterium terregens*, *Microbacterium trichothecenolyticum* and *Microbacterium keratanolyticum* (Behrendt *et al.*, 2001). The major polar lipids detected for strain XIL02^T were phosphatidylglycerol, diphosphatidylglycerol (cardiolipin) and an unknown glycolipid. Chemotaxonomic differences found between strain XIL02^T and its closest phylogenetic relatives are shown in Table 1.

The G+C content of strain XIL02^T was 69 mol%. This value is similar to those obtained in species from the genus *Microbacterium*.

DNA–DNA reassociation studies were used to confirm the species status of the novel isolate in relation to its closest phylogenetic neighbours. The results of DNA–DNA hybridization showed that strain XIL02^T presented 43·9 and 32·6 % relatedness, respectively, with *M. arborescens* DSM 20754^T and *M. imperiale* DSM 20530^T. These results indicate that isolate XIL02^T does not belong to either of these species

when the recommendation of a threshold value of 70 % DNA–DNA similarity for species definition is considered (Wayne *et al.*, 1987).

Therefore, on the basis of phylogenetic, chemotaxonomic and phenotypic data, we propose that isolate XIL02^T should be classified as the type strain a novel species, for which the name *Microbacterium ulmi* sp. nov. is proposed.

Description of *Microbacterium ulmi* sp. nov.

Microbacterium ulmi (ul′mi. L. gen. fem. n. *ulmi* of the elm tree).

Gram-positive, aerobic or facultatively anaerobic, non-motile, non-spore-forming rods. It grows between 15 and 37 °C. The pH range for growth is 5–8. Catalase- and oxidase-negative. Utilizes D-arabinose, carboxymethylcellulose, cellobiose, D-fructose, gentiobiose, maltose, mannitol, D-mannose, phenylacetate, starch and xylan as carbon sources. Does not use L-arabinose, malate, *N*-acetyl glucosamine or citrate as carbon sources. Aesculin, casein and gelatin are hydrolysed; does not reduce nitrate. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, tryptophan deaminase and urease are not produced. Chemotaxonomic properties are listed in Table 1.

The type strain is strain XIL02^T (=LMG 20991^T=CECT 5976^T), isolated from sawdust of a decayed tree of *Ulmus nigra*.

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