Xylanimonas cellulosilytica gen. nov., sp. nov., a xylanolytic bacterium isolated from a decayed tree (*Ulmus nigra*)

Raúl Rivas, Manuel Sánchez, Martha E. Trujillo, José Luis Zurdo-Piñeiro, Pedro F. Mateos, Eustoquis Martínez-Molina and Encarna Velázquez

Correspondence
Encarna Velázquez
evp@gugu.usal.es

Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, Lab. 209, Universidad de Salamanca, 37007 Salamanca, Spain

A bacterial strain, designated XIL07^T, isolated from a decayed tree, *Ulmus nigra*, in Salamanca (Spain) produced abundant cellulases and xylanases. The micro-organism was Gram-positive, aerobic, coccoid and non-motile. Growth was observed on many carbohydrates, including cellulose and xylan as the sole carbon sources. No growth was observed with acetate, citrate, gluconate, inositol, malate or mannitol as carbon sources. The strain showed very weak catalase activity. HPLC analysis of menaquinones revealed two peaks: the main peak corresponded with MK-9(H₄) and the smaller one with MK-8(H₄). The major fatty acid found was anteiso-C15:0 (12-methyl tetradecanoic acid). Mycolic acids were absent. The polar lipids detected were phosphatidyl-glycerol, diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides. Peptidoglycan type was A4 α , L-Lys–D-Asp. The cell-wall sugars detected were galactose and rhamnose. The complete 16S rDNA sequence of strain XIL07^T was obtained and phylogenetic analysis based on the neighbour-joining method indicated that this bacterium belongs to the high-G + C-content Gram-positive bacteria and that the closest related genera are *Promicromonospora* and *Cellulosimicrobium*. The DNA G + C content was 73 mol%. According to the data obtained in this work, this bacterium belongs to a new genus in the family *Promicromonosporaceae* and the name *Xylanimonas cellulosilytica* gen. nov., sp. nov. is proposed; the type strain is strain XIL07^T (=LMG 20990^T =CECT 5975^T).

INTRODUCTION

The families *Cellulomonadaceae* and *Promicromonosporaceae* are closely related and are Gram-positive bacteria with high G + C content within the order *Actinomycetales*. The taxonomy of these two families has been the subject of important changes in recent years: species from the genus *Oerskovia* have been reclassified into the genus *Cellulomonas* (Stackebrandt *et al.*, 1980, 1982); the genus *Rarobacter* has been included in the new family *Rarobacteraceae* (Stackebrandt & Schumann, 2000); and *Cellulomonas cellulans* has been reclassified in the new genus *Cellulosimicrobium* (Schumann *et al.*, 2001). This latter genus has been separated from the family *Cellulomonadaceae* (Schumann *et al.*, 2001) and included in the family *Promicromonosporaceae* (Stackebrandt *et al.*, 1997), which also includes the genus *Promicromonospora*. One of the most common characteristics of species included in the families *Cellulomonadaceae* and *Promicromonosporaceae* is

their ability to hydrolyse cellulose, often linked to the hydrolysis of xylan and other polysaccharides. Cellulolytic bacteria are commonly isolated from soil, but taking into account that these micro-organisms can destroy wood, cellulolytic and xylanolytic species may be found in decayed trees. From these sources, a strain has been isolated, strain XIL07^T, that hydrolyses xylan. The present investigation was designed to establish the taxonomic position of isolate XIL07^T; phylogenetic data obtained suggest that this micro-organism belongs to a new genus closely related to members of the family *Promicromonosporaceae*. The chemotaxonomic and phenotypic data support these results. The name *Xylanimonas cellulosilytica* gen. nov., sp. nov. is proposed for strain XIL07^T.

METHODS

Isolation of strains and culture conditions. A sample of sawdust from a decayed tree, *Ulmus nigra*, was collected under aseptic conditions and 1 g was suspended in sterile water and stirred for 30 min. From this suspension, 100 μ l was spread onto XED medium (xylan, 0.7%; yeast extract, 0.3%; agar, 2.5%) and incubated at 28 °C. A bacterial strain, strain XIL07^T, was isolated that hydrolysed xylan. This strain was maintained as a glycerol suspension (25%, v/v) at –80 °C.

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain XIL07^T is AF403541.

Morphology. Strain XIL07^T was grown in YED medium (yeast extract, 0.5%; glucose, 0.7%; agar, 1.5%) for 48 h to check for motility by phase-contrast microscopy. The cells were also stained according to the classical Gram procedure described by Doetsch (1981). For EM, the cells were grown in liquid YED for 3 days at 180 r.p.m. and 28 °C. The samples were fixed overnight in 2% glutaraldehyde. Sections were mounted according to Reynolds (1963) and were examined at 80 kV with a Zeiss EM 209 TEM.

Chemotaxonomic characterization. Strain XIL07^T was cultivated in TSB (Becton Dickinson, BBL) 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 and the presence of mycolic acids. Amino acid and cell-wall sugar analyses were performed according to described procedures (Staneck & Roberts, 1974). Menaquinone and cellular fatty acid composition were determined as described by Zimmermann *et al.* (1998).

Phenotypic tests and determination of DNA base composition. Isolate XIL07^T was grown in YED plates for 48 h. Catalase production was assayed using 0.3% hydrogen peroxide with one colony taken from YED agar plates. Oxidase activity was detected using N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride. Cellulases were detected after 7 days incubation in plates containing 0.5% carboxymethylcellulose as the carbon source, 0.3% yeast extract and 1.5% agar. Plates were stained with a 1% Congo Red water solution. Casein activity was detected on skimmed milk agar after 7 days incubation. Other physiological and biochemical tests were done using the API 20NE, API 20E and API 50CH strips (bioMérieux) according to the manufacturer's instructions.

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

16S rDNA analysis. DNA extraction was carried out as described previously (Rivas *et al.*, 2001). PCR amplification of 16S rDNA was carried out using primers 5'-AGAGTTTGATCTGGCTCAG-3' and 5'-AAGGAGGTGATCCANCCRCA-3' under conditions described previously (Velázquez *et al.*, 2001). The sequence reaction was performed on an ABI377 sequencer (Applied Biosystems) using a BigDye terminator v3.0 cycle sequencing kit as supplied by the manufacturer. The following primers were used (corresponding positions in the *Escherichia coli* small-subunit rDNA sequence are given in parentheses): 5'-AGAGTTTGATCTGGCTCAG-3' (8–27); 5'-CTCCTACGGGAGGCGAGCAGT-3' (339–358); 5'-CAGCAGCCGC GGTAATAC-3' (519–537); 5'-GCCTGGGGAGTACGGCCGCA-3' (849–869); 5'-ACTGCTGCCTCCCGTAGGAG-3' (1093–1112); and 5'-AAGGAGGTGATCCANCCRCA-3' (1498–1522). The sequence obtained was compared with those from GenBank using the FASTA program (Pearson & Lipman, 1988). Sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1997). The distances were calculated according to Kimura's 2-parameter method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The PHYLIP package (Felsenstein, 1993) was used for all analyses. The trees were rooted using *Rarobacter faecitabidus* DSM 4813^T as the outgroup.

RESULTS AND DISCUSSION

Morphology

The strain isolated from sawdust was a Gram-positive, non-motile, coccoid organism (1.1 × 0.8 µm). Fig. 1 shows the

Fig. 1. EMs of thin sections of strain XIL07^T grown in YED medium for 48 h. The cells are coccoid (a; bar, 0.15 µm) and at high magnification (b; bar, 0.09 µm), cell division is seen, with membrane and cell-wall formation.

cell morphology of strain XIL07^T as observed by TEM. Colonies were white- to cream-coloured on XED medium. They were smooth and mostly flat.

Phenotypic tests

Strain XIL07^T showed high cellulase and xylanase activity. Phenotypic characteristics of strain XIL07^T and species of closely related genera are shown in Table 1. According to the data, this strain differs from *Promicromonospora* in mycelium formation, caseinase, catalase and urease production, and growth on acetate, citrate, malate, mannitol and D-raffinose as sole carbon source. Strain XIL07^T differs from *Cellulosimicrobium* in mycelium formation, catalase production and growth with acetate, gluconate, inositol and mannitol as sole carbon source.

Table 1. Characters that differentiate the genus *Xylanimonas* from other genera of the family *Promicromonosporaceae*

Data from Bakalidou *et al.* (2002), Goodfellow (1989), Kalakoutsii *et al.* (1989) and Schumann *et al.* (2001). +, Positive; –, negative; v, variable; ND, not determined; w, weak.

Character	<i>Xylanimonas</i>	<i>Promicromonospora</i>	<i>Cellulosimicrobium</i>
Shape	Coccoid	Rod-like, coccoid, Y- or V-shaped	Short rods
Aerial hyphae	–	+	–
Catalase	w	+	+
Fermentation	w	Rare	+
Peptidoglycan composition	L-Lys–D-Asp	L-Lys–Ala	L-Lys–D-Ser–D-Asp
Cell-wall sugars*	Gal, Rha	Gal	Rha, Fuc, Gal, Man, Glu
Major menaquinones	MK-9(H ₄), MK-8(H ₄)	MK-9(H ₄)	MK-9(H ₄)
Predominant cellular fatty acids	ai-C _{15:0} , i-C _{15:0}	ai-C _{15:0} , i-C _{15:0}	ai-C _{15:0} , i-C _{15:0} , C _{16:0} , i-C _{16:0}
Phospholipid composition†	PG, DPG, PI, PIM‡	PG, PI	PG, PI, PIM, PET
Growth on sole carbon source:			
Acetate	–	+	+
Malate	–	+	–
Gluconate	–	ND	+
Citrate	–	+	ND
D-Raffinose	–	+	–
Mannitol	–	+	+
Inositol	–	v	+
Hydrolysis of:			
Cellulose	+	+	+
Casein	–	+	ND
Urea	–	+	ND

*Gal, Galactose; Rha, rhamnose; Fuc, fucose; Man, mannose; Glu, glucose.

†PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PET, phosphatidylethanolamine.

‡Other unidentified phosphoglycolipids were detected.

Chemotaxonomic characteristics and DNA base composition

The results of the chemotaxonomic analyses are shown in Table 1. The peptidoglycan of strain XIL07^T contained the amino acids L-lysine and D-aspartic acid. This composition differs from that reported for peptidoglycan of the genus *Promicromonospora* (Kalakoutsii *et al.*, 1989), which contains L-lysine and alanine, and from peptidoglycan of the genus *Cellulosimicrobium* (Schumann *et al.*, 2001), where L-lysine, D-serine and D-aspartic acid have been detected.

The cell-wall sugars detected for strain XIL07^T were galactose and rhamnose. In many strains of the genus *Promicromonospora*, only galactose has been detected (Kalakoutsii *et al.*, 1989). In the case of *Cellulosimicrobium* species, fucose, galactose, glucose, mannose and rhamnose have been found (Bakalidou *et al.*, 2002; Schumann *et al.*, 2001).

As expected for members of the family *Promicromonosporaceae* (Kalakoutsii *et al.*, 1989; Bakalidou *et al.*, 2002), mycolic acids were not detected.

The cellular fatty acid pattern of strain XIL07^T was composed of iso- and anteiso-branched fatty acids. The main

fatty acid detected was anteiso-C15:0 (12-methyl tetradecanoic acid). According to the published data, the cellular fatty acid pattern of the genus *Promicromonospora* is made up of iso- and anteiso-C15:0 (Kalakoutsii *et al.*, 1989), whereas for the genus *Cellulosimicrobium* the fatty acid pattern reported contains anteiso-C15:0, iso-C15:0, C16:0 and iso-C16:0 (Schumann *et al.*, 2001).

HPLC analysis of the menaquinones revealed two peaks; the main peak corresponded to MK-9(H₄) and the smaller one to MK-8(H₄). MK-9(H₄) is the major menaquinone in both *Promicromonospora* and *Cellulosimicrobium* (Kalakoutsii *et al.*, 1989; Schumann *et al.*, 2001).

The major polar lipids detected for strain XIL07^T were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and other unidentified phosphoglycolipids. The main difference between *Promicromonospora* and XIL07^T is the presence of phosphatidylinositol mannosides (Goodfellow, 1989). With respect to *Cellulosimicrobium*, strain XIL07^T contains diphosphatidylglycerol, but not phosphatidylethanolamine (Bakalidou *et al.*, 2002).

The DNA G+C content was 73 mol%. This value is

similar to those obtained for species of the genera *Cellulosimicrobium* and *Promicromonospora*.

16S rDNA analysis

The complete 16S rDNA sequence for isolate XIL07^T was obtained. A comparison with 16S rDNA sequences held in GenBank indicated that the organism is phylogenetically related to members of the family *Promicromonosporaceae*. The 16S rDNA sequence of strain XIL07^T contained the nucleotide signatures of this family according to Stackebrandt & Schumann (2000). Fig. 2 shows the phylogenetic tree obtained by the neighbour-joining method. Strain XIL07^T formed a separate group from the *Cellulomonas* species. This group included strain XIL07^T, *Promicromonospora citrea* DSM 43110^T, *Promicromonospora sukumoe* DSM 44121^T and *Cellulosimicrobium cellulans* DSM 43879^T. The 16S rDNA sequence of strain XIL07^T showed 95.02 % similarity with that of *P. citrea*, 95.03 % with that of *P. sukumoe* and 94.67 % with that of *C. cellulans*. These results indicate that the isolate merits genus status.

Recovery of *Promicromonospora enterophila* DSM 43852^T in the branch corresponding to *Cellulomonas* species indicates that this strain probably belongs to the genus *Cellulomonas* (Bakalidou *et al.*, 2002; M. Takeuchi and others, unpublished results).

Therefore, on the basis of phylogenetic, chemotaxonomic and phenotypic data, it is proposed that isolate XIL07^T

should be classified in a new genus, *Xylanimonas*, as *Xylanimonas cellulosilytica* gen. nov., sp. nov.

Description of *Xylanimonas* gen. nov.

Xylanimonas (Xy.lan.i.mo.nas. N.L. n. *xylanum* xylan, a polysaccharide; Gr. n. *monas* a unit; N.L. n. *Xylanimonas* a monad from xylan).

Gram-positive, non-spore-forming, coccoid cells. Aerobic or facultatively anaerobic, chemo-organotrophic and xylanolytic. Aerial mycelium is not formed. Optimal growth temperature is 30 °C; pH 7 is optimal for growth. Oxidase is produced, but catalase production is weak. Phylogenetically related to members of the family *Promicromonosporaceae*. Peptidoglycan type is A4 α , L-Lys-D-Asp. Cell-wall sugars are galactose and rhamnose. Major menaquinones are MK-9(H₄) and MK-8(H₄); predominant fatty acid is anteiso-C15:0 (12-methyl tetradecanoic acid). Mycolic acids are absent. Contains phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and other unidentified phosphoglycolipids. The type species of the genus is *Xylanimonas cellulosilytica*.

Description of *Xylanimonas cellulosilytica* sp. nov.

Xylanimonas cellulosilytica (ce.l.lu.lo.si.ly.ti.ca. N.L. n. *cellulosum* cellulose; Gr. adj. *lytikos* dissolving; N.L. adj. *lyticus* dissolving; N.L. fem. adj. *cellulosilytica* cellulose-dissolving).

Fig. 2. Comparative sequence analysis of 16S rDNA from *Xylanimonas cellulosilytica* XIL07^T and representative strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 5 nt substitutions per 100 nt.

Gram-positive, aerobic or facultatively anaerobic, non-motile, non-spore-forming coccoid cells of $1.1 \times 0.8 \mu\text{m}$. Colonies on YED are circular, convex, white, opaque and usually 1–3 mm in diameter within 7 days at 28 °C. Utilizes L-arabinose, carboxymethylcellulose, mannose, maltose, rhamnose, starch and xylan as sole carbon sources. Does not grow in acetate, adonitol, D-arabinose, citrate, dulcitol, erythritol, gluconate, inositol, inulin, malate, mannitol, raffinose, sorbitol, L-sorbose, L-xylose, methyl α -D-glucoside, methyl α -D-mannoside, N-acetylglucosamine or methyl β -xyloside. Produces acid from amygdalin, L-arabinose, arbutin, cellobiose, fructose, galactose, gentobiose, glucose, glycerol, glycogen, lactose, lyxose, maltose, mannose, melizitose, rhamnose, salicin, sucrose, trehalose, turanose and D-xylose. Actively produces amylases, cellulases, gelatinase, xylanases and β -galactosidase. Aesculin is hydrolysed. Reduces nitrate and produces acetoin (Voges–Proskauer medium). Does not produce arginine dihydrolase, caseinase, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase or urease. The type strain is XIL07^T (= LMG 20990^T = CECT 5975^T), which has a DNA G + C content of 73 mol%.

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