Tepidimicrobium xylanilyticum sp. nov., an anaerobic xylanolytic bacterium, and emended description of the genus *Tepidimicrobium*

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A novel, xylanolytic, anaerobic, moderately thermophilic bacterium, strain PML14^T, was isolated from the sludge of a thermophilic anaerobic digester treating municipal solid waste and sewage in Beijing, China. The strain was a Gram-positive, spore-forming and motile rod. Growth of the novel strain was observed at 25–67 °C (optimum 60 °C) and pH 5.8–9.3 (optimum pH 8.5). Strain PML14^T grew on a number of carbohydrates, including xylan, xylose, glucose and cellobiose, and a variety of proteinaceous compounds, including peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, L-cysteine, L-serine, L-lysine, L-glycine, L-threonine, L-methionine and pyruvate. The fermentation products from glucose included acetate, ethanol, butyrate, hydrogen and carbon dioxide. Propionate was produced from xylan in addition to other compounds. Fe(III), 9,10-anthraquinone 2,6-disulfonate and thiosulfate were reduced with peptone as the electron donor. NH_3 was produced. Indole was not produced. Gelatin was not hydrolysed. The DNA G+C content of strain PML14^T was 36.2±0.8 mol% (T_m). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain PML14^T was related to the members of cluster XII of the clostridia, most closely to *Tepidimicrobium ferriphilum* SB91^T with 93.8 % 16S rRNA gene sequence similarity. On the basis of polyphasic evidence from this study, it is suggested that strain PML14^T (=CGMCC 1.5080^T=JCM 15035^T) represents a novel species of the genus *Tepidimicrobium*, for which the name *Tepidimicrobium xylanilyticum* sp. nov., is proposed. An emended description of the genus *Tepidimicrobium* is also provided.

Complex organic matter is degraded completely to CO_2 and CH_4 by the synergy of several trophic micro-organisms in methanogenic environments (Zehnder, 1978). In comparison with thermochemical and electrochemical hydrogen production processes, hydrogen production through biological processes could be a cost-effective, pollution-free and energy-saving alternative energy source in the future (Das & Veziroglu, 2001). Moreover, bio-hydrogen processes utilize renewable resources such as plant biomass, thus making hydrogen production an attractive and environmentally friendly process. Many strict anaerobes and facultative anaerobic chemoheterotrophs, such as clostridia and enteric bacteria, are efficient hydrogen producers (Nandi & Sengupta, 1998; Das & Veziroglu, 2001). During our survey of hydrogen-producing bacteria from a variety of polysaccharide- and protein-rich environments, several strains producing abundant hydrogen were isolated. A strain, designated PML14^T, was isolated from the sludge of a thermophilic anaerobic digester treating municipal solid waste and sewage and its characteristics were determined.

Tepidimicrobium ferriphilum DSM 16624^T was purchased from the DSMZ. Strain PML14^T was isolated in pre-reduced peptone-yeast extract-glucose (PYG) medium (Holdeman *et al.*, 1977) by serial dilution and the Hungate roll-tube technique (Hungate, 1969). Single colonies were picked, transferred to the same broth and incubated at 60 °C for 2 days. The roll-tube procedure was repeated several times until a pure culture was obtained. The purity of strain PML14^T was confirmed by observing similar morphologies of colonies on agar and of cells under a microscope. Routine cultivation was performed with PYG broth in anaerobic tubes sealed with butyl rubber stoppers under a gaseous atmosphere of N_2 (1.01×10^5 Pa).

Spore staining was performed using the Schaeffer and Fulton staining method as described by Barrow & Feltham (1993). Substrate utilization tests, light microscopy and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PML14^T is EF522948.

analytical techniques were performed as described previously (Niu *et al.*, 2008). Physiological studies on the temperature, pH and salinity ranges for growth were carried out in PYG broth. Genomic DNA was extracted and purified using the method of Marmur (1961). The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a DU 800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as the reference strain. The 16S rRNA gene sequence amplification, sequencing and sequence analysis were performed as described previously (Chen & Dong, 2004).

Cells of strain PML14^T were straight or slightly curved rods with slightly pointed ends, 0.4–0.5 μm in diameter and 4.0–10.0 μm in length. The cells occurred singly or in pairs, were peritrichously flagellated and exhibited slight twitching motility. Terminal spherical spores were observed when grown on PY medium (PYG without glucose) and the novel strain could survive heat treatment at 80 °C for 10 min. The cells stained Gram-negative in all of the growth phases, however a Gram-positive bacterial cell-wall structure was revealed by electron microscopy. Colonies on PYG agar were white, round and 0.5 mm in diameter after cultivation at 60 °C for 72 h.

Strain PML14^T grew strictly anaerobically. Growth occurred at 25–67 °C and pH 5.8–9.3, with optimum growth at 60 °C and pH 8.5, respectively. The strain could grow in the presence of 0–4.5 % (w/v) NaCl, with optimum growth at 3.0 % (w/v). The doubling time of strain PML14^T was 4.1 h when growing on PYG at 60 °C. The strain used a number of proteinaceous compounds and carbohydrates, including peptone, tryptone, Casamino acids, yeast extract, beef extract and casein hydrolysate (each at 10 g l^{-1}), L-cysteine, L-serine, L-lysine, L-glycine, L-threonine, L-methionine and pyruvate (each at 20 mM), and xylan, xylose, glucose and cellobiose (each at 5 g l^{-1}). The following substrates were not utilized: mannose, lactose, galactose, sucrose, fructose, maltose, L-arabinose, rhamnose, glycerol, lactate, malate, fumarate, L-alanine, L-arginine, L-proline, casein, starch, CM-cellulose, filter paper and chitin. The fermentation products from glucose included hydrogen, carbon dioxide, acetate, ethanol, butyrate and a trace amount of propionate. While growing in xylan, strain PML14^T produced substantial amounts of acetate, ethanol, propionate, hydrogen and carbon dioxide. Strain PML14^T did not hydrolyse gelatin or curdle milk. NH_3, but not indole, was produced. When using peptone as electron donor, strain PML14^T reduced 9,10-anthraquinone 2,6-disulfonate (AQDS) (20 mM) to 9,10-anthrahydroquinone 2,6-disulfonate and thiosulfate (20 mM) to hydrogen sulfide. Fe(III) citrate and nitrate were also reduced. However, sulfate, selenite and fumarate were not used as electron acceptors with peptone (10 g l^{-1}) as the electron donor. The G+C content of the genomic DNA of strain PML14^T was 36.2±0.8 mol% (T_m).

To ascertain the phylogenetic position of strain PML14^T, an almost-complete 16S rRNA gene sequence (1528 bp) was compared with the most similar sequences and those of the representatives of clostridia retrieved from GenBank. On the basis of a consensus 1345 bp, a 16S rRNA gene sequence phylogenetic tree rooted with *Peptostreptococcus hydrogenalis* GIFU 7662^T was constructed (Fig. 1). Phylogenetic analysis showed that strain PML14^T was affiliated to the phylum *Firmicutes*, and belonged to cluster XII of the clostridia (Collins *et al.*, 1994). The closest relative was *T. ferriphilum* SB91^T with a sequence similarity of 93.8 %. The 16S rRNA gene sequence similarity levels of the novel strain with those of other members of cluster XII ranged between 86.5 and 92.8 %. Phylogenetic trees constructed by the neighbour-joining, UPMGA and minimum-evolution algorithms showed the same topology (Fig. 1).

As strain PML14^T was found to be phylogenetically related to *T. ferriphilum* SB91^T, the phenotypic characteristics of the two strains were determined in parallel. The phenotypic characterization (Table 1) revealed that strain PML14^T could represent a novel species of the genus *Tepidimicrobium*. Morphologically, strain PML14^T formed spores in PY medium, while *T. ferriphilum* did not. Strain PML14^T also differed from *T. ferriphilum* by producing hydrogen and utilizing glucose, pyruvate and xylan. In

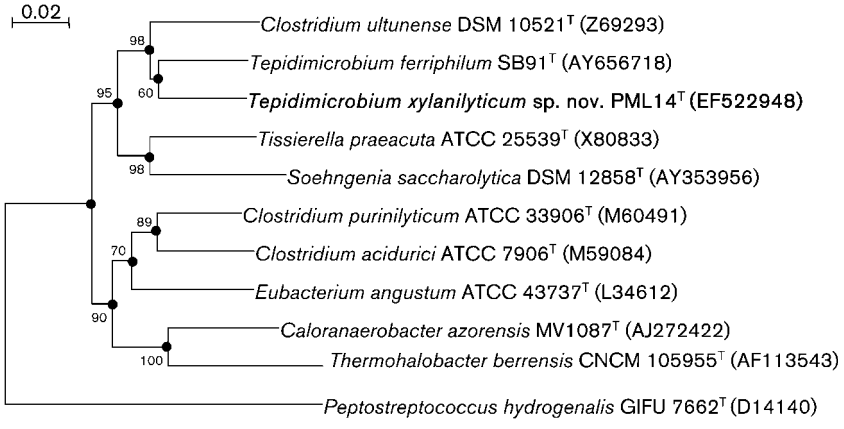

Fig. 1. Phylogenetic dendrogram of strain PML14^T and related species, based on 16S rRNA gene sequences and constructed using the neighbour-joining method. Percentages at nodes (>50 %) represent levels of bootstrap support based on 1000 resamplings. Solid circles indicate that the corresponding nodes were also recovered with UPMGA and minimum-evolution methods. *Peptostreptococcus hydrogenalis* GIFU 7662^T was used as the outgroup. Bar, 2 % sequence divergence.

Table 1. Differential characteristics of strain PML14^T and its closest phylogenetic relatives

Strains: 1, PML14^T (data from this study); 2, *Tepidimicrobium ferriphilum* DSM 16624^T (this study); 3, *Clostridium ultunense* DSM 10521^T (Schnürer *et al.*, 1996).

Characteristic	1	2	3
Colonies	Round, whitish	Spherical, brown	Disk-shaped, whitish
Spore formation	+	−	+
Optimum growth at:			
Temperature (°C)	60	50	37
pH	8.5	8.0	7.0
Glucose fermentation	+	−	+
Pyruvate utilization	+	−	+
Xylan utilization	+	−	−
Hydrogen production	+	−	+
Fe(III) reduction	+	+	−
Indole production	−	−	+
DNA G+C content (mol%)	36.2	33.0	32.0

addition, the optimum growth temperature of strain PML14^T differed by 10 °C from that of *T. ferriphilum* (Slobodkin *et al.*, 2006 and this study). Therefore, we propose strain PML14^T as the type strain of a novel species of the genus *Tepidimicrobium*, *Tepidimicrobium xylanilyticum* sp. nov. Accordingly, the description of the genus *Tepidimicrobium* is emended.

Emended description of the genus *Tepidimicrobium* (Slobodkin *et al.* 2006)

The genus description is the same as that given by Slobodkin *et al.* (2006) except that abilities to form spores and to reduce elemental sulfur, fumarate and selenite are variable.

Description of *Tepidimicrobium xylanilyticum* sp. nov.

Tepidimicrobium xylanilyticum (xy.la.ni.ly′ti.cum. N.L. n. *xylanum* xylan, a plant polymer; Gr. adj. *lytikos* able to loosen, able to dissolve; N.L. adj. *lyticus -a -um* dissolving; N.L. neut. adj. *xylanilyticum* xylan-dissolving).

Cells are Gram-positive, straight or slightly curved rods with slightly pointed ends, 0.4–0.5 μm in diameter and 4.0–10.0 μm in length. Cells occur singly or in pairs and exhibit twitching motility due to peritrichous flagella. Occasionally, terminal spherical spores are formed. Colonies on PYG agar are white, round and 0.5 mm in diameter after cultivation at 60 °C for 72 h. Growth occurs at 25–67 °C (optimum 60 °C) and pH 5.8–9.3 (optimum pH 8.5). Growth occurs in the range of 0–4.5 % (w/v) NaCl, with optimum growth at 3.0 % (w/v). Utilizes a variety of substrates, including peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, L-cysteine, L-serine, L-lysine, L-glycine, L-threonine, L-methionine, pyruvate, xylan, xylose, glucose and cellobiose. Acetate, ethanol, butyrate and hydrogen are the main products when glucose is available as the sole substrate, and a trace amount of propionic acid is also produced. The following substrates are not used: L-histidine, L-leucine, L-phenylalanine, L-valine, L-glutamine, L-tyrosine, tryptophan, L-isoleucine, L-proline, aspartate, L-alanine, L-arginine, ribose, fructose, mannose, lactose, galactose, L-arabinose, rhamnose, sucrose, maltose, melibiose, raffinose, aesculin, glycogen, inulin, casein, starch, glycerol, lactate, malate, fumarate, salicin, sorbose, trehalose, adonitol, amygdalin, dulcitol, erythritol, inositol, mannitol, sorbitol, ribitol, methanol, ethanol, 1-propanol, citrate, fumarate, malate, malonate, hippurate, sodium gluconate, butane diacid, β-hydroxybutyric acid, phenylacetic acid, CM-cellulose, chitin and filter paper. Milk is not curdled and gelatin is not liquefied. Produces NH_3 from PYG and H_2S from thiosulfate. Indole is not produced.

The type strain, PML14^T (=CGMCC 1.5080^T=JCM 15035^T), was isolated from the sludge of a thermophilic anaerobic digester treating municipal solid waste and sewage in Beijing, China. The G+C content of the genomic DNA of the type strain is 36.2±0.8 mol% (T_m).

Acknowledgements

This study was supported by National Basic Research Program of China (2004CB719602).

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