Cellulomonas phragmiteti sp. nov., a cellulolytic bacterium isolated from reed (*Phragmites australis*) periphyton in a shallow soda pond

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An alkalitolerant and moderately halophilic strain, designated KB23^T, characterized by optimal growth at pH 8.0–9.0 and in the presence of 5–7 % (w/v) NaCl, was isolated from a reed (*Phragmites australis*) periphyton sample originating from an extremely shallow, alkaline soda pond located in Hungary. Cells of strain KB23^T were Gram-stain-positive, motile straight rods. Strain KB23^T was facultatively anaerobic, catalase-positive, oxidase-negative and contained peptidoglycan type A4β (L-Orn–D-Asp). MK-9(H4) was the predominant isoprenoid quinone and anteiso-C_{15:0}, C_{16:0} and anteiso-C_{15:1} were the major cellular fatty acids. The DNA G+C content of strain KB23^T was 74.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that this strain belongs to the genus *Cellulomonas* and that it is related most closely to *Cellulomonas flavigena* DSM 20109^T (97.35 % similarity), *Cellulomonas terrae* DB5^T (96.81 %), *Cellulomonas iranensis* O^T (96.75), *Cellulomonas chitinilytica* X.bu-b^T (96.60 %), *Cellulomonas persica* I^T (96.53 %), *Cellulomonas composti* TR7-06^T (96.45 %), *Cellulomonas biazotea* DSM 20112^T (96.34 %) and *Cellulomonas fimi* DSM 20113^T (96.20 %). According to these results, together with DNA–DNA hybridization and physiological data, strain KB23^T is considered to represent a novel species of the genus *Cellulomonas*, for which the name *Cellulomonas phragmiteti* sp. nov. is proposed. The type strain is KB23^T (=DSM 22512^T =NCAIM B002303^T).

Soda lakes and ponds are characterized by very high productivity as well as high levels of activity of the various decomposition processes which take place (Grant *et al.*, 1990). Bacteria isolated from such environments show considerable phylogenetic and physiological diversity (Zavarzin *et al.*, 1999; Jones *et al.*, 1998; Duckworth *et al.*, 1996; Zhilina & Zavarzin, 1994). Much of the physiological diversity of these bacteria is based on their disposal of various enzymes, influencing the efficiency of self-purification processes, including the degradation of

autochthonous and allochthonous organic matter in aquatic environments.

During a polyphasic study of plant-associated bacterial communities, a large number of bacterial strains were isolated (Rusznyák *et al.*, 2008) from reed periphyton originating from a shallow soda pond (Kelemen-szék, Kiskunság National Park, Hungary, 46° 47' N 19° 11' E) in April 2004. Conductivity and pH of the pond water at the time of sampling were 1960 µS cm⁻¹ and pH 9.1. On the basis of preliminary 16S rRNA gene sequence data, one strain, designated KB23^T, was found to be a member of the genus *Cellulomonas* in the suborder *Micrococccineae*, being able to digest cellulose. The aim of this study was to determine the taxonomic position of strain KB23^T. Further study of this strain, based on a polyphasic approach that included chemotaxonomic, physiological and DNA–DNA hybridization analyses, confirmed its taxonomic position as a representative of a novel species within the genus *Cellulomonas*.

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The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain KB23^T is AM902253.

Two supplementary figures are available with the online version of this paper.

From the sampling site, 10-cm-long reed (*Phragmites australis*) stem pieces were collected in five replicates from about 10–20 cm below the water surface. Biofilm samples were washed from the reed stems into sterile saline solution (0.89 g NaCl l⁻¹, pH 9) by using sterile brushes. Cultivation was from composite samples prepared from aliquots of the sample and serial dilutions were made. Strain KB23^T was isolated on King's B medium (Barrow & Feltham, 2003), adjusted to pH 9, following a 7 day incubation period at 25 °C under aerobic conditions. After isolation, strain KB23^T was cultivated by being transferred monthly onto King's B medium. Stock cultures were preserved as suspensions in glycerol (20 %, v/v) at -70 °C.

Colony morphology as well as cell morphology of Hucker's Gram-stained smears was observed. Motility of cells was examined in wet-mount preparations by phase-contrast microscopy. Standard biochemical tests were performed (catalase and oxidase reactions, oxidative and fermentative utilization of D-glucose, nitrate reduction, methyl red and Voges-Proskauer reaction, Simmon's citrate test, Baird-Parker's phosphatase activity, aesculin, casein, gelatin, starch, Tween 80 and DNA hydrolysis, hydrogen sulphide and indole production) according to Barrow & Feltham (2003). Hydrolysis of cellulose was tested according to Horikoshi (1995). Other physiological and biochemical properties were further determined with API ZYM and API 50CH test kits (bioMérieux) according to the manufacturer's instructions. The pH range for growth was determined in nutrient broth (DSMZ Medium 1, www.dsmz.de) (supplemented with 50 g NaCl l⁻¹) for 7 days that was adjusted to pH 7.0–11.0 with KOH at intervals of 1.0 pH unit. The NaCl requirement for growth was studied in nutrient broth adjusted to pH 9.0 and supplemented with 0, 2.0, 5.0, 7.0, 10.0 and 12.0 % (w/v) NaCl for 7 days. Optical density alterations were observed at 600 nm over 1–7 days. Growth under anaerobic conditions was determined after incubating the strain on King's B agar in a GasPak jar (BBL) at 25 °C for 7 days. *Cellulomonas flavigena* DSM 20109^T was used as reference strain for the investigation of phenotypic properties of strain KB23^T under the same laboratory conditions.

Isolation of chromosomal DNA, PCR amplification and direct sequencing of the purified product were carried out as described previously (Rusznayk *et al.*, 2008). The complete 16S rRNA gene sequence of strain KB23^T was assembled by using the BioEdit program (Hall, 1999). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL X program (Thompson *et al.*, 1997). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1983) and the Jukes-Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were reconstructed by using the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Fitch, 1971) methods in the MEGA4 program (Tamura *et al.*, 2007), with bootstrap values based on 2000 replications (Felsenstein, 1985). The neighbour-joining

phylogenetic tree with Kimura's two-parameter model is shown in Fig. 1. Phylogenetic trees reconstructed with additional models are presented in Supplementary Fig. S1 available in IJSEM Online.

The peptidoglycan of strain KB23^T was isolated after disintegration of cells with glass beads in a Vibrogen cell mill (Johanna Otto GmbH) and subsequent trypsin digestion according to the method of Schleifer & Seidl (1985), and its structure was analysed by using the methods of Schleifer (1985), MacKenzie (1987) and Groth *et al.* (1996). For analysis of cell-wall sugars, the peptidoglycan preparation was hydrolysed in 0.5 M H₂SO₄ (2 h at 100 °C). H₂SO₄ was removed by 20 % (N,N)-diocetyl-methylamine in chloroform according to Whiton *et al.* (1985) and the sugars in the hydrolysate were analysed by TLC on cellulose plates according to Stanek & Roberts (1974). Isoprenoid quinones were extracted from cells cultivated in liquid Rich medium (Yamada & Komagata, 1972) according to the method of Collins *et al.* (1977) and the profile was analysed by HPLC (HP 9001) (Groth *et al.*, 1997). Cellular fatty acids were extracted from cells cultivated on trypticase soy agar (Difco) at 28 °C according to Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996).

The G+C content of the chromosomal DNA was determined as described by Mesbah *et al.* (1989) by using reversed-phase HPLC (Shimadzu). For spectroscopic DNA-DNA hybridization, DNA was isolated with a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA-DNA hybridizations were carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) by using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

Strain KB23^T was facultatively anaerobic. Cells stained Gram-positive, were rod-shaped (0.4–0.6 × 1.0–1.5 µm), motile and did not form a mycelium. Strain KB23^T contained L-Orn-D-Asp as an interpeptide bridge in the peptidoglycan (type A4β; DSMZ type A21.4, http://www.dsmz.de/microorganisms/main.php?content_id=35). The predominant quinone was MK-9(H4). The sugars analysed in the purified cell wall were rhamnose, mannose and fucose. The DNA G+C content of strain KB23^T was 74.8 mol%.

High levels of 16S rRNA gene sequence similarity between the type strains of recognized species of the genus *Cellulomonas* and strain KB23^T (almost-complete 16S rRNA gene sequence, 1413 bp) supported its inclusion in the genus *Cellulomonas*. It was evident from Fig. 1 that strain KB23^T was related most closely to *C. flavigena* DSM 20109^T (97.35 % 16S rRNA gene sequence similarity), supported by a high bootstrap value. However, it was evident from DNA-DNA hybridization data that strain KB23^T and *C. flavigena* DSM 20109^T should not be

Fig. 1. Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationships between strain KB23^T and representatives of the genus *Cellulomonas*. Numbers at nodes indicate levels of bootstrap support (percentages) based on a neighbour-joining analysis of 2000 resampled datasets; only values >50% are shown. Bar, 0.005 substitutions per nucleotide site.

classified within the same species as they shared a DNA–DNA hybridization value of 18.5% [repetition 23.1%; in 2 × standard saline citrate buffer with 10% (v/v) formamide at 71 °C], well below the 70% cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987). The organism also had a profile of phenotypic properties that distinguished it from the type strains of recognized species of the genus *Cellulomonas* (Yoon *et al.*, 2008; Kang *et al.*, 2007; An *et al.*, 2005). Additionally, a number of characteristics distinguished strain KB23^T from *C. flavigena*, for example methyl-red reaction, nitrate reduction, hydrolysis of urea, casein and Tween 80, and activities of a number of enzymes (Table 1). Furthermore, strain KB23^T was capable of utilizing a number of carbon sources in contrast to the type strain of *C. flavigena* (Table 1).

The genotypic and phenotypic data indicate that strain KB23^T merits recognition as a representative of a novel species of the genus *Cellulomonas*, for which the name *Cellulomonas phragmiteti* sp. nov. is proposed.

Description of *Cellulomonas phragmiteti* sp. nov.

Cellulomonas phragmiteti (phrag.mi'te.ti. N.L. gen. n. *phragmiteti* of *Phragmitetum*, referring to plant association *Scirpo-Phragmitetum*, the habitat from where the type strain was isolated).

Cells are Gram-stain-positive, facultatively anaerobic, motile rods. Colonies on King's B agar plates are, smooth, yellow to pale orange and circular within 3 days at 25 °C. Catalase-positive and oxidase-negative. Growth occurs at 15–37 °C, at pH 7.0–9.0 and in the presence of 2.0–7.0% (w/v) NaCl. Optimum growth occurs at 25 °C, at pH 8.0 and with 5.0% (w/v) NaCl. Growth occurs under anaerobic conditions on King's B agar. Utilizes L-arabinose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, melezitose, starch, glycogen and turanose as sole carbon source, but not glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol,

inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, lactose, inulin, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Hydrolyses casein, gelatin, aesculin and Tween 80. Cellulase is produced. Does not produce indole from tryptophan or hydrogen sulphide from cysteine.

Table 1. Differential characteristics between strain KB23^T and its nearest phylogenetic neighbour, *Cellulomonas flavigena* DSM 20109^T

Strains: 1, KB23^T; 2, *C. flavigena* DSM 20109^T. All data are from the present study.

Characteristic	1	2
Colony colour	Yellow–orange	Yellow
Motility	+	–
Methyl-red test	+	–
Reduction of nitrate to nitrite	–	+
Hydrolysis of:		
Urea	+	–
Tween 80	+	–
Casein	+	–
Enzyme activities		
Esterase lipase (C8)	+	–
Valine arylamidase	+	–
α-Chymotrypsin	–	+
Acid phosphatase	–	+
Naphthol-AS-BI-phosphohydrolase	–	+
Carbon source utilization		
Methyl β-D-xylopyranoside	+	–
D-Mannitol	+	–
Arbutin	+	–
Melibiose	+	–
Trehalose	+	–
Melezitose	+	–
Turanose	+	–

Table 2. Fatty acid profiles of strain KB23^T and *Cellulomonas flavigena* DSM 20109^T

Data are from the present study. Values are percentages of the total fatty acids.

Fatty acid	KB23 ^T	<i>C. flavigena</i> DSM 20109 ^T
C _{12:0}	—	0.12
C _{13:0}	0.34	0.35
C _{14:0}	6.85	10.17
C _{15:0}	2.05	—
C _{16:0}	23.31	20.85
C _{17:0}	0.20	0.44
C _{18:0}	0.21	0.16
iso-C _{14:0}	1.63	3.21
iso-C _{15:0}	2.52	3.34
iso-C _{16:0}	1.65	3.29
iso-C _{17:0}	0.15	0.39
iso-C _{15:1}	0.22	0.41
anteiso-C _{13:0}	0.55	0.31
anteiso-C _{15:0}	37.83	38.92
anteiso-C _{17:0}	3.46	3.87
anteiso-C _{15:1}	19.03	14.17

Positive in the methyl-red test but negative for the Voges–Proskauer reaction. Negative for nitrate reduction. Positive for urease but negative for phosphatase. The peptidoglycan is of type A4β (L-Orn–D-Asp). MK-9(H4) is the predominant menaquinone. The major cellular fatty acids are anteiso-C_{15:0}, C_{16:0} and anteiso-C_{15:1} (Table 2). The G+C content of the genomic DNA of the type strain is 74.8 mol%.

The type strain, KB23^T (=DSM 22512^T =NCAIM B002303^T), was isolated from *Phragmites australis*-associated biofilm originating from a soda pond in Kiskunság National Park, Hungary.

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