Klugiella xanthotipulae gen. nov., sp. nov., a novel member of the family *Microbacteriaceae*

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An actinobacterium, designated strain 44C3^T, was isolated in Michigan, USA, from the hindgut of the larvae of *Tipula abdominalis*, an aquatic crane fly, and was subjected to a polyphasic taxonomic investigation. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain represented a separate clade within the family *Microbacteriaceae*. It showed highest 16S rRNA gene sequence similarity with *Cryobacterium psychrotolerans* 0549^T (96.5%). Strain 44C3^T had a novel B-type peptidoglycan. The peptidoglycan contained the diamino acid lysine, the peptide Gly–D–Glu was detected in the partial hydrolysate and alanine was the N terminus of the interpeptide bridge. No other amino acids found in other B-type peptidoglycans (including diaminobutyric acid, ornithine, homoserine and hydroxyglutamic acid) could be detected. The major menaquinones were MK-12 and MK-11, the major fatty acids were ai-C_{15:0}, ai-C_{17:0} and i-C_{16:0} and the DNA G + C content was 60.9 mol%. Analysis of the chemotaxonomic and phylogenetic data suggested that strain 44C3^T represented a novel species of a new genus within the family *Microbacteriaceae*, for which the name *Klugiella xanthotipulae* gen. nov., sp. nov. is proposed. The type strain of *Klugiella xanthotipulae* is 44C3^T (=DSM 18031^T =ATCC BAA-1524^T).

Strain 44C3^T was isolated from the hindgut of *Tipula abdominalis* larvae as described by Cook *et al.* (2007). *T. abdominalis* is an aquatic crane fly, larvae of which are primary shredders of leaf litter in small, riparian streams. The hindgut of *T. abdominalis* larvae hosts a dense and diverse bacterial community (Klug & Kotarski, 1980), which is suggested to facilitate digestion of their ligno-cellulosic diet (Lawson & Klug, 1989).

Strain 44C3^T was maintained as 40% (w/v) glycerol suspensions at –20 °C. Culture for biochemical and molecular studies was obtained by cultivation on trypticase soy agar (TSA; Difco) or in trypticase soy broth (TSB; Difco) at 28 °C for 48 h. Cultures were incubated at 4, 10, 22, 28, 30, 37 and 45 °C to determine the range and optimum temperature for growth. At 28 °C, growth was tested at pH 6–12 and in the presence of NaCl concentrations of 0.5–9% to determine the pH and NaCl optima and range. Colony morphology was observed on TSA after 48 h growth at 28 °C. Gram staining was performed and standard physiological tests were performed with API NE, API Staph, API Strep and API Coryne test kits

(bioMérieux). For phase-contrast microscopy observation, cells were viewed at ×100 magnification with a Leica SP2 upright microscope (Leica Microsystems Inc.) and images were captured with a Zeiss AxioCam (Carl Zeiss Micro-Imaging, Inc.) at the Center for Advanced Ultrastructure Research at the University of Georgia. A pixel to micrometre ratio was calculated via imaging software and this ratio was used to determine cell size.

Strain 44C3^T stained Gram-variable, but was Gram-type positive. It was aerobic, grew optimally at 28 °C and was able to grow at 4–30 °C. Although limited growth did occur at 4 °C, this was not observed until 672 h (4 weeks) of incubation. Irregular rod-shaped cells (0.6–3.4 × 0.4–0.8 µm) were observed, but spores were not found. Small, smooth, yellow colonies formed on TSA. Detailed biochemical and physiological characteristics of the strain are given in the genus and species descriptions below.

Analyses of cell-wall sugars, menaquinones, amino acids and acyl type were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the direction of Dr Peter Schumann according to recognized methods (Groth *et al.*, 1996; Schleifer, 1985; Schleifer & Kandler, 1972; Stanek & Roberts, 1974; Uchida *et al.*, 1999). Total cellular fatty acids were analysed by using the MIDI-FAME procedure essentially as described by Haack *et al.* (1994); gas chromatographs were compared

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 44C3^T is AY372075.

A minimum-evolution phylogenetic dendrogram based on 16S rRNA gene sequence similarity is available as supplementary material with the online version of this paper.

against profiles generated with authenticated standards and archived profiles from known cultures grown under standard conditions by using the MIDI Microbial Identification software (MIDI Inc.).

Rhamnose was the only cell-wall sugar. The major menaquinones were MK-12 and MK-11. The fatty acid profile contained ai-C_{15:0} (54.71 %), ai-C_{17:0} (18.28 %), i-C_{16:0} (17.92 %), i-C_{15:0} (1.37 %) and i-C_{14:0} (1.03 %). No glycosyl residues were found in the peptidoglycan, and thus the peptidoglycan was of the acetyl type. Analysis of the cell-wall amino acids revealed that the peptidoglycan of strain 44C3^T contained alanine, glycine, glutamate and lysine at a molar ratio of 1.6:0.9:1.0:1.0. No other amino acids found in some B-type peptidoglycans (including diaminobutyric acid, ornithine, homoserine and hydroxyglutamic acid) could be detected. As usual for B-type peptidoglycans, the peptide Gly-D-Glu was detected in the partial hydrolysate. The peptide D-Ala-Ala was found, confirming alanine as the N terminus of the interpeptide bridge. Three additional peptides were found, which probably comprised lysine and alanine residues. Although these data were not sufficient to propose a definite structure, they do not concur with published peptidoglycan structures, and support the conclusion that the peptidoglycan of strain 44C3^T represents a novel B-type.

Sequencing of the 16S rRNA gene was performed at MIDI Laboratories. Putative strain identity was determined by searching catalogued sequences in GenBank (Benson *et al.*, 2005) by using the BLAST tool (Altschul *et al.*, 1990). Sequence alignments among strain 44C3^T and the type

strains of the most closely related actinobacteria were created by using the CLUSTAL_X program (Thompson *et al.*, 1997), and these alignments were edited in GeneDoc (Nicholas *et al.*, 1997). Distances were calculated by using the Jukes-Cantor algorithm (Jukes & Cantor, 1969), and branching order was calculated with the neighbour-joining method (Saitou & Nei, 1987). A phylogenetic tree was constructed by using the program MEGA 3.1, which calculates bootstrap values internally (Kumar *et al.*, 2004). To confirm the phylogenetic position of strain 44C3^T, a minimum-evolution algorithm analysis was also performed with the MEGA 3.1 program (see Supplementary Fig. S1 in IJSEM Online). Extraction of genomic DNA was performed by using French pressure cell lysis (Thermo Spectronic), followed by purification via chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The DNA G+C content was determined according to Mesbah *et al.* (1989), and was confirmed by the DSMZ under the direction of P. Schumann according to standard methods (Cashion *et al.*, 1977; Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984; Visuvanathan *et al.*, 1989).

The nearest phylogenetic neighbours of strain 44C3^T, as determined based on analysis of its 16S rRNA gene sequence (1501 bp), were distantly related members of the family *Microbacteriaceae* (similarities ranging from 92.5 to 96.5 %). Strain 44C3^T formed a distinct subclade within the family, and showed highest 16S rRNA gene sequence similarity to *Cryobacterium psychrotolerans* 0549^T (96.5 %) (Fig. 1). The G+C content of the genomic DNA of strain 44C3^T was 60.9 mol%.

Fig. 1. Neighbour-joining, Jukes-Cantor phylogenetic dendrogram based on 16S rRNA gene sequence similarity, showing the position of strain 44C3^T among its phylogenetic neighbours. Numbers at branch nodes are bootstrap percentages (based on 1000 resamplings; only values >50 are shown). *Brevibacterium linens* DSM 20425^T served as an outgroup. GenBank accession numbers are given in parentheses. Bar, 1 % sequence divergence. A minimum-evolution tree is available as Supplementary Fig. S1.

Genera of the family *Microbacteriaceae* contain rhamnose as well as one or more other sugars in the cell wall (see references in Table 1), whereas rhamnose was the only sugar detected in strain 44C3^T. Strain 44C3^T was similar to members of the genera *Mycetocola*, *Frigoribacterium* and *Microcella* in having lysine as a cell-wall diamino acid, but differed in its major menaquinones, major fatty acids and DNA G+C content. In terms of the quinone system, members of the genus *Agrococcus* (Groth *et al.*, 1996) have menaquinones similar to those of strain 44C3^T, but they differ in other chemotaxonomic characteristics, including peptidoglycan amino acids, major fatty acid composition and DNA G+C content (Table 1).

Chemotaxonomic characteristics that differentiate strain 44C3^T from representatives of its nearest phylogenetic neighbours detected based on 16S rRNA gene sequence analysis are reported in Table 1. It is evident from the genotypic and phenotypic data presented that strain 44C3^T represents a novel species of a new genus within the family *Microbacteriaceae*, for which the name *Klugiella xanthotipulae* gen. nov., sp. nov. is proposed. *Klugiella* can be distinguished from other genera of the *Microbacteriaceae* based on its major menaquinones (MK-12 and MK-11) and cell-wall diamino acid (lysine). Some species of the genus *Microbacterium* (Table 1) have the above characteristics, but *Klugiella* can be differentiated from them by having rhamnose as the only detectable cell-wall sugar and having a lower DNA G+C content, of approximately 61 mol%.

Description of *Klugiella* gen. nov.

Klugiella (Klu.gi.el'la. N.L. fem. n. *Klugiella* named after Michael J. Klug, an American entomologist/microbiologist who, along with S. Kotarski, first described the microbial

community of the *Tipula abdominalis* larval gut, from which strain 44C3^T was isolated).

Gram-type positive, Gram-reaction variable, mesophilic and aerobic. Cells are non-motile, non-spore-forming, irregular rods (0.6–3.4 × 0.4–0.8 µm). The peptidoglycan type is B, lysine is the diamino acid of the peptidoglycan and alanine is the N terminus of the interpeptide bridge. The major menaquinones are MK-12 and MK-11. The major fatty acids are ai-C_{15:0}, ai-C_{17:0} and i-C_{16:0}. The G+C content of the genomic DNA is about 61 mol%. 16S rRNA gene sequence similarity indicates membership of the family *Microbacteriaceae*. The type species is *Klugiella xanthotipulae*.

Description of *Klugiella xanthotipulae* sp. nov.

Klugiella xanthotipulae (xan'tho.ti'pu.lae. Gr. adj. *xanthos* yellow; N.L. fem. gen. n. *tipulae* of *Tipula*, a zoological genus name; N.L. fem. gen. n. *xanthotipulae* yellow from *Tipula*, referring to the isolation of a yellow-colony-forming organism from *Tipula abdominalis*).

Colonies are convex, circular and yellow. Growth occurs at 4–30 °C with an optimum at 28 °C and at a pH range of 6–11 with an optimum at pH 8. Can grow in the presence of 1 % (w/v) NaCl but not 3 %. Catalase-positive and oxidase-negative. Positive for pyrazinamidase, β-glucuronidase, β-galactosidase and α-glucosidase. Negative for α-galactosidase, arginine dihydrolase, leucine arylamidase, pyrrolidonyl arylamidase, alkaline phosphatase, *N*-acetyl-β-glucosaminidase and urease, indole production, acetoin production, hydrolysis of gelatin and reduction of nitrates. Negative for acid production from L-arabinose, lactose, ribose and sorbitol. Acid is produced from fructose,

Table 1. Differential chemotaxonomic characteristics between strain 44C3^T and related genera of the family *Microbacteriaceae*

Data for reference taxa were taken from Tsukamoto *et al.* (2001) (*Mycetocola*), Groth *et al.* (1996) (*Agrococcus*), Kämpfer *et al.* (2000) (*Frigoribacterium*), Suzuki *et al.* (1997) (*Cryobacterium*), Tiago *et al.* (2005) (*Microcella*) and Takeuchi & Hatano (1998) and Yokota *et al.* (1993a, b) (*Microbacterium dextranolyticum*, *Microbacterium lacticum*, *Microbacterium laevaniformans* and *Microbacterium hominis*). ND, No data available.

Characteristic	<i>Klugiella</i> (strain 44C3 ^T)	<i>Mycetocola</i>	<i>Agrococcus</i>	<i>Frigoribacterium</i>	<i>Cryobacterium</i>	<i>Microcella</i>	<i>Microbacterium</i> species (<i>n</i> =4)
Diamino acid*	Lys	Lys	DAB	Lys	DAB	Lys or Orn	Lys
Major cell-wall sugar(s)†	Rha‡	ND	Glc, Rha	ND	Rha, Fuc	ND	Gal, Rha, (Man, 6dT, Xyl, Glc)
Major fatty acids	ai-C _{15:0} , ai-C _{17:0} , i-C _{16:0}	ai-C _{15:0} , ai-C _{17:0}	ai-C _{15:0} , i-C _{16:0} , i-C _{15:0}	ai-C _{15:0} , i-C _{16:0}	ai-C _{15:0} , i-C _{15:0} , ai-C _{17:0}	i-C _{16:0} , ai-C _{15:0} , i-C _{14:0} , i-C _{15:0}	ai-C _{15:0} , ai-C _{17:0} , i-C _{16:0}
Major menaquinone(s)	11, 12	10	11, 12	9	10	12, 13 or 13, 14	11, 12
DNA G+C content (mol%)	60.9	63.9–65.2	74	71.7	65	68.8	68.3–71.2

*DAB, Diaminobutyric acid; Lys, lysine; Orn, ornithine.

†6dT, 6-Deoxalose; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose. Presence of compounds in parentheses is variable between species.

‡Only sugar detected.

mannose, maltose, trehalose, mannitol, xylitol, melibiose, raffinose, xylose, sucrose and methyl α -D-glucoside. Rhamnose is the only sugar of the cell wall. The cell wall acyl type is acetyl. The menaquinones are MK-12, MK-11, MK-10, MK-13 and MK-9 (43:38:7:6:1 in the type strain). The G + C content of the genomic DNA of the type strain is 60.9 mol%.

The type strain, 44C3^T (=DSM 18031^T =ATCC BAA-1524^T), was isolated from the hindgut of *Tipula abdominalis* larvae collected in Michigan, USA.

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