Nesterenkonia flava sp. nov., isolated from paper-mill effluent

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A Gram-positive, non-motile, rod-shaped, non-spore-forming bacterium, designated CAAS 251^T, was isolated from paper-mill effluent in Wuhan, China. The organism grew optimally at 40–42 °C and at pH 9.0–10.0. The major menaquinones were MK-7, MK-8 and MK-9. The predominant cellular fatty acids were anteiso-C_{15:0} (34.78 %), anteiso-C_{17:0} (25.24 %) and C_{16:0} (13.37 %). The G + C content of the genomic DNA was 65.5 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAAS 251^T belongs to the genus *Nesterenkonia*, having sequence identities ranging from 96.0 to 97.0 % with respect to eight recognized species of the genus *Nesterenkonia*. Data from DNA–DNA hybridization and physiological and biochemical tests indicated that strain CAAS 251^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia flava* sp. nov. is proposed. The type strain is CAAS 251^T (=CCTCC AB 207010^T=JCM 14814^T).

The genus *Nesterenkonia*, belonging to the family *Micrococcaceae*, was first proposed by Stackebrandt *et al.* (1995) through the reclassification of *Micrococcus halobius* (Onishi & Kamekura, 1972) as *Nesterenkonia halobia* (Stackebrandt *et al.*, 1995). At the time of writing, the names of seven other *Nesterenkonia* species have been validly published, including *N. lacusekhoensis* (Collins *et al.*, 2002), *N. halotolerans* (Li *et al.*, 2004), *N. xinjiangensis* (Li *et al.*, 2004), *N. lutea* (Li *et al.*, 2005), *N. sandarakina* (Li *et al.*, 2005), *N. aethiopica* (Delgado *et al.*, 2006) and *N. jeotgali* (Yoon *et al.*, 2006). In this study, we present the taxonomic characterization of strain CAAS 251^T, a novel alkaliphilic member of the genus *Nesterenkonia* that was obtained from paper-mill effluent in Wuhan, China.

Strain CAAS 251^T was isolated using Luria–Bertani (LB) medium at 37 °C at pH 10.0. Subculturing was performed on PYA medium (containing, l⁻¹: peptone, 8.0 g; yeast extract, 3.0 g; K₂HPO₄, 1.0 g; EDTA, 3.5 mg; ZnSO₄ · 7H₂O,

3.0 mg; FeSO₄ · 7H₂O, 3.0 mg; MnSO₄ · H₂O, 2.0 mg; CuSO₄ · 5H₂O, 1.0 mg; H₃BO₃, 1.0 mg; agar, 15.0 g; NaHCO₃/Na₂CO₃, 0.1 mol l⁻¹, pH 10.0) at 40 °C until a pure culture was obtained. Morphological properties of cells grown for 12–18 h were observed under a light microscope (BH-2; Olympus) and by transmission electron microscopy (H-600; Hitachi). Colony characteristics were examined by growing colonies on PYA and on solid LB medium at 40 °C and pH 10.0 (Li *et al.*, 2004). Cell motility was studied on LB swarming agar (0.4 %, w/v). The Gram reaction was determined using a Gram-stain kit (bioMérieux) according to the manufacturer's instructions.

The optimum temperature for growth was determined by growing the isolate in liquid PYA medium at 4, 10, 25, 30, 37, 40, 42, 45, 50 and 55 °C. The optimal pH for growth was tested in PYA medium at 40 °C at pH 7.0–12.0 using the following buffers: NaH₂PO₄/Na₂HPO₄ (pH 7.0–8.0), 0.1 mol l⁻¹; NaHCO₃/Na₂CO₃ (pH 9.0–10.0), 0.1 mol l⁻¹; Na₂HPO₄/NaOH (pH 11.0), 0.05 mol l⁻¹; and KCl/NaOH (pH 12.0), 0.2 mol l⁻¹. Halotolerance was tested in PYA medium supplemented with 0–17 % (w/v) NaCl at pH 10.0 and 45 °C. Catalase and oxidase activities were determined in 3 % (v/v) H₂O₂ and 1 % (w/v) tetramethyl-*p*-phenylenediamine, respectively. The hydrolysis of starch, gelatin and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAAS 251^T is EF680886.

The fatty acid profiles of strain CAAS 251^T and related *Nesterenkonia* species are presented in a supplementary table available with the online version of this paper.

Table 1. Comparison of phenotypic characteristics of strain CAAS 251^T and recognized species of the genus *Nesterenkonia*

Taxa: 1, strain CAAS 251^T; 2, *N. aethiopica*; 3, *N. xinjiangensis*; 4, *N. lacusekhoensis*; 5, *N. halobia*; 6, *N. lutea*; 7, *N. halotolerans*; 8, *N. sandarakina*; 9, *N. jeotgali*. Data for reference strains were taken from Delgado *et al.* (2006) and Yoon *et al.* (2006). +, Positive; –, negative; w, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Morphology	Short rods	Short rods	Short rods	Short rods	Cocci	Cocci	Cocci	Cocci	Cocci
Colony pigmentation	Yellow	Yellow	Light yellow	Bright yellow	Colourless	Light yellow to primrose yellow	Orange-yellow	Orange-yellow	Light yellow
Motility	–	–	–	–	–	+	+	–	–
Optimal temperature (°C)	40–42	30–37	28	27–33.5	30	28	28	28	25–30
pH tolerance	8.0–12.0	7.0–11.0	7.0–12.0	7.5–9.5	<6.0–10.0	6.5–10.0	7.0–9.0	5.0–12.0	6.0–8.5
NaCl tolerance (%)	0–10	3–12	0–25	0–15	5–23	0–20	0–25	1–16	0–16
Catalase activity	+	+	+	+	+	+	+	+	ND
Oxidase activity	–	+	–	–	+	–	–	–	–
Nitrate reduction	–	ND	–	–	–	+	–	–	–
Citrate test	–	–	ND	w	–	ND	ND	ND	ND
Voges–Proskauer reaction	–	ND	ND	–	+	–	+	–	+
Hydrolysis of:									
Starch	+	+	–	–	+	–	–	–	–
Gelatin	+	+	+	–	–	–	+	+	–
Tween 80	+	ND	ND	–	–	–	–	–	–
Utilization of:									
D-Glucose	+	ND	+	+	ND	w	+	+	+
D-Xylose	–	–	+	ND	+	+	–	+	+
L-Arabinose	+	+	+	ND	+	w	–	w	+
Cellobiose	+	ND	+	ND	ND	+	–	+	+
Trehalose	–	–	–	+	–	–	–	+	+
D-Fructose	+	ND	+	+	ND	+	+	+	+
D-Mannose	+	–	+	+	–	+	+	+	w
Sucrose	+	w	+	+	–	+	+	+	+
Maltose	+	ND	+	+	ND	+	+	+	+
Acid production from:									
D-Galactose	–	–	–	–	w	+	–	+	+
Lactose	–	–	–	–	+	+	–	–	–
Trehalose	–	–	–	+	–	+	–	–	w
D-Xylose	–	–	–	–	+	+	–	+	+
Mannitol	–	–	–	–	+	+	–	+	+
Major menaquinones	MK-7, MK-8, MK-9	ND	MK-7, MK-8, MK-9	MK-7, MK-8	MK-8, MK-9	MK-8, MK-7	MK-7, MK-8	MK-7, MK-8	MK-7, MK-8, MK-9
Peptidoglycan type	Lys–Gly–D–Asp	Lys–Gly–L–Glu	Lys–Gly–L–Glu	Lys–Gly–L–Glu	Lys–Gly–L–Glu	Lys–Gly–D–Asp	Lys–Gly–D–Asp	Lys–Gly–D–Asp	Lys–Gly–D–Asp
Polar lipids*	PG, DPG, PI, GL	ND	PG, DPG, PC	PG, DPG, PC, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL
DNA G + C content (mol%)	65.5	69.0	66.7	66.1	71.5	64.5	64.4	64.0	68.0

*Abbreviations: DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PG, phosphatidylglycerol; PI, phosphatidylinositol.

Tween 80 were determined as described by Cowan & Steel (1965). Nitrate reduction was determined according to the method of Lányi (1987). Acid production from carbohy-

drates was tested as described by Leifson (1963). Other physiological and biochemical tests were performed with the API 20E system (bioMérieux).

Cell mass of strain CAAS 251^T grown on PYA plates at 40 °C was collected for the analysis of the cell wall, menaquinones and polar lipids and for DNA extraction. Menaquinones were isolated by following the method of Collins (1985) and then analysed using HPLC (Wu *et al.*, 1989). The cellular fatty acid composition was determined using the Sherlock system (MIDI). Polar lipids were analysed and identified using the method of Minnikin *et al.* (1984). Peptidoglycan was purified from the cell wall and its composition determined at the China Center for Type Culture Collection (Wuhan University, PR China) by using the methods described by Schleifer & Kandler (1972) and Schleifer (1985).

Genomic DNA was extracted and purified as described by Johnson (1985) and Marmur (1961). PCR amplification and sequencing of the 16S rRNA gene were performed as described by Chun & Goodfellow (1995) and Lane (1991). Phylogenetic affiliation to sequences available in GenBank was determined using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul *et al.*, 1990). Phylogenetic trees were constructed by using three different methods, namely the neighbour-joining, maximum-parsimony and minimum evolution algorithms with MEGA, version 3.1 (Kumar *et al.*, 2004). Bootstrap values based on 1000 replications were used to evaluate the topology of the neighbour-joining tree. The DNA G+C content was determined by using the thermal denaturation method of De Ley (1970) with *Escherichia coli* K-12 as the control. DNA–DNA hybridization was determined by using established procedures (De Ley *et al.*, 1970; Huß *et al.*, 1983).

Young cells of strain CAAS 251^T were Gram-positive, short rods approximately 0.6–0.8 µm in width and 1.2–1.4 µm in length. Colonies were yellow, smooth, circular and glistening on LB medium. Growth of strain CAAS 251^T was observed at 10–50 °C, the optimum temperature being 40–42 °C. Strain CAAS 251^T was able to grow at pH 8.0–12.0, the optimal pH being 9.0–10.0 (NaHCO₃/Na₂CO₃ at 0.1 mol l⁻¹). Growth occurred in PYA medium supplemented with 0–10 % (w/v) NaCl, maximal growth being observed in the absence of NaCl. Other phenotypic features of strain CAAS 251^T are presented in Table 1.

Analysis of the almost-complete 16S rRNA gene sequence (1412 bp) placed strain CAAS 251^T in the genus *Nesterenkonia* (Fig. 1). The highest sequence similarities were found with *N. lacusekhoensis* JCM 11953^T (97.0 %), *N. aethiopica* DSM 17733^T (97.0 %) and *N. xinjiangensis* DSM 15475^T (97.0 %). The sequence similarities with respect to other *Nesterenkonia* species were as follows: 96.8 % for *N. halotolerans*, 96.8 % for *N. sandarakina*, 96.7 % for *N. lutea*, 96.7 % for *N. jeotgali* and 96.0 % for *N. halobia*. The DNA G+C content of strain CAAS 251^T was 65.5 mol%, closely corresponding with the range for *Nesterenkonia* species (64–72 mol%) (Delgado *et al.*, 2006). Furthermore, the DNA–DNA hybridization analysis of strain CAAS 251^T revealed 47.7 % relatedness with respect to *N. lacusekhoensis* JCM 11953^T, 14.3 % with respect to *N. aethiopica* DSM 17733^T and 66.0 % with respect to *N. xinjiangensis* CCTCC AA 001025^T. In view of the threshold values for the delineation of genomic species, i.e. 97 % 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994) and 70 % DNA–DNA relatedness (Wayne *et al.*, 1987), it is evident that the isolate from paper-mill effluent represents a previously unknown species. Therefore strain CAAS 251^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia flava* sp. nov. is proposed.

Description of *Nesterenkonia flava* sp. nov.

Nesterenkonia flava (fla'va. L. fem. adj. *flava* yellow, referring to the colour of colonies of the organism).

Cells are Gram-positive, non-spore-forming, non-motile, short rods (approx. 0.6–0.8 µm in width and 1.2–1.4 µm in length). Colonies are smooth, circular, opaque and yellow in colour on LB medium and approx. 1.0–1.5 mm in diameter at 40 °C after 24 h. Optimum temperature for growth is 40–42 °C and the optimal pH is 9.0–10.0. Growth occurs in the presence of 0–10 % NaCl. Catalase-positive and oxidase-negative and the methyl red reaction is negative. Utilizes almost all carbon sources tested, including glucose, arabinose, cellulose, fructose, mannose, sucrose and maltose; xylose and trehalose are not utilized. The cell-wall peptidoglycan type is A4α, Lys–Gly–D–Asp. The main polar lipids are phosphatidylglycerol, diphos-

Fig. 1. Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequences, showing the position of strain CAAS 251^T with respect to eight *Nesterenkonia* species and five other related taxa. Bootstrap percentages (based on 1000 replications) are shown at branch points. The sequence of *Streptomyces megasporus* DSM 41476^T (Z68100) was used as the root. Bar, 1 % sequence divergence.

phatidylglycerol, phosphatidylinositol and an unidentified glycolipid. The predominant menaquinones are MK-7 (17.8 %), MK-8 (43.6 %) and MK-9 (37.7 %). Major fatty acids are anteiso- $C_{15:0}$ (34.78 %), anteiso- $C_{17:0}$ (25.24 %) and $C_{16:0}$ (13.37 %) (see Supplementary Table S1 in IJSEM Online). The DNA G+C content of the type strain is 65.5 mol%.

The type strain, CAAS 251^T (=CCTCC AB 207010^T=JCM 14814^T), was isolated from paper-mill effluent collected in Wuhan, China.

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