Nesterenkonia alba sp. nov., an alkaliphilic actinobacterium isolated from the black liquor treatment system of a cotton pulp mill

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An alkaliphilic actinobacterium, designated strain CAAS 252^T, was isolated from the black liquor treatment system of a cotton pulp mill in Wuhan, China. Cells of strain CAAS 252^T were Gram-positive, non-motile, non-endospore-forming, short rod-shaped, and grew optimally at 42 °C and pH 9–10 in the presence of 3 % (w/v) NaCl. Strain CAAS 252^T contained MK-7, MK-8 and MK-9 as the major menaquinones and anteiso-C_{17:0}, anteiso-C_{15:0} and C_{16:0} as the predominant cellular fatty acids and had a peptidoglycan type of A4 α , Lys–Gly–D–Asp. The DNA G + C content was 60.2 mol%. Based on analysis of 16S rRNA gene sequences (94.7–96.8 % similarity), DNA–DNA hybridization (<70 % relatedness) and chemotaxonomic characteristics, strain CAAS 252^T belonged to the genus *Nesterenkonia*, but differed from all recognized species. Therefore, it is proposed that strain CAAS 252^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia alba* sp. nov. is proposed. The type strain is CAAS 252^T (=CCTCC AB 207011^T=DSM 19423^T).

A number of novel species of the genus *Nesterenkonia* have been described in recent years. Currently, the genus comprises ten recognized species: *Nesterenkonia halobia* (Stackebrandt *et al.*, 1995), *Nesterenkonia lacusekhoensis* (Collins *et al.*, 2002), *Nesterenkonia halotolerans* (Li *et al.*, 2004), *Nesterenkonia xinjiangensis* (Li *et al.*, 2004), *Nesterenkonia lutea* (Li *et al.*, 2005), *Nesterenkonia sandarakina* (Li *et al.*, 2005), *Nesterenkonia aethiopica* (Delgado *et al.*, 2006), *Nesterenkonia jeotgali* (Yoon *et al.*, 2006), *Nesterenkonia halophila* (Li *et al.*, 2008) and *Nesterenkonia flava* (Luo *et al.*, 2008). Most of these species were isolated from hypersaline or alkaline habitats such as saline soil, solar salt, seafood, soda lake or alkaline wastewater. This paper focuses on the taxonomic characterization of a novel alkaliphilic actinobacterium, designated strain CAAS 252^T, isolated from the black liquor treatment system of a cotton pulp mill in Wuhan, China.

Strain CAAS 252^T was isolated using the dilution plating method at 42 °C with carboxymethylcellulose sodium salt

medium (CMC; containing, 1^{−1}: peptone, 10 g; yeast extract, 10 g; NaCl, 5 g; carboxymethylcellulose sodium salt, 10 g; KH₂PO₄, 1 g; pH 10, adjusted with NaOH). Subculturing was conducted on peptone yeast extract agar (PYA; containing, 1^{−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/NaCO₃, 0.1 mol; pH 10.0) and Luria–Bertani (LB) plates at 42 °C until a pure culture was obtained.

The morphological, physiological and biochemical characteristics of strain CAAS 252^T were investigated using cultures grown on PYA. Cells of strain CAAS 252^T were grown at 42 °C for 24–36 h and their morphology was observed using a light microscope (BH-2; Olympus) and transmission electron microscopy (H-600; Hitachi). Colony characteristics were examined by growing the bacterium on PYA and on solid LB medium at 42 °C and pH 10 for 2 days (Li *et al.*, 2004). The optimum temperature for growth was determined by growing the isolate in liquid LB medium at temperatures ranging from 4 to 55 °C. The pH optimum for growth was tested in LB

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAAS 252^T is EU566871.

Table 1. Comparison of phenotypic characteristics of strain CAAS 252^T and recognized species of the genus *Nesterenkonia*

Taxa: 1, strain CAAS 252^T (*Nesterenkonia alba* sp. nov.); 2, *N. aethiopica* DSM 17733^T (data from Delgado *et al.*, 2006); 3, *N. xinjiangensis* (Yoon *et al.*, 2006); 4, *N. flava* JCM 14814^T (Luo *et al.* (2008)); 5, *N. halophila* YIM 70179^T (Li *et al.*, 2008); 6, *N. halobia* (Yoon *et al.*, 2006); 7, *N. lacusekhoensis* (Yoon *et al.*, 2006); 8, *N. lutea* (Yoon *et al.*, 2006); 9, *N. jeotgali* (Yoon *et al.*, 2006); 10, *N. sandarakina* (Yoon *et al.*, 2006); 11, *N. halotolerans* (Yoon *et al.*, 2006) +, Positive; −, negative; w, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Morphology	Short rods	Short rods	Short rods	Short rods	Cocci	Cocci	Short rods	Cocci	Cocci	Cocci	Cocci
Colony pigmentation	White	Yellow	Light yellow	Yellow	Ivory white	Colourless	Bright yellow	Light yellow to primrose yellow	Light yellow	Orange-yellow	Orange-yellow
Motility	−	−	−	−	−	−	−	+	−	−	+
Optimal temperature (°C)	42	30–37	28	40–42	28	30	27–33.5	28	25–30	28	28
pH tolerance	8.0–12.0	7.0–11.0	7.0–12.0	8.0–12.0	6.0–10.5	<6.0–10.0	7.5–9.5	6.5–10.0	6.0–8.5	5.0–12.0	7.0–9.0
NaCl tolerance (%)	0–6	3–12	0–25	0–10	0.5–30	5–23	0–15	0–20	0–16	1–16	0–25
Oxidase activity	−	+	−	−	−	+	−	−	−	−	−
Urease	−	−	+	−	−	−	ND	−	−	−	+
H ₂ S production	−	−	−	−	−	−	w	−	−	−	−
Indole production	−	−	ND	−	−	−	−	−	−	−	−
ONPG test*	+	−	ND	−	+	ND	ND	+	−	+	+
Nitrate reduction	−	ND	−	−	+	−	−	+	−	−	−
Citrate test	−	−	ND	−	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	w	−	−	−	ND	−	+	−	+	+	−
D-Fructose	−	ND	+	+	+	ND	+	+	+	+	+
D-Mannose	−	−	+	+	+	−	+	+	w	+	+
Sucrose	+	w	+	+	ND	−	+	+	+	+	+
Maltose	+	ND	+	+	ND	ND	+	+	+	+	+
Acid production from:											
D-Galactose	−	−	−	−	ND	w	−	+	+	+	−
Lactose	−	−	−	−	−	+	−	+	−	−	−
Trehalose	w	−	−	−	ND	−	+	+	w	−	−
D-Xylose	−	−	−	−	−	+	−	+	+	+	−

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Mannitol	–	–	–	–	–	+	–	+	+	+	–
Major menaquinones	MK-7, MK-8, MK-9	ND	MK-7, MK-8, MK-9, MK-9	MK-7, MK-8, MK-9, MK-9	MK-7, MK-8, MK-9, MK-9	MK-8, MK-9	MK-7, MK-8, MK-9	MK-8, MK-9	MK-7, MK-8, MK-9	MK-7, MK-8, MK-9	MK-7, MK-8, MK-9
Peptidoglycan type	L-Lys-Gly- D-Asp	L-Lys-Gly- L-Glu	L-Lys-Gly- L-Glu	L-Lys-Gly- D-Asp	L-Lys-Gly- L-Glu	L-Lys-Gly- L-Glu	L-Lys-Gly- L-Glu	L-Lys-Gly- D-Asp	L-Lys-Gly- D-Asp	L-Lys-Gly- D-Asp	L-Lys-Gly- D-Asp
Polar lipids†	PG, DPG, PI, GL	ND	PG, DPG, PC	PG, DPG, PI, GL	PI, PG, DPG, GL	PG, DPG, PI, GL	PG, DPG, PC, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL	PG, DPG, PI, GL
DNA G+C content (mol%)	60.2	69.0	66.7	65.5	68.4	71.5	66.1	64.5	68.0	64.0	64.4

*ONPG, o-Nitrophenyl-β-D-galactopyranoside.

†DPG, Diphenylphosphatidylglycerol; GL, unidentified glycolipid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

medium at 42 °C and pH 7–12 using the buffer system given by Luo *et al.* (2008). Halotolerance was tested in LB medium supplemented with 0–15 % (w/v) NaCl at pH 10 and 42 °C. Catalase and oxidase activities were determined in 3 % (v/v) H₂O₂ and 1 % (w/v) tetramethyl-*p*-phenylenediamine, respectively. The utilization of various carbon sources was determined as described by Shirling & Gottlieb (1966). Hydrolysis of starch, gelatin and Tween 80 were determined as described by Cowan & Steel (1965). Nitrate reduction was determined according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Other physiological and biochemical tests were performed with the API 20E system (bioMérieux).

Biomass for analysis of menaquinones, cell wall and polar lipids and DNA extraction was grown on LB plates at 42 °C for 2 days. The cells were harvested and washed twice with sterile distilled water. The isolation and analysis of menaquinones, cellular fatty acids, polar lipids, peptidoglycan and the 16S rRNA gene were conducted according to Luo *et al.* (2008). Phylogenetic trees were generated using three methods, namely the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) algorithms with MEGA version 4 (Tamura *et al.*, 2007). The evolutionary distance method used for the neighbour-joining and minimum-evolution methods was based on the Jukes–Cantor model (Jukes & Cantor, 1969). Bootstrap values based on 1000 replications were used to evaluate the topology of the neighbour-joining tree. The DNA G+C content was determined using the thermal denaturation method of De Ley (1970) with *Escherichia coli* K-12 as a control. DNA–DNA hybridization with the type strains of four closely related recognized *Nesterenkonia* species was determined by using the spectrophotometric method (De Ley *et al.*, 1970; Huß *et al.*, 1983). *N. lacusekhoensis* JCM 11953^T was obtained from Japan Collection of Microorganisms and *N. aethiopica* DSM 17733^T and *N. xinjiangensis* DSM 15475^T were obtained from the German Collection of Microorganisms and Cell Cultures. *N. flava* JCM 14814^T was maintained in our laboratory (Luo *et al.*, 2008).

The phenotypic and biochemical features of strain CAAS 252^T in comparison with those of other *Nesterenkonia* species are given in Table 1. Strain CAAS 252^T could be distinguished from other *Nesterenkonia* species by means of the colour of the colonies, NaCl requirement and low DNA G+C content. The cellular fatty acids of strain CAAS 252^T and recognized *Nesterenkonia* species are compared in Table 2. The culture conditions used for the analysis of the cellular fatty acids were not the same as those described by Yoon *et al.* (2006), but the major fatty acid profile was generally similar to those of other *Nesterenkonia* species.

Multiple alignments of a nearly complete 16S rRNA gene sequence (1494 nt) of strain CAAS 252^T with those of other recognized *Nesterenkonia* species were carried out using CLUSTAL_X (Thompson *et al.*, 1997) and the sequence

Table 2. Cellular fatty acid composition of strain CAAS 252^T and recognized species of the genus *Nesterenkonia*

Taxa: 1, strain CAAS 252^T (*Nesterenkonia alba* sp. nov.); 2, *N. xinjiangensis* (Yoon *et al.*, 2006); 3, *N. flava* JCM 14814^T (Luo *et al.*, 2008); 4, *N. halophila* YIM 70179^T (Li *et al.*, 2008); 5, *N. halobia* (Yoon *et al.*, 2006); 6, *N. lacusekhoensis* (Yoon *et al.*, 2006); 7, *N. lutea* (Yoon *et al.*, 2006); 8, *N. jeotgali* (Yoon *et al.*, 2006); 9, *N. sandarakina* (Yoon *et al.*, 2006); 10, *N. halotolerans* (Yoon *et al.*, 2006). Values are percentages of the total fatty acids. —, Not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10
C _{10:0}	0.56	—	—	—	—	—	—	—	—	—
C _{12:0}	0.55	—	—	—	—	—	—	—	—	—
iso-C _{14:0}	—	0.20	—	0.4	—	0.50	1.80	0.20	0.90	0.60
C _{14:0}	1.15	—	1.67	—	—	2.50	0.40	0.40	2.80	—
iso-C _{15:1} G	—	1.20	0.62	—	—	—	1.00	—	—	—
anteiso-C _{15:1} A	—	0.40	0.86	—	—	0.60	7.80	0.40	—	—
iso-C _{15:0}	1.61	6.20	2.1	2.4	1.70	1.30	5.50	1.40	0.80	2.20
anteiso-C _{15:0}	11.02	21.90	34.80	47.1	33.80	62.10	53.60	43.70	59.70	72.80
C _{15:0} 3-OH	—	—	1.26	—	—	—	—	—	—	—
C _{15:0}	—	0.20	—	—	—	—	—	0.90	2.10	—
iso-C _{16:1} G	1.16	4.80	0.80	—	—	—	0.40	—	—	—
iso-C _{16:0}	3.71	9.60	7.30	11.2	8.30	6.70	17.80	13.00	4.40	9.80
C _{16:0}	8.10	0.70	13.40	3.3	1.60	6.10	2.60	2.60	14.90	1.10
anteiso-C _{17:1} A	5.46	8.50	—	—	—	—	—	—	—	—
iso-C _{17:0}	1.76	6.20	0.80	1.3	2.30	—	0.50	1.70	—	0.40
anteiso-C _{17:0}	53.56	39.70	25.20	31.1	52.40	19.80	8.00	33.80	7.10	12.20
C _{18:0}	3.70	—	3.79	—	—	—	—	0.70	5.10	—
C _{18:1} ω ₉ c	3.03	—	1.80	—	—	—	—	—	—	—
Summed features*										
3	0.30	—	2.13	—	—	—	—	—	—	—
5	0.50	—	0.70	—	—	—	—	—	—	—
8	3.65	—	2.20	—	—	—	—	—	—	—

*Summed features comprise a group of two or more fatty acids that could not be separated under the given conditions. Summed feature 3 comprises C_{16:1}ω₇c and/or C_{16:1}ω₆c. Summed feature 8 comprises C_{18:2}ω₆,9c and/or anteiso-C_{18:0}. Summed feature 5 comprises C_{18:1}ω₇c and/or C_{18:1}ω₆c.

similarities were calculated using vector NTI software (Lu & Moriyama, 2004). Based on the phylogenetic tree (Fig. 1), strain CAAS 252^T was most closely related to *N. aethiopica* DSM 17733^T (96.8% gene sequence similarity), *N. xinjiangensis* DSM 15475^T (96.6%) and *N. flava* JCM 14814^T

(96.1%). The 16S rRNA gene sequence similarities to other *Nesterenkonia* species were 95.8% to *N. halophila* YIM 70179^T, 95.5% to *N. halobia* JCM 11483^T, 95.4% to *N. lacusekhoensis* JCM 11953^T, 95.2% to *N. lutea* YIM 70081^T, 95.0% to *N. jeotgali* JG-241^T, 94.9% to *N. sandarakina* YIM

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain CAAS 252^T and related taxa. *Streptomyces megasporus* DSM 41476^T (Z68100) was used as an outgroup. Bootstrap values (>50%) based on 1000 replications are shown at nodes. Bar, 1% sequence divergence.

70009^T and 94.7 % to *N. halotolerans* YIM 70084^T. The DNA G+C content of strain CAAS 252^T was 60.2 mol%. Furthermore, the DNA–DNA hybridization analysis of strain CAAS 252^T revealed 60.7 % DNA–DNA relatedness with respect to *N. aethiopica* DSM 17733^T, 57.2 % with respect to *N. lacusekhoensis* JCM 11953^T, 41.7 % with respect to *N. flava* JCM 14814^T and 35.0 % 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 similarity (Stackebrandt & Goebel, 1994) and 70 % DNA–DNA relatedness (Wayne *et al.*, 1987), it is evident that strain CAAS 252^T, isolated from a black liquor-treatment system, represents a previously unknown species. Therefore, strain CAAS 252^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia alba* sp. nov. is proposed.

Description of *Nesterenkonia alba* sp. nov.

Nesterenkonia alba (al'ba. L. fem. adj. *alba* white, referring to the colour of colonies of the organism).

Cells are Gram-positive, non-motile, non-spore-forming, short rods, 0.4–0.6 µm in width and 0.8–1.2 µm in length. Colonies on solid LB medium are white, smooth, circular and approximately 0.9–1.2 mm in diameter at 42 °C at 48 h. The surface of the colonies is moist and glistening. Growth occurs at 20–50 °C and pH 8–12, and with 0–6 % (w/v) NaCl. Optimum conditions for growth are 42 °C and pH 9–10, with 3 % NaCl. Catalase-positive but negative for oxidase, urease and the methyl red reaction. Does not produce H₂S or indole. Glucose, arabinose, sucrose, maltose, xylitol, melibiose, melezitose, raffinose, trehalose and glycerol are utilized, but mannose, fructose, xylose, D-galactose, α-D-lactose, arabinol, cellobiose, adonitol and sorbitol are not. Negative for hydrolysis of starch and positive for hydrolysis of Tween 80 and gelatin. Acid is not produced from D-galactose, lactose, xylose or mannitol. Major menaquinones are MK-7 (9.7 %), MK-8 (54.8 %) and MK-9 (35.5 %). Fatty acids are anteiso-C_{17:0}, anteiso-C_{15:0}, C_{16:0}, anteiso-C_{17:1A}, iso-C_{16:0}, C_{18:0}, summed feature 8 (C_{18:1ω7c} and/or C_{18:1ω6c}), C_{18:1ω9c}, iso-C_{17:0}, iso-C_{15:0}, C_{14:0}, C_{10:0}, C_{12:0}, summed feature 5 (C_{18:2ω6,9c} and/or anteiso-C_{18:0}) and summed feature 3 (C_{16:1ω7c} and/or C_{16:1ω6c}). Cell-wall peptidoglycan type is A4_z, Lys–Gly–D–Asp. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and unidentified glycolipids. The DNA G+C content of the type strain is 60.2 mol%.

The type strain, CAAS 252^T (=CCTCC AB 207011^T=DSM 19423^T), was isolated from the black liquor treatment system of a cotton pulp mill in Wuhan, China.

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