Nesterenkonia aurantiaca sp. nov., an alkaliphilic actinobacterium isolated from Antarctica

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A Gram-stain-positive, non-endospore-forming, haloalkaliphilic actinobacterium, strain CK5^T, was isolated from a soil sample, collected at Cape King (Antarctica), and its taxonomic position was investigated by using a polyphasic approach. Cells were cocci with orange pigmentation, non-motile and grew optimally at 25 °C and pH 9.0–9.5 in the presence of 2 % (w/v) NaCl. Cellular membrane contained MK-7 (72 %) and MK-8 (28 %), and anteiso-$C_{15:0}$ (64.8 %), iso-$C_{16:0}$ (13.3 %), *n*-$C_{17:0}$ (9.9 %), *n*-$C_{16:0}$ (4.0 %), *n*-$C_{14:0}$ (3.7 %) as major cellular fatty acids. The DNA G+C content was 64.8 mol%. Strain CK5^T, based on the 16S rRNA gene sequence similarity, was most closely related to *Nesterenkonia jeotgali* JG-241^T (99.5 %), *Nesterenkonia sandarakina* YIM 70009^T (99.4 %), *Nesterenkonia lutea* YIM 70081^T (99.4 %), *Nesterenkonia halotolerans* YIM 70084^T (99.3 %), *Nesterenkonia xinjiangensis* YIM 70097^T (97.2 %), *Nesterenkonia flava* CAAS 251^T (97.1 %) and *Nesterekonia aethiopica* CCUG 48939^T (97.1 %). Strain CK5^T revealed 31 % DNA–DNA relatedness with respect to *N. sandarakina* DSM 15664^T, 29 % with respect to *N. jeotgali* DSM 19081^T, 10 % with respect to *N. lutea* DSM 15666^T and 1 % with respect to *N. halotolerans,* DSM 15474^T, *N. xinjiangensis* DSM 15475^T, *N. aethiopica* DSM 17733^T and *N. flava* DSM 19422^T. On the basis of 16S rRNA gene sequences, DNA–DNA hybridization and chemotaxonomic characteristics, strain CK5^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia aurantiaca* sp. nov. is proposed. The type strain is CK5^T (=DSM 27373^T=JCM 19723^T).

The genus *Nesterenkonia* was first proposed by Stackebrandt *et al.* (1995) when *Micrococcus halobius* (Onishi & Kamekura, 1972) was reclassified in a new genus, as *Nesterenkonia halobia*. This genus belongs to the family *Micrococcaceae* within the phylum *Actinobacteria* and is most closely related to the genera *Micrococcus*, *Arthrobacter* and *Kocuria* (Stackebrandt *et al.*, 1995). The genus *Nesterenkonia* includes Gram-stain-positive, non-endospore-forming, catalase-positive, oxidase-negative, aerobic, mesophilic bacteria that may be halotolerant or halophilic.

At the time of writing, this genus includes fourteen species, *N. 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) *Nesterenkonia flava* (Luo *et al.*, 2008), *Nesterenkonia alba* (Luo *et al.*, 2009), *Nesterenkonia suensis* (Govender *et al.*, 2013), *Nesterenkonia rhizosphaerae* (Wang *et al.*, 2014) and *Nesterenkonia alkaliphila* (Zhang *et al.*, 2015). These species have been isolated from saline and alkaline soils or lakes, but also from paper and cotton pulp mill effluents, fermented seafoods and a rhizosphere soil sample. Species of the

Abbreviations: FAME, fatty acid methyl ester; PNPG, 4-nitrophenyl β-D-glucopyranoside.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CK5^T is HG795012.

One supplementary table and one supplementary figure are available with the online Supplementary Material.

genus *Nesterenkonia* have been described as Gram-stain-positive micro-organisms showing menaquinones as predominant quinones and anteiso-$C_{15:0}$ as the major cellular fatty acid. The DNA G+C content of species belonging to this genus ranges from 60.2 to 71.5 mol% (Zhang *et al.*, 2015).

The aim of this work was to determine the exact taxonomic position of strain CK5^T isolated from Cape King, Antarctica, during the XXI Italian Antarctic Research Expedition (2005–2006) based on its phylogenetic, genotypic and chemotaxonomic characteristics.

Soil samples (0.2 g) collected from Cape King (73° 05′ S 166° 40′ E) in Antarctica with a conductivity value of 86.7 μS cm^{-1} and pH 6.5–7.0 were used to inoculate 10 ml growth medium A (Romano *et al.*, 2008) containing (l^{-1}) yeast extract, 10 g; trisodium citrate, 3 g; KCl, 2 g; $MgSO_4 . 7H_2O$, 1 g; NaCl, 100 g; $MnCl_2 . 4H_2O$, 0.36 g; $FeSO_4 . 7H_2O$, 0.05 g, Na_2CO_3, 3.0 g (pH 9.0) and 10 ml enrichment growth medium B having the same composition as medium A without Na_2CO_3 (pH 7.0).

Solid medium was obtained by adding 1.8 % (w/v) agar. After 1 week of incubation at a temperature of 10 °C, growth had occurred only in liquid medium A. This growth, spread on solid medium A, revealed the presence of orange colonies, which were purified by using the serial dilution-plating method at 10 °C followed by restreaking on the same solid medium. Strain CK5^T represented the faster-growing micro-organism in enrichment medium A and it was the only colony-forming strain at the highest dilutions. Subculturing was performed on the same medium for 24 h at 25 °C in shaken flask culture (shaking speed 150 r.p.m.), and culture was maintained as a glycerol stock at −20 °C for further studies.

The 16S rRNA gene sequence was determined at the BMR Genomics Service (Padova University, Italy) by using the following primers for amplification: 3′-AGTTTGATCCTGGCTCAG-5′, 3′-CCAGCAGCCGCGGTAAT-5′, 3′-CACGGTTGTCGTCAGCTCGT-5′, 5′-ATTACCGCGGCTGCTGG-3′, 5′-ACGAGCTGACGACAACCGTG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′. A phylogenetic tree was reconstructed by using the software package MEGA version 5 (Tamura *et al.*, 2011) after multiple alignment of the data by CLUSTAL X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980) and clustering were based on the neighbour-joining, maximum-likelihood and maximum-parsimony methods (Saitou & Nei, 1987; Nei & Kumar, 2000; Tamura *et al.*, 2011). Tree topology was re-examined by the bootstrap method of resampling (Felsenstein, 1985) using 1000 replications.

The novel isolate was identified using the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012) on the basis of 16S rRNA sequence data. Strain CK5^T belonged to the genus *Nesterenkonia* and was most closely related, based on gene sequence similarity, to *Nesterenkonia jeotgali* JG-241^T (99.5 %), *N. sandarakina* YIM 70009^T (99.4 %), *N. lutea* YIM 70081^T (99.4 %), *N. halotolerans* YIM 70084^T (99.3 %), *N. xinjiangensis* YIM 70097^T (97.2 %), *N. flava* CAAS 251^T (97.1 %) and *N. aethiopica* CCUG 48939^T (97.1 %). The 16S rRNA gene sequence similarities to other species of the genus *Nesterenkonia* ranged between 96.8 and 95.4 %, where *N. alkaliphila* F10^T showed the lowest gene sequence similarity and the type strain of the species that defines the genus, *N. halobia* 28-3^T, showed 96.71 % similarity. The phylogenetic tree reconstructed using the neighbour-joining method showed that strain CK5^T was a member of the genus *Nesterenkonia* and formed a clade with *N. halotolerans*, *N. jeotgali*, *N. sandarakina* and *N. lutea* (Fig. 1). Analogous phylogenetic information for strain CK5^T were obtained by using the maximum-likelihood and maximum-parsimony algorithms, as indicated by filled circles at branches stable with all the algorithms used (Fig. 1).

Cell morphology was determined by phase-contrast microscopy (Zeiss). Colony morphology was analysed on solid medium by using a stereomicroscope (M8, Leica). Gram staining was performed according to the method of Dussault (1955). The NaCl requirement for growth was studied in medium A in which the NaCl concentration was supplemented up to 25 % (w/v) using concentrations of 0, 2, 4, 6, 8, 10, 15, 20 % (w/v). Unless stated otherwise, growth medium A was supplemented with 2 % (w/v) NaCl. Growth was tested at different temperatures (2, 4, 10, 15, 20, 25, 30 °C). The pH range for growth was tested at 25 °C at different pH by using medium A supplemented with 2 % NaCl and buffered with 50 mM MES (for pH 5.5, 6.0 and 6.7) or with 50 mM HEPES (for pH 7.5 and 8.2) or with 50 mM TAPS (for pH 8.5 and 9.0) or with 50 mM CAPS (for pH 9.7, 10.0 and 11.0). Unless stated otherwise, the pH of optimal growth medium was pH 9.0. To study the utilization of single carbon and energy sources, the novel isolate was grown using liquid medium C containing (l^{-1}): NaCl, 20 g; $MgSO_4 . 7H_2O$, 0.1 g; Na_2CO_3, 3 g; K_2HPO_4, 7 g; KH_2PO_4 2 g; $(NH_4)_2SO_4$ 1 g, pH 9.0 (Romano *et al.*, 2008). Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l^{-1} except for carbohydrates, which were used at 10 g l^{-1}. When the substrates were amino acids, they should be tested as a nitrogen source, and medium C was therefore prepared without $(NH_4)_2SO_4$. Acid production was determined in liquid medium A according to the protocol of Arahal *et al.* (1996). All phenotypic and biochemical tests were performed at the optimal growth temperature, pH and NaCl concentration: 25 °C, pH 9.0 and 2 % (w/v) NaCl, respectively. Sensitivity of the strain to antibiotics was tested by using solid medium A and Sensi-discs (6 mm; Oxoid) (Romano *et al.*, 1993); oxidase activity was determined by assessing the oxidation of tetramethyl-*p*-phenylenediamine, and catalase activity was determined by assessing bubble production in a 3 % (v/v) hydrogen peroxide solution. For nitrate and nitrite reduction, optimal medium

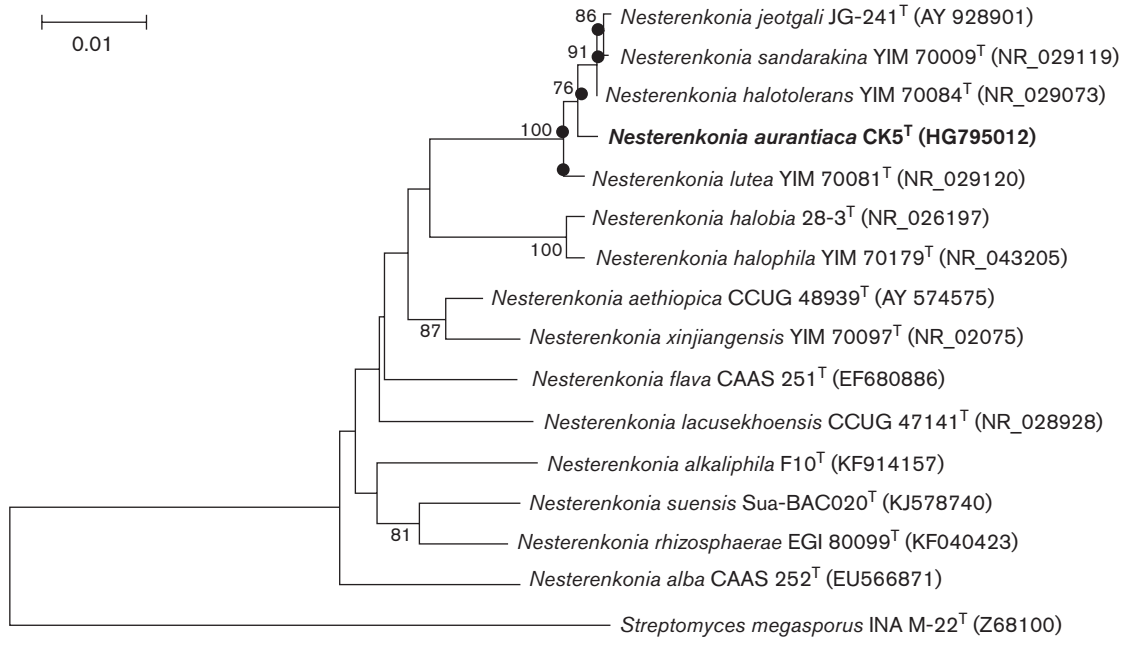

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain CK5^T and related taxa. *Streptomyces megasporus* INA M-22^T (Z68100) was used as an outgroup. Bootstrap values based on 1000 replications are shown at nodes. Bootstrap values higher than 70 are included in the figure. Filled circles indicate branches stable with all the algorithms used. Bar, 0.01 substitutions per nucleotide position.

A containing 0.1 % (w/v) KNO_3 or 0.001 % (w/v) $NaNO_2$, respectively, was used. Hydrolysis of hippurate was tested by using 1.0 % (w/v) sodium hippurate. Tyrosine decomposition was tested on solidified growth medium containing 0.45 % (w/v) L-tyrosine. For the indole test, the novel strain was grown in the presence of 1.0 % (w/v) tryptophan using Kovacs' reagent.

4-Nitrophenyl β-D-glucopyranoside (PNPG) and ONPG tests were performed according to the protocols of De Rosa *et al.* (1980). Starch and pectin hydrolysis were tested by flooding cultures with Lugol's iodine on solid medium A containing 0.2 % (w/v) starch or 0.05 % (w/v) pectin, respectively. Xylan and cellulose hydrolysis were tested by flooding cultures with 0.1 % Congo red followed by 1 M NaCl on solid medium A containing 1.0 % (w/v) xylan or 0.5 % (w/v) CM-cellulose, respectively. Lipase activity was tested on solid medium A containing 2.5 % (w/v) olive oil and 0.001 % rhodomine B (Kouker & Jaeger, 1987). For casein hydrolysis, solid medium A plus 5.0 % (w/v) skimmed milk was used. For gelatin hydrolysis and sensitivity to lysozyme, 1.0 % (w/v) gelatin or 0.001 % (w/v) lysozyme were used, respectively. The urease activity test was performed according to the method of Rustigian & Stuart (1941). For the endospore formation test, 0.001 % (w/v) $MnCl_2 . 4H_2O$ was added in liquid medium as a stimulating agent and checked by using optical microscopy after 24 and 48 h of incubation. The Voges–Proskauer test was performed according to the method of Clark & Lubs (1915). Motility and Tween 80 hydrolysis were tested as previously described (Poli *et al.*, 2007).

Cells of strain CK5^T were Gram-stain positive cocci (1.5 μm), non-motile and non-endospore- forming. Colonies on solid medium A were orange, smooth, circular and approximately 1 mm in diameter at 25 °C after 48 h. The surface of the colonies was shiny and glistening. Growth occurred in medium A at a temperature range of 4–30 °C, when supplemented with 0.003–20 % (w/v) NaCl, and in the range of pH 6.5–10. Optimum conditions for growth in media A were 25 °C and pH 9, with 2 % NaCl. Strain CK5^T was catalase-positive but negative for oxidase, urease and indole formation. It was able to hydrolyse xylan and pectin but not starch, casein, gelatin, hippurate, phenylalanine, urea or Tween 80. Strain CK5^T did not reduce nitrate or nitrite and it was found to be positive for the ONPG, PNPG, lipase and aminopeptidase tests and negative for the Voges–Proskauer reaction. It was able to utilize D-glucose, D-xylose, galactose, lactose, trehalose, D-fructose and sucrose as sole carbon sources but did not produce acid from them. It did not utilize L-arabinose, raffinose or D-mannose as sole carbon sources. It was able to utilize L-alanine and it did not utilize L-cysteine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-serine or L-valine. Strain CK5^T was able to utilize acetate, citrate, propionate, succinate and malonate and it did not utilize formate, gluconate or fumarate. Strain CK5^T was susceptible to (amount per disc): nystatin (100 U), streptomycin (25 μg), bacitracin (10 U), novobiocin (30 μg),

penicillin (2 U), tetracycline (30 μg), fusidic acid (10 μg), lincomycin (15 μg), vancomycin (30 μg) and chloramphenicol (10 μg). The results of the comparison of the physiological, biochemical and molecular properties of strain CK5^T with its nearest phylogenetic neighbour species of the genus *Nesterenkonia* is reported in Table 1.

Genomic DNA was extracted and purified from bacterial cell cultures (approx. 250 mg dry pellet) using the Genomic-DNA-Buffer Set and Genomic-tip-100/G columns (Qiagen), according to the manufacturer's instructions as reported by Poli *et al.*, (2009). Evaluation of DNA G+C content (mol%) and DNA–DNA filter hybridization analysis were performed as previously described (Poli *et al.*, 2009; Romano *et al.*, 2010). The type strains *N. halotolerans* DSM 15474^T (Li *et al.*, 2004), *N. lutea* DSM 15666^T (Li *et al.*, 2005), *N. sandarakina* DSM 15664^T (Li *et al.*, 2005) *N. jeotgali* DSM 19081^T (Yoon *et al.*, 2006), *N. xinjiangensis* DSM 15475^T (Li *et al.*, 2004), *N. aethiopica* DSM 17733^T (Delgado *et al.*, 2006) and *N. flava* DSM 19422^T (Luo *et al.*, 2008) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Strains were grown in the media recommended by the culture collection and they were used for comparative purposes.

DNA–DNA hybridizations, evaluated by filter hybridization, were carried out between strain CK5^T and the closely related species. Strain CK5^T revealed 31 % DNA–DNA relatedness with respect to *N. sandarakina* DSM 15664^T, 29 % with respect to *N. jeotgali* DSM 19081^T, 10 % with respect to *N. lutea* DSM 15666^T and 1 % with respect to *N. halotolerans* DSM 15474^T, *N. xinjiangensis* DSM 15475^T, *N. aethiopica* DSM 17733^T and *N. flava* DSM 19422^T. DNA–DNA relatedness values were below then 70 % threshold generally accepted for species delineation, hence supporting the distinct position of strain CK5^T within the genus *Nesterenkonia*. The DNA G+C content of strain CK5^T was 64.8 mol%, which was within the range reported for species of the genus *Nesterenkonia* (Stackebrandt *et al.*, 1995).

Table 1. Differential features of strain CK5^T and closely related species of the genus *Nesterenkonia*

Strains: 1, strain CK5^T; 2, *N. sandarakina* DSM 15664^T; 3, *N. jeotgali* DSM 19081^T; 4, *N. lutea* DSM 15666^T; 5, *N. halotolerans* DSM 15474^T. All tests were performed at the authors' laboratory under identical conditions. +, Positive; −, negative; w, weakly positive result.

Characteristic	1	2	3	4	5
Colony pigmentation	Orange	Orange–yellow	Light yellow	Light yellow to primrose yellow	Orange–yellow
Motility	−	−	−	+	+
Optimal temperature for growth (°C)	25	28	25–30	28	28
pH range for growth	6.5–10.0	5.0–12.0	6.0–8.5	6.5–10.0	7.0–9.0
NaCl range for growth (%)	0.003–20	1–16[a]*	0–16[b]	0–20[a]	0–25[c]
Urease	−	−	−	−	+
Lipase	+	+	−	+	+
Nitrate reduction	−	−	−	+	−
ONPG test	+	+	−	−	−
Voges–Proskauer reaction	−	−	+	−	+
Hydrolysis of:					
Gelatin	−	+	−	−	−
Xylan	+	+	−	+	−
Pectin	w	−	−	−	−
CM-cellulose	−	w	−	w	−
Utilization of:					
D-Glucose	+	+	+	w	+
D-Xylose	+	+	+	+	−
L-Arabinose	−	w	+	w	−
Galactose	+	+	+	+	−
Trehalose	+	+	+	−	−
D-Mannose	−	+	w	+	+
Acid production from:					
D-Galactose	−	+	+	+	−
Trehalose	−	−	w	+	−
D-Xylose	−	+	+	+	−
Major menaquinones	MK-7, MK-8	MK-7, MK-8	MK-7, MK-8, MK-9	MK-7, MK-8	MK-7, MK-8, MK-5
DNA G+C content (mol%)	64.8	64.0[a]	68.0[b]	64.5[a]	64.4[c]

*Data from: *a*, Li *et al.* (2005) *b*, Yoon *et al.* (2006) *c*, Li *et al.* (2004).

CK5^T lipid extract was obtained from 0.74 g freeze-dried cells harvested at stationary growth phase after growth conducted in a shaken flask culture at a temperature of 25 °C for 24 h in medium A supplemented with 2 % (w/v) NaCl. Representatives of the genus *Nesterenkonia* used for chemotaxonomic comparison were *N. halotolerans* DSM 15474^T (Li *et al.*, 2004), *N. lutea* DSM 15666^T (Li *et al.*, 2005), *N. sandarakina* DSM 15664^T (Li *et al.*, 2005) and *N. jeotgali* DSM 19081^T (Yoon *et al.*, 2006). Quinones were extracted form freeze-dried cells with *n*-hexane and were purified by TLC on silica gel (0.25 mm; F254, Merck) and eluted with *n*-hexane/ethylacetate (96 : 4, v/v). The purified UV-bands from TLC were then analysed by LC/MS on a reverse-phase RP-18 Lichrospher column eluted with *n*-hexane/ethylacetate (99 : 1, v/v) with a flow rate of 1.0 ml min^{-1} and identified by electrospray ionization (ESI)-MS and ^{1}H-NMR spectrometry (Nicolaus *et al.*, 2001). NMR spectra, recorded at the NMR Service of Institute of Biomolecular Chemistry of CNR (Pozzuoli, Italy), were acquired on a Bruker DPX-300 operating at 300 MHz, using a dual probe. The residual cellular pellet, after *n*-hexane extraction of freeze-dried cells, was subjected to extraction with $CHCl_3$/CH_3OH/H_2O (65 : 25 : 4, by vol.) for polar lipids recovery. The polar lipid extract was analysed by TLC on silica gel (0.25 mm, F254, Merck) eluted in the first dimension with $CHCl_3$/CH_3OH/H_2O (65 : 25 : 4, by vol.) and in the second dimension with $CHCl_3$/CH_3OH/acetic acid/H_2O (80 : 12 : 15 : 4, by vol.). All polar lipids were detected by spraying the plates with 0.1 % (w/v) $Ce(SO_4)_2$ in 1 M H_2SO_4 or with 3 % (w/v) methanolic solution of molybdophosphoric acid followed by heating at 100 °C for 5 min. Phospholipids and aminolipids were detected by spraying TLC plates with the Dittmer–Lester and ninhydrin reagents, respectively, and glycolipids were visualized with α-naphthol (Nicolaus *et al.*, 2001). Peptidoglycan analyses were performed as described by Erbs *et al.* (2008). In order to study the structure of the glycolipid present in strain CK5^T, 300 mg of polar lipid extract was fractionated by flash chromatography on silica gel (80 ml column volume) with chloroform/methanol (95 : 5, v/v), chloroform/methanol (90 : 10, v/v), chloroform/methanol (85 : 15, v/v) and chloroform/methanol (80 : 20, v/v). The fraction that was α-naphthol positive was eluted with chloroform/methanol (85 : 15, v/v) and was purified by preparative TLC eluting with chloroform/methanol/water (65 : 25 : 4, by vol.). The purified glycolipid (3.7 mg) dissolved in deuterated methanol was analysed by ^{1}H-^{13}C NMR-HSQC (heteronuclear single quantum correlation). The NMR spectra of glycolipids were recorded at the NMR Service of Institute of Biomolecular Chemistry of CNR (Pozzuoli, Italy) at 600 MHz (Bruker) with cryo-probe.

Cells of species of the genus *Nesterenkonia* used for comparison in the fatty acid methyl esters (FAMEs) analysis were grown under standard conditions as recommended by the culture collection and harvested at stationary growth phase. Cells of CK5^T were harvested at stationary growth phase after growth in shaken flask culture at a temperature of 25 °C for 24 h in medium A supplemented with 2 % (w/v) NaCl. FAMEs were obtained from complex lipids by acid methanolysis and analysed using a Hewlett Packard 5890A gas chromatograph fitted with a flame ionization detector, as previously reported (Nicolaus *et al.*, 2001). The FAME composition of CK5^T was anteiso-$C_{15:0}$ (64.8 %), iso-$C_{16:0}$ (13.3 %), *n*-$C_{17:0}$ (9.9 %), *n*-$C_{16:0}$ (4.0 %), *n*-$C_{14:0}$ (3.7 %), $C_{15:1}$ (2.0 %), *n*-$C_{15:0}$ (1.3 %) and iso-$C_{15:0}$ (0.95 %). The cellular fatty acids of strain CK5^T and recognized species of the genus *Nesterenkonia* are compared in Table S1 (available in the online Supplementary Material). anteiso-$C_{15:0}$ was the common and most abundant fatty acid in strain CK5^T and the most closely related species of the genus *Nesterenkonia*; moreover, although anteiso-$C_{17:0}$ was present in a high relative percentage in *N. sandarakina* DSM 15664^T (39.6 %), *N. jeotgali* DSM 19081^T (30.2 %) and *N. halotolerans* DSM 15474^T (27.6 %), and in *N. lutea* DSM 15666^T at a lower percentage (7.4 %), it was absent in CK5^T. In this latter strain, *n*-$C_{14:0}$, *n*-$C_{15:0}$ and *n*-$C_{17:0}$ were present in contrast to the nearest phylogenetically related species.

The total lipid content in strain CK5^T was 12.5 % of the total dry weight of cells grown at 25 °C in standard conditions and harvested in the stationary phase of growth. Chromatographic analysis of quinones revealed the presence of two UV-absorbing bands. The ^{1}H-NMR spectrum showed the presence of menaquinone signals similarly reported in the recognized species of the genus *Nesterenkonia*. The LC/MS analysis gave two molecular peaks: the predominant respiratory quinone was identified as MK-7 (72 %); MK-8 was also detected (28 %). The cell-wall peptidoglycan type was L-Lys-Gly-L-Asp. Three major phospholipids were found: phosphatidylinositol, diphosphatidylglycerol and phosphatidylglycerol (Fig. S1A). These phospholipids were also found in the related species of the genus *Nesterenkonia* tested, even if in a different ratio, as confirmed by TLC analysis (Fig. S1B). The TLC analysis of glycolipids of strain CK5^T showed a main band, visualized by using α-naphthol reagent, that presented a retention factor (R_f) similar to that found for the other species of the genus *Nesterenkonia* tested (Fig. S1C). The glycolipid of strain CK5^T was purified and then analysed by means of mono- and bi-dimensional NMR techniques revealing the presence of two mannose residues.

On the basis of physiological, biochemical and phylogenetic properties, considering that the DNA–DNA hybridization values were lower than the recommended threshold value accepted for defining a novel species (ranging from 31 to 1 %), strain CK5^T represents a novel species within the genus *Nesterenkonia*, for which the name *Nesterenkonia aurantiaca* sp. nov. is proposed.

Description of *Nesterenkonia aurantiaca* sp. nov.

Nesterenkonia aurantiaca (au.ran.ti.a′ca. N.L. fem. adj. *aurantiaca* orange-coloured, referring to the colour of colonies of the type strain).

Cells are Gram-stain-positive cocci (1.5 μm), non-motile and non-endospore-forming. Colonies on solid medium are orange, smooth, circular and approximately 1 mm in diameter at 25 °C after 48 h. The surface of the colonies is shiny and glistening. Growth occurs at 4–30 °C and pH 6.5–10 and with 0.003–20 % (w/v) NaCl. Optimum conditions for growth are 25 °C and pH 9, with 2 % NaCl. Catalase-positive but negative for oxidase, urease and indole formation. Does not reduce nitrate or nitrite. Hydrolyses xylan and pectin but not starch, casein, gelatin, hippurate, phenylalanine, urea or Tween 80. Positive for the ONPG, PNPG, lipase and aminopeptidase tests. Negative result in Voges–Proskauer reaction. Utilizes D-glucose, D-xylose, galactose, lactose, trehalose, D-fructose and sucrose as sole carbon and energy sources and does not produce acid from them. Does not utilize L-arabinose, raffinose or D-mannose. It is able to utilize L-alanine as sole nitrogen and energy source but not L-cysteine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-serine or L-valine. Utilizes acetate, citrate, propionate, succinate and malonate but not formate, gluconate or fumarate. The predominant menaquinones are MK-7 and MK-8; polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and a glycolipid containing the presence of two mannose residues. The cell-wall peptidoglycan type is L-Lys-Gly-L-Asp. The major FAMEs are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and *n*-$C_{17:0}$; the minor fatty acids are *n*-$C_{16:0}$, *n*-$C_{14:0}$, $C_{15:1}$, *n*-$C_{15:0}$ and iso-$C_{15:0}$.

The type strain, CK5^T (=DSM 27373^T=JCM 19723^T), was isolated from a soil sample, collected at Cape King (73° 05′ S 166° 40′ E, Antarctica). The DNA C+G content of the type strain is 64.8 mol%.

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References

Arahal, D. R., Dewhirst, F. E., Paster, B. J., Volcani, B. E. & Ventosa, A. (1996). Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl Environ Microbiol* **62**, 3779–3786.

Clark, W. M. & Lubs, H. A. (1915). The differentiation of bacteria of the *Colonaerogenes* family by the use of indicators. *J Infect Dis* **17**, 160–173.

Collins, M. D., Lawson, P. A., Labrenz, M., Tindall, B. J., Weiss, N. & Hirsch, P. (2002). *Nesterenkonia lacusekhoensis* sp. nov., isolated from hypersaline Ekho Lake, East Antarctica, and emended description of the genus *Nesterenkonia*. *Int J Syst Evol Microbiol* **52**, 1145–1150.

De Rosa, M., Gambacorta, A., Nicolaus, B., Buonocore, V. & Poerio, E. (1980). Immobilized bacterial cell containing a thermostable β-galactosidase. *Biotechnol Lett* **2**, 29–34.

Delgado, O., Quillaguamán, J., Bakhtiar, S., Mattiasson, B., Gessesse, A. & Hatti-Kaul, R. (2006). *Nesterenkonia aethiopica* sp. nov., an alkaliphilic, moderate halophile isolated from an Ethiopian soda lake. *Int J Syst Evol Microbiol* **56**, 1229–1232.

Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.

Erbs, G., Silipo, A., Aslam, S., De Castro, C., Liparoti, V., Flagiello, A., Pucci, P., Lanzetta, R., Parrilli, M. & other authors (2008). Peptidoglycan and muropeptides from pathogens *Agrobacterium* and *Xanthomonas* elicit plant innate immunity: structure and activity. *Chem Biol* **15**, 438–448.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Govender, L., Naidoo, L. & Setati, M.-E. (2013). *Nesterenkonia suensis* sp. nov., a haloalkaliphilic actinobacterium isolated from a salt pan. *Int J Syst Evol Microbiol* **63**, 41–46.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kouker, G. & Jaeger, K.-E. (1987). Specific and sensitive plate assay for bacterial lipases. *Appl Environ Microbiol* **53**, 211–213.

Li, W.-J., Chen, H.-H., Zhang, Y.-Q., Schumann, P., Stackebrandt, E., Xu, L.-H. & Jiang, C.-L. (2004). *Nesterenkonia halotolerans* sp. nov. and *Nesterenkonia xinjiangensis* sp. nov., actinobacteria from saline soils in the west of China. *Int J Syst Evol Microbiol* **54**, 837–841.

Li, W.-J., Chen, H.-H., Kim, C.-J., Zhang, Y.-Q., Park, D.-J., Lee, J.-C., Xu, L.-H. & Jiang, C.-L. (2005). *Nesterenkonia sandarakina* sp. nov. and *Nesterenkonia lutea* sp. nov., novel actinobacteria, and emended description of the genus *Nesterenkonia*. *Int J Syst Evol Microbiol* **55**, 463–466.

Li, W.-J., Zhang, Y.-Q., Schumann, P., Liu, H.-Y., Yu, L.-Y., Zhang, Y.-Q., Stackebrandt, E., Xu, L.-H. & Jiang, C.-L. (2008). *Nesterenkonia halophila* sp. nov., a moderately halophilic, alkalitolerant actinobacterium isolated from a saline soil. *Int J Syst Evol Microbiol* **58**, 1359–1363.

Luo, H.-Y., Miao, L.-H., Fang, C., Yang, P.-L., Wang, Y.-R., Shi, P.-J., Yao, B. & Fan, Y.-L. (2008). *Nesterenkonia flava* sp. nov., isolated from paper-mill effluent. *Int J Syst Evol Microbiol* **58**, 1927–1930.

Luo, H.-Y., Wang, Y.-R., Miao, L.-H., Yang, P.-L., Shi, P.-J., Fang, C.-X., Yao, B. & Fan, Y.-L. (2009). *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium isolated from the black liquor treatment system of a cotton pulp mill. *Int J Syst Evol Microbiol* **59**, 863–868.

Nei, M. & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Nicolaus, B., Manca, M. C., Lama, L., Esposito, E. & Gambacorta, A. (2001). Lipid modulation by environmental stresses in two models of extremophiles isolated from Antarctica. *Polar Biol* **24**, 1–8.

Onishi, H. & Kamekura, M. (1972). *Micrococcus halobius* sp. nov. *Int J Syst Bacteriol* **22**, 233–236.

Poli, A., Esposito, E., Orlando, P., Lama, L., Giordano, A., de Appolonia, F., Nicolaus, B. & Gambacorta, A. (2007). *Halomonas alkaliantarctica* sp. nov., isolated from saline lake Cape Russell in

Antarctica, an alkalophilic moderately halophilic, exopolysaccharide-producing bacterium. *Syst Appl Microbiol* **30**, 31–38.

Poli, A., Romano, I., Cordella, P., Orlando, P., Nicolaus, B. & Ceschi Berrini, C. (2009). *Anoxybacillus thermarum* sp. nov., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs, Abano Terme, Italy. *Extremophiles* **13**, 867–874.

Romano, I., Manca, M. C., Lama, L., Nicolaus, B. & Gambacorta, A. (1993). A method for antibiotic assay on *Sulfolobales*. *Biotechnol Tech* **7**, 439–440.

Romano, I., Finore, I., Nicolaus, G., Huertas, F. J., Lama, L., Nicolaus, B. & Poli, A. (2008). *Halobacillus alkaliphilus* sp. nov., a halophilic bacterium isolated from a salt lake in Fuente de Piedra, southern Spain. *Int J Syst Evol Microbiol* **58**, 886–890.

Romano, I., Dipasquale, L., Orlando, P., Lama, L., d'Ippolito, G., Pascual, J. & Gambacorta, A. (2010). *Thermoanaerobacterium thermostercus* sp. nov., a new anaerobic thermophilic hydrogen-producing bacterium from buffalo-dung. *Extremophiles* **14**, 233–240.

Rustigian, R. & Stuart, C. A. (1941). Decomposition of urea by *Proteus*. *Proc Soc Exp Biol Med* **47**, 108–112.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E., Koch, C., Gvozdiak, O. & Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *Int J Syst Bacteriol* **45**, 682–692.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Wang, H.-F., Zhang, Y.-G., Chen, J.-Y., Hozzein, W. N., Li, L., Wadaan, M. A. M., Zhang, Y.-M. & Li, W.-J. (2014). *Nesterenkonia rhizosphaerae* sp. nov., an alkaliphilic actinobacterium isolated from rhizosphere soil in a saline-alkaline desert. *Int J Syst Evol Microbiol* **64**, 4021–4026.

Yoon, J.-H., Jung, S.-Y., Kim, W., Nam, S.-W. & Oh, T.-K. (2006). *Nesterenkonia jeotgali* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **56**, 2587–2592.

Zhang, G., Zhang, Y., Yin, X. & Wang, S. (2015). *Nesterenkonia alkaliphila* sp. nov., an alkaliphilic, halotolerant actinobacteria isolated from the western Pacific Ocean. *Int J Syst Evol Microbiol* **65**, 516–521.