

Nesterenkonia halophila sp. nov., a moderately halophilic, alkalitolerant actinobacterium isolated from a saline soil

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A Gram-positive, non-motile, moderately halophilic, alkalitolerant actinobacterium, designated strain YIM 70179^T, was isolated from a saline soil sample collected from Xinjiang Province, north-west China, and was subjected to a polyphasic taxonomic study. The cell-wall peptidoglycan type of strain YIM 70179^T was A4 α , L-Lys–Gly–L–Glu. Cells of the isolate contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown glycolipid, MK-8 as major menaquinone and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as major fatty acids. The DNA G + C content was 68.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 70179^T fell within the radiation of species of the genus *Nesterenkonia*. Levels of 16S rRNA gene sequence similarity between strain YIM 70179^T and the type strains of recognized *Nesterenkonia* species were below 97%, except to *Nesterenkonia halobia* DSM 20541^T (99.6%), but these two strains exhibited a low level of DNA–DNA relatedness (18.4%). Based on genetic and phenotypic evidence, it is proposed that strain YIM 70179^T represents a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia halophila* sp. nov. is proposed. The type strain is YIM 70179^T (=DSM 16378^T =KCTC 19048^T).

The genus *Nesterenkonia* was first proposed by Stackebrandt *et al.* (1995) with the reclassification of *Micrococcus halobius* (Onish & Kamekura, 1972) as *Nesterenkonia halobia*. The description of the genus was later emended by Collins *et al.* (2002) and Li *et al.* (2005). At the time of writing, the genus comprises seven recognized species, namely *N. halobia* (Stackebrandt *et al.*, 1995), *N. lacusekhoensis* (Collins *et al.*, 2002), *N. halotolerans* and *N. xinjiangensis* (Li *et al.*, 2004), *N. sandarakina* and *N. lutea* (Li *et al.*, 2005) and *N. aethiopica* (Delgado *et al.*, 2006). Here we present the description of strain YIM 70179^T, which is shown to represent a novel species of the genus *Nesterenkonia*.

Strain YIM 70179^T was isolated from a saline soil sample collected from Xinjiang Province, north-west China, by using the dilution plating method. For isolation, MSG medium supplemented with 25% KCl (w/v) was used. The MSG medium was modified from S-G medium (Sehgal & Gibbons, 1960) and contained (per litre distilled water): 7.5 g Casamino acids, 10.0 g yeast extract, 3.0 g trisodium citrate, 2.0 g NaCl, 2.0 g MgCl₂ · 6H₂O, 1.0 g MgSO₄ · 7H₂O, 0.05 g FeSO₄ · 7H₂O and 0.2 mg MnSO₄ · 7H₂O (pH 9.0). Sterilized saturated NaOH was used to adjust the pH. Plates were incubated at 28 °C for 2 weeks. Strain YIM 70179^T was maintained on MSG agar slants that contained 10% KCl (w/v) at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Biomass for chemical and molecular systematic studies was obtained from MSG agar supplemented with 10% KCl (w/v) incubated at 28 °C for about 4–5 days.

Gram staining and the KOH lysis test were carried out according to Gram (1884) and Cerny (1978), respectively.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 70179^T is AY820953.

Morphology and motility of cells (tested at various growth stages) on MSG agar containing 10% KCl were examined by light microscopy (model BH 2; Olympus) and transmission electron microscopy (H-800; Hitachi). For transmission electron microscopy observation, cells were negatively stained with 1% (w/v) phosphotungstic acid after air drying. Colony morphology and colour were observed on MSG and PYGV agar media (Staley, 1968) containing 10% KCl after incubation at 28 °C for 2 days. Colour was determined by comparing the cultures with the most suitable colour chips from Kelly (1964). Growth was tested at 0, 4, 10, 28, 37, 40, 45 and 55 °C on trypticase soy agar (TSA) containing 10% KCl. Experiments investigating tolerance to NaCl, KCl and MgCl₂·6H₂O were carried out by using non-salt-containing trypticase soy broth (TSB) as the basal medium. The following NaCl, KCl and MgCl₂·6H₂O concentrations (w/v) were tested: 0, 0.5, 1, 3, 5, 10, 15, 20, 25 and 30%. For pH endurance experiments, the following buffer solutions were used: pH 5.0–5.5, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 10 mM HEPES/0.5 M NaOH; pH 8.5–11.5, 0.5 M NaHCO₃/0.5 M Na₂CO₃. Liquid TSB (containing 10% KCl) cultures were cultivated in tubes at 28 °C for 14 days. Tests investigating the ability of the novel strain to use sole carbon sources and peptonization activity and coagulation of milk were performed according to the methods described by Shirling & Gottlieb (1966). Tests for hydrolysis of casein, starch and Tweens 20 and 80, H₂S production and the methyl red and Voges–Proskauer reactions were performed as described by Smibert & Krieg (1981), by using the basal medium supplemented with 10% (w/v) KCl. Activity of several enzymes, such as lipase, lysine decarboxylase and arginine dihydrolase, was determined by using API ID32 E test kits. Nitrate reduction, gelatin liquefaction and acid production were tested by using the API Coryne system according to the manufacturer's instructions (bioMérieux), except that the cell suspension to inoculate the systems was prepared by using sterilized artificial seawater supplemented with 10% (w/v) KCl. The oxidase reaction was tested by using API oxidase reagent. Catalase activity was determined based on production of bubbles after the addition of a drop of 3% H₂O₂.

Purified peptidoglycan preparations were obtained according to the method described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates (Merck) by using the solvent systems of Schleifer & Kandler (1972). The amino-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985). Molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyl amino acid isobutyl esters (MacKenzie, 1987). Analysis of enantiomers of peptidoglycan amino acids was performed by GC of *N*-pentafluoropropionyl amino acid isopropyl esters (Frank *et al.*, 1980) on an L-chirasil Val column (Macherey-Nagel) as described by Groth *et al.* (1997). Analyses of polar lipids and menaquinones followed published procedures (Groth *et al.*,

1999). Analysis of the whole-cell fatty acid pattern followed the methods of Miller (1982) by using the MIDI system (Microbial ID, Inc.).

Extraction of genomic DNA was performed as described by Xu *et al.* (2003). Primers PA (forward; positions 8–27 according to the *Escherichia coli* numbering system; 5'-AGAGTTTGATCCTGGCTCAG-3') and PB (reverse; 1523–1504; 5'-AAGGAGGTGATCCAGCCGCA-3') were used for amplification of the 16S rRNA gene sequence of the novel isolate, and the PCR products were purified and sequenced as described by Cui *et al.* (2001).

Phylogenetic analysis was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignments of the data with CLUSTAL_X (Thompson *et al.*, 1997). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980, 1983) and cluster analysis was performed with the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985).

The DNA base composition of strain YIM 70179^T was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). DNA–DNA hybridization was carried out applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions.

When grown on SMG agar for 48 h, cells of strain YIM 70179^T were aerobic, Gram-positive, non-motile, non-spore-forming cocci, about 1.2–1.8 µm in diameter. Colonies were ivory white, circular, lubricious and opaque. Growth occurred from 4 to 45 °C on TSA containing 10% KCl. Strain YIM 70179^T was able to grow in TSB supplemented with 10% KCl (w/v) or NaCl, when the initial pH ranged from 6.0 to 10.5. In TSB broth lacking salts, growth was observed with 0.5–30% KCl or NaCl or MgCl₂·6H₂O (w/v) added. Good growth occurred at 28 °C, at an initial pH of 7.0–8.0 and in the presence of 10.0% KCl or NaCl. Detailed physiological properties of strain YIM 70179^T are listed in Table 1 and in the species description below.

Chemotaxonomically, the peptidoglycan type of strain YIM 70179^T was A4α (Schleifer & Kandler, 1972), L-Lys–Gly–L-Glu, in which L-lysine is in position 3 of the peptide subunit and glycine and L-glutamic acid form the interpeptide bridge. The isoprenoid quinones were menaquinones of type MK-8, MK-9 and MK-7 (ratio 81:7:6). Polar lipids consisted of phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. Major cellular fatty acids were anteiso-C_{15:0} (47.1%), anteiso-C_{17:0} (31.1%) and iso-C_{16:0} (11.2%); the detailed fatty acid profile of strain YIM 70179^T is given in the species description below.

Analysis of the almost-complete 16S rRNA gene sequence (1484 nt) of strain YIM 70179^T with those of a broad range

Table 1. Comparison of phenotypic properties of strain YIM 70179^T and its closest phylogenetic neighbour, *N. halobia* DSM 20541^T

Data for strain YIM 70179^T were obtained in the present study with TSB as the basal medium. Data for *N. halobia* DSM 20541^T were taken from Stackebrandt *et al.* (1995) and Mota *et al.* (1997) except where indicated. The two strains share the following characteristics. Cells are Gram-positive, non-motile, non-spore-forming cocci. Positive for catalase, ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, α -glucosidase, β -glucosidase, β -galactosidase, α -maltosidase and aesculin hydrolysis, but negative for α -galactosidase, *N*-acetylglucosaminidase, β -glucuronidase, *L*-aspartic arylamidase, urease, gelatin liquefaction, methyl red and Voges–Proskauer tests and H₂S and indole production. The two strains have the same peptidoglycan type and polar lipid composition.

| Characteristic | YIM 70179 ^T | <i>N. halobia</i> DSM 20541 ^T |
|-----------------------------------|---|---|
| Cell arrangement | Single, in pairs or in clusters | Pairs, tetrads or irregular clusters |
| Colony pigmentation (PYGV medium) | Ivory white | Colourless |
| NaCl range for growth (%) | 0.5–30.0 | 5.0–25* |
| pH tolerance | 6.0–10.5 | 5.5–10.0* |
| Lipase | + | – |
| Oxidase activity | – | + |
| Starch hydrolysis | – | + |
| Nitrate reduction | + | – |
| Major menaquinone(s) | MK-8 | MK-8, MK-9 |
| Major fatty acids (>10%) | anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{16:0} | anteiso-C _{15:0} , iso-C _{17:0} |
| DNA G + C content (mol%) | 68.4 | 71.5 |

*Determined in the present study with TSB as basal medium.

of homologous actinobacterial taxa revealed that the closest relative of strain YIM 70179^T was *N. halobia* DSM 20541^T (sequence similarity of 99.6%); levels of 16S rRNA gene sequence similarity between strain YIM 70179^T and the other actinobacterial test strains were less than 97%. Fig. 1 shows the phylogenetic position of strain YIM 70179^T within the radiation of type strains of all recognized *Nesterenkonia* species.

The level of DNA–DNA relatedness of 18.4% (mean value of two experiments) between strain YIM 70179^T and its closest phylogenetic neighbour, *N. halobia* DSM 20541^T, was significantly below the value of 70% which is considered to be the threshold for the delineation of genomic species (Wayne *et al.*, 1987). Additionally, strain YIM 70179^T could be differentiated from *N. halobia* DSM 20541^T on the basis of several phenotypic characteristics (Table 1).

On the basis of its phenotypic and genotypic properties, strain YIM 70179^T is considered to represent a novel species of the genus *Nesterenkonia*, for which the name *Nesterenkonia halophila* sp. nov. is proposed.

Description of *Nesterenkonia halophila* sp. nov.

Nesterenkonia halophila (ha.lo'phi.la. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* loving; N.L. fem. adj. *halophila* salt-loving, referring to the ability to grow at high salt concentrations).

Cells are Gram-positive, non-motile, non-spore-forming cocci. Colony colour is ivory white. Colonies are circular, opaque and approximately 0.5–1.0 mm in diameter after 48 h at 28 °C. Growth occurs at 0.5–30% (w/v) NaCl, KCl or MgCl₂·6H₂O (optimum growth at 10%, w/v). Good growth occurs at initial pH of 7.0–8.0 and at 28 °C. Positive for peptonization and coagulation of milk, but negative for production of H₂S, ammonia and melanin. Other phenotypic characteristics are given in Table 1. Utilizes *L*-arabinose, xylose, *D*-fructose, cellulose, *D*-mannose, glucose, malonate, mannitol, salicin and acetamide, but not arabitol, adonitol, cellobiose, inositol or rhamnose as the only carbon source. Negative for hydrolysis of casein and Tweens 20 and 80. No acid production from glucose, ribose, xylose, mannitol, malonate, lactose, sucrose or glycogen. The peptidoglycan type is A4 α , *L*-Lys–Gly–*L*-Glu.

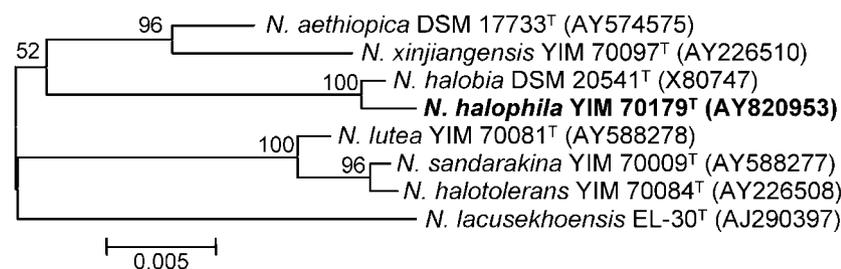


Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 70179^T among its phylogenetic neighbours. Numbers on branch nodes are bootstrap values (1000 resamplings; only values >50% are given). Bar, 0.5% sequence divergence.

The polar lipids contain phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid. The isoprenoid quinones are menaquinones of type MK-8, MK-9 and MK-7 (ratio 81:7:6). The cellular fatty acid profile comprises anteiso-C_{15:0} (47.1%), anteiso-C_{17:0} (31.1%), iso-C_{16:0} (11.2%), C_{16:0} (3.3%), iso-C_{15:0} (2.4%), iso-C_{17:0} (1.3%), iso-C_{16:1} (0.9%), anteiso-C_{17:1}ω9c (0.5%), iso-C_{14:0} (0.4%), anteiso-C_{15:1} (0.4%), C_{16:1}ω7c (0.3%) and iso-C_{15:1} (0.2%). The DNA G+C content of the type strain is 68.4 mol%.

The type strain, YIM 70179^T (=DSM 16378^T =KCTC 19048^T), was isolated from a saline soil sample in Xinjiang Province, north-west China.

Note added on revision

Since this article was submitted for publication, an additional species of the genus has been described, *Nesterenkonia jeotgali* (Yoon *et al.*, 2006).

Acknowledgements

This research was supported by the National Facilities and Information Infrastructure for Science and Technology (grant no. 2006DKA21203), National Basic Research Program of China (project no. 2004CB719601), National Natural Science Foundation of China (project no. 30600001) and a Key Project of the Chinese Ministry of Education (no. 206139). W.-J. L. was also supported by the Program for New Century Excellent Talent in University (NCET).

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