Nesterenkonia rhizosphaerae sp. nov., an alkaliphilic actinobacterium isolated from rhizosphere soil in a saline-alkaline desert

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An alkaliphilic actinobacterial strain, designated EGI 80099T, was isolated from a rhizosphere soil sample of Reaumuria soongorica found in the desert soils of Fukang, Xinjiang, north-west China. Cells of strain EGI 80099T were Gram-stain-positive, non-motile, non-endospore-forming cocci. The predominant menaquinones were MK-7, MK-8 and MK-9. The major cellular fatty acids (>10%) were anteiso-C15:0 and anteiso-C17:0. Analysis of the cell wall showed the presence of peptidoglycan of the type L-Lys–Gly–L-Glu, variation A4α. Cells of the isolate contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, an unknown phospholipid and an unidentified glycolipid as polar lipids. The genomic DNA G+C content was 63.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain EGI 80099T belongs to the genus Nesterenkonia, sharing 95.68–97.37% sequence similarities with the type strains of recognized species within this genus. DNA–DNA hybridization of strain EGI 80099T with the type strains of species that showed the highest sequence similarities, Nesterenkonia aethiopica DSM 17733T (97.37%), Nesterenkonia flava CAAS 251T (97.23%) and Nesterenkonia xinjiangensis YIM 70097T (97.02%), gave relatedness values of 8.7–62.2%. Data from DNA–DNA hybridizations and physiological and biochemical tests indicated that strain EGI 80099T represents a novel species of the genus Nesterenkonia, for which the name Nesterenkonia rhizosphaerae sp. nov. is proposed. The type strain is EGI 80099T (=BCRC 16947T=JCM 19129T).

The genus Nesterenkonia was first proposed by Stackebrandt et al. (1995). A number of novel species of the genus Nesterenkonia have been described in recent years. At the time of writing, the genus comprises 12 recognized species:

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EGI 80099T is KF040423.

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.
rhizosphere soil sample of Reaumuria soongorica and proposed that it should be assigned to a novel species based on data from a polyphasic taxonomic study.

Strain EGI 80099T was isolated using the dilution plating method at 30 °C with glycerol–asparagine agar medium (Shirling & Gottlieb, 1966). After sterilization, the medium was adjusted to pH 10.0 with autoclaved 10 M NaOH. Subculturing was conducted on Horikoshi-I medium (HI) containing (g l⁻¹): glucose (10 g), yeast extract (5 g), peptone (5 g) K₂HPO₄ (1 g), MgSO₄·7H₂O (0.2 g), agar 1(5 g), adjusted to pH 10.0 with 10 M NaOH instead of Na₂CO₃ (Horikoshi, 1999) and plates were incubated at 30 °C until a pure culture was obtained. The purified strain, EGI 80099T, was preserved in glycerol suspensions (20 %, w/v, glycerol) at −80 °C.

Gram staining was carried out using the standard Gram stain. The morphological, physiological and biochemical characteristics of strain EGI 80099T were investigated using cultures grown on trypticase soy agar (TSA, Difco Company). Cells of strain EGI 80099T were grown on TSA medium (adjusted to pH 10.0 with 10 M NaOH instead of Na₂CO₃) at 30 °C for 24–48 h and their morphology was observed using light microscopy (BH-2; Olympus) and transmission electron microscopy (JEM-2100; JEOL). Colony characteristics were examined by growing the bacterium on TSA medium at 30 °C at pH 10 for 2 days (Li et al., 2004). Temperature range for growth was determined by growing the isolate in TSA medium (pH 10.0) at 5, 10, 15, 20, 25, 30, 35, 40, 45, 55 and 60 °C. The optimum pH for growth was tested in trypticase soy broth (TSB, Difco Company) at 30 °C and pH 4–13 (at intervals of 1.0 pH unit) using the buffer system described by Xu et al. (2005). NaCl tolerance was tested in TSA medium supplemented with 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 and 30 % (w/v) NaCl at pH 10 and 30 °C. Catalase and oxidase activities were determined using 3 % (v/v) H₂O₂ and 1 % (w/v) tetramethyl-p-phenylenediamine, respectively. Carbon source utilization tests were performed according to the methods described by Shirling & Gottlieb (1966). Nitrogen source utilization tests were carried out as described by Williams et al. (1983). Acid production from carbohydrates was tested as described by Leifson (1963), supplemented with 0.01 % (w/v) yeast extract. Other physiological and biochemical characteristics were examined as described by Goodfellow (1971) and Williams et al. (1983).

Antibiotic sensitivity was determined by the agar diffusion test. One hundred microlitres of a 48 h culture was spread on a TSA agar plate (pH 10.0) and antibiotic discs were then placed on the agar followed by incubation for 5 days at 30 °C. The antimicrobial compounds tested were (concentration per disc): ampicillin (10 µg), cephalosporin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (10 µg), novobiocin (30 µg), rifampicin (5 µg), roxithromycin (15 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). Antibiotic sensitivity or resistance was defined in accordance with Performance Standards for Antimicrobial Susceptibility Testing, 16th Informational Supplement (CLSI, 2006).

Cells of strain EGI 80099T were Gram-stain-positive, non-motile, non-spore-forming cocci of 0.5 to 0.7 µm diameter (Fig. 1). Colonies were light yellow, smooth, glossy, circular with entire margins and approximately 1.0–1.2 mm in

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**Fig. 1.** Transmission electron micrograph of cellular morphological characteristics of strain EGI 80099T grown on solid TSA medium adjusted to pH 10.0 for 4 days at 30 °C. Bar, 500 nm. Magnification ×15 000.
diameter on TSA medium at 30 °C after 48 h. Growth occurred at 15–45 °C and no growth was observed at 5, 10, 15, 50, 55 or 60 °C. The strain grew at pH 6–11, but not at pH 4, 5 or 12. Growth was observed in the absence of NaCl and in the presence of up to 15 % (w/v) NaCl, while no growth occurred at 5, 10, 15, 50, 55 or 60 °C. The strain grew at pH 6–11, but not at pH 4, 5 or 12. Growth was observed in the absence of NaCl and in the presence of up to 15 % (w/v) NaCl. Cells grew optimally in TSA medium at 30–35 °C at pH 9–10 and with 0–5 % (w/v) NaCl. Cells were sensitive to ampicillin, gentamicin, kanamycin, neomycin, nalidixic acid, streptomycin, tetracycline and vancomycin; and resistant to cephalosporin, chloramphenicol, erythromycin, novobiocin, rifampicin and roxithromycin. Other differential characteristics between strain EGI 80099T and type reference strains (N. flava CAAS 251T, N. alba CAAS 252T, N. aethiopica DSM 17733T, N. xinjiangensis YIM 70097T) are shown in Table 1, while the detailed characteristics of the strain EGI 80099T are given in the species description.

Biomass for analysis of menaquinones, cell wall and polar lipids, and for DNA extraction was prepared by growth on solid HI medium adjusted to pH 10.0 with NaOH at 30 °C for 4 days. A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer & Kandler (1972). Amino acids in cell-wall hydrolysates were analysed by HPLC as described by Tang et al. (2009). Polar lipids were extracted and identified by two-dimensional TLC following the method of Minnikin et al. (1984). Menaquinones were extracted and analysed by HPLC as described by Collins et al. (1977) and Kroppenstedt (1982). For fatty acid analysis, strain EGI 80099T was cultured on TSA (Difco) medium adjusted to pH 10.0 with NaOH at 30 °C for 4 days. Cellular fatty acid analysis was performed as described by Sasser (1990) according to the standard protocol of the Sherlock Microbial Identification System (MIDI; version 6.1, MIDI database TSBA6).

### Table 1. Comparison of phenotypic characteristics of strain EGI 80099T with closely related species of the genus Nesterenkonia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>Cocci</td>
<td>Short rods*</td>
<td>Short rods†</td>
<td>Short rods‡</td>
<td>Short rods§</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Yellow</td>
<td>Yellow</td>
<td>White</td>
<td>Yellow</td>
<td>Light yellow</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Degradation of starch</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Urease</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Degradation of Tween 60</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Degradation of Tween 80</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Temperature range (°C)</td>
<td>10–45</td>
<td>15–50</td>
<td>15.0–50.0</td>
<td>10–45</td>
<td>10.0–35.0</td>
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<tr>
<td>pH tolerance</td>
<td>6–11</td>
<td>7–11</td>
<td>6.0–10.0</td>
<td>6–11</td>
<td>6.0–10.0</td>
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<td>NaCl tolerance (%) (%, w/v)</td>
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<td>0–10</td>
<td>0–7.5</td>
<td>0–15</td>
<td>0–17.5</td>
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<tr>
<td>Growth on sole carbon source</td>
<td>d-Galactose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Trehalose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>Raffinose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Xyitol</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>d-Fructose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>a-Lactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Inositol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>d-Mannitol</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>d-Sorbitol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Growth on sole nitrogen source</td>
<td>l-Arginine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:</td>
<td>Trehalose</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
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<tr>
<td></td>
<td>d-Glucose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>d-Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>63.4</td>
<td>65.5*</td>
<td>60.2†</td>
<td>69.0‡</td>
<td>66.7§</td>
</tr>
</tbody>
</table>

*Data were taken from Luo et al. (2008).†Data were taken from Luo et al. (2009).‡Data were taken from Delgado et al. (2006).§Data were taken from Li et al. (2004).
The peptidoglycan type of strain EGI 80099T was A4a, L-Lys–Gly–L-Glu. The predominant menaquinones were MK-7 (13.4 %), MK-8 (31.5 %) and MK-9 (53.0 %), with minor amounts of MK-10 (2.1 %). Polar lipids of strain EGI 80099T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, an unknown phospholipid and an unidentified glycolipid (see Fig. S1, available in the online Supplementary Material). The major cellular fatty acids (≥10 %) were anteiso-C15:0 (45.1 %) and anteiso-C17:0 (40.1 %); the detailed fatty acid profile of strain EGI 80099T is given in Table S1.

The DNA base composition of strain EGI 80099T was determined by HPLC analysis and the G+C content calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah et al. (1989). The genomic DNA G+C content of strain EGI 80099T was 63.4 mol%.

An almost-complete 16S rRNA gene fragment was amplified from genomic DNA using the primer set PA (forward, positions 8–27 according to the Escherichia coli numbering system, 5′-AGAGTTTGATCCTGGCTCAG-3′) and PB (reverse, 1523–1504, 5′-AAGGAGGTGATCCAGCCGCA-3′). The PCR amplicon was cloned into E. coli DH5α cells using the pEASY T1 simple vector and was sequenced at Sangon Biotech (Shanghai). Next, the 16S rRNA gene sequence was compared with sequences from EzBioCloud using BLAST (http://www.ezbiocloud.net/eztaxon) (Kim et al., 2012). The gene sequence was aligned with corresponding sequences (retrieved from the GenBank/EMBL/DDBJ database) using CLUSTAL X, version 1.83 (Thompson et al., 1997).

Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 5 (Tamura et al., 2011). Evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1983). Phylogenetic relationships were analysed by using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings.

**Fig. 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain EGI 80099T in relation to recognized species of the genus Nesterenkonia. Numbers at nodes indicate the level of bootstrap support (≥50 %) based on 1000 resamplings. Asterisks indicate the corresponding nodes that were also recovered in the tree generated with the maximum-parsimony algorithm. The maximum-likelihood method generated the same tree topology as the neighbour-joining algorithm. Bar, 0.01 changes per nucleotide position.
Morphological, biochemical and chemical features, strain EGI

delineation of genomic species (Wayne et al., 1987).

Comparative analysis of the almost-complete 16S rRNA
gene sequence (1521 bp) revealed that strain EGI 80099T
belonged to the genus *Nesterenkonia*. Phylogenetic analysis
based on the neighbour-joining algorithm and evaluated by
bootstrap analysis of 1000 resamplings indicated that these
strains clustered together in a distinct branch (Fig. 2).

Strain EGI 80099T was most closely related to
*Nesterenkonia renkonia rhizosphaerae* (rhi.zo.sphae-rae. N.L. gen. n.
rhizosphaerae of the rhizosphere).

According to the significant differences between strain EGI
80099T and those members of the genus *Nesterenkonia* in
morphological, biochemical and chemical features, strain
EGI 80099T represents a previously unknown novel species
of the genus *Nesterenkonia*, for which the name *Nesteren-
konia rhizosphaerae* sp. nov. is proposed.

**Description of Nesterenkonia rhizosphaerae sp. nov.**

*Nesterenkonia rhizosphaerae* (rhi.zo.spha’erae. N.L. gen. n.
rhizosphaerae of the rhizosphere).

Cells are Gram-stain-positive, non-motile, non-sporo-
forming coccii. The colony colour on modified TSA
medium is light yellow. Colonies are smooth, glossy,
circular with entire margins, and approximately 1.0–
1.2 mm in diameter on solid TSA medium at 30 °C after
48 h. Growth occurs at 10–45 °C, pH 6–11 and with 0–
15 % (w/v) NaCl. Positive for oxidase, catalase and gel
liquefaction, while negative for urease, production of H2S,
nitrate reduction and the methyl red test. Does not degrade
Tryptophan. Cells are sensitive to ampicillin, gentamicin,
Tween 20, Tween 40, Tween 60, Tween 80, starch or
nitrate reduction and the methyl red test. Does not degrade
galactose, D-glucose, glycerol, maltose, mannose, D-sorbi-
tol, sorbose, starch, sucrose, trehalose and xylose are
utilized, but inositol, α-lactose, Δ-mannitol, raffinose and
xylitol are not. The peptidoglycan type is A4
and MK-9. The major cellular fatty acids (>10 %) are
anteiso-C15:0 and anteiso-C17:0. Phospholipids are phos-
phatidylglycerol, diphosphatidylglycerol, phosphatidy-
choline, an unknown phospholipid and an unidentified
glycerolipid.

The type strain is EGI 80099T (=BCRC 16947T=JCM
19129T), isolated from the rhizosphere soil sample of
*Rhaumuria soongorica* found in the desert soil of Fukang,
Xinjiang, north-west China. The DNA G+C content of the
type strain is 63.4 mol%.

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