Nesterenkonia suensis sp. nov., a haloalkaliphilic actinobacterium isolated from a salt pan

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A Gram-positive, non-motile, non-sporo-forming actinobacterium designated strain Sua-BAC020T was isolated from brine from Sua salt pan in Botswana. The strain was alkaliphilic and moderately halophilic, displaying optimal growth at 35–37 °C, pH 9 and 2.5 % (w/v) NaCl. Comparative 16S rRNA gene sequence analysis showed that strain Sua-BAC020T belonged to the genus Nesterenkonia, sharing 96.2–99.0 % sequence similarity with the type strains of recognized species within this genus. DNA–DNA hybridization with the type strains of species that showed the closest phylogenetic affiliation, Nesterenkonia xinjiangensis (16S rRNA gene sequence similarity, 98.9 %), Nesterenkonia aethiopica (99.0 %), Nesterenkonia halophila (97.5 %), Nesterenkonia flava (97.4 %) and Nesterenkonia halobia (97.2 %), gave relatedness values of 10–45 %. The peptidoglycan type of strain Sua-BAC020T was A4γ, L-Lys–Gly–D-Asp. Cells of the isolate contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and unidentified glycolipids as major polar lipids, MK-8, MK-9 and MK-7 were the predominant menaquinones, and the major fatty acids (>10 %) were anteiso-C₁₅:₀ and anteiso-C₁₇:₀. The DNA G+C content of strain Sua-BAC020T was 64.8 mol%. Based on DNA–DNA hybridization, and physiological and biochemical tests, strain Sua-BAC020T is distinct from all recognized Nesterenkonia species, suggesting that this strain represents a novel species, for which the name Nesterenkonia suensis sp. nov. is proposed. The type strain is Sua-BAC020T (=DSM 22748T =NCCB 100309T).

The genus Nesterenkonia comprises Gram-positive non-sporo-forming actinobacteria that may be either halotolerant or halophilic. Some species may also be alkalitolerant or alkaliphilic. Nesterenkonia species have been isolated from various environments including saline soil, hypersaline lakes, soda lakes, solar salt works and fermented seafood, as well as paper and cotton pulp mills. At the time of writing, the genus comprises 11 recognized species: Nesterenkonia halobia (Stackebrandt et al., 1995; Mota et al., 1997), Nesterenkonia lacusekoehensis (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 alba (Luo et al., 2009). Members of the genus Nesterenkonia generally contain peptidoglycan of the A4γ type and have DNA G+C contents in the range 64–72 mol% (Li et al., 2005). The current study describes the taxonomic characterization of strain Sua-BAC020T, a novel haloalkaliphilic member of the genus Nesterenkonia which was isolated from an evaporator pond at Sua salt pan in Botswana.

Strain Sua-BAC020T was isolated from brine collected from evaporator pond CS1 at Sua salt pan in Botswana. The brine was enriched with 2 % yeast extract and incubated with shaking at 150 r.p.m. for 7 days, followed by direct plating onto 10 % salt water medium (SW-10) containing 80 g NaCl, 11.7 g MgSO₄⋅7H₂O, 10 g MgCl₂⋅6H₂O, 0.17 g CaCl₂⋅H₂O, 2.3 g KCl and 20 g bacteriological agar, per litre of distilled water (Dyall-Smith, 2000). The purified strain was maintained in 40 % glycerol at −70 °C, and routinely cultivated on Luria–Bertani medium supplemented with 5 % (w/v) NaCl (LB-NaCl). Morphological characteristics were examined by light microscopy at ×1000 magnification and scanning electron microscopy (SEM) at ×25 000 magnification. Strain Sua-BAC020T was

### Abbreviation
SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Sua-BAC020T is FJ948172.

Two supplementary figures are available with the online version of this paper.
cultivated for 48–72 h at 37 °C on LB-NaCl agar medium. Single colonies were picked and stained by Gram staining for light microscopy observations. For SEM analysis, the samples were fixed in glutaraldehyde and osmium tetroxide, then dehydrated through an alcohol series and dried using a critical point dryer (Hitachi CPD HCP-1). The dried samples were then loaded onto aluminium stubs using double-sided carbon tape, and coated with gold using a 500-Sputter coater. The samples were viewed with a Leo 1450 scanning electron microscope using SmartSEM software, version 5.03.05. Cell motility was examined from freshly grown cultures according to Lee et al. (2004). The optimum growth temperature was determined in liquid LB-NaCl, with shaking at 150 r.p.m., at 4, 8, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C. A temperature of 37 °C was included due to its regular use for bacterial cultivation. Optimum pH for growth was determined within the range pH 6–11 (at intervals of 0.5 pH units) in buffered LB using 50 mM NaOH buffer (pH 8.5, 9 and 9.5) and 50 mM phosphate-NaOH buffer (pH 10, 10.5 and 11). Tolerance to NaCl was evaluated in LB medium supplemented with 0, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 18, 19 and 20 % (w/v) NaCl. Growth was monitored by determining OD600 and viable counts by plating on LB-NaCl at 3 h intervals for 48 h. The aerobic growth was determined on 2 % skimmed milk.

The production of acid and gas from carbohydrates (glucose, mannose, galactose, lactose, sucrose, fructose, maltose and xylose) was determined in SW-10 supplemented with 0.5 % yeast extract following the method of Leifson (1963). Methyl red and Voges–Proskauer tests were performed using the MR-VP medium as described by Cowan & Steel (1965). Citrate utilization was tested on Simmons citrate agar. Nitrate reduction and phenylalanine deamination were assessed in nitrate broth and phenylalanine agar, respectively. Catalase and oxidase activity was determined using 3 % H2O2 and 1 % tetramethylphenylenediamine, following standard procedures.

Production of xylanase was determined on LB-NaCl agar supplemented with 0.2 % birchwood xylan. The plates were incubated at 37 °C for 48 h followed by staining with 0.1 % (w/v) Congo red solution (Teather & Wood, 1982). Starch hydrolysis was determined on 1 % soluble starch, followed by staining with iodine solution, while casein hydrolysis was determined on 2 % skimmed milk.

Antibiotic sensitivity was determined by the agar diffusion test. One hundred microlitres of a 48 h culture was spread on LB-NaCl agar, and antibiotic discs were then placed on the agar and incubated for 5 days at 37 °C. The antimicrobial compounds tested were: ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), novobiocin (30 µg), penicillin G (10 µg), streptomycin (10 µg) and tetracycline (30 µg). Antibiotic sensitivity or resistance was defined in accordance with Performance Standards for Antimicrobial Susceptibility Testing: 16th Informational Supplement (CLSI, 2006).

Strain Sua-BAC020T formed non-motile, non-spore-forming short rods of 0.4–0.55 µm width and 0.8–1.4 µm length (see Fig. S1 available in IJSEM Online). Cells were Gram-positive. Colonies were light yellow, smooth-glossy, circular with entire margins after 72 h of growth on LB agar amended with 5 % NaCl, and became intensely yellow with extended incubation. Growth occurred at 20–40 °C; however, at 15 and 45 °C the cells only reached OD600 0.5 after 48 h, and no growth was observed at 4, 8 or 50 °C. The strain grew at pH 7–11, but not at pH 6 or 6.5. Growth was observed in the absence of NaCl and in the presence of up to 18 % (w/v) NaCl. However, at 15, 17.5 and 18 % (w/v) NaCl, a 24 h lag phase was observed, while no growth occurred at 19 and 20 % (w/v) NaCl. Optimal growth occurred at 35–37 °C and pH 9, at 2.5 % NaCl. Cells were sensitive to ampicillin, cephalothin, chloramphenicol and tetracycline, and resistant to gentamicin, novobiocin, penicillin G and streptomycin. Cells were catalase-positive, oxidase-negative, methyl red- and Voges–Proskauer-negative. Citrate was not utilized. A phenotypic comparison of strain Sua-BAC020T and related species of the genus Nesterenkonia is presented in Table 1.

The peptidoglycan structure, menaquinone, polar lipid and cellular fatty acid pattern analyses were carried out by the identification services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The peptidoglycan was determined by using the modified methods of Rhuland et al. (1955), Schleifer & Kandler (1972) and Schleifer (1985), and the molar ratio of [fractional change] to [fractional change] was determined [by method]. Menaquinones were extracted as described by Collins et al. (1977) and analysed by HPLC, while mycolic acids were extracted by the method of Minnikin et al. (1975) and identified by two-dimensional TLC, developed with specific reagents as described by Collins & Jones (1980). Mycolic acids were extracted and analysed according to the protocol of Klatt et al. (1994). Polar lipids were extracted from 100 mg of freeze-dried cells using the modified method of Bligh & Dyer (1959), and separated by two-dimensional silica gel TLC according to Tindall et al. (2007). Cellular fatty acids were analysed from 40 mg of actively growing cells on agar medium. The cells were scraped off and fatty acid methyl esters were obtained by saponification, methylation and extraction following the method of Miller (1982) and Kuykendall et al. (1988) with minor modifications. The fatty acid methyl esters were separated using the Sherlock Microbial Identification System (MIS) (MIDI). The cell wall of strain Sua-BAC020T contained murein of the L-Lys–Gly–D-Asp type, variation A4z. The menaquinone
Table 1. Comparison of phenotypic characteristics of strain Sua-BAC020<sup>T</sup> with closely related species of the genus Nesterenkonia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>5</th>
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<th>7</th>
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<tr>
<td>Morphology</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Cocci</td>
<td>Short rods</td>
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<tr>
<td>Colony pigmentation</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Ivory white</td>
<td>Colourless</td>
<td>Yellow</td>
<td>White</td>
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<td>Oxidase</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Nitrate reduction</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>NaCl (% w/v) for growth</td>
<td>0–18</td>
<td>1–15</td>
<td>0–18</td>
<td>0.5–30</td>
<td>5–23</td>
<td>0–10</td>
<td>0–6</td>
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<td>Optimal temperature (°C)</td>
<td>35–37</td>
<td>35</td>
<td>30</td>
<td>28</td>
<td>30</td>
<td>40–42</td>
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<td>pH range for growth</td>
<td>7.0–11.0</td>
<td>6.5–11.0</td>
<td>7.0–11.0</td>
<td>6.0–10.5</td>
<td>6.0–10.0</td>
<td>8.0–12.0</td>
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<td>Starch hydrolysis</td>
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<td>–</td>
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<td>–</td>
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<td>Casein hydrolysis</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<td>Voges–Proskauer reaction</td>
<td>–</td>
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<td>Xylose</td>
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<td>–</td>
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<td>Lactose</td>
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<td>D-Galactose</td>
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<td>ND</td>
<td>+</td>
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<td>Utilization of:</td>
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<td>D-Mannose</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>Chemical characteristics</td>
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<tr>
<td>Polar lipids*</td>
<td>PG, DPG, PI, GL</td>
<td>ND</td>
<td>DPG, PC, PG</td>
<td>Anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (39.1 %); anteiso-C&lt;sub&gt;15:0&lt;/sub&gt; (36.94 %)</td>
<td>DPG, PC, PG</td>
<td>PI, PG, DPG, GL</td>
<td>Anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (38.1 %); anteiso-C&lt;sub&gt;15:0&lt;/sub&gt; (47.1 %); anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (31.1 %); C16:0 (11.2 %)</td>
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<tr>
<td>Major cellular fatty acids (&gt;10%)</td>
<td>Anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (31.1 %); C16:0 (11.2 %)</td>
<td>DPG, PC, PG</td>
<td>Anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (31.1 %); C16:0 (11.2 %)</td>
<td>DPG, PC, PG</td>
<td>PI, PG, DPG, GL</td>
<td>Anteiso-C&lt;sub&gt;17:0&lt;/sub&gt; (31.1 %); C16:0 (11.2 %)</td>
<td>DPG, PC, PG</td>
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<tr>
<td>Major menaquinones</td>
<td>MK-8, MK-7, MK-9</td>
<td>ND</td>
<td>MK-8, MK-7, MK-9</td>
<td>MK-8, MK-7, MK-9</td>
<td>MK-8, MK-9, MK-7</td>
<td>MK-8, MK-9, MK-7</td>
<td>MK-7, MK-8, MK-9, MK-9</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>64.8</td>
<td>69</td>
<td>66.7</td>
<td>66.4</td>
<td>71.5</td>
<td>65.5</td>
<td>60.2</td>
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</table>

*PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; GL, unidentified glycolipids.
composition was MK-7 (30.4 %), MK-8 (62.6 %) and MK-9 (7 %). Mycolic acids were absent. The peptidoglycan and menaquinone were similar to those reported in N. flava CCTCC AB 207010\(^T\) (Luo et al., 2008). The new isolate contained three phospholipids: phosphatidylglycerol, diphosphatidylglycerol and phosphatidylglycinol. In addition, two unidentified glycolipids were revealed by TLC (see Fig. S2). The major cellular fatty acids (>10 %) were anteiso-C\(_{15:0}\) (36.94 %) and anteiso-C\(_{17:1}\) (39.10 %), while other groups were only present in lower amounts. The menaquinone and fatty acid composition of strain Sua-BAC020\(^T\) closely resembled that of N. xinjiangensis DSM 15475\(^T\). The detailed fatty acid profile of strain Sua-BAC020\(^T\) is given in the species description below.

To calculate the G+C content, DNA was isolated by chromatography on hydroxyapatite (Cashion et al., 1977). The G+C content was determined by HPLC analysis and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah et al. (1989), at the identification service of the DSMZ. The DNA G+C base composition of strain Sua-BAC020\(^T\) was 64.8 mol%. The high DNA G+C content is typical of Nesterenkonia species.

An almost-complete 16S rRNA gene fragment was amplified from genomic DNA using the primer set E8F (5'-AGAGTTTGATCCTGGCTCAG-3') and E1541R (5'-AAGGAGGTGATCCANCCRCA-3') (Baker et al., 2003). The PCR amplicon was cloned into Escherichia coli DH5\(\alpha\) using the pGEM-T Easy vector and sequenced using the ABI BigDye Terminator Cycle Sequencing kit 3.1. Separation of bases was carried out on a 3130XL genetic analyser (Applied Biosystems). A BLAST search was performed to obtain 16S rRNA gene sequences of related taxa from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). Pairwise alignments were performed in BioEdit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted 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 maximum-parsimony (Fitch, 1971), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) methods. The neighbour-joining tree was constructed from evolutionary distances computed with bootstrap values based on 1000 replications (Felsenstein, 1985).

To determine DNA–DNA relatedness, cells were disrupted by using a French pressure cell (Thermo Spectronic), and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out according to the method of De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983).

Comparative analyses of the almost-complete 16S rRNA gene sequence (1421 bp) revealed that strain Sua-BAC020\(^T\) belonged to the genus Nesterenkonia. The novel strain displayed 98.9 and 99.0 % similarity to N. xinjiangensis DSM 15475\(^T\) and N. aethiopica DSM 17733\(^T\), respectively. 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. 1). Levels of 16S rRNA gene sequence similarity between strain Sua-BAC020\(^T\) and recognized Nesterenkonia species were 97.5 % (N. halophila DSM 16378\(^T\)), 97.4 % (N. flava CCTCC AB 207010\(^T\)), 97.2 % (N. halobia JCM 11483\(^T\)), 96.8 % (N. alba CAAS 252\(^T\)), 96.9 % (N. lutea YIM 70081\(^T\)), 97.0 % (N. lacusekhoensis IFAM EL-30\(^T\)), 96.6 % (N. jeotgali JG-241\(^T\)) and 96.19 % (N. halotolerans YIM 70084\(^T\) and N. sandarakina YIM 70009\(^T\)).

Furthermore, DNA–DNA hybridization (mean values of two experiments) between strain Sua-BAC020\(^T\) and its closest relatives revealed 45.4 % DNA–DNA relatedness to N. xinjiangensis DSM 15475\(^T\), 10.5 % to N. aethiopica DSM 17733\(^T\), 31.6 % to N. halophila DSM 16378\(^T\), 15.5 % to N. halobia JCM 11483\(^T\) and 42.7 % to N. flava CCTCC AB 207010\(^T\). Since the threshold values for the delineation of genomic species are 97 % 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994) and 70 % DNA–DNA relatedness (Wayne et al., 1987), it can be concluded that the new isolate from Sua salt pan does not belong to any of the known Nesterenkonia species. This is further supported by morphological, biochemical and chemical differences between strain Sua-BAC020\(^T\) and the type strains of these species. We therefore propose that strain Sua-BAC020\(^T\) be included in the genus Nesterenkonia as representing a novel species, Nesterenkonia suensis sp. nov.

**Description of Nesterenkonia suensis sp. nov.**

Nesterenkonia suensis (su.en’sis. N.L. fem. adj. suensis pertaining to Sua salt pan, Botswana, where the type strain was isolated).

Cells are Gram-positive, non-motile, non-spore-forming short rods of 0.4–0.55 \(\mu\)m width and 0.8–1.4 \(\mu\)m length. Colonies on LB agar medium supplemented with 5 % NaCl are smooth, circular and 1–1.2 mm in diameter after growth at 37 °C for 72 h. The surface of the colonies is moist and shiny, and the colonies become yellow with extended storage. Optimal growth occurs at 35–37 °C and pH 9, at 2.5 % NaCl. Catalase-positive, oxidase-negative and reduces nitrate. Starch and xylan are hydrolysed while casein is not hydrolysed. Citrate is not utilized. Utilizes fructose, sucrose, xylose, L-serine, L-alanine, L-asparagine, L-glutamic acid, glyceral, mannitol, arabinose, sorbitol, D-gluconic acid, x-D-glucose, Tween 40 and Tween 80, with acid production from fructose and xylose. Trehalose, maltose, galactose, xylitol, arabinot, dextrin, succinic acid, glycogen, mannann, cellulbiose and D-alanine are not utilized. The polar lipids contain diphosphatidylglycerol, phosphoryl-dglycerol, phosphatidylglycinol and unidentified glycolipids. The isoprenoid quinones are menaquinones of type MK-8, MK-7 and MK-9. The cellular fatty acid profile comprises iso-C\(_{14:0}\) (0.28 %), C\(_{14:0}\) (0.20 %), iso-C\(_{15:1}\) (0.90 %), anteiso-C\(_{15:0}\) (0.71 %), iso-C\(_{15:0}\) (4.95 %), anteiso-C\(_{15:0}\) (36.94 %), iso-C\(_{16:1}\) (1.84 %), iso-C\(_{16:0}\) (5.92 %),
C_{16:0} (1.07 %), anteiso-C_{17:0} (4.45 %), iso-C_{17:0} (3.65 %) and anteiso-C_{17:0} (39.10 %).

The type strain, Sua-BAC020\(^\text{T}\) (=DSM 22748\(^\text{T}\)=NCCB 100309\(^\text{T}\)), was isolated from a brine sample from evaporator pond CS1, at Sua salt pan in Botswana. The DNA G+C content of the type strain is 64.8 mol%.

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**References**


