Bacillus halosaccharovorans sp. nov., a moderately halophilic bacterium from a hypersaline lake

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A novel Gram-stain-positive, moderately halophilic bacterium, designated strain E33T, was isolated from water of the hypersaline lake Aran-Bidgol in Iran and characterized taxonomically using a polyphasic approach. Cells of strain E33T were motile rods and produced ellipsoidal endospores at a central or subterminal position in swollen sporangia. Strain E33T was a strictly aerobic bacterium, catalase- and oxidase-positive. The strain was able to grow at NaCl concentrations of 0.5–25 % (w/v), with optimum growth occurring at 5–15 % (w/v) NaCl. The optimum temperature and pH for growth were 40 °C and pH 7.5–8.0, respectively. On the basis of 16S rRNA gene sequence analysis, strain E33T was shown to belong to the genus Bacillus within the phylum Firmicutes and showed the closest phylogenetic similarity with the species Bacillus niabensis 4T19T (99.2 %), Bacillus herbersteinensis D-1-5aT (97.3 %) and Bacillus litoralis SW-211T (97.2 %). The DNA G+C content of the type strain of the novel species was 42.6 mol%. The major cellular fatty acids of strain E33T were anteiso-C15 : 0 and iso-C15 : 0, and the polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids, an unknown lipid and an unknown phospholipid. The isoprenoid quinones were MK-7 (97 %), MK-6 (2 %) and MK-8 (0.5 %). The peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid. All these features confirm the placement of isolate E33T within the genus Bacillus. DNA–DNA hybridization experiments revealed low levels of relatedness between strain E33T and Bacillus niabensis IBRC-M 10590T (22 %), Bacillus herbersteinensis CCM 7228T (38 %) and Bacillus litoralis DSM 16303T (19 %). On the basis of polyphasic evidence from this study, a novel species of the genus Bacillus, Bacillus halosaccharovorans sp. nov. is proposed, with strain E33T (=IBRC-M 10095T=DSM 25387T) as the type strain.

The genus Bacillus in the family Bacillaceae, belonging to the phylum Firmicutes, includes obligate aerobic or facultative anaerobic, endospore-forming bacilli and is metabolically and ecologically a diverse group. Among the species of this genus, several moderately halophilic or halotolerant endospore-forming species have been described (de la Haba et al., 2011). These bacteria are frequently isolated from saline and hypersaline environments, such as saline soils and saline aquatic habitats (Arahal & Ventosa, 2002; Márquez et al., 2011; Ventosa, 2006; Ventosa et al., 1998). During the study of the diversity of halophilic and halotolerant prokaryotes in Aran-Bidgol Lake (34° 18’–34° 45’ N 51° 33’–52° 10’ E, 2400 km²), Esfehan Province, Iran, a large number of novel taxa have been isolated (Amoozegar et al., 2012; Bagheri...
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et al., 2012; Didari et al., 2012; Makhdoumi-Kakhki et al., 2012). The pH of the water of the lake is neutral (about pH 7.0–7.5) and its salinity reaches saturation. Here we describe the taxonomic properties of a halophilic bacterial strain, designated E33\textsuperscript{T}, which was isolated from this hypersaline lake.

The strain was isolated by plating the water sample on 7.5\% HM medium and incubating at 35 °C aerobically. The 7.5\% HM medium contained (g l\textsuperscript{−1}): NaCl, 60.7; MgCl\textsubscript{2}, 6H\textsubscript{2}O, 5.2; MgSO\textsubscript{4}, 7H\textsubscript{2}O, 7.2; CaCl\textsubscript{2}, 2H\textsubscript{2}O, 0.27; KCl, 1.5; NaHCO\textsubscript{3}, 0.045; NaBr, 0.0195; proteose–peptone number 3, 5; yeast extract, 10 and glucose, 1; 1.5 \% (w/v) agar was added when necessary (Ventosa et al., 1982). The pH of this medium was adjusted to pH 7.5. The strain was subsequently purified three times by plating on the same medium. It was maintained on the same medium and also at −80 °C in 7.5\% HM without agar and supplemented with 30 \% (v/v) glycerol. Bacillus niabensis IBRC-M 10590\textsuperscript{T} was obtained from the IBRC culture collection and was used as reference strain for comparison in our study. It was cultured under the same growth conditions as strain E33\textsuperscript{T}.

In order to phenotypically characterize strain E33\textsuperscript{T}, standard phenotypic tests were performed. The recommended Minimal Standards for describing new taxa of aerobic, endospore-forming bacteria were followed (Logan et al., 2009). Cell morphology was examined using an Olympus BX41 microscope equipped with phase-contrast optics using cells from exponentially growing cultures. Gram staining was performed by the Burke method (Murray et al., 1994). The presence of endospores was investigated by using the Schaeffer–Fulton staining method (Murray et al., 1994). Motility was analysed by the wetmount method (Murray et al., 1994). Catalase, oxidase and urease activities, nitrate and nitrite reduction, hydrolysis of ascelin, production of indole, methyl red and Voges–Proskauer tests were tested as recommended by Smibert & Krieg (1994) using media supplemented with 7.5\% NaCl. Hydrolysis of Tween 20, 40, 60 or 80 was examined as described by Harrigan & McCance (1976). Determination of acid production from carbohydrates, as well as utilization of carbon and energy sources, was performed as recommended by Ventosa et al. (1982). Antimicrobial susceptibility tests were performed on Mueller–Hinton agar plus 7.5\% (w/v) sea salts (Ventosa et al., 1982) seeded with a bacterial suspension containing 1.5 × 10\textsuperscript{8} c.f.u. ml\textsuperscript{−1} using discs (HiMedia) impregnated with various antimicrobial compounds. The plates were incubated at 35 °C for 48 h and the inhibition zone was interpreted according the manufacturer’s manual. To determine the optimal temperature and pH for growth of the strain, broth cultures were incubated at temperatures of 15–50 °C at intervals of 5 °C and at pH 5–10 at intervals of 0.5 pH units. pH values below 6, pH 6–9 and pH values above 9 were obtained using sodium acetate/acetic acid, Tris/HCl and glycine/ sodium hydroxide buffers, respectively. Growth at different NaCl concentrations (0, 0.5, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 25.0 and 27.5 \%, w/v) was tested on HM medium at pH 7.5. Growth was monitored by turbidity (OD\textsubscript{600}) using a spectrophotometric method (model UV-160 A; Shimadzu).

Other physiological and biochemical tests were performed as described previously (Mata et al., 2002; Quesada et al., 1984; Ventosa et al., 1982). Strain E33\textsuperscript{T} was Gram-positive, non-motile and strictly aerobic. Cells were rods with a width of 0.5 \(μ\)m and length of 3.0–8.0 \(μ\)m. Ellipsoidal endospores were formed at a central or subterminal position in swollen sporangia. When grown for 2 days at 40 °C on 7.5\% HM medium, the colonies were punctiform, convex, entire, smooth, cream and with a diameter of 2 mm. This isolate was moderately halophilic, growing in media containing 0.5–25 \% (w/v) NaCl and optimally in media containing 5–15 \% (w/v) NaCl. Strain E33\textsuperscript{T} grew at pH 6.0–9.0 and optimally in media at pH 7.5–8.0. Strain E33\textsuperscript{T} was sensitive to bacitracin (10 U), carbenicillin (100 \(μ\)g), cephalothin (30 \(μ\)g), chloromphenicol (30 \(μ\)g), erythromycin (15 \(μ\)g), gentamicin (30 \(μ\)g), kanamycin (30 \(μ\)g), nalidixic acid (30 \(μ\)g), nitrofurantoin (300 \(μ\)g), tetracycline (30 \(μ\)g) and tobramycin (10 \(μ\)g) but resistant to amoxicillin (30 \(μ\)g) and polymixin B (100 \(U\)). Other phenotypic features are included in Table 1 and the species description.

Genomic DNA from strain E33\textsuperscript{T} was isolated using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488. The amplification was performed by initial denaturation at 95 °C for 5 min followed by: 10 cycles of 93 °C for 1 min, 63 °C for 1 min, 71 °C for 1.5 min; 20 cycles of 93 °C for 1 min, 67 °C for 1 min, 71 °C for 2 min; and a final extension at 71 °C for 5 min. The purified PCR product was sequenced in both directions using an automated sequencer by the SeqLab Laboratory (Göttingen, Germany). The 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al., 2004). The 16S rRNA gene sequence was aligned with the published sequences of the closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were reconstructed using three different methods: maximum-parimony (Fitch, 1971), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987), algorithms integrated in the ARB software for phylogenetic inference. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

An almost–complete 16S rRNA gene sequence (1449 bp) of strain E33\textsuperscript{T} was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://www.eztaxon-e.ezbiocloud.net; Kim et al., 2012). Phylogenetic analysis based on the maximum-parsimony algorithm revealed that strain E33\textsuperscript{T}
was included in a cluster constituted by species of the genus *Bacillus* (Fig. 1). The most closely related species of strain E33<sup>T</sup> were *B. niabensis* 4T19<sup>T</sup> (99.2% 16S rRNA gene sequence similarity), *Bacillus herbersteinensis* D-1-5a<sup>T</sup> (97.3%) and *Bacillus litoralis* SW211<sup>T</sup> (97.2%). The 16S rRNA gene sequence similarity between strain E33<sup>T</sup> and other species of the genus *Bacillus* was lower than 96.7%. Maximum-likelihood and neighbour-joining methods resulted in highly similar tree topologies (Fig. 1) and confirmed the phylogenetic cluster formed by strain E33<sup>T</sup> and strains of species of the genus *Bacillus*. Based on the sequence divergence, it was evident that strain E33<sup>T</sup> constituted a different taxon separated from other species of the genus *Bacillus*.

For determination of DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) incorporating the modifications described by Huß et al. (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostat-regulated 6 × 6 multiecell changer and a temperature controller with an *in situ* temperature probe (Varian).

The percentages of DNA–DNA reassociation of strain E33<sup>T</sup> to *B. niabensis* IBRC-M 10590<sup>T</sup>, *B. herbersteinensis* DSM 16534<sup>T</sup> and *B. litoralis* DSM 16303<sup>T</sup> were 22%, 38% and 19%, respectively. According to Wayne et al. (1987), DNA–DNA relatedness lower than 70% is considered to be the threshold value for the delineation of genospecies, so the result obtained is low enough to separate strain E33<sup>T</sup> from *B. niabensis* IBRC-M 10590<sup>T</sup> and the other most closely relates species of the genus *Bacillus*.

The DNA G + C content was determined by reversed-phase HPLC of nucleosides according to the protocol of Mesbah et al. (1989). The G + C content of the DNA of strain E33<sup>T</sup> was 42.6 mol%. This value is within the range for the genus *Bacillus* and higher than that of *B. niabensis* (Table 1).

Cell biomass for analysis of the peptidoglycan, isoprenoid quinones and polar lipids was obtained by cultivation on

### Table 1. Characteristics that distinguish strain E33<sup>T</sup> from members of the most closely phylogenetically related species of the genus *Bacillus*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>Cream</td>
<td>Yellowish-white</td>
<td>Cream†</td>
</tr>
<tr>
<td>Endospore shape</td>
<td>Ellipsoidal</td>
<td>Oval</td>
<td>Oval</td>
</tr>
<tr>
<td>Sporangium</td>
<td>Swollen</td>
<td>Swollen*</td>
<td>Non-swollen†</td>
</tr>
<tr>
<td>Endospore position</td>
<td>Central/subterminal</td>
<td>Terminal</td>
<td>Terminal†</td>
</tr>
<tr>
<td>NaCl range (%)</td>
<td>0.5–25</td>
<td>0–5*</td>
<td>0–5†</td>
</tr>
<tr>
<td>Optimum NaCl (%)</td>
<td>5.0–15</td>
<td>0</td>
<td>0–0.5</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>20–45</td>
<td>15–50*</td>
<td>4–28†</td>
</tr>
<tr>
<td>pH range</td>
<td>6.0–9.0</td>
<td>6.0–8.0*</td>
<td>7.0–9.0†</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.5–8.0</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tween 20</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tween 40</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Urease activity</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>42.6</td>
<td>40.9*</td>
<td>36.2†</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>anteiso-C&lt;sub&gt;15:0&lt;/sub&gt;, iso-C&lt;sub&gt;15:0&lt;/sub&gt;, anteiso-C&lt;sub&gt;17:0&lt;/sub&gt;</td>
<td>iso-C&lt;sub&gt;15:0&lt;/sub&gt;, anteiso-C&lt;sub&gt;17:0&lt;/sub&gt;, iso-C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>iso-C&lt;sub&gt;15:0&lt;/sub&gt;, anteiso-C&lt;sub&gt;15:0&lt;/sub&gt;, iso-C&lt;sub&gt;16:0&lt;/sub&gt;†</td>
</tr>
</tbody>
</table>

*Data from Kwon et al. (2007).
†Data from Wieser et al. (2005).
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Fig. 1. Phylogenetic tree, based on the maximum-parsimony algorithm of the 16S rRNA sequences, showing the position of strain E33T and the closely related species of the genus Bacillus. GenBank accession numbers are shown in parentheses. Bootstrap values &gt;70 % are indicated at branch-points. Filled circles indicate branches also found in phylogenetic consensus trees generated with the maximum-likelihood, neighbour-joining methods. Bacillus subtilis subsp. subtilis DSM 10T (GenBank accession no. AJ276351) was used as outgroup. Bar, 1 % sequence divergence.

7.5 % HM agar at 35 °C. The whole-cell hydrolysate (4 M HCl, 100 °C, 16 h) was analysed by chromatography on a cellulose thin-layer plate (Merck) using the solvent system herbersteinensis E33T was MK-7 (97.5 %), although MK-6 and MK-8 were also present (2 % and 0.5 %, respectively. The respiratory lipoquinone and peptidoglycan diamino acid of the cell wall of strain E33T were typical of those found in members of the genus Bacillus (Bagheri et al., 2012; Chen et al., 2011; Wieser et al., 2005; Xue et al., 2008). The whole-cell fatty acid composition was determined by GC using the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID). Cells were cultured on marine agar containing 5 % (w/v) NaCl at 30 °C following the protocol of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). The cellular fatty acid profile of strain E33T was characterized by the fatty acids anteiso-C15 : 0 (43.5 %), iso-C15 : 0 (21.0 %) and anteiso-C17 : 0 (9.5 %) as the major fatty acids (Table S1). The fatty acid composition could not be compared under standardized conditions because of different growth conditions to those used for Bacillus subtilis DSM 10T (AJ747813). Both of them are lacking phosphatidylethanolamine and have two glycolipids with similar chromatographic motility. Their similar polar lipid profile might be considered in the future for the reclassification of these taxa within a new genus when more data support it. The major isoprenoid quinone of strain E33T was MK-7 (97.5 %), although MK-6 and MK-8 were also present (2 % and 0.5 %, respectively. The respiratory lipoquinone and peptidoglycan diamino acid of the cell wall of strain E33T were typical of those found in members of the genus Bacillus (Bagheri et al., 2012; Chen et al., 2011; Wieser et al., 2005; Xue et al., 2008). The whole-cell fatty acid composition was determined by GC using the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID). Cells were cultured on marine agar containing 5 % (w/v) NaCl at 30 °C following the protocol of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). The cellular fatty acid profile of strain E33T was characterized by the fatty acids anteiso-C15 : 0 (43.5 %), iso-C15 : 0 (21.0 %) and anteiso-C17 : 0 (9.5 %) as the major fatty acids (Table S1). The fatty acid composition could not be compared under standardized conditions because of different growth conditions to those used for Bacillus subtilis DSM 10T (AJ747813). Both of them are lacking phosphatidylethanolamine and have two glycolipids with similar chromatographic motility. Their similar polar lipid profile might be considered in the future for the reclassification of these taxa within a new genus when more data support it. The major isoprenoid quinone of strain E33T was MK-7 (97.5 %), although MK-6 and MK-8 were also present (2 % and 0.5 %, respectively. The respiratory lipoquinone and peptidoglycan diamino acid of the cell wall of strain E33T were typical of those found in members of the genus Bacillus (Bagheri et al., 2012; Chen et al., 2011; Wieser et al., 2005; Xue et al., 2008). The whole-cell fatty acid composition was determined by GC using the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID). Cells were cultured on marine agar containing 5 % (w/v) NaCl at 30 °C following the protocol of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). The cellular fatty acid profile of strain E33T was characterized by the fatty acids anteiso-C15 : 0 (43.5 %), iso-C15 : 0 (21.0 %) and anteiso-C17 : 0 (9.5 %) as the major fatty acids (Table S1). The fatty acid composition could not be compared under standardized conditions because of different growth conditions to those used for Bacillus subtilis DSM 10T (AJ747813). Both of them are lacking phosphatidylethanolamine and have two glycolipids with similar chromatographic motility. Their similar polar lipid profile might be considered in the future for the reclassification of these taxa within a new genus when more data support it. The major isoprenoid quinone of strain E33T was MK-7 (97.5 %), although MK-6 and MK-8 were also present (2 % and 0.5 %, respectively.

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taxa (Tables 1 and S1). Also, the DNA–DNA hybridization data clearly separate strain E33 from the most closely related species. Therefore, on the basis of these polyphasic taxonomic data, we propose that the novel strain represents a novel species of the genus *Bacillus*, for which the new name *Bacillus halosaccharovorans* sp. nov. is proposed.

**Description of *Bacillus halosaccharovorans* sp. nov.**


Cells are Gram-positive, motile, endospore-forming rods, 0.5 × 3.0–8.0 μm in size. Ellipsoidal endospores are produced at a central or subterminal position in swollen sporangia. Colonies are circular, convex, entire, smooth, cream and 2 mm in diameter on 7.5 % HM agar medium after 48 h of incubation at 35 °C. Strictly aerobic. Moderately halophilic, growing over a wide range of NaCl concentrations (from 0.5 to 25 % w/v NaCl), with optimal growth at 5–15 % (w/v) NaCl. Grows at 20–45 °C (optimally at 40 °C) and pH 6.0–9.0 (optimally at pH 7.5–8.0). Catalase and oxidase-positive. Aesculin, casein, gelatin, DNA, starch and Tweens 20 and 40 are hydrolysed but Tweens 60 and 80 are not hydrolysed. Nitrate is not reduced. Indole or H₂S are not produced. Acid is produced from D-glucose, D-fructose, galactose, lactose, sucrose, maltose, D-mannitol, ribose and D-xylose. Methyl red, Voges–Proskauer, urease, β-galactosidase, lysine and ornithine decarboxylase, arginine dihydrolase and phenylalanine deaminase tests are negative. The following compounds are not utilized as sole source of carbon and energy: arabinose, D-glucose, galactose, D-mannose, maltose, melibiose, D-ribose, sucrose, mannitol, raffinose D-fructose, cellobiose, trehalose, salicin, starch, D-xylose, glycerol, alanine and proline. The following compounds are not hydrolysed but Tweens 60 and 80 are not hydrolysed. Optimal growth at 5–15 % (w/v) NaCl. Grows at 20–45 °C, 61

The type strain is E33 (=IBRC-M 10095T=DSM 25387T) isolated from Aran-Bidgol hypersaline lake in Iran. The DNA G+C content of the type strain is 42.6 mol% (determined by HPLC).

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