**Piscibacillus halophilus** sp. nov., a moderately halophilic bacterium from a hypersaline Iranian lake

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A Gram-positive, moderately halophilic bacterium, designated strain **HS224**T, was isolated from the hypersaline lake Howz-Soltan in Iran. Cells of strain **HS224**T were rod-shaped, motile and produced oval endospores. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain **HS224**T was affiliated to the genus **Piscibacillus**, exhibiting 98.5 % sequence similarity to the type strain of **Piscibacillus salipiscarius**. Strain **HS224**T was also related closely to the type strains of **Aquisalibacillus elongatus** (98.0 % 16S rRNA gene sequence similarity), **Filobacillus milosensis** (97.9 %) and **Tenuibacillus multivorans** (97.0 %). Strain **HS224**T was able to grow at NaCl concentrations of 1–20 % (w/v), with optimum growth occurring at 10 % (w/v) NaCl. The optimum temperature and pH for growth were 35 °C and pH 7.5. Major polar lipids were phosphatidylglycerol and diphosphatidylglycerol, the isoprenoid quinone was MK-7 and the peptidoglycan type was A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid; these characteristics were shared with **P. salipiscarius**. The major cellular fatty acids of strain **HS224**T were anteiso-C15 : 0, iso-C15 : 0, anteiso-C17 : 0 and iso-C16 : 0. The G+C content of the DNA was 37.5 mol%. The level of DNA–DNA relatedness between strain **HS224**T and **P. salipiscarius** **JCM 13188**T was 30.8 %. It is evident from the genotypic, chemotaxonomic and phenotypic data presented that strain **HS224**T represents a novel species of the genus **Piscibacillus**, for which the name **Piscibacillus halophilus** sp. nov. is proposed. The type strain is **HS224**T (=CCM 7596T=DSM 21633T=JCM 15721T=LMG 24786T).

The genus **Piscibacillus** was proposed by Tanasupawat et al. (2007) to accommodate a moderately halophilic bacterium isolated from fermented fish (pla-ra) in Thailand. The only recognized species of the genus is **Piscibacillus salipiscarius**. Cells of **P. salipiscarius** are Gram-positive, motile rods that produce oval endospores at terminal positions in swollen sporangia. It is a moderately halophilic bacterium that is able to grow at 2–30 % (w/v) NaCl, with optimal growth at 10–20 % (w/v) NaCl. This species contains meso-diaminopimelic acid in the cell-wall peptidoglycan, menaquinone with seven isoprene units (MK-7), and phosphatidylglycerol and diphosphatidylglycerol as polar lipids. Its major cellular fatty acids are iso-C15 : 0 and anteiso-C15 : 0 and the DNA G+C content is 36.7 mol%. Phylogenetically, **P. salipiscarius** is a member of the family **Bacillaceae**, within a cluster including species of the genera **Aquisalibacillus**, **Filobacillus** and **Tenuibacillus**. These genera are currently represented by single species, namely **Aquisalibacillus elongatus** (Márquez et al., 2008), **Filobacillus milosensis** (Schlesner et al., 2001) and **Tenuibacillus multivorans** (Ren & Zhou, 2005).

In this paper we describe the isolation, and phenotypic, chemotaxonomic, phylogenetic and genotypic features of a novel, moderately halophilic bacterium, designated strain **HS224**T, isolated from a hypersaline lake in Iran.

Strain **HS224**T was isolated from water of the hypersaline lake Howz-Soltan, which is located near Qom city in central Iran, during the course of studies focusing on determination of the biodiversity of the lake. This lake covers an area of about 240 and 280 km² during the dry and wet seasons, respectively. The depth of the salt layer...
that covers almost the entire surface of the playa varies between 20 and 46 m, and the pH of the water, saline soil and salt sediments differs between 6.5 and 8.2. The major chemical components of the soil, brine, mud and salt are NaCl, KCl, MgSO4, MgCl2 and Na2SO4. Ions present in the lake water are at the following concentrations: Na+, 8.56%; Ca2+, 0.079%; Mg2+, 4.54%; K+, 0.056%; SO42-, 0.63%; Cl-, 16.77%; HCO3-, 0.022%; Mn2+, 1.73 p.p.m.; Li+, 2.55 p.p.m. At the time of sampling, the temperature of the water was 32 °C and the pH was 7.6.

Strain HS224T was isolated by diluting a water sample in sterile 10% (w/v) salt solution, plating on 10% HM medium and incubating aerobically at 37 °C. The 10% HM medium contained (per litre distilled water): 81 g NaCl, 7 g MgCl2, 9.6 g MgSO4, 0.36 g CaCl2, 2 g KCl, 0.06 g NaHCO3, 0.026 g NaBr, 5 g proteose peptone no. 3, 10 g yeast extract and 1 g glucose (Ventosa et al., 1982). The pH of this medium was adjusted to 7.5. Growth was monitored based on turbidity at 750 nm. 10% HM medium and was maintained on the same 10% HM medium and was subsequently purified three times by plating on the same 10% HM medium and at −80 °C on this medium without agar but supplemented with 30% (v/v) glycerol. P. salipiscarius JCM 13188T was used as a reference strain and was cultured under the same conditions as strain HS224T.

Cell morphology was examined by using light microscopy (model CX 3; Olympus) with cells from exponentially growing cultures. Gram staining was performed by using the Burke method (Murray et al., 1994) and the result was confirmed by using the KOH test (Baron & Finegold, 1990). The presence of endospores was investigated by using the Schaeffer–Fulton staining method (Murray et al., 1990). Motility was analysed by using the wet-mount method (Murray et al., 1994). Motility was analyised by using the wet-mount method (Murray et al., 1994). To determine the optimal temperature and pH for growth, broth cultures were incubated at 10–60 °C at intervals of 5 °C and at pH 5–11 at intervals of 0.5 pH units. pH values below 6, 6–9 and above 9 were obtained by using sodium acetate/acetate acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth at different salt concentrations (0, 2.5, 5, 7.5, 10, 15, 20, 25 and 30%, w/v) was tested on HM medium at pH 7.5. Growth was monitored based on turbidity at OD600 by using a spectroscopic method (model UV-160 A; Shimadzu). Catalase, oxidase and urease activities, nitrate reduction, hydrolysis of aesculin, production of indole, and methyl red and Voges–Proskauer tests were investigated according to Smibert & Krieg (1994). Hydrolysis of Tween 80 was examined as described by Harrigan & McCance (1976). Determination of acid production from carbohydrates, as well as utilization of carbon and nitrogen sources, was performed according to Ventosa et al. (1982). Antibiotic susceptibility tests were performed on Mueller–Hinton agar plus 10% (w/v) sea salts (Ventosa et al., 1982) seeded with a bacterial suspension containing 1.5 × 107 c.f.u. ml−1 by using discs (HiMedia) impregnated with various antibiotics. The plates were incubated at 35 °C for 48 h and the inhibition zone was interpreted according to the manufacturer’s manual. Other physiological and biochemical tests were performed as described by Mata et al. (2002), Quesada et al. (1984) and Ventosa et al. (1982).

Strain HS224T was a Gram-positive, motile rod able to produce oval endospores at terminal positions in swollen sporangia. When grown for 2 days at 35 °C on 10% HM medium, the colonies were circular, entire, smooth, cream and with a diameter of 2 mm. This isolate was moderately halophilic, growing in media containing 1–20% (w/v) NaCl and optimally in media containing 10% (w/v) NaCl. No growth was observed in the absence of NaCl. Strain HS224T grew between pH 7.0 and 10.0, and optimally in media with pH 7.5, and at temperatures ranging from 15 to 55 °C, with optimal growth at 35 °C. Other phenotypic features are included in Table 1 and the species description.

Genomic DNA from strain HS224T was prepared according to the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 [AGAGTTTGATC(T)AGGCTCAG] and the reverse primer 16R1488 [CGGTTACCTTGTAGACTTCACC]. Direct sequence determination of the PCR-amplified DNA was carried out by using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al., 2004). The 16S rRNA gene sequence was aligned with published sequences of closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule by using the alignment tool of the ARB software package. Phylogenetic trees were constructed by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms integrated within the ARB software package. Phylogenetic analysis, based on the maximum-parsimony algorithm, revealed that strain HS224T formed a phylogenetic group with P. salipiscarius RBU1-1T (Fig. 1), and these two taxa shared 98.5% 16S rRNA gene sequence similarity. Strain HS224T was also related closely to the type strains of A. elongatus (98.0% 16S rRNA gene sequence similarity), F. milosensis (97.9%) and T. multivorans (97.0%). The neighbour-joining (Supplementary Fig. S1, available in IJSEM Online) and maximum-likelihood (Supplementary Fig. S2) methods resulted in highly similar tree topologies, confirming the phylogenetic cluster formed by strain HS224T and P. salipiscarius RBU1-1T.
The G+C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979), as previously described in detail (Ventosa et al., 1999). The G+C content of the DNA of strain HS224T was 37.5 mol%, very similar to that reported for SH4sT (Tanasupawat et al., 2007) as well as for other closely related species except A. elongatus, P. salipiscarius for the type strain of (Ventosa Owen & Hill (1979), as previously described in detail by Johnson (1994). Experiments were carried out in triplicate. The level of DNA–DNA relatedness between strain HS224T and SH4sT (Schlesner milosensis (Ma´rquez et al., 2001), 15–55 (35) 15–48 (37) 20–55 (37) ND–42 (33–38) 21–42 (36–41).

Table 1. Characteristics that differentiate strain HS224T from P. salipiscarius and other closely related species

<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>Cell size (µm)</td>
<td>0.5–0.7 × 2.5–4.0</td>
<td>0.4–0.5 × 1.5–4.0</td>
<td>0.5 × 2.0–10.0</td>
<td>0.3–0.4 × 3.0–7.0</td>
<td>0.3–0.5 × 2.0–6.0</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Anaerobic growth</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Spore shape</td>
<td>Oval, terminal in swollen sporangia</td>
<td>Oval, terminal in swollen sporangia</td>
<td>–</td>
<td>Spherical, terminal in swollen sporangia</td>
<td>Spherical, terminal in swollen sporangia</td>
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<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Temperature range (optimum) (°C)</td>
<td>15–55 (35)</td>
<td>15–48 (37)</td>
<td>20–55 (37)</td>
<td>ND–42 (33–38)</td>
<td>21–42 (36–41)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>7.0–10.0 (7.5)</td>
<td>5.0–9.0 (7)</td>
<td>7.0–10.0 (7.5)</td>
<td>6.5–8.9 (7.3–7.8)</td>
<td>6.5–9.0 (7.0–8.0)</td>
</tr>
<tr>
<td>Acid production from: Galactose</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
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<tr>
<td>d-Glucose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>d-Fructose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>Nitrite reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Hydrolysis of: Aesculin</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Tween 80</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNAse</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>37.5</td>
<td>36.7</td>
<td>45.9</td>
<td>35.0</td>
<td>36.5–37.0</td>
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</table>

*meso-DAP, meso-diaminopimelic acid.

Analysis of polar lipids, quinones and peptidoglycan of the cell wall was carried out by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Major polar lipids were phosphatidyglycerol and diphostatidyglycerol; two phospholipids were detected as minor components. Strain HS224T contained MK-7 as the unique menaquinone and a cell-wall peptidoglycan type A1β with meso-diaminopimelic acid as the diagnostic diamino acid. These features are the same as those reported for P. salipiscarius (Tanasupawat et al., 2007). However, these two taxa can be distinguished from species of other closely related genera based on peptidoglycan type: the cell wall of A. elongatus contains peptidoglycan of the A4β type, with L-Orn as the diamino acid and D-Asp as the dicarboxylic amino acid present in the cross-linkage (Márquez et al., 2008), and F. milosensis also has peptidoglycan of the A4β type, but with D-Glu in the cross-linkage (Schlesner et al., 2001).
system. This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG bacterial collection (Gent, Belgium). The cellular fatty acid profile of strain HS224^T was characterized by anteiso-C\textsubscript{15:0} (35.3 % of the total), iso-C\textsubscript{15:0} (26.3 %), anteiso-C\textsubscript{17:0} (13.8 %) and iso-C\textsubscript{16:0} (9.4 %) as major components; iso-C\textsubscript{17:0} (5.8 %), iso-C\textsubscript{14:0} (2.6 %), C\textsubscript{16:1} \textit{v} \textsubscript{7c} (2.5 %), C\textsubscript{16:0} (1.4 %), C\textsubscript{16:1} \textit{v} \textsubscript{11c} (0.5 %) and summed feature 4 (iso-C\textsubscript{17:1} and/or anteiso-C\textsubscript{17:1}; 1.3 %) were detected as minor components. This profile is similar to that reported for \textit{P. salipiscarius} (Supplementary Table S1, available in IJSEM Online), which also contained anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0}, anteiso-C\textsubscript{17:0} and iso-C\textsubscript{16:0} as major fatty acids (Tanasupawat et al., 2007).

The characteristics that can be used to differentiate between strain HS224^T and \textit{P. salipiscarius} and other related species are summarized in Table 1. The results show clearly that strain HS224^T is related closely to \textit{P. salipiscarius} but can be differentiated based on several phenotypic and chemotaxonomic features. DNA–DNA hybridization data support the placement of strain HS224^T as a member of species distinct from \textit{P. salipiscarius}. On the basis of the data obtained from this polyphasic taxonomy study, we suggest that strain HS224^T represents a novel species of the genus \textit{Piscibacillus}, for which the name \textit{Piscibacillus halophilus} sp. nov. is proposed.

**Description of \textit{Piscibacillus halophilus} sp. nov.**

\textit{Piscibacillus halophilus} (ha.lo.phi’ilus. Gr. n. hals, halos salt; Gr. adj. philos loving; N.L. masc. adj. halophilus salt-loving).

Cells are Gram-positive, motile rods (0.5–0.7 × 2.5–4.0 \textmu m). Cells produce oval endospores terminally positioned within swollen sporangia. Colonies are circular, entire, smooth, cream in colour and 2 mm in diameter on 10 % HM agar medium after 48 h incubation at 35 °C. Facultatively anaerobic. Moderately halophilic. The optimum NaCl concentration for growth is 10 % (w/v), with a range of 1–20 % (w/v) NaCl for growth. No growth occurs in the absence of NaCl. Growth is observed at 15–55 °C (optimum at 35 °C) and at pH 7.0–10.0 (optimum at pH 7.5). Catalase- and oxidase-positive. Indole and H\textsubscript{2}S are not produced. Gelatin, casein, aesculin, and Tweens 20, 40, 60 and 80 are hydrolysed. Starch and DNA are not hydrolysed. Nitrate is not reduced to nitrite. Acid is not produced from D-glucose, D-fructose, galactose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylose or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-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: D-fructose, lactose, maltose, melibiose, D-mannose, trehalose, D-xylene or myo-inositol. Methyl red, Voges–Proskauer, urease, \beta-galactosidase, lysine and ornithine decarboxylase, arginine dihydrolase and phenylalanine deaminase tests are negative.
A1\textsubscript{v}, with meso-diaminopimelic acid as the diagnostic diamino acid. Cellular fatty acids are anteiso-C\textsubscript{15:0}, iso-C\textsubscript{15:0}, anteiso-C\textsubscript{17:0} iso-C\textsubscript{16:0}, iso-C\textsubscript{17:0} and summed feature 4 (iso-C\textsubscript{16:1} and/or anteiso-C\textsubscript{17:1}). The DNA G+C content of the type strain is 37.5 mol\% ($T_m$).

The type strain, HS224\textsuperscript{T} (=CCM 7596\textsuperscript{T}=DSM 21633\textsuperscript{T}=JCM 15721\textsuperscript{T}=LMG 24786\textsuperscript{T}), was isolated from the hypersaline lake Howz-Soltan in Iran.

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