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 A1 γ , 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-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. 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 7596^T=DSM 21633^T=JCM 15721^T=LMG 24786^T).

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-C_{15:0} and anteiso-C_{15:0} 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HS224^T is FM864227.

Neighbour-joining and maximum-likelihood phylogenetic trees, based on 16S rRNA gene sequences, showing the position of strain HS224^T among related species, and a table giving the cellular fatty acid profiles of strain HS224^T and *Piscibacillus salipiscarius* RBU1-1^T are available as supplementary material with the online version of this paper.

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 HS224^T 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. The strain was subsequently purified three times by plating on the same medium and was maintained 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 13188^T was used as a reference strain and was cultured under the same conditions as strain HS224^T.

Cell morphology was examined by using light microscopy (model CX 31; 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.*, 1994). Motility was analysed 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/acetic 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 1.5×10^6 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 HS224^T 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 HS224^T 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 HS224^T was prepared according to the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 [AGAGTTTGATC(AC)TGGCTCAG] and the reverse primer 16R1488 (CGGTTACCTTGTAGGACTTCACC). 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 for phylogenetic inference. 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 (1472 bp) of strain HS224^T was obtained and used for initial BLAST searches in GenBank and for phylogenetic analysis. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic analysis, based on the maximum-parsimony algorithm, revealed that strain HS224^T formed a phyletic group with *P. salipiscarius* RBUI-1^T (Fig. 1), and these two taxa shared 98.5 % 16S rRNA gene sequence similarity. Strain HS224^T 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 HS224^T and *P. salipiscarius* RBUI-1^T.

Table 1. Characteristics that differentiate strain HS224^T from *P. salipiscarius* and other closely related species

Taxa: 1, strain HS224^T; 2, *P. salipiscarius* RBU1-1^T (data from Tanasupawat *et al.*, 2007); 3, *A. elongatus* SH4s^T (Márquez *et al.*, 2008); 4, *F. milosensis* SH 714^T (Schlesner *et al.*, 2001); 5, *T. multivorans* (Ren & Zhou, 2005). +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Cell size (µm)	0.5–0.7 × 2.5–4.0	0.4–0.5 × 1.5–4.0	0.5 × 2.0–10.0	0.3–0.4 × 3.0–7.0	0.3–0.5 × 2.0–6.0
Oxidase	+	+	–	–	+
Anaerobic growth	+	w	–	–	–
Spore shape	Oval, terminal in swollen sporangia	Oval, terminal in swollen sporangia	–	Spherical, terminal in swollen sporangia	Spherical, terminal in swollen sporangia
Motility	+	+	–	+	–
NaCl range (optimum) (% w/v)	1–20 (10)	2–30 (10–20)	3–20 (10)	2–23 (8–14)	1–20 (5)
Temperature range (optimum) (°C)	15–55 (35)	15–48 (37)	20–55 (37)	ND–42 (33–38)	21–42 (36–41)
pH range (optimum)	7.0–10.0 (7.5)	5.0–9.0 (7)	7.0–10.0 (7.5)	6.5–8.9 (7.3–7.8)	6.5–9.0 (7.0–8.0)
Acid production from:					
Galactose	–	+	ND	–	–
D-Glucose	–	+	–	–	–
D-Fructose	–	+	–	–	–
Nitrate reduction	–	–	+	ND	–
Nitrite reduction	–	–	+	ND	ND
Hydrolysis of:					
Aesculin	+	ND	–	–	+
Starch	–	+	–	–	–
Casein	+	+	–	–	+
Gelatin	+	+	–	–	+
Tween 80	+	–	–	ND	–
DNase	–	+	ND	+	ND
Peptidoglycan type/variant	A1γ/ <i>meso</i> -DAP*	A1γ/ <i>meso</i> -DAP	A4β/Orn-D-Asp	A4β/Orn-D-Glu	A1γ/ <i>meso</i> -DAP
DNA G+C content (mol%)	37.5	36.7	45.9	35.0	36.5–37.0

**meso*-DAP, *meso*-diaminopimelic acid.

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 HS224^T was 37.5 mol%, very similar to that reported for the type strain of *P. salipiscarius* (Tanasupawat *et al.*, 2007) as well as for other closely related species except *A. elongatus* (Márquez *et al.*, 2008) (Table 1).

DNA–DNA hybridization studies were performed by using the competition procedure of the membrane method (Johnson, 1994), as described in detail by Arahal *et al.* (2001a, b). The hybridization temperature used was 46.3 °C, which is within the limit of validity for the filter method (De Ley & Tijtgate, 1970) and the percentage DNA–DNA relatedness was calculated according to Johnson (1994). Experiments were carried out in triplicate. The level of DNA–DNA relatedness between strain HS224^T and *P. salipiscarius* JCM 13188^T was 30.8 %, well below the 70% cut-off generally accepted for species delineation (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

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 phosphatidylglycerol and diphosphatidylglycerol; two phospholipids were detected as minor components. Strain HS224^T contained MK-7 as the unique menaquinone and a cell-wall peptidoglycan type A1γ, 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).

Fatty acid analysis was performed by using the MIDI system (Microbial Identification System). Cells were cultured on marine agar 2216 (Difco) containing 10 % (w/v) NaCl at 37 °C for 24 h. Extraction and analysis were performed according to the recommendations of the MIDI

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequence comparisons, showing the relationship of strain HS224^T with related species. Accession numbers of the sequences used in this study are given in parentheses. *Bacillus licheniformis* DSM 13^T was used as an outgroup. Bootstrap percentages (based on 1000 replicates) of >50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

system. This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCMTM/LMG bacterial collection (Gent, Belgium). The cellular fatty acid profile of strain HS224^T was characterized by anteiso-C_{15:0} (35.3% of the total), iso-C_{15:0} (26.3%), anteiso-C_{17:0} (13.8%) and iso-C_{16:0} (9.4%) as major components; iso-C_{17:0} (5.8%), iso-C_{14:0} (2.6%), C_{16:1}ω7c (2.5%), C_{16:0} (1.4%), C_{16:1}ω11c (0.5%) and summed feature 4 (iso-C_{17:1} and/or anteiso-C_{17:1}; 1.3%) were detected as minor components. This profile is similar to that reported for *P. salipiscarius* (Supplementary Table S1, available in IJSEM Online), which also contained anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as major fatty acids (Tanasupawat *et al.*, 2007).

The characteristics that can be used to differentiate between strain HS224^T and *P. salipiscarius* and other related species are summarized in Table 1. The results show clearly that strain HS224^T is related closely to *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 *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 *Piscibacillus*, for which the name *Piscibacillus halophilus* sp. nov. is proposed.

Description of *Piscibacillus halophilus* sp. nov.

Piscibacillus halophilus (ha.lo.phi'lus. 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 μm). 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 H₂S 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, 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: D-fructose, galactose, D-glucose, lactose, maltose, D-mannose, melibiose, D-ribose, sucrose, glycerol, *myo*-inositol, alanine, arginine, asparagine, cysteine, glycine, leucine, lysine, methionine, proline and valine. Sensitive to carbenicillin (100 μg), nitrofurantoin (300 μg), tetracycline (30 μg) and rifampicin (5 μg). Resistant to amoxicillin (30 μg), gentamicin (30 μg), tobramycin (10 μg), kanamycin (30 μg) and polymyxin B (100 U). Polar lipids are phosphatidylglycerol and diphosphatidylglycerol as well as two minor phospholipids. The isoprenoid quinone is MK-7. The peptidoglycan type is

Al γ , with *meso*-diaminopimelic acid as the diagnostic diamino acid. Cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0}, iso-C_{17:0}, iso-C_{14:0}, C_{16:1} ω 7c, C_{16:0}, C_{16:1} ω 11c and summed feature 4 (iso-C_{17:1} and/or anteiso-C_{17:1}). The DNA G+C content of the type strain is 37.5 mol% (T_m).

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

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