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

M. A. Amoozegar,¹ C. Sánchez-Porro,² R. Rohban,³ M. Hajighasemi¹ and A. Ventosa²

Correspondence

A. Ventosa

ventosa@us.es

¹Extremophiles Laboratory, Department of Microbiology, Faculty of Biology, College of Science, University of Tehran, Tehran, Iran

²Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

³Department of Biology, Islamic Azad University, Science and Research Campus, Tehran, Iran

A Gram-positive, moderately halophilic, endospore-forming bacterium, designated strain HS136^T, was isolated from the hypersaline lake Howz-Soltan in Iran. Cells were motile rods, producing ellipsoidal endospores at a central–subterminal position in non-swollen sporangia. Strain HS136^T, a strictly aerobic bacterium, grew between pH 7.0 and 10.0 (optimal growth at pH 8.0–8.5), between 25 and 45 °C (optimal growth at 40 °C) and at salinities of 5–20 % (w/v) NaCl, growing optimally at 10 % (w/v) NaCl. On the basis of 16S rRNA gene sequence analysis, strain HS136^T was shown to belong to the genus *Bacillus* within the phylum *Firmicutes* and showed closest phylogenetic similarity to *Bacillus salarius* BH169^T (95.2%) and *Bacillus qingdaonensis* CM1^T (94.5%). The DNA G+C content of this new isolate was 37.1 mol%. The major cellular fatty acids of strain HS136^T were iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}, and its polar lipid pattern consisted of phosphatidylglycerol and diphosphatidylglycerol. The isoprenoid quinone was MK-7. The peptidoglycan type is A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid. On the basis of polyphasic evidence from this study, *Bacillus persepolensis* sp. nov. is proposed, with strain HS136^T (=CCM 7595^T=DSM 21632^T=JCM 15720^T=LMG 25222^T) as the type strain.

Endospore-forming, moderately halophilic, Gram-positive rods are frequently isolated from saline and hypersaline environments such as soils and aquatic habitats (Arahal & Ventosa, 2002; Ventosa, 2006). They are characterized by optimal growth in media containing 3–15 % (w/v) NaCl (Ventosa *et al.*, 1998). They belong to species included in the genus *Bacillus*, as well as related genera. The moderately halophilic species belonging to the genus *Bacillus* are *Bacillus aurantiacus* (Borsodi *et al.*, 2008), *Bacillus chagannorensis* (Carrasco *et al.*, 2007a), *Bacillus coahuilensis* (Cerritos *et al.*, 2008), *Bacillus isabeliae* (Albuquerque *et al.*, 2008), *Bacillus oshimensis* (Yumoto *et al.*, 2005), *Bacillus polygoni* (Aino *et al.*, 2008), *Bacillus qingdaonensis* (Wang *et al.*, 2007), *Bacillus salarius* (Lim *et al.*, 2006c) and *Bacillus taeanaensis* (Lim *et al.*, 2006a). Other genera related phylogenetically to the genus *Bacillus* that include moderately halophilic species are *Alkalibacillus* (Jeon *et al.*, 2005; Usami *et al.*, 2007), *Aquisalibacillus* (Márquez *et al.*, 2008), *Filobacillus* (Schlesner *et al.*, 2001), *Gracilibacillus* (Wainø *et al.*, 1999; Carrasco *et al.*, 2006; Jeon *et al.*, 2008), *Halalkalibacillus* (Echigo *et al.*, 2007), *Halobacillus* (Spring

et al., 1996; Amoozegar *et al.*, 2003; Yoon *et al.*, 2003, 2005, 2007a, 2008; Liu *et al.*, 2005; An *et al.*, 2007b; Hua *et al.*, 2007; Romano *et al.*, 2008; Soto-Ramírez *et al.*, 2008), *Halolactibacillus* (Cao *et al.*, 2008), *Lentibacillus* (Yoon *et al.*, 2002; Namwong *et al.*, 2005; Pakdeeto *et al.*, 2007; Lee *et al.*, 2008), *Marinibacillus* (Yoon *et al.*, 2004a), *Oceanobacillus* (Lee *et al.*, 2006), *Piscibacillus* (Tanasupawat *et al.*, 2007), *Pontibacillus* (Lim *et al.*, 2005), *Salimicrobium* (Ventosa *et al.*, 1989; Yoon *et al.*, 2007b), *Salinibacillus* (Ren & Zhou, 2005b), *Salsuginibacillus* (Carrasco *et al.*, 2007b), *Sediminibacillus* (Carrasco *et al.*, 2008), *Tenuibacillus* (Ren & Zhou, 2005a), *Thalassobacillus* (García *et al.*, 2005) and *Virgibacillus* (Garabito *et al.*, 1997; Arahal *et al.*, 1999; Heyrman *et al.*, 2003; Yoon *et al.*, 2004b; Lee *et al.*, 2006; An *et al.*, 2007a; Chen *et al.*, 2008; Hua *et al.*, 2008; Wang *et al.*, 2008). This paper describes a novel endospore-forming, moderately halophilic, Gram-positive bacterium, strain HS136^T, isolated from the hypersaline lake Howz-Soltan in the centre of Iran.

Strain HS136^T was isolated from water of the hypersaline lake Howz-Soltan, which is located near Qom city in the centre of Iran, with an area of about 240 and 280 km² in dry and wet seasons, respectively. The depth of the salt layer that covers almost all of the surface of the playa varies between 20 and 46 m and the pH of the water, saline soil

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HS136^T is FM244839.

and salt sediments differs between 6.5 and 8.2. The major chemical composition of the soil, brine, mud and salt consists of NaCl, KCl, MgSO₄, MgCl₂ and Na₂SO₄. At the time of sampling, the temperature of the water was 32 °C and the pH was 7.6. Strain HS136^T was isolated during studies focused on the determination of the biodiversity of the lake and constituted a single isolate of the total population. It 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 (g l⁻¹): NaCl, 81; MgCl₂, 7; MgSO₄, 9.6; CaCl₂, 0.36; KCl, 2; NaHCO₃, 0.06; NaBr, 0.026; proteose peptone no. 3 (Difco), 5; yeast extract, 10; glucose, 1 (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 maintained on the same HM medium and at -80 °C on this medium without agar and supplemented with 30 % (v/v) glycerol. In order to characterize strain HS136^T phenotypically, standard phenotypic tests were performed.

Cell morphology was examined by light microscopy (model BH41; Olympus) using cells from exponentially growing cultures. Gram staining was performed by the Burke method (Murray *et al.*, 1994) and the result was confirmed by 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 the wet-mount method (Murray *et al.*, 1994). Catalase, oxidase and urease activities, nitrate reduction, hydrolysis of aesculin and production of indole were tested as recommended by Smibert & Krieg (1994). Hydrolysis of Tween 80 was examined as described by Harrigan & McCance (1976). Methyl red and Voges–Proskauer tests were done as recommended by Smibert & Krieg (1994). Determination of acid production from carbohydrates, as well as utilization of carbon and nitrogen sources, was performed as recommended by 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 × 10⁶ c.f.u. ml⁻¹ by using discs (HiMedia) impregnated with various antibiotics. The plates were incubated at 37 °C for 48 h and the inhibition zone was interpreted according to the manufacturer's manual. To determine the optimal temperature and pH for growth of the strain, broth cultures were incubated at 10–50 °C at intervals of 5 °C and at pH 5–11 at intervals of 0.5 pH units. pH values of <6, 6–9 and >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, 5.0, 7.5, 10, 15, 20, 25 and 30 %, w/v) was tested on HM medium at pH 8.0. Growth was monitored by turbidity at OD₆₀₀ by using a spectroscopic method (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; Mata *et al.*, 2002).

Strain HS136^T was Gram-positive, motile and strictly aerobic. Cells were rods with a width of 1.0–1.5 µm and a length of 2.5–3.0 µm. Ellipsoidal endospores were formed at the central–subterminal position in non-swollen sporangia. When grown for 2 days at 37 °C on 10 % HM medium, colonies were circular, entire, smooth and cream and had a diameter of 2 mm. This isolate was moderately halophilic and slightly alkaliphilic, growing in media containing 5–20 % (w/v) NaCl and optimally in medium containing 10 % (w/v) NaCl. No growth was observed in the absence of NaCl. Strain HS136^T grew between pH 7.0 and 10.0 and optimally in media with pH 8.0–8.5. Other phenotypic features are included in the species description.

Genomic DNA from strain HS136^T was prepared by 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 (Márquez *et al.*, 2008). Direct sequence determination of the PCR-amplified DNA was carried out 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 the 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 three different methods: the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) 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 GenBank and their strain designations and accession numbers are shown in Fig. 1.

The almost-complete 16S rRNA gene sequence (1474 bp) of strain HS136^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 by using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic analysis based on the maximum-parsimony algorithm revealed that strain HS136^T formed a phyletic group with *B. salarius* BH169^T (95.2 % similarity) and *B. qingdaonensis* CM1^T (94.5 %) (Fig. 1). 16S rRNA sequence similarity with other *Bacillus* species was ≤94.5 %. Neighbour-joining and maximum-likelihood methods resulted in highly similar tree topologies and only the maximum-parsimony results are shown. Based on the sequence divergence, it was evident that strain HS136^T constituted a different taxon separated from other *Bacillus* species. DNA–DNA hybridization between strain HS136^T and the nearest phylogenetic neighbours was not attempted, as strains differing by >3 % of the 16S rRNA gene sequence are unlikely to exhibit >70 % relatedness at

Fig. 1. Maximum-parsimony tree based on 16S rRNA gene sequence comparison, showing the relationship of strain HS136^T to related species. The GenBank accession numbers of the sequences used in this study are shown in parentheses after the strain designation. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

the whole-genome level (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002; Stackebrandt & Ebers, 2006).

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). The G+C content of the DNA of strain HS136^T was 37.1 mol%. This value is within the range for the genus *Bacillus*, but is lower than those of *B. salarius* and *B. qingdaonensis* (Table 1).

Fatty acid analysis was performed by using the MIDI system (Microbial ID). Cells were cultured on marine agar (Difco) containing 10% (w/v) NaCl at 30 °C for 48 h. Extraction and analysis were performed conforming to the recommendations of the MIDI system. This analysis was carried out by the Identification Service of the BCCM/LMG, Gent, Belgium. The cellular fatty acid profile of strain HS136^T was characterized by the fatty acids iso-C_{15:0} (41.6%) and anteiso-C_{15:0} (35.8%) as the major fatty acids (Table 2). The fatty acid composition is similar to those found in *B. salarius* BH169^T and *B. qingdaonensis* CM1^T, except that the proportion of iso-C_{15:0} was lower and that of anteiso-C_{17:0} was higher for strain HS136^T than for these two *Bacillus* species. In order to complete the chemotaxonomic characterization of strain HS136^T, analysis of polar lipids, quinones and peptidoglycan of the cell wall was carried out by the Identification Service of the DSMZ, Braunschweig, Germany. The polar lipids detected were phosphatidylglycerol and diphosphatidylglycerol. Strain HS136^T contained MK-7 as the unique menaquinone and a cell wall type based on meso-diaminopimelic acid. The respiratory lipoquinone, polar lipids and peptidoglycan type of the cell wall of strain HS136^T were typical of those found in members of the genus *Bacillus* (Priest *et al.*, 1988; Heyrman *et al.*, 2004, 2005; Wieser *et al.*, 2005; Lim *et al.*, 2006a, b, c).

The characteristics that differentiate strain HS136^T from related *Bacillus* species are summarized in Table 1. The differences in some features, such as endospore position and shape, motility, optimum temperature, acid production from carbohydrates, genomic DNA G+C content and fatty acid composition, can be used to distinguish this strain from phylogenetically related taxa (Tables 1 and 2). Therefore, on the basis of these polyphasic taxonomic data, we propose that the new strain represents a novel species of the genus *Bacillus*, for which the name *Bacillus persepolensis* sp. nov. is proposed, with the type strain HS136^T.

Description of *Bacillus persepolensis* sp. nov.

Bacillus persepolensis (per.se.po.len'sis. N.L. masc. adj. *persepolensis* related to the ancient city of Persepolis, in modern-day Iran).

Cells are Gram-positive, motile, endospore-forming rods, 1.0–1.5 × 2.5–3.0 µm in size. Ellipsoidal endospores are produced at a central–subterminal position in non-swollen sporangia. Colonies are circular, entire, smooth, cream and 2 mm in diameter on 10% HM medium after 48 h incubation at 37 °C. Strictly aerobic. Slightly alkaliphilic. Moderately halophilic, growing over a wide range of NaCl concentrations [from 5 to 20% (w/v) NaCl], with optimal growth at 10% (w/v) NaCl. No growth occurs in the absence of NaCl. Grows at 25–45 °C (optimally at 40 °C) and pH 7.0–10.0 (optimally at pH 8.0–8.5). Catalase- and oxidase-positive. Casein and Tween 80 are hydrolysed, but starch, gelatin, DNA and aesculin are not. Nitrate is not reduced to nitrite. Indole and H₂S are not produced. Acid is produced from D-glucose, trehalose and *m*-inositol, but not from D-fructose, galactose, lactose, maltose, melibiose, D-mannose or D-xylose. Methyl red, Voges–Proskauer, urease,

Table 1. Characteristics that distinguish strain HS136^T from related species of the genus *Bacillus*

Strains: 1, HS136^T; 2, *B. salarius* BH169^T (Lim *et al.*, 2006c); 3, *B. qingdaonensis* CM1^T (Wang *et al.*, 2007). +, Positive; – negative; ND, not determined.

Characteristic	1	2	3
Cell size	1.0–1.5 × 2.5–3.0	0.5–0.7 × 1.3–1.9	0.3–0.4 × 1.8–3.2
Pigmentation	Cream	Cream-white to yellow	Cream
Endospore formation	+	+	–
Endospore position/shape	Central-subterminal/ellipsoidal	Spherical/terminal	–
Oxidase	+	+	–
Motility	+	–	–
NaCl concentration for growth (% w/v):			
Range	5–20	3–20	2.5–20
Optimum	10	10–12	12
Temperature for growth (°C):			
Range	25–45	15–40	25–45
Optimum	40	30	37
pH for growth:			
Range	7.0–10.0	6.8–9.5	6.5–10.5
Optimum	8.0–8.5	8.0	9.0
Acid production from:			
D-Fructose	–	+	+
D-Mannose	–	+	+
Maltose	–	+	ND
Melibiose	–	+	ND
D-Xylose	–	+	+
Nitrate reduction	–	–	+
Arginine dehydrolase	–	ND	+
Hydrolysis of:			
Casein	+	–	ND
Aesculin	–	+	ND
Urease activity	–	–	+
DNA G + C content (mol%)	37.1	43	48

β -galactosidase, lysine and ornithine decarboxylase, arginine dihydrolase and phenylalanine deaminase tests

Table 2. Cellular fatty acid compositions (%) of strain HS136^T and closely related *Bacillus* species

Strains: 1, HS136^T; 2, *B. salarius* BH169^T (Lim *et al.*, 2006c); 3, *B. qingdaonensis* CM1^T (Wang *et al.*, 2007).

Fatty acid	1	2	3
Iso-C _{14:0}	0.5	–	1.6
C _{14:0}	1.3	–	1.3
C _{15:0}	–	–	1.2
Iso-C _{15:0}	41.6	8.9	5.9
Anteiso-C _{15:0}	35.8	53.1	34.8
Iso-C _{16:0}	1.3	6.6	14.0
C _{16:1} ω11c	1.5	–	–
C _{16:0}	5.8	–	7.7
Iso-C _{17:0}	3.7	–	4.4
Anteiso-C _{17:0}	8.4	18.7	21.4
C _{18:0}	–	–	2.1

are negative. The following compounds are utilized as sole sources of carbon and energy: D-fructose, *m*-inositol, maltose, asparagine and cysteine. The following compounds are not utilized as sole sources of carbon and energy: D-glucose, galactose, lactose, D-mannose, melibiose, D-ribose, sucrose, glycerol, alanine, arginine, glycine, leucine, lysine, methionine, proline and valine. Sensitive to amoxicillin (30 µg), carbenicillin (100 µg), nitrofurantoin (300 µg), tetracycline (30 µg) and rifampicin (5 µg). Resistant to gentamicin (30 µg), tobramycin (10 µg), kanamycin (30 µg) and polymyxin B (100 U). Polar lipids are phosphatidylglycerol and diphosphatidylglycerol. The isoprenoid quinone is MK-7. The peptidoglycan type is A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid. Cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0}, C_{16:0}, iso-C_{17:0}, C_{16:1}ω11c, iso-C_{16:0}, C_{14:0} and iso-C_{14:0}. The DNA G + C content of the type strain is 37.1 mol% (*T_m*).

The type strain is HS136^T (=CCM 7595^T=DSM 21632^T=JCM 15720^T=LMG 25222^T), isolated from Howz-Soltan hypersaline lake in Iran.

Acknowledgements

We thank J. Euzéby for his help with the etymology of the novel species' name. This study was supported by grants from the Iranian Environment Department (3/42274), the Spanish Ministerio de Educación y Ciencia (BIO2006-06927) and Junta de Andalucía (P06-CVI-01829).

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