Bacillus iranensis sp. nov., a moderate halophile from a hypersaline lake

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A Gram-positive, moderately halophilic rod, designated X5B^T, was isolated from saline mud of the hypersaline lake Aran-Bidgol in Iran. Strain X5B^T was a strictly aerobic, motile bacterium that produced ellipsoidal endospores at a central-subterminal position in non-swollen sporangia. The isolate grew at pH 7.0–10.0 (optimum pH 7.5), at 25–45 °C (optimum 35 °C) and with 2.5–15 % (w/v) NaCl (optimum 5–7.5 %). On the basis of 16S rRNA gene sequences, strain X5B^T belonged to the genus *Bacillus* and showed highest similarity with *Bacillus persepolis* HS136^T (95.6 % 16S rRNA gene sequence similarity) and *Bacillus salarius* BH169^T (95.5 %). The DNA G + C content was 42.4 mol%. The major cellular fatty acids were anteiso-C_{15:0} and iso-C_{15:0} and the polar lipid profile consisted of phosphatidylglycerol, diphosphatidylglycerol, three phospholipids and two glycolipids. The diamino acid found in the cell-wall peptidoglycan was meso-diaminopimelic acid and the isoprenoid quinones were MK-7 (92 %), MK-6 (6 %) and MK-5 (2 %). On the basis of phylogenetic, chemotaxonomic and phenotypic data, a novel species of the genus *Bacillus* is proposed, with the name *Bacillus iranensis* sp. nov. The type strain is X5B^T (=IBRC 10446^T =DSM 23995^T).

Moderately halophilic bacteria are represented by physiologically and taxonomically heterogeneous groups that include both Gram-negative and Gram-positive microorganisms (Ventosa *et al.*, 1998; Ventosa, 2006). Among the Gram-positive organisms, several aerobic, endospore-forming, halophilic genera and species have been reported (Arahal & Ventosa, 2002; Ventosa, 2006; de la Haba *et al.*, 2011). These bacteria are frequently isolated from saline and hypersaline environments such as soils and aquatic habitats (Arahal & Ventosa, 2002; Ventosa, 2006; Márquez *et al.*, 2011), are characterized by optimal growth in media containing 3–15 % (w/v) NaCl (Ventosa *et al.*, 1998) and belong to species of the genus *Bacillus* or other *Bacillus*-related genera (de la Haba *et al.*, 2011). Studies of such bacteria are of great importance as they may produce compounds of industrial interest, such as extracellular hydrolytic enzymes that have diverse potential uses in

biotechnological processes (Margesin & Schinner, 2001; Mellado & Ventosa, 2003; Sánchez-Porro *et al.*, 2003; Amoozegar *et al.*, 2008; Kiran & Chandra, 2008; Rohban *et al.*, 2009; Karbalaei-Heidari *et al.*, 2009; Shafiei *et al.*, 2010).

During the study of the microbial population in hypersaline lakes in Iran, an aerobic, Gram-positive, moderately halophilic bacterium, designated strain X5B^T, was isolated from mud of the hypersaline lake Aran-Bidgol, the largest playa in the centre of Iran, 1000 km distant from the coast and at an altitude of 800 m (sampling site: 34.64378° N 51.83838° E). This playa was formed by deposition of halite sediments in different geological periods. Most salts in this lake are NaCl, Na₂SO₄, MgCl₂ and MgSO₄ and there are traces of carbonate ions. The pH of the water of the lake is neutral (about pH 7.0–7.5) and its salinity reaches saturation.

Strain X5B^T was isolated by diluting the mud sample in sterile 10 % (w/v) salt solution, plating onto 7.5 % HM agar (containing l⁻¹: 60.75 g NaCl, 5.25 g MgCl₂ · 6H₂O, 7.2 g MgSO₄ · 7H₂O, 0.27 g CaCl₂ · 2H₂O, 1.5 g KCl, 0.045 g NaHCO₃, 0.0195 g NaBr, 5 g proteose-peptone no. 3, 10 g

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain X5B^T is HQ433452.

Three supplementary figures are available with the online version of this paper.

yeast extract, 1 g glucose, 20 g agar; pH 7.5; Ventosa *et al.*, 1982) and incubating at 35 °C under aerobic conditions. The isolate was subsequently purified three times and maintained on the same medium and was stored in 7.5 % HM broth supplemented with 30 % (v/v) glycerol at –80 °C.

In order to phenotypically characterize strain X5B^T, the recently published minimal standards for describing new taxa of aerobic, endospore-forming bacteria were followed (Logan *et al.*, 2009). Unless stated otherwise, the morphological, physiological and biochemical characteristics of the isolate were studied in 7.5 % HM broth at pH 7.5 and 35 °C. *Bacillus persepolensis* IBRC 10436^T and *Bacillus salarius* DSM 16461^T were obtained from the IBRC and DSMZ culture collections, cultured under the same growth conditions as strain X5B^T and used as reference strains.

Cell morphology was examined by light microscopy (model CX31-2; 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 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, aesculin hydrolysis, indole production and methyl red and Voges-Proskauer tests were done as recommended by Smibert & Krieg (1994). Hydrolysis of Tweens 40, 60 and 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). Antibiotic susceptibility tests were performed using discs impregnated with various antimicrobial compounds (HiMedia) on Mueller-Hinton agar plus 7.5 % (w/v) sea salts (Ventosa *et al.*, 1982) seeded with a bacterial suspension containing 1.5×10^6 c.f.u. ml⁻¹. The plates were incubated at 35 °C for 48 h and inhibition zones were interpreted according to the manufacturer's instructions. Growth at 10–50 °C (at intervals of 5 °C) and at pH 5–11 (at intervals of 0.5 pH unit) was determined in nutrient broth supplemented with 7.5 % (w/v) NaCl, with the pH adjusted using the following buffer systems, as recommended by Amoozegar *et al.* (2009): sodium acetate/acetic acid (pH <6), Tris/HCl (pH 6–9) and glycine/sodium hydroxide (pH >9). Growth with 0, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v) NaCl was tested in HM broth at pH 7.5. Growth was monitored by measuring turbidity at A₆₀₀ (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described elsewhere (Mata *et al.*, 2002; Quesada *et al.*, 1984; Ventosa *et al.*, 1982).

Strain X5B^T was Gram-positive, motile and strictly aerobic. Ellipsoidal endospores were formed at the central-subterminal position in non-swollen sporangia. Cells were rods (0.5–0.9 µm wide and 1.2–2.5 µm long). After growth for 2 days at 35 °C on 7.5 % HM agar, colonies were circular, entire, smooth, cream and 2 mm in diameter. The isolate was moderately halophilic and grew in media containing

2.5–15 % (w/v) NaCl (optimum 5.0–7.5 %). No growth was observed in the absence of NaCl. Strain X5B^T grew at pH 7.0–10.0 (optimum pH 7.5). Other phenotypic features are included in Table 1 and the species description.

Genomic DNA from strain X5B^T was prepared 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 as described by Mellado *et al.* (1995). Direct sequence determination of the amplified DNA was carried out using an automated DNA sequencer (ABI 3130 XL; Applied Biosystems) at the SeqLab Laboratory (Göttingen, Germany). An almost-complete 16S rRNA gene sequence (1439 bp) of strain X5B^T was obtained and used for initial BLAST searches in GenBank

Table 1. Characteristics that distinguish strain X5B^T from its closest phylogenetic neighbours

Strains: 1, *Bacillus iranensis* sp. nov. X5B^T; 2, *B. persepolensis* IBRC 10436^T; 3, *B. salarius* DSM 16461^T. Data were taken from this study unless otherwise indicated.

Characteristic	1	2	3
Cell dimensions (µm)			
Width	0.5–0.9	1.0–1.5	0.5–0.7*
Length	1.2–2.5	2.5–3.0	1.3–1.9*
Pigmentation†	C	C	CW to Y
Endospore position/shape‡	CST/E	CST/E	T/S
Motility	+	+	–
NaCl concentration (% w/v)			
Range	2.5–15	5–20	3–20
Optimum	5–7.5	10	10–12
Temperature (°C)			
Range	25–45	25–45	15–40*
Optimum	35	40	30*
pH			
Range	7–10	7–10	6.8–9.5*
Optimum	7.5	8.0–8.5	8.0*
Nitrate reduction	+	–	–
Hydrolysis of:			
Tween 80	–	+	–
Casein	–	+	–
Aesculin	–	–	+
Acid production from:			
D-Glucose	–	+	+
D-Galactose	–	–	+
D-Fructose	–	–	+
Lactose	–	–	+
Maltose	–	–	+
D-Mannitol	+	–	+
D-Xylose	–	–	+
Quinones	MK-7, MK-6, MK-5	MK-7	MK-7*
DNA G + C content (mol%)	42.4	37.1	43*

*Data were taken from Lim *et al.* (2006c).

†C, Cream; CW, cream-white; Y, yellow.

‡CST/E, Central-subterminal/ellipsoidal; T/S, terminal/spherical.

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://eztaxon-e.ezbiocloud.net>; Kim *et al.*, 2012). Sequence analysis was performed using 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 using the alignment tool of the ARB software package. Phylogenetic trees were reconstructed using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms in the ARB software.

The closest relative of strain X5B^T was *B. persepolensis* HS136^T (95.6 % 16S rRNA gene sequence similarity). Other strains closely related to strain X5B^T were *B. salarius* BH169^T (95.5 %), *Bacillus aidingensis* 17-5^T (94.8 %) and *Bacillus qingdaonensis* CM1^T (94.3 %). 16S rRNA gene sequence similarity between strain X5B^T and other Gram-positive endospore-forming bacilli was <94 %. The three treeing algorithms resulted in phylogenetic trees with similar topologies (Fig. 1 and Supplementary Figs S1 and S2, available in IJSEM Online). On the basis of sequence divergence, it was evident that strain X5B^T constituted a taxon separate from the other known species of the genus *Bacillus*. DNA–DNA hybridization between strain X5B^T and its nearest phylogenetic neighbours was not done since strains exhibiting <97 % 16S rRNA gene sequence similarity are unlikely to exhibit >70 % DNA–DNA relatedness at the whole-genome level, a criteria necessary for the delineation of separate species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

To determine the DNA base composition, DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The DNA G+C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). The G+C content of the DNA of strain X5B^T was 42.4 mol%. This value is within the range for the genus *Bacillus* and similar to that of the type species of the genus, *Bacillus subtilis* (42.9 mol%; Logan & De Vos, 2009); however, the G+C content of the DNA of strain X5B^T was higher than that of *B. persepolensis* IBRC 10436^T and slightly lower than that of *B. salarius* DSM 16461^T (Table 1).

Cell biomass for analysis of the cell-wall peptidoglycan, polar lipids and isoprenoid quinones was obtained by cultivation on 7.5 % HM agar at 35 °C. Whole-cell hydrolysates (100 °C, 4 M HCl, 16 h) were examined for diaminopimelic acid isomers by TLC on cellulose plates using the solvent system of Rhuland *et al.* (1955). Polar lipids were analysed as described by Groth *et al.* (1996) and isoprenoid quinones were analysed as described by Monciardini *et al.* (2003).

The cell-wall peptidoglycan was based on *meso*-diaminopimelic acid and the polar lipids detected were phosphatidylglycerol, diphosphatidylglycerol, three phospholipids and two glycolipids (Supplementary Fig. S3). The isolate displayed a complex polar lipid profile similar to that of *B. subtilis* (Kämpfer *et al.*, 2006), while *B. persepolensis* displayed a simple polar lipid pattern consisting of phosphatidylglycerol and diphosphatidylglycerol (Amoozegar *et al.*, 2009). However, phosphatidylethanolamine, which has been reported for *B. subtilis*, was not detected in strain X5B^T or *B. persepolensis* (Amoozegar *et al.*, 2009; Kämpfer *et al.*, 2006). The major isoprenoid quinone of strain X5B^T was

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain X5B^T and closely related type strains of species of the genus *Bacillus*. Bootstrap values (>70 %) based on 1000 replications are given at branch nodes. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

MK-7 (92 %), which is typical of the genus *Bacillus*, and MK-6 and MK-5 were also present (6 and 2 %, respectively). In summary, the data for the respiratory quinones, polar lipids and the peptidoglycan type of the cell wall of strain X5B^T were typical for members of the genus *Bacillus* (Priest *et al.*, 1988; Heyrman *et al.*, 2004, 2005; Wieser *et al.*, 2005; Lim *et al.*, 2006a, b, c).

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 (Difco) containing 5 % (w/v) NaCl at 30 °C for 48 h. The cellular fatty acid composition of strain X5B^T was characterized by the major fatty acids anteiso-C_{15:0} (43.4 %) and iso-C_{15:0} (21.8 %). The fatty acid composition was similar to that of *B. persepolensis* IBRC 10436^T except that strain X5B^T contained higher proportions of anteiso-C_{17:0} and iso-C_{17:0} and also contained C_{18:1}ω9c (Table 2).

The isolate could be distinguished from closely related members of the genus *Bacillus* by a combination of differences in endospore shape and position, motility, NaCl range and optimum for growth, acid production from carbohydrates, nitrate reduction and other phenotypic tests, as well as by the genomic DNA G + C content. The fatty acid composition could also be used to distinguish strain X5B^T from phylogenetically related taxa. Therefore, we propose that the isolate represents a novel species of the genus *Bacillus*, for which the name *B. iranensis* sp. nov. is proposed.

Description of *Bacillus iranensis* sp. nov.

Bacillus iranensis (i.ra.nen'sis. N.L. masc. adj. *iranensis* from Iran, where the type strain was isolated).

Cells are Gram-positive, motile, endospore-forming rods (0.5–0.9 × 1.2–2.5 µm). Ellipsoidal endospores are produced at a central-subterminal position in non-swollen sporangia.

Table 2. Cellular fatty acid compositions of strain X5B^T and its closest phylogenetic neighbours

Strains: 1, *Bacillus iranensis* sp. nov. X5B^T; 2, *B. persepolensis* IBRC 10436^T; 3, *B. salarius* DSM 16461^T. Data were taken from this study under the same conditions.

Fatty acid (%)	1	2	3
iso-C _{14:0}	0.5	0.9	2.4
C _{14:0}	1.2	1.0	1.0
iso-C _{15:0}	21.8	62.0	7.8
anteiso-C _{15:0}	43.4	21.0	49.7
iso-C _{16:0}	1.6	1.4	6.8
C _{16:0}	7.9	3.6	5.5
iso-C _{17:0}	10.2	3.9	3.4
anteiso-C _{17:0}	9.4	3.5	18.9
C _{16:1} ω11c	0.8	2.0	0.5
C _{18:1} ω9c	8.0	—	—

Colonies are circular, entire, smooth, cream and 2 mm in diameter after 48 h at 35 °C on 7.5 % HM agar. Strictly aerobic. Moderately halophilic; grows with 2.5–15 % (w/v) NaCl (optimum 5–7.5 %), but not without NaCl. Grows at 25–45 °C (optimum 35 °C) and pH 7.0–10.0 (optimum pH 7.5). Catalase- and oxidase-positive. Casein, gelatin, DNA, starch and Tweens 40, 60 and 80 are not hydrolysed. Nitrate is reduced to nitrite, but nitrite is not reduced. Indole and H₂S are not produced. Acid is produced from D-mannitol, but not from D-glucose, D-fructose, galactose, lactose, sucrose, maltose, trehalose, D-mannose or D-xylose. Methyl red, Voges–Proskauer, urease, β-galactosidase, lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase tests are negative. The following compounds are utilized as sole source of carbon and energy: D-fructose, cellobiose and alanine. The following compounds are not utilized as sole source of carbon and energy: D-glucose, galactose, D-mannose, melibiose, sucrose, glycerol, trehalose, raffinose, arabinose, arginine, glycine, leucine, methionine, proline, cysteine, asparagine, tyrosine, histidine and valine. Sensitive to (µg per disc unless otherwise stated) bacitracin (10 U), chloramphenicol (30), erythromycin (15), nitrofurantoin (300), penicillin G (10 U), tetracycline (30) and rifampicin (5); resistant to gentamicin (30), kanamycin (30) and polymyxin B (100 U). The diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, three phospholipids and two glycolipids. The isoprenoid quinones are MK-7, MK-6 and MK-5. The predominant cellular fatty acids (>5 %) are anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{17:0}, anteiso-C_{17:0}, C_{18:1}ω9c and C_{16:0}. The DNA G + C content of the type strain is 42.4 mol% (HPLC).

The type strain is X5B^T (=IBRC 10446^T =DSM 23995^T), isolated from the hypersaline lake Aran-Bidgol in Iran.

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