Saliterribacillus persicus gen. nov., sp. nov., a moderately halophilic bacterium isolated from a hypersaline lake

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A novel Gram-positive, moderately halophilic bacterium, designated strain X4Bᵀ, was isolated from soil around the hypersaline lake Aran-Bidgol in Iran and characterized taxonomically using a polyphasic approach. Cells of strain X4Bᵀ were motile rods and formed ellipsoidal endospores at a terminal or subterminal position in swollen sporangia. Strain X4Bᵀ was a strictly aerobic bacterium, catalase- and oxidase-positive. The strain was able to grow at NaCl concentrations of 0.5–22.5 % (w/v), with optimum growth occurring at 7.5 % (w/v) NaCl. The optimum temperature and pH for growth were 35 °C and pH 7.0. Analysis of 16S rRNA gene sequence revealed that strain X4Bᵀ is a member of the family Bacillaceae, constituting a novel phyletic lineage within this family. Highest sequence similarities were obtained with the 16S rRNA gene sequences of the type strains of Sediminibacillus albus (96.0 %), Paraliobacillus ryukyuensis (95.9 %), Paraliobacillus quinghaiensis (95.8 %) and Sediminibacillus halophilus (95.7 %), respectively.

The DNA G+C content of this novel isolate was 35.2 mol%. The major cellular fatty acids of strain X4Bᵀ were anteiso-C₁₅ : 0 and anteiso-C₁₇ : 0 and its polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, two aminolipids, an aminophospholipid and an unknown phospholipid. The isoprenoid quinones were MK-7 (89 %) and MK-6 (11 %). The peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid. On the basis of 16S rRNA gene sequence analysis in combination with chemotaxonomic and phenotypic data, strain X4Bᵀ represents a novel species in a new genus in the family Bacillaceae, order Bacillales for which the name Saliterribacillus persicus gen. nov., sp. nov. is proposed. The type strain of the type species (Saliterribacillus persicus) is X4Bᵀ (= IBRC-M 10629ᵀ = KCTC 13827ᵀ).

The family Bacillaceae in the order Bacillales, is a large taxonomic group containing more than 39 genera with many different physiological features. Many of these genera include halophilic or halotolerant endospore-forming species, such as the genera Halobacillus (Spring et al., 1996), Lentibacillus (Yoon et al., 2002), Thalassobacillus (García et al., 2005), Ornithinibacillus (Mayr et al., 2006), Terribacillus (An et al., 2007), Piscibacillus (Tanasupawat et al., 2007) Sediminibacillus (Carrasco et al., 2008), Streptohalobacillus (Wang et al., 2011), Natribacillus (Echigo et al., 2012) and Alteribacillus (Didari et al., 2012). Members of these genera have been isolated from different environments such as saline or hypersaline lakes, saline or non-saline soils, seawater, soda lakes, mineral pools, food and salt-fermented foods (Arahal & Ventosa, 2002; de la Haba et al., 2011).

In our previous studies, we isolated some halophilic and halotolerant micro-organisms from hypersaline lakes in Iran and identified species from three novel haloarchaeal
genera, Halovenus aranensis (Makhdoumi-Kakhki et al., 2012a), Haloarchaeobius iranensis (Makhdoumi-Kakhki et al., 2012b) and Halopenitus persicus (Amoozegar et al., 2012), two novel extremely halophilic species belonging to the genus Salinibacter, Salinibacter iranicus and Salinibacter luteus (Makhdoumi-Kakhki et al., 2012c) and nine moderately halophilic bacterial species including Halobacillus karajensis (Amoozegar et al., 2003) Salinivibrio proteolyticus (Amoozegar et al., 2008a), Salinicoccus iranensis (Amoozegar et al., 2008b), Bacillus persepolensis (Amoozegar et al., 2009b), Thalassobacillus cyi (Sánchez-Porro et al., 2009) Piscibacillus halophilus (Amoozegar et al., 2009a), Lentibacillus persicus (Sánchez-Porro et al., 2010), Bacillus iranensis (Bagheri et al., 2012) and Alteribacillus bidgolensis (Didari et al., 2012). During the study of the microbial population in Aran-Bidgol lake, a hypersaline lake in Iran (35° 70’ N 51° 39’ E), an aerobic, Gram-positive, moderately halophilic bacterium, designated strain X4B, was isolated and characterized. On the basis of a polyphasic taxonomic characterization, strain X4B was assigned to a new genus of the family Bacillaceae, and its differential features permitted us to propose it as a representative of a novel species in a new genus, Salterribacillus persicus gen. nov., sp. nov.

The strain was isolated from soil from the area around the lake by diluting a sample in sterile 7.5 % (w/v) salt solution, plating on 7.5 % halophiles moderate (HM) medium and incubating at 35 °C aerobically. The 7.5 % HM medium contained (g l⁻¹): NaCl, 60.7; MgCl₂·6H₂O, 5.2; MgSO₄·7H₂O, 7.2; CaCl₂·2H₂O, 0.27; KCl, 1.5; NaHCO₃, 0.045; NaBr, 0.0195; proteose-peptone no. 3, 5; yeast extract, 10; and glucose, 1 (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 and maintained on the same medium. Long-term preservation was performed at ~80 °C in 7.5 % HM broth medium, supplemented with 30 % (v/v) glycerol.

In order to phenotypically characterize strain X4B, standard phenotypic tests were performed. The recommended minimal standards for describing new taxa of aerobic, endospore-forming bacteria were followed (Logan et al., 2009). Strains Sediminibacillus halophilus CCM 7364T and Paralibacillus ryukyuensis DSM 15140T were obtained from the Czech Collection of Microorganisms (CCM) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collections, respectively, and were used as reference strains for comparison in our study. They were cultured under the same growth conditions as strain X4B.

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) 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 and nitrite reduction, hydrolysis of aesculin, production of indole, methyl red and Voges–Proskauer tests were tested as recommended by Snibert & Krieg (1994). Hydrolysis of Tween 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 × 10⁶ c.f.u. ml⁻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 5–9 at intervals of 0.5 pH units. pH values below 6 and pH values above 6 were obtained using sodium acetate/acetate acid and Tris/HCl buffers, respectively. Growth at different NaCl concentrations (0, 0.5, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 22.5 and 25.0 %, w/v) was tested on HM medium at pH 7.5. Growth was monitored by turbidity at OD₆₀₀ 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 X4B was Gram-positive, non-motile and strictly aerobic. Cells were rods with a width of 0.5 μm and length of 3.0–8.0 μm. Ellipsoidal endospores were formed at the terminal or subterminal position in swollen sporangia. When grown for 2 days at 35 °C on 7.5 % HM medium, the colonies were pustuliform, convex, entire, smooth and cream-coloured with a diameter of 2 mm. This isolate was moderately halophilic, growing in media containing 0.5–22.5 % (w/v) NaCl; optimal growth occurred in media containing 7.5 % (w/v) NaCl. Strain X4B grew between pH 6.0 and 8.0 (optimum pH 7.0). Other phenotypic features are included in the species description and Table 1.

Genomic DNA of strain X4B was extracted 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. PCR conditions included three temperature cycles: initial denaturation at 95 °C for 5 min; 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer model ABI 3130XL (Applied Biosystems) 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 constructed using three different methods: maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981), 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 (1472 bp) of strain X4B\textsuperscript{T} was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. 16S rRNA gene sequence analysis showed that strain X4B\textsuperscript{T} is a member of the family Bacillaceae, but there were low similarities to type strains of the type species of other members of this family. The most closely related taxa were the type strains of: *Sediminibacillus albus* (96.0 % similarity), *Paraliobacillus ryukyuensis* (95.9 %), *Paraliobacillus quinghaiensis* (95.8 %) and *Sediminibacillus halophilus* (95.7 %), respectively. Phylogenetic analysis using the maximum-likelihood algorithm revealed that strain X4B\textsuperscript{T} clustered in a separate clade (Fig. 1). The phylogenetic position of strain X4B\textsuperscript{T} was also confirmed by trees generated using the neighbour-joining and maximum-parsimony algorithms (Fig. 1). The 16S rRNA gene sequence similarity with members of other genera of the family Bacillaceae was equal or lower than 95.6 %, e.g. *Virgibacillus* (≤95.6 %), *Gracilibacillus* (≤95.6 %), *Natronobacillus* (≤95.1 %), *Halobacillus* (≤95.0 %), *Thalassobacillus* (≤95.0 %) or *Amphibacillus* (≤94.8 %), indicating that the isolate could represent a novel species in a new separate genus.

For determination of the DNA base composition, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). The DNA G+C content of the DNA of strain X4B\textsuperscript{T} was 35.2 mol%. This value is within the range for species of the genus *Paraliobacillus* but lower than that of species of the genus *Sediminibacillus* (Table 1).

Cell biomass for analysis of the peptidoglycan, isoprenoid quinones and polar lipids was obtained by cultivation on 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 plates (Merck) using the solvent system of

**Table 1.** Characteristics that distinguish strain X4B\textsuperscript{T} from other phylogenetically related taxa

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Cell size (μm)</td>
<td>0.5 × 3.0–8.0</td>
<td>0.9 × 1.5–7*</td>
<td>0.4–0.5 × 2.3–4.5†</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Cream</td>
<td>Cream</td>
<td>Yellow</td>
</tr>
<tr>
<td>Endospores:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formation</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Ellipsoidal</td>
<td>–</td>
<td>Ellipsoidal or spherical</td>
</tr>
<tr>
<td>Position</td>
<td>Terminal–subterminal</td>
<td>–</td>
<td>Terminal</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose requirement (aerobic growth)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NaCl concentration for growth (%, w/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.5–22.5</td>
<td>0–20*</td>
<td>0–22†</td>
</tr>
<tr>
<td>Optimum</td>
<td>7.5</td>
<td>5–7.5*</td>
<td>0.75–3†</td>
</tr>
<tr>
<td>Growth pH</td>
<td>6–8</td>
<td>5.0–9.5*</td>
<td>5.5–9.5†</td>
</tr>
<tr>
<td>Optimum</td>
<td>7</td>
<td>7.5*</td>
<td>7–8.5†</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>20–45</td>
<td>15–55*</td>
<td>10–47.5†</td>
</tr>
<tr>
<td>Optimum</td>
<td>35</td>
<td>37*</td>
<td>37–40†</td>
</tr>
<tr>
<td>Reduction of nitrate to nitrite</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gelatin</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Major isoprenoid quinone (%)</td>
<td>MK-7 (89), MK-6 (11)</td>
<td>MK-7 (83), MK-6 (17)*</td>
<td>MK-7†</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>35.2</td>
<td>47.5*</td>
<td>35.6†</td>
</tr>
</tbody>
</table>

*Data from Carrasco et al. (2008).
†Data from Ishikawa et al. (2002).
Rhuland et al. (1955). Polar lipids were analysed as described by Groth et al. (1996). Isoprenoid quinone analysis was carried out as described by Monciardini et al. (2003).

The peptidoglycan was based on meso-diaminopimelic acid and the polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, two aminolipids, an aminophospholipid and an unknown phospholipid (Fig. 2). Strain X4BT shared the occurrence of the peptidoglycan type A1\textsuperscript{c} based on meso-diaminopimelic acid with members of the genera Paraliobacillus and Sediminibacillus as well as several other genera of the Bacillaceae (Carrasco et al., 2008; Chen et al., 2009; Ishikawa et al., 2002; Wang et al., 2009). The major isoprenoid quinone of strain X4BT was MK-7 (89 %), although MK-6 was also present (11 %). The respiratory lipoquinone pattern with MK-7 as the major and MK-6 as the minor component of strain X4BT corresponds with that of members of the genus Sediminibacillus, while this characteristic distinguishes strain X4BT from members of the genus Paraliobacillus, which have been described as having MK-7 as the sole respiratory lipoquinone.

The polar lipid composition of strain X4BT\textsuperscript{T} supports the discrimination from its phylogenetic relatives, i.e. the absence of phosphatidylmethyl ethanolamine and phosphatidylcholine differentiates strain X4BT\textsuperscript{T} from Paraliobacillus quinghaiensis (Chen et al., 2009) and the glycolipid typical of members of the genus Sediminibacillus (Carrasco et al., 2008; Wang et al., 2009) could not be detected in strain X4BT\textsuperscript{T}.

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 7 % (w/v) NaCl.

Fig. 1. Phylogenetic tree, based on the maximum-likelihood algorithm of the 16S rRNA sequences, showing the position of strain X4BT\textsuperscript{T} and the closely related species of genera within the Bacillaceae. Sequence accession numbers used are shown in parentheses. Bootstrap values higher than 70 % are indicated at branch-points. Filled circles indicate branches found in phylogenetic consensus trees generated with the maximum-parsimony, neighbour-joining and maximum-likelihood methods. 

Virgibacillus koreensis BH30097\textsuperscript{T} was used as outgroup. Bar, 1 % sequence divergence.

Fig. 2. TLC of polar lipids of strain X4BT\textsuperscript{T}. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; AL1–AL2, unknown aminolipids; APL, unknown aminophospholipid; PL, unknown phospholipid.
at 35 °C following the protocol of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). Strain X4Bᵀ, *Sediminibacillus halophilus* CCM 7364ᵀ and *Paralibacillus ryukyuensis* DSM 15140ᵀ had similar growth behaviour and sufficient cells of comparable physiological age could be harvested from the third streak quadrant of the agar plates after cultivation under the applied conditions. The cellular fatty acid profile of strain X4Bᵀ was characterized by the fatty acids anteiso-C₁₅:₀ (66.6 %) and anteiso-C₁₇:₀ (19.9 %) as the major fatty acids. The fatty acid composition is similar to those found in phylogenetically closely related taxa, except that the level of anteiso-C₁₅:₀ was higher while those of iso-C₁₅:₀ and C₁₆:₀ were lower for strain X4Bᵀ than for species of the genera *Sediminibacillus* and *Paralibacillus* (see Table S1 available in IJSEM Online).

Phylogenetic analysis of the 16S rRNA gene sequence showed that strain X4Bᵀ represents a novel branch within the Gram-positive, endospore-forming bacteria in the family *Bacillaceae*. In addition, some chemotaxonomic, biochemical and physiological properties, such as components of polar lipids, respiratory lipoquinones and fatty acids, DNA G+C content, anaerobic growth, reduction of nitrate to nitrite, glucose requirement in aerobic growth, NaCl concentration for growth (range and optimum), temperature for growth (range and optimum), hydrolysis of gelatin, starch and Tween 80, and acid production from carbohydrates, distinguish strain X4Bᵀ from its closely related phylogenetic neighbours, the members of the genera *Paralibacillus* and *Sediminibacillus*. In conclusion, the results of examination of the morphological and physiological properties of the isolate, the low levels of 16S rRNA gene sequence similarity with other genera within the family *Bacillaceae* and distinctive components of polar lipids suggest that strain X4Bᵀ represents a novel species of a new genus within the family *Bacillaceae*, for which the name *Saliterribacillus persicus* gen. nov., sp. nov. is proposed.

**Description of Saliterribacillus persicus gen. nov.**

*Saliterribacillus persicus* (per’si.cus. L. masc. adj. persicus of Persia).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.5 × 3.0–8.0 μm in size. Colonies are punctiform, convex, entire, smooth, cream and grow to 2 mm in diameter on 7.5 % HM agar medium after 48 h of incubation at 35 °C. Able to grow over a wide range of NaCl concentrations [from 0.5 to 22.5 % (w/v) NaCl], with optimal growth at 7.5 % (w/v) NaCl. Grows at 20–45 °C (optimally at 35 °C) and pH 6.0–8.0 (optimally at pH 7.0). Casein, gelatin, DNA, starch, and Tweenes 40, 60 and 80 are not hydrolysed. Indole and H₂S are not produced. Acid is produced from D-glucose, D-fructose, galactose, lactose, sucrose, maltose, D-mannose, D-mannitol and D-xylene, but not from ribose. Methyl red, Voges–Proskauer, urease, β-galactosidase, lysine and ornithine decarboxylases, arginine dihydrolase and phenylalanine deaminase tests are negative. The following compounds are utilized as sole sources of carbon and energy: D-arabinose, D-galactose, D-ribose, sucrose, raffinose, D-fructose, cellobiose, trehalose, glycerol, melibiose and mannitol. The following compounds are not utilized as sole source of carbon and energy: L-asparagine, L-arginine, L-histidine, L-leucine, glycine, alanine, L-methionine, L-phenylalanine, L-proline, cysteine, L-tyrosine and L-valine. Sensitive to amikacin (30 μg), amoxicillin (30 μg), bacitracin (10 U), carbenicillin (100 μg), gentamycin (30 μg), nitrofurantoin (300 μg), tetracycline (30 μg), rifampicin (5 μg) and tobramycin (10 μg). Resistant to kanamycin (30 μg), polymixin B (100 U) and streptomycin (10 μg).

The type strain is X4Bᵀ (=IBRC-M 10629ᵀ=KCTC 13827ᵀ) isolated from soil at Aran-Bidgol hypersaline lake in Iran. The DNA G+C content of the type strain is 35.2 mol% (HPLC).

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