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Oceanobacillus limi sp. nov., a moderately halophilic bacterium from a salt lake

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A Gram-stain-positive, endospore-forming, rod-shaped, strictly aerobic, moderately halophilic bacterium, designated strain H9B^T, was isolated from a mud sample of the hypersaline lake Aran-Bidgol in Iran. Cells of strain H9B^T were motile and produced colonies with a yellowish-grey pigment. Growth occurred between 2.5 and 10 % (w/v) NaCl and the isolate grew optimally at 7.5 % (w/v) NaCl. The optimum pH and temperature for growth of the strain were pH 7.0 and 35 °C, respectively, while it was able to grow over pH and temperature ranges of pH 6–10 and 25–45 °C, respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain H9B^T is a member of the genus *Oceanobacillus*. The closest relative to this strain was *Oceanobacillus profundus* CL-MP28^T with 97.1 % 16S rRNA gene sequences similarity. The level of DNA–DNA relatedness between the novel isolate and this phylogenetically related species was 17 %. The major cellular fatty acids of the isolate were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The polar lipid pattern of strain H9B^T consisted of phosphatidylglycerol, diphosphatidylglycerol, four phospholipids and an aminolipid. It contained MK-7 as the predominant menaquinone and *meso*-diaminopimelic acid in the cell-wall peptidoglycan. The G+C content of the genomic DNA of this strain was 37.1 mol%. Phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness data suggest that this strain represents a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus limi* sp. nov. is proposed. The type strain of *Oceanobacillus limi* is strain H9B^T (=IBRC-M 10780^T=KCTC 13823^T=CECT 7997^T).

The genus *Oceanobacillus* was first described by Lu *et al.* (2001, 2002) and emended by Yumoto *et al.* (2005), Lee *et al.* (2006) and Hirota *et al.* (2013a) to accommodate Gram-stain-positive, endospore-forming rods which are motile by means of peritrichous flagella. Members of this genus produce ellipsoidal endospores at the subterminal or terminal position within swollen sporangia. They are obligately aerobic or facultatively anaerobic, obligately or facultatively alkaliphilic and grow in the presence of 0–22 % (w/v) NaCl. The type species, *Oceanobacillus iheyensis*, was isolated from deep-sea sediment collected at a depth of 1050 m on the Iheya Ridge, Japan (Lu *et al.*, 2001). At the time of writing (July 2013), 13 other species have been described in this genus as facultative or obligate alkaliphiles with different levels of salt tolerance, from the halotolerant species *Oceanobacillus iheyensis* (Lu *et al.*, 2001), *Oceanobacillus oncorhynchi* (Yumoto *et al.*, 2005) *Oceanobacillus chironomi* (Raats & Halpern, 2007), *Oceanobacillus profundus* (Kim *et al.*, 2007), *Oceanobacillus caeni* (Nam *et al.*, 2008), *Oceanobacillus sojae* (Tominaga *et al.*, 2009) and *Oceanobacillus kimchii* (Whon *et al.*,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H9B^T is HQ433455.

One supplementary table and one supplementary figure are available with the online version of this paper.

2010), to moderately halophilic ones: *Oceanobacillus kapialis* (Namwong *et al.*, 2009), *Oceanobacillus locisalsi* (Lee *et al.*, 2010), *Oceanobacillus indicireducens* (Hirota *et al.*, 2013a) and *Oceanobacillus polygoni* (Hirota *et al.*, 2013b). Although this genus includes a species which grows optimally at pH 5, *Oceanobacillus chungangensis* (Lee *et al.*, 2013), most species are neutrophilic, alkaliphilic or alkalitolerant. Here we describe the isolation and polyphasic characterization of a novel moderately halophilic, alkalitolerant micro-organism from a mud sample of the hypersaline lake Aran-Bidgol in Iran and propose this strain as a representative of a novel species of the genus *Oceanobacillus*.

Strain H9B^T was isolated from a mud sample (pH 7.5, salinity 22 %) of the hypersaline lake Aran-Bidgol in Iran (34.31° N 51.40° E). We sampled saline mud (up to 40 cm in depth). The samples were collected in sterile plastic containers and kept in the dark at environmental temperature for four hours until analysed in the laboratory. The strain was isolated by diluting the sample in sterile 10 % (w/v) salt solution up to 10^{-5} dilution, plating on 7.5 % HM medium and incubating at 35 °C aerobically for one month. The 7.5 % HM medium contained (g l^{-1}): NaCl, 60.75; $MgCl_2 . 6H_2O$, 5.25; $MgSO_4 . 7H_2O$, 7.2; $CaCl_2 . 2H_2O$, 0.27; KCl, 1.5; $NaHCO_3$, 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. After successive cultivation, a pure isolate, designated strain H9B^T was obtained and routinely grown on 7.5 % HM medium agar at 35 °C. Characterization of this strain was achieved by following a polyphasic approach, including conventional phenotypic features, chemotaxonomic data (polar lipid, fatty acid, quinone and cell-wall composition) and molecular analysis (16S rRNA gene sequence similarity and DNA–DNA relatedness) (Logan *et al.*, 2009).

The genomic DNA of strain H9B^T was extracted with a DNA extraction kit (High Pure PCR Template Preparation kit; Roche) according to the manufacturer's protocol and the 16S rRNA gene was amplified using the bacterial universal primers 27F and 1492R (Lane *et al.*, 1985). Direct sequence determination of the PCR-amplified DNA was conducted on an ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Phylogenetic 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 using the alignment tool of the ARB software package (Ludwig *et al.*, 2004).

The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e database (Kim *et al.*, 2012).

Phylogenetic trees were reconstructed using three different methods: maximum-parsimony (Fitch, 1971), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) algorithms integrated in the ARB software, for phylogenetic inference.

An almost complete 16S rRNA gene sequence of strain H9B^T (1478 nt) was obtained. 16S rRNA gene sequence analysis revealed that strain H9B^T is a member of the genus *Oceanobacillus*. The closest phylogenetic relative of strain H9B^T was *Oceanobacillus profundus* CL-MP28^T, with a sequence similarity of 97.1 %. The sequence similarities of the novel strain to *Oceanobacillus polygoni* SA9^T, *Oceanobacillus caeni* S-11^T, *Oceanobacillus chungangensis* CAU 1051^T and *Oceanobacillus kimchii* X50^T were 96.7, 96.2, 96.7 and 96.7 %, respectively. Phylogenetic analysis using the maximum-parsimony algorithm revealed that the novel strain clustered with the members of this genus although in a separate clade (Fig. 1). 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. The phylogenetic position was also confirmed in trees generated using the neighbour-joining and maximum-likelihood algorithms.

In order to phenotypically characterize strain H9B^T, standard phenotypic tests were selected according to the recommendations of the Minimal Standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009). *Oceanobacillus profundus* IBRC-M 10567^T *Oceanobacillus kapialis* IBRC-M 10565^T and *Oceanobacillus caeni* KCTC 13061^T were obtained from Iranian Biological Resource Center and Korean Collection for Type Cultures and were used as reference strains for comparison in our study. They were cultured following the recommendations of the culture collection.

Cell morphology was examined by light microscopy (model BX41; 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).

Physiological tests were conducted using HM broth or agar, unless stated otherwise. Broth cultures were incubated at 35 °C in an orbital incubator at 150 r.p.m. Growth was monitored by turbidity at OD_{600} using a spectroscopic method (model UV-160 A; Shimadzu). To determine the optimal temperature and pH for growth of strain H9B^T, broth cultures were incubated at temperatures of 10–50 °C at intervals of 5 °C and at pH 5.5–10.5 at intervals of 0.5 pH units. pH values below 6, pH 6–9 and pH values above 9 were obtained using sodium acetate/acetic acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth at different NaCl concentrations (1.0, 2.5, 5, 7.5, 10, 12.5 and 15 %, w/v) was tested on HM medium at pH 7.5.

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, production of indole,

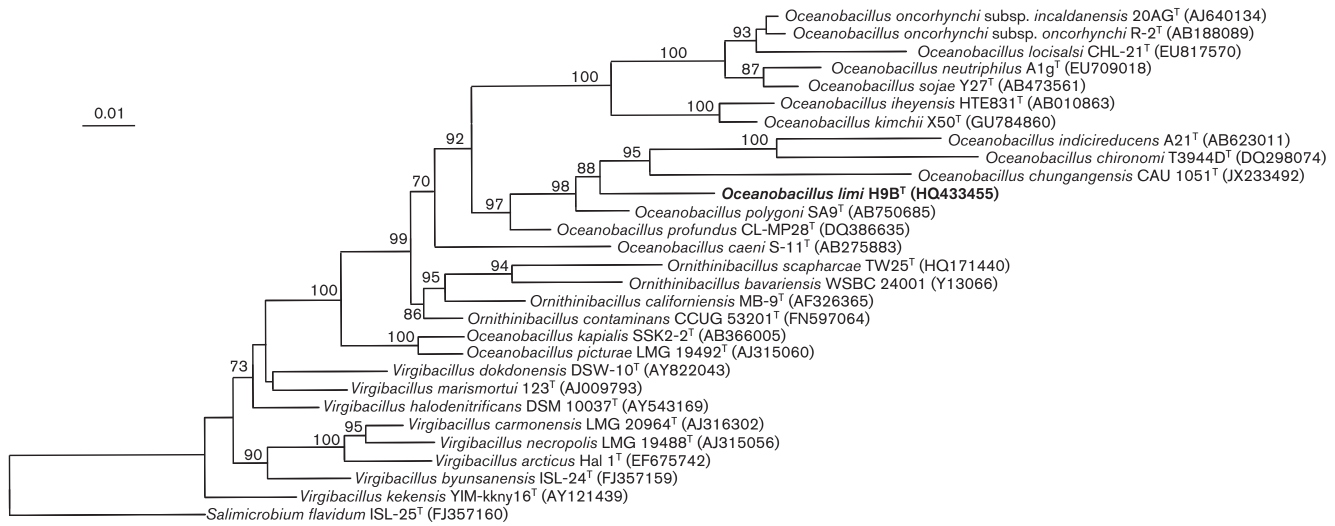

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain H9B^T with other species of the genus *Oceanobacillus* and related genera. Accession numbers of the sequences are given in parentheses. The sequence of *Salimicrobium flavidum* ISL-25^T (FJ357160) was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Only values greater than 70 % are shown. Bar, 0.01 substitutions per nucleotide position.

methyl red and Voges–Proskauer tests were done as recommended by Smibert & Krieg (1994). Hydrolysis of Tweens 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 as recommended by Ventosa *et al.* (1982). Antibiotic 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 1.5×10^6 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 to the manufacturer's manual. Other physiological and biochemical tests were performed as described previously (Mata *et al.*, 2002; Quesada *et al.*, 1984; Ventosa *et al.*, 1982).

Strain H9B^T was Gram-stain-positive, motile and strictly aerobic, catalase- and oxidase-positive and produced oval terminal endospores in swollen sporangia. Cells were rods with a width of 0.4 μm and length of 2–7 μm. This isolate was moderately halophilic, no growth was observed in the absence of NaCl. Strain H9B^T was sensitive to ampicillin (10 μg per disc), amoxicillin (25 μg), bacitracin (10 U), carbenicillin (100 μg), chloramphenicol (30 μg), gentamicin (10 μg), penicillin G (10 U), rifampicin (5 μg), streptomycin (10 μg) and tetracycline (30 μg) but resistant to amikacin (30 μg), cephalotin (30 μg), erythromycin (5 μg), kanamycin (5 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), polymixin B (100 U) and tobramycin (10 μg). Other phenotypic features are included in Table 1 and the species description.

For determination of DNA base composition and DNA–DNA hybridization, cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments) and the DNA in the crude lysate was 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 H9B^T was 37.1 mol%. This value is within the range described for species of the genus *Oceanobacillus* (Lu *et al.*, 2001). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) incorporating the modifications described by Huss *et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). DNA–DNA hybridization experiments between strain H9B^T and its closest phylogenetic relative, *Oceanobacillus profundus* CL-MP28^T, was 17 % (13 % in duplicate). According to the 70 % threshold proposed by Wayne *et al.* (1987) for the discrimination of prokaryotic species using DNA–DNA relatedness, this result confirmed that the new isolate represents a new genomic species.

Cell biomass for fatty acid, isoprenoid quinone, polar lipid and cell-wall peptidoglycan analyses was obtained by cultivation in 7.5 % HM broth at 150 r.p.m. and 35 °C. Cells were harvested in the mid-exponential growth phase. The whole-cell fatty acid composition of strain H9B^T was determined according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; Identification Library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kämpfer & Kroppenstedt (1996). Fatty acid

Table 1. Differential characteristics between strain H9B[T] and phylogenetically related species within the genus *Oceanobacillus*

Strains: 1, H9B[T] (*Oceanobacillus limi* sp. nov.); 2, *Oceanobacillus profundus* IBRC-M 10567[T] ; 3, *Oceanobacillus kapialis* IBRC-M 10565[T]; 4, *Oceanobacillus caeni* KCTC 13061[T]; 5, *Oceanobacillus chungangensis* CAU 1051[T] (data from Lee *et al.*, 2013). All data from this study unless otherwise indicated. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5
Endospore position	Terminal	Terminal	Terminal	Central	Subterminal or central
Temperature for growth (°C):					
Range	25–45	15–40	10–45	20–45	20–37
Optimum	35	35	37	35	30
pH for growth:					
Range	6–10	6.5–9.5	6–9	6–9	4.5–10
Optimum	7	7.5–8.5	8	7–7.5	5.0
NaCl concentration for growth (%):					
Range	2.5–10	0–14	0.5–20	0–10	0–10
Optimum	7.5	3	10	3	0
NaCl requirement	+	−	+	−	−
Nitrate reduction	−	+	−	−	−
Hydrolysis of:					
Aesculin	+	+	−	−	+
Gelatin	−	+	+	+	−
Tween 80	+	−	−	−	−
Acid production from:					
Glycerol	−	+	−	+	−
Lactose	−	+	−	−	−
Mannose	−	+	+	+	+
Mannitol	−	+	+	+	W
DNA G+C content (mol%)	37.1	40.2[a*]	39.7[b]	33.6[c]	36.3

*Data taken from: *a*, Kim *et al.* (2007); *b*, Namwong *et al.* (2009); *c*, Nam *et al.* (2008).

peaks were identified using the TSBA40 database. The fatty acid profile of strain H9B[T] was characterized by the fatty acids anteiso-$C_{15:0}$ (38.8 %), anteiso-$C_{17:0}$ (18.9 %), iso-$C_{15:0}$ (14.4 %) and iso-$C_{16:0}$ (12.6 %) as the major fatty acids followed by iso-$C_{14:0}$ (6.9 %), $C_{16:1}\omega7c$ alcohol (4.6 %), iso-$C_{17:0}$ (1.6 %) and $C_{16:0}$ (1.2 %). The fatty acids profile was in agreement with those of some other members of the genus *Oceanobacillus*, with branched fatty acids, including anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$, as dominant fatty acids (Kim *et al.*, 2007; Raats & Halpern, 2007; Namwong *et al.*, 2009). However, the amounts of these fatty acids as well as those of iso-$C_{15:0}$ and iso-$C_{16:0}$ were different from those observed in other phylogenetically related species (Table S1, available in the online Supplementary Material).

The polar lipids and respiratory quinones of strain H9B[T] were analysed as described by Groth *et al.* (1996). The polar lipids detected in strain H9B[T] were phosphatidylglycerol, diphosphatidylglycerol, four phospholipids and one aminolipid (Fig. S1). The major isoprenoid quinone was MK-7. Strain H9B[T] contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, which distinguished this isolate from the other members of the genus *Ornithinibacillus*, which contain L-ornithine in the cell-wall peptidoglycan (Mayr *et al.*, 2006).

In conclusion, the results obtained from this polyphasic study indicate that strain H9B[T] represents a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus limi* sp. nov. is proposed.

Description of *Oceanobacillus limi* sp. nov.

Oceanobacillus limi (li′mi. L. gen. masc. n. *limi* of/from mud).

Cells of the single isolate are Gram-stain-positive, motile, rods, 0.4 by 2–7 µm in size. Terminal ellipsoidal endospores are observed in swollen sporangia. Colonies are punctiform, umbonate, undulate, contoured, yellowish-grey-pigmented and 3.9–4.7 mm in diameter after incubation for 48 h at 35 °C on 7.5 % HM agar. Strictly aerobic. Moderately halophilic, growing at NaCl concentrations from 2.5 to 10 % (w/v), with optimal growth at 7.5 % (w/v) NaCl. No growth occurs in the absence of NaCl. Grows at 25–45 °C (optimally at 35 °C) and pH 6.0–10.0 (optimally at pH 7.0). Catalase- and oxidase-positive. Tweens 20, 40, 60 and 80, aesculin, casein and DNA are hydrolysed, while gelatin, starch and urea are not. Nitrate and nitrite are not reduced. Indole or H_2S are not produced. Methyl red, Voges–Proskauer, citrate utilization, lysine and ornithine decarboxylase, and arginine dihydrolase tests are negative.

Produces acid from D-glucose, D-fructose, maltose, sucrose and D-xylose but not from galactose, lactose, D-mannitol or D-ribose. The following compounds are utilized as sole sources of carbon and energy: L-arabinose, cellobiose, D-glucose and sucrose. The following compounds are not utilized as sole sources of carbon and energy: D-galactose, D-fructose, glycerol, D-mannitol, D-mannose, melibiose, raffinose, D-ribose, trehalose, xylose, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glycine, L-histidine, L-methionine, L-phenylalanine, L-proline, L-tyrosine and L-valine. The major isoprenoid quinone is MK-7. *meso*-Diaminopimelic acid is the diagnostic diamino acid. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, four phospholipids and one aminolipid. The predominant fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$.

The type strain is H9B^{T} (=IBRC-M 10780^{T}=KCTC 13823^{T}=CECT 7997^{T}), isolated from Aran-Bidgol hypersaline lake, Iran. The DNA G+C content of the type strain is 37.1 mol% (HPLC).

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