Lentibacillus salarius sp. nov., isolated from saline sediment in China, and emended description of the genus *Lentibacillus*

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A Gram-positive, spore-forming and moderately halophilic bacterium, strain BH139^T, was isolated from saline sediment of Xinjiang Province, China. Cells of strain BH139^T were motile, flagellated rods. The organism grew optimally at 30–35 °C in the presence 12–14 % (w/v) NaCl. The major fatty acids were branched saturated fatty acids such as iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and anteiso-C_{17:0}. The G + C content of the genomic DNA was about 43 mol% and the predominant isoprenoid quinone was MK-7. Comparative 16S rRNA gene sequence analyses showed that strain BH139^T was most closely related to *Lentibacillus salicampi* KCCM 41560^T (96.9 % 16S rRNA gene sequence similarity) and formed a distinct phyletic line from that species. On the basis of physiological and molecular properties, the isolate represents a novel species within the genus *Lentibacillus*, for which the name *Lentibacillus salarius* sp. nov. is proposed. The type strain is BH139^T (=KCTC 3911^T = DSM 16459^T).

Moderately halophilic bacteria that grow optimally in media containing 1–15 % (w/v) NaCl are widely distributed in different marine environments (Oren, 2002). They are attracting interest because they have great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner, 2001). Taxonomically, this group of bacteria includes both Gram-positive and Gram-negative micro-organisms. Aerobic, spore-forming, moderately halophilic, Gram-positive rods are also taxonomically very diverse and were originally assigned to the genus *Bacillus* (Ventosa *et al.*, 1998; Nielsen *et al.*, 1994). However, molecular and chemical analyses have shown that they form several phylogenetically distinct lineages such as the genera *Halobacillus* (Spring *et al.*, 1996), *Gracilbacillus* (Wainø *et al.*, 1999), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Filobacillus* (Schlesner *et al.*, 2001) and *Pontibacillus* (Lim *et al.*, 2005) within the group classically defined as the

genus *Bacillus* (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994).

It has been reported that some members of the genera *Filobacillus* and *Halobacillus* show Gram-negative or Gram-variable reactions despite belonging phylogenetically to a Gram-positive group (Schlesner *et al.*, 2001; Yoon *et al.*, 2003). Yoon *et al.* (2002) indicated that *Lentibacillus salicampi* isolated from the Yellow sea in Korea also produced a Gram-variable reaction. In the course of screening of halophilic bacteria, an aerobic, Gram-positive, moderately halophilic bacterium, strain BH139^T, was isolated from Xinjiang Province, China. In this study, this isolate is described as a novel species within the genus *Lentibacillus*, on the basis of phylogenetic and phenotypic characteristics.

Strain BH139^T was isolated from soil sediment of a salt lake in Xinjiang Province, China, using marine agar 2216 (MA; Difco), to which 15 % (w/v) NaCl had been added (final NaCl concentration: 16.94 %, w/v), at 28 °C for 3 days. NaCl requirements and tolerance were determined in trypticase soy broth (per litre: 17.0 g casein, 3.0 g soy-bean meal and 2.5 g glucose, 5.0 g sodium chloride and 2.5 g dipotassium phosphate) supplemented with modified artificial sea water (per litre: 0–30 %, w/v, NaCl; 5.94 g MgSO₄·7H₂O; 4.53 g MgCl₂·6H₂O; 0.64 g KCl; 1.3 g CaCl₂). To investigate its morphological and physiological characteristics, strain

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH139^T is AY667493.

A transmission electron micrograph showing the general morphology and flagella of strain BH139^T is available as supplementary material in IJSEM Online.

BH139^T was cultivated on MA with 12 % (w/v) NaCl at 30 °C, except where indicated otherwise. Anaerobic growth was determined by incubation in an anaerobic chamber for 5 days at 30 °C on MA supplemented with 10 % (w/v) NaCl. Optimum growth was tested at different temperatures (4–55 °C) on MA containing 12 % (w/v) NaCl and at different pH values (5.0–10.0) in trypticase soy broth supplemented with artificial sea water containing 12 % (w/v) NaCl.

Cell morphology was studied using light microscopy and transmission electron microscopy. Motility was observed on agar-coated wet mounts by using a light microscope (Nikon E600) after 12 and 36 h cultivation. The flagellum type was examined by transmission electron microscopy using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids (Electron Microscopy Science) and negatively stained with 2 % (w/v) uranyl acetate for 15 s, then subjected to transmission electron microscopy (JEM-1010; JEOL) operated at 80 kV. Endospores were stained using Schaeffer–Fulton stain (Smibert & Krieg, 1981).

Gram staining was performed using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Catalase activity was determined from the production of oxygen bubbles in a 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested for by the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine and xanthine was determined on MA according to methods described previously (Cowan & Steel, 1965; Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction was performed according to the method of Lanyi (1987). Acid production from carbohydrates was determined as described by Leifson (1963); all suspension media were supplemented with artificial sea water containing 12 % (w/v) NaCl.

For quantitative analysis of whole-cell fatty acids, strain BH139^T was cultivated on MA and on MA with the addition of 10 % (w/v) NaCl, at 30 °C for 5 and 2 days, respectively. The preparation of fatty acid methyl esters and their analysis were performed according to the instructions of the Microbial Identification System (Microbial ID; MIDI). Analysis of the peptidoglycan and the polar lipids of the strain was carried out using the methods described by Komagata & Suzuki (1987). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using HPLC with a reversed-phase column (GROM-SIL 100 ODS-2FE, GROM). The DNA G + C content of strain BH139^T was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984).

Sequencing and assembly of 16S rRNA genes were carried out as described previously (Bakermans & Madsen, 2002). The resultant sequences of 16S rRNA genes were compared with available 16S rRNA sequences from GenBank, using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>), and aligned with closely related members by using CLUSTAL

W software (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; <http://35.8.164.52/html/>; Cole *et al.*, 2003). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. The phylogenetic trees were constructed using three different algorithms – neighbour-joining, maximum-likelihood and maximum-parsimony – available within PHYLIP software, version 3.6 (Felsenstein, 2002). Distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package.

On MA medium with 12 % (w/v) NaCl, strain BH139^T formed smooth, creamy, circular colonies and the colony morphologies were consistent at various salinities in the range 1–20 % (w/v) NaCl. Strain BH139^T showed moderate halophily, growing in media containing 1–20 % (w/v) NaCl; optimum growth occurred on media with 12–14 % (w/v) NaCl. Growth was observed at temperatures between 15 and 45 °C; the optimum growth temperature was 30–35 °C. Strain BH139^T grew in the pH range 6.0–8.5 in 12 % (w/v) NaCl-containing trypticase soy broth; optimal growth was observed at pH 7.0–7.5. Cells of strain BH139^T from early and late growth phases revealed obvious Gram-positive reactions; however, it was reported that *L. salicampi* KCCM 41560^T showed a Gram-variable reaction (Yoon *et al.*, 2002). Cells of the isolate were slender and strictly rod-shaped,

Table 1. Cellular fatty acid compositions of strain BH139^T and *L. salicampi* KCCM 41560^T on MA and/or MA with 10 % (w/v) NaCl

Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	Strain BH139 ^T on:		<i>L. salicampi</i> KCCM 41560 ^T on MA
	MA	MA + 10 % NaCl	
Straight-chain fatty acids			
C _{14:0}	0.3	0.2	ND
C _{15:0}	ND	0.3	ND
C _{16:0}	2.8	1.0	1.4
Branched fatty acids			
iso-C _{13:0}	ND	0.2	ND
iso-C _{14:0}	4.1	13.9	12.0
iso-C _{15:0}	7.1	16.5	3.4
anteiso-C _{15:0}	39.4	25.3	38.6
iso-C _{16:0}	15.2	26.5	30.1
iso-C _{17:0}	3.6	4.4	0.7
anteiso-C _{17:0}	26.5	11.5	13.4
iso-C _{18:0}	0.6	0.2	ND
Unsaturated fatty acid			
C _{16:1} ω7 <i>c</i> alcohol	ND	ND	0.5

being 0.2–0.3 µm wide and 1.5–3.0 µm long after 2 days incubation at 30 °C on MA with 12 % (w/v) NaCl. The isolate produced a spherical terminal endospore within a swollen sporangium. Cell motility was facilitated by two flagella on the sides of the cells (see the Supplementary Figure in IJSEM Online). In contrast, Namwong *et al.* (2005) reported that *Lentibacillus juripiscarius* JCM 12147^T was non-motile and lacked flagella. Anaerobic growth was not observed after 5 days at 30 °C on MA with 12 % (w/v) NaCl under anaerobic conditions.

The major isoprenoid quinone of strain BH139^T was MK-7. The fatty acid profile of the strain was characterized by the presence of branched and saturated fatty acids such as iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and anteiso-C_{17:0} (the major fatty acids). Although this fatty acid profile was shared by *L. salicampi* KCCM 41560^T (currently the closest relative, on the basis of 16S rRNA gene sequence), the fatty acid compositions of the two micro-organisms were somewhat different (Table 1). Analysis of cell-wall

peptidoglycan showed that strain BH139^T did not contain any amino acids. Attempts to analyse the peptidoglycan of the isolate failed, even after several re-examinations and analysis by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). However, it was reported that the phylogenetically closest strain, *L. salicampi* KCCM 41560^T, possesses *meso*-diaminopimelic acid as the diagnostic diamino acid (Yoon *et al.*, 2002). The major polar lipids of strain BH139^T were diphosphatidylglycerol and phosphatidylglycerol, which were the same as those of *L. salicampi* KCCM 41560^T. The genomic DNA G+C content of strain BH139^T was about 43 mol%. In Table 2, typical phenotypes of strain BH139^T are summarized and compared with those of the type strains of closely related taxa.

The phylogenetic relatedness based on 16S rRNA gene sequences showed that strain BH139^T formed a distinct line from the species *L. salicampi* KCCM 41560^T with a bootstrap value of 9 % in the neighbour-joining analysis

Table 2. Characteristics of strain BH139^T and related species

Data are from Yoon *et al.* (2002), Arahal *et al.* (1999, 2000), Heyrman *et al.* (2003), Namwong *et al.* (2005) and this study. Strains: 1, BH139^T; 2, *L. salicampi* KCCM 41560^T; 3, *Virgibacillus marismortui* DSM 12325^T; 4, *L. juripiscarius* JCM 12147^T. Symbols: +, positive; –, negative; W, weak; NA, not available. All strains were catalase-positive rods.

Characteristic	1	2	3	4
Size (µm)	0.2–0.3 × 1.5–3.0	0.4–0.7 × 2.0–4.0	0.5–0.7 × 2.0–3.6	0.4–0.5 × 1.5–6.0
Sporangia				
Spore shape	Spherical/oval	Spherical/oval	Ellipsoidal	Oval
Spore position	Terminal	Terminal	Terminal/subterminal	Terminal
NaCl range (% w/v)	1–20	2–23	5–25	3–30
Oxidase	–	+	+	+
Nitrate reduction	+	NA	–	+
Hydrolysis of:				
Aesculin	+	–	+	NA
Casein	–	+	+	+
Tween 80	–	+	–	+
Urea	–	–	+	–
Acid production from:				
D-Glucose	+	–	+	+
D-Fructose	+	–	+	+
D-Mannitol	W	–	–	–
D-Ribose	+	–	NA	+
D-Xylose	+	–	–	+
Maltose	+	–	+	–
D-Trehalose	W	–	–	–
Major fatty acids on MA	anteiso-C _{15:0} , anteiso-C _{17:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{17:0}	NA	anteiso-C _{15:0} , iso-C _{16:0}
Cell wall type*	–	<i>meso</i> -DAP	<i>meso</i> -DAP	<i>meso</i> -DAP
Major quinone	MK-7	MK-7	NA	MK-7
Polar lipids†	PG, DPG	PG, DPG	NA	PG, DPG, GL
DNA G+C content (mol%)	43	44	39–42.8	43

* *meso*-DAP, *meso*-Diaminopimelic acid.

† DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PG, phosphatidylglycerol.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain BH139^T and other related taxa. Bootstrap values are given as percentages of 1000 replicates, if greater than 50%. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 change per nucleotide position.

(Fig. 1). The topologies of phylogenetic trees obtained using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed using neighbour-joining analysis (data not shown). The strain shared 16S rRNA gene sequence similarity values of 96.9 and 95.5 %, respectively, with the closely related strains *L. salicampi* KCCM 41560^T and *L. juripiscarius* JCM 12147^T; these levels of 16S rRNA gene sequence similarity are sufficient to justify defining a novel species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002).

On the basis of the phenotypic and molecular properties that have been reported to date, it is proposed that strain BH139^T represents a novel species of the genus *Lentibacillus*, for which the name *Lentibacillus salarius* sp. nov. is proposed.

Description of *Lentibacillus salarius* sp. nov.

Lentibacillus salarius (sa.la'ri.us. L. masc. adj. *salarius* of, or belonging to, salt, because of the isolation of this micro-organism from saline sediment).

Cells are approximately 0.2–0.3 µm wide and 1.5–3.0 µm long and are strictly aerobic motile rods with flagella. Colonies are cream-coloured, low convex, smooth and circular on MA supplemented with 10 % (w/v) NaCl. Oxidase-negative and catalase-positive. Growth occurs at 15–50 °C (optimum: 30–35 °C), pH 6.0–8.5 (optimum: pH 7.0–7.5) and 1–20 % (w/v) NaCl (optimum: 12–14 %). No growth occurs without NaCl or in the presence of more than 20 % (w/v) NaCl. Nitrate is reduced to nitrite. Aesculin is hydrolysed. Hydrolysis of urea, L-tyrosine, hypoxanthine, casein, starch, Tween 80 and xanthine is not observed. Acids are produced from D-xylose, D-ribose, glycerol, D-glucose, maltose, D-trehalose, L-arabinose, α-D-lactose, D-mannitol,

D-fructose and D-mannose, but not from rhamnose, adonitol, raffinose, arbutin, D-salicin or D-melibiose. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The major fatty acids are iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and anteiso-C_{17:0}. The DNA G + C content is about 43 mol% (HPLC).

The type strain is BH139^T (= KCTC 3911^T = DSM 16459^T), isolated from saline soil of Xinjiang Province, China.

Emended description of the genus *Lentibacillus* Yoon *et al.* 2002

Lentibacillus (Len.ti.ba.cil'lus. L. adj. *lentus* slow; L. dim. n. *bacillus* small rod; N.L. masc. n. *Lentibacillus* slowly growing bacillus).

Cells are rods. Gram-variable. Spherical or oval endospores lie terminally in swollen sporangia. Motile or non-motile. Colonies are cream-coloured, smooth and circular to slightly irregular. Catalase-positive and oxidase-variable. Urease-negative. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The major fatty acids are anteiso-C_{15:0} and iso-C_{16:0}. The G + C content of the DNA is in the range 42–44 mol%.

The type species is *Lentibacillus salicampi*.

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