Bacillus salarius sp. nov., a halophilic, spore-forming bacterium isolated from a salt lake in China

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A moderately halophilic bacterium, strain BH169^T, capable of growing at salinities of 3–20% (w/v) NaCl was isolated from a saline lake in China. Strain BH169^T was strictly aerobic, short-rod-shaped and non-motile (non-flagellated). Its major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}. The genomic DNA G+C content was about 43 mol% and the predominant quinone was MK-7. The cell-wall peptidoglycan was of the A1 γ type, containing meso-diaminopimelic acid as the diagnostic diamino acid. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a distinct phylogenetic line within the spore-forming rods of the genus *Bacillus*. The levels of 16S rRNA gene sequence similarity to the type strains of *Bacillus* species were below 93%. On the basis of phenotypic and molecular properties, strain BH169^T (=KCTC 3912^T = DSM 16461^T) represents the type strain of a novel species within the genus *Bacillus*, for which the name *Bacillus salarius* sp. nov. is proposed.

Aerobic, spore-forming, halophilic, Gram-positive rods are taxonomically very diverse and have been isolated from different saline habitats such as salterns, estuarine water, salt lakes, salty foods, sea ice and deep-sea hydrothermal vents (Agnew *et al.*, 1995; Arahall *et al.*, 1999; Nielsen *et al.*, 1994; Ventosa *et al.*, 1989; Yoon *et al.*, 2004). They are attracting interest because this group of bacteria has great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner, 2001). On the basis of molecular and chemical analyses, the halophilic Gram-positive bacteria that were originally assigned to the genus *Bacillus* have been reclassified as members of novel genera or transferred to other genera (Stackebrandt & Liesack, 1993; Spring *et al.*, 1996; Heyndrickx *et al.*, 1999; Wainø *et al.*, 1999; Schlesner *et al.*, 2001; Yoon *et al.*, 2001, 2002). In particular, from the results of 16S rRNA gene

sequence analysis, it was reported that the genus *Bacillus* contains six phylogenetically distinct groups and that many alkaliphilic/halophilic bacilli belong to rRNA *Bacillus* group 6 (Ash *et al.*, 1991; Nielsen *et al.*, 1994).

In the course of screening halophilic bacteria from the surface sediment of a saline lake in China, an aerobic, Gram-positive, obligately halophilic bacterium, designated strain BH169^T, was isolated and characterized. On the basis of its phylogenetic and phenotypic characteristics, strain BH169^T was assigned to a novel species of the genus *Bacillus*.

Strain BH169^T was isolated from soil sediment of a salt lake in Xinjiang Province in China after incubation on marine agar 2216 (MA; Difco) containing 15% (w/v) NaCl [final concentration 16.94% (w/v) NaCl] at 34 °C for 3 days. NaCl requirements/tolerance were determined in tryptic soy broth (17.0 g casein, 3.0 g soybean meal, 2.5 g glucose, 5.0 g sodium chloride and 2.5 g dipotassium phosphate l⁻¹) supplemented with modified artificial sea water [artificial sea water comprises 0–30% (w/v) NaCl, 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂ l⁻¹]. The strain was routinely grown aerobically on MA containing 10% (w/v) NaCl for 3 days at 30 °C, except where indicated otherwise. Anaerobic growth was determined by incubation in an anaerobic chamber at 30 °C for 5 days on MA

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A transmission electron micrograph showing the general morphology of stained cells of strain BH169^T is available as a supplementary figure in IJSEM Online.

containing 10 % (w/v) NaCl. Optimum growth was determined by testing different temperatures (4–55 °C) on MA containing 10 % (w/v) NaCl and by testing different pH values (pH 5.0–11.0) in tryptic soy broth supplemented with artificial sea water containing 10 % (w/v) NaCl. Cell morphology was studied using light microscopy and transmission electron microscopy. Motility was observed at 24 and 36 h in wet mounts under a light microscope (Nikon E600). For visualization of the flagella, cells were mounted on Formvar-coated copper grids (Electron Microscopy Science) and negatively stained with 2 % (w/v) uranyl acetate for 15 s and then subjected to transmission electron microscopy (JEM-1010; JEOL). Endospores were stained according to the Schaeffer–Fulton method (Smibert & Krieg, 1981).

Gram staining was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase activity was determined from the production of

oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by means of the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine, gelatin and xanthine was determined on MA according to the methods described previously (Cowan & Steel, 1965; Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction was determined according to the method of Lanyi (1987). Acid production from carbohydrates was tested as described by Leifson (1963).

GC analysis of fatty acid methyl esters was performed using cells grown on MA containing 10 % (w/v) NaCl for 3 days at 30 °C according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Preparation of cell walls from the test strain and analysis of peptidoglycan structures were carried out using the methods described by Schleifer (1985), with the modification that TLC on cellulose sheets was performed instead of paper chromatography.

Table 1. Characteristics of strain BH169^T and related species

Strains: 1, BH169^T; 2, *Bacillus smithii* DSM 4216^T; 3, *B. agaradhaerens* DSM 8721^T; 4, *B. clarkii* DSM 8720^T; 5, *B. vedderi* DSM 9768^T; 6, *Bacillus subtilis* NCDO 1769^T. Data are from Nakamura *et al.* (1988), Li *et al.* (2002), Agnew *et al.* (1995), Fujita *et al.* (1996), Heyrman *et al.* (2003) and this study. All strains are rods and grow at 40 °C. +, Positive; –, negative; W, weak; V, variable; ND, no data.

Characteristic	1	2	3	4	5	6
Cell size (µm)	0.5–0.7 × 1.3–1.9	0.8–1.0 × 5.0–6.0	0.5–0.6 × 2.0–5.0	0.6–0.7 × 2.0–5.0	ND	ND
Flagellation	–	Peritrichous	ND	ND	Peritrichous	+
Gram reaction	+	+	+	+	+	ND
Spore shape†	S	O	E	E	S*	O
Spore position‡	T	T/ST	ST	ST	T	C
Growth at:						
50 °C	–	+	–	–	+	–
60 °C	–	+	–	–	–	–
pH 7	+	–	–	–	–	ND
pH 9	+	–	+	+	–	ND
pH 10	–	–	+	+	+	ND
Growth in NaCl at:						
8 % (w/v)	+	–	+	+	+	+
15 % (w/v)	+	–	+	+	–	ND
20 % (w/v)	+	–	–*	–*	–	ND
Anaerobic growth	–	ND	–*	–*	+	–
Catalase activity	+	ND	+	+	+	+
Oxidase activity	+	+	–*	–*	+	+
Nitrate reduction	–	–	+	+	+	+
Hydrolysis of:						
Aesculin	+	V	+	+	+	ND
Casein	–	–	+	+	–	+
Starch	–	W	+	–	–	+
DNA G + C content (mol%)	43	38.7–39.7	39.3–39.5	42.4–43.0	38.3	43

*Data from this study (all data for strain BH169^T were also from this study).

†E, Ellipsoidal; O, oval; S, spherical.

‡C, Central; ST, subterminal; T, terminal.

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 BH169^T was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984).

The 16S rRNA gene was amplified by a PCR using primers Eubac 27F and 1492R (DeLong, 1992) and then sequenced. The resultant 16S rRNA gene sequence was aligned with those of representative members of selected genera by using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix, version 1.1 (Ribosomal Database Project II; <http://rdp.cme.msu.edu/>) (Cole *et al.*, 2003). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Phylogenetic trees were constructed using three different methods: neighbour-joining analysis (Saitou & Nei, 1987), the maximum-likelihood algorithm (Felsenstein, 1981) and the maximum-parsimony algorithm (Fitch, 1971) available in PHYLIP software, version 3.6 (Felsenstein, 2002). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. Bootstrap analyses (1000 replications) were performed to evaluate the stability of the phylogenetic tree produced with the neighbour-joining method in the PHYLIP package.

Strain BH169^T grew at salt concentrations in the range 3–20 % (w/v) NaCl; optimum growth occurred on media containing 10–12 % (w/v) NaCl. Colonies of the strain were cream in colour, smooth, low-convex and circular/slightly irregular on MA medium containing 10 % (w/v) NaCl.

Strain BH169^T grew at pH values in the range 6.8–9.5 in 10 % (w/v) NaCl-containing tryptic soy broth; optimal growth was observed at pH 8.0. The morphological and phenotypic characteristics suggest that the isolate is a halophilic member of the genus *Bacillus*. Growth was observed at temperatures between 15 and 40 °C; optimum growth was at 30 °C. Cells of the isolate were slender, short rods, 0.3–0.5 µm wide and 1.3–1.9 µm long (see Supplementary Fig. S1 available in IJSEM Online), and were strictly aerobic. Spherical terminal endospores were produced within swollen sporangia. Anaerobic growth was not observed under anaerobic conditions after 5 days incubation at 30 °C on MA with 10 % (w/v) NaCl.

Analysis of the cell-wall peptidoglycan showed that strain BH169^T possessed the A1_γ type, with *meso*-diaminopimelic acid as the diagnostic diamino acid, in common with the great majority of the members of the genus *Bacillus* (Priest *et al.*, 1988). The predominant isoprenoid quinone of strain BH169^T was MK-7 and the G+C content of the genomic DNA was about 43 mol%, a value that falls within the range defined for the genus *Bacillus* (Nielsen *et al.*, 1995; Priest *et al.*, 1988). The cellular fatty acid profile of the strain grown on MA containing 10 % (w/v) NaCl was characterized as containing saturated branched fatty acids such

as anteiso-C_{15:0} (53.13 %), anteiso-C_{17:0} (18.67 %), iso-C_{15:0} (8.95 %) and iso-C_{16:0} (6.6 %). The major fatty acids, the major lipoquinone and the DNA G+C content were typical of those of members of the genus *Bacillus* (Arahal *et al.*, 1999; Fritze, 1996; Garabito *et al.*, 1997; Nielsen *et al.*, 1995; Priest *et al.*, 1988). Phenotypic and physiological properties of strain BH169^T are summarized and compared with those of phylogenetically related type strains in Table 1.

Comparative analysis of 16S rRNA gene sequences showed that strain BH169^T was a member of the phyletic group classically defined as the genus *Bacillus*, and was associated with *Bacillus* group 6, which includes many alkaliphilic bacilli (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994; Schlesner *et al.*, 2001). Phylogenetic analysis using the neighbour-joining method showed that the isolate formed a distinct phylogenetic line, with a 66 % bootstrap value, from *Bacillus agaradhaerens* DSM 8721^T, *Bacillus vedderi* DSM 9768^T and *Bacillus clarkii* DSM 8720^T (Fig. 1). The topologies of phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed using neighbour-joining analysis (data not shown). Strain BH169^T shared very low 16S rRNA gene sequence similarities (less than 93 %) with the closely related type strains of *Bacillus* species; this is sufficient to permit classification of this strain as a different species (Stackebrandt *et al.*, 2002). Phenotypic properties of strain BH169^T, such as flagellation and spore

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain BH169^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates, when greater than 50 %. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

shape, also supported the view that the isolate was distinguishable from closely related *Bacillus* species (Table 1). On the basis of the above results, it is proposed that strain BH169^T should be placed in the genus *Bacillus* as the type strain of a novel species, for which the name *Bacillus salarius* sp. nov. is proposed.

Description of *Bacillus salarius* sp. nov.

Bacillus salarius (sa.la'ri.us. L. masc. adj. *salarius* of or belonging to salt).

Colonies are cream, smooth and circular. Cells are approximately 0.3–0.5 µm wide and 1.3–1.9 µm long. Strictly aerobic, short-rod-shaped and non-motile (non-flagellated). Stains Gram-positive and gives a negative result in the KOH test. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Growth occurs at 15–40 °C (optimum 30 °C), pH 6.8–9.5 (optimum pH 8.0) and 3–20% (w/v) NaCl (optimum 10–12%). Aesculin is hydrolysed. Hydrolysis of casein, starch, Tween 80, L-tyrosine, hypoxanthine, xanthine and urea is not observed. Acids are produced from D-glucose, maltose, D-trehalose, D-xylose, L-arabinose, glycerol, L-rhamnose, D-fructose, D-mannitol, D-salicin, D-mannose, D-ribose, α-D-lactose and D-melibiose, but not from adonitol, D-raffinose or arbutin. Cell wall contains meso-diaminopimelic acid (A1γ type). The major isoprenoid quinone is MK-7. The DNA G+C content is about 43 mol% (HPLC). The predominant cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}.

The type strain is BH169^T (= KCTC 3912^T = DSM 16461^T), which was isolated from a saline soil in China.

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