Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China

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A spore-forming, halophilic bacterium, designated strain BH163^T, was isolated from a salt lake in China. Cells were motile, strictly aerobic rods that contained type A1 γ peptidoglycan with meso-diaminopimelic acid as the diagnostic diamino acid. The isolate showed Gram- and catalase-positive reactions and formed a terminal endospore with a swollen sporangium. The major cellular fatty acids were anteiso-C₁₅:0, iso-C₁₅:0, anteiso-C₁₇:0 and iso-C₁₆:0. The genomic DNA G + C content of the strain was 41.0 mol%. Comparative analysis of 16S rRNA gene sequences showed that strain BH163^T formed a distinct line within the phyletic group classically defined as the genus *Bacillus* and was most closely related to the taxa [*Bacillus*] *haloalkaliphilus* DSM 5271^T and *Filobacillus milosensis* DSM 13259^T, with 16S rRNA gene sequence similarities of 95.9 and 94.5 %, respectively. On the basis of physiological and molecular properties, it is proposed that [*Bacillus*] *haloalkaliphilus* DSM 5271^T is reclassified in the new genus *Alkalibacillus* as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. Strain BH163^T (= KCTC 3916^T = DSM 16460^T) was assigned as the type strain of the novel species *Alkalibacillus salilacus*.

Moderately halophilic bacteria that grow optimally in media containing 3–15 % (w/v) NaCl are widely distributed in different saline habitats such as salterns, estuarine water, salt lakes, salty foods, sea ice and deep-sea hydrothermal vents. These micro-organisms are taxonomically very diverse and are spread over each of the three domains: *Archaea*, *Bacteria* and *Eucarya* (Oren, 2002). They are also metabolically diverse. Aerobic, spore-forming, moderately halophilic, Gram-positive rods are also taxonomically diverse and have been isolated from marine environments and related habitats. They were originally assigned to the genus *Bacillus*,

but they have been reclassified as new genera by the application of molecular methods and improved phenotypic approaches (Heyndrickx *et al.*, 1999; Spring *et al.*, 1996; Wainø *et al.*, 1999; Yoon *et al.*, 2001, 2004).

In the course of screening halophilic bacteria, an aerobic Gram-positive, obligately halophilic bacterium, designated strain BH163^T, was isolated from soil sediment of a salt lake. Comparative analysis of 16S rRNA gene sequences indicated that the closest relative of the strain was [*Bacillus*] *haloalkaliphilus* WN13^T (95.9 % 16S rRNA gene sequence similarity), which was classified as a novel species within the genus *Bacillus* (Fritze, 1996). However, we found that strain WN13 should be reclassified in a new genus on the basis of phylogenetic and phenotypic characteristics. Therefore, we propose the reclassification of [*Bacillus*] *haloalkaliphilus* in the new genus *Alkalibacillus* as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov., with WN13^T as the type strain. A novel species, *Alkalibacillus salilacus* sp. nov., type strain BH163^T, is also described.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH163^T is AY671976.

An electron micrograph of strain BH163^T and a table detailing cellular fatty acid compositions for strain BH163^T and related type strains are available as supplementary material in IJSEM Online.

Strain BH163^T was isolated from soil sediment of Ai-Ding Lake in Xin-jiang province in China. Ai-Ding Lake is a typical chloride-sulphate saline lake with a neutral pH and a 20–26 % (w/v) salt concentration. Surface soil sediment was sampled from a shallow area of the lakeside. To isolate halophilic bacteria, soil sediment was serially diluted, spread on marine agar 2216 (MA; Difco) supplemented with 15 % (w/v) NaCl [final concentration 16.94 % NaCl (w/v)] and incubated for 5 days at 28 °C. Requirement for and tolerance of NaCl were determined in tryptic soy broth (TSB) supplemented with modified artificial sea water [ASW; 0–30 % (w/v) NaCl, 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂ per litre]. 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 containing 10 % (w/v) NaCl. Optimum growth was tested at different temperatures (4–55 °C) on MA containing 10 % (w/v) NaCl and at different pH values (5.0–10.0) in TSB supplemented with ASW containing 10 % (w/v) NaCl.

Cell morphology was studied using light and transmission electron microscopy (TEM). Motility was observed at 24 and 48 h in agar-coated wet mounts using a light microscope (Nikon E600). Each agar-coated wet mount was prepared by placing 10 µl culture under a cover glass on a glass slide that had previously been coated with a film of 0.5 % (w/v) agarose (Cambrex) in distilled water and air-dried. For visualization of 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 TEM (JEM-1010; JEOL). Endospores were stained according to the method of Schaeffer-Fulton (Smibert & Krieg, 1981).

Catalase activity was determined by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by 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 previously described methods (Cowan & Steel, 1965; Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction was performed according to Lanyi (1987). Acid production from carbohydrates was tested as described by Leifson (1963); all suspension media were supplemented with ASW containing 10 % (w/v) NaCl.

GC analysis of fatty acid methyl esters was performed with cells grown on MA containing 5 or 10 % (w/v) NaCl for 3 days at 30 °C according to the manufacturer's instructions for the Microbial Identification System (MIDI; Microbial ID). Preparation of cell walls from the test strain and analyses of peptidoglycan structures were carried out using the methods described by Schleifer (1985) with the modification that TLC was performed on cellulose sheets instead of using paper chromatography. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using

an HPLC fitted with a reverse-phase column (GROM-SIL 100 ODS-2FE; GROM). The DNA G + C content of strain BH163^T was determined by reverse-phase HPLC according to Tamaoka & Komagata (1984).

The 16S rRNA gene was amplified by PCR using Eubac 27F and 1492R primers (DeLong, 1992) and sequenced. The resulting sequences were compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to determine the approximate phylogenetic affiliation and then aligned with closely related members by using CLUSTAL W software (Thompson *et al.*, 1994). Sequence similarity values were computed using the Similarity Matrix tool, 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 methods, neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971), using algorithms available in PHYLIP software, version 3.6 (Felsenstein, 2002). For the neighbour-joining method, evolutionary distance matrices were calculated according to the Kimura two-parameter model (Kimura, 1980). 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 software package.

Strain BH163^T showed moderate halophilic properties, growing in media containing 5–20 % (w/v) NaCl; optimum growth occurred in media with 10–12 % (w/v) NaCl. Colonies of the strain were cream, smooth, low-convex and circular/slightly irregular on MA containing 10 % (w/v) NaCl. Growth was observed at temperatures between 15 and 40 °C, with an optimum growth temperature of 30 °C. Cells of the isolate were slender, strictly aerobic and motile rods (width of 0.4–0.5 µm and length 1.6–3.0 µm) with peritrichous flagella (see Supplementary Fig. S1 in IJSEM Online).

Strain BH163^T grew well in the slightly alkaline conditions of pH 7.0–9.0 in TSB containing 10 % (w/v) NaCl; optimal growth was observed at pH 8.0. [*Bacillus*] *haloalkaliphilus* DSM 5271^T, the previously determined closest relative based on 16S rRNA gene sequence similarities, also grows at concentrations of up to 25 % NaCl and shows good growth at relatively high pH (pH 9.7) (Fritze, 1996). The isolate produced a spherical terminal endospore with a swollen sporangium, as found in the phylogenetically close species [*Bacillus*] *haloalkaliphilus* and *Filobacillus milosensis*. Growth was not observed after 5 days of incubation under anaerobic conditions at 30 °C on MA with 10 % (w/v) NaCl.

Strain BH163^T was catalase-positive, oxidase-negative and reduced nitrate to nitrite. Gram reaction and KOH tests of cells from early and late growth phases were positive and negative, respectively. However, it has been reported that [*Bacillus*] *haloalkaliphilus* and *F. milosensis*

show Gram-negative reactions despite having been described as Gram-positive bacteria by Fritze (1996) and Schlesner *et al.* (2001) and confirmed by our tests. The analysis of cell-wall peptidoglycan showed that strain BH163^T possessed A1 γ -type peptidoglycan with meso-diaminopimelic acid (*m*-DAP) as the diagnostic diamino acid, which was identical to that of [*Bacillus*] *haloalkaliphilus*. However, *F. milosensis* possesses the A4 β -type peptidoglycan, L-Orn-D-Glu, which clearly distinguishes strain BH163^T from *F. milosensis* (Fritze, 1996; Schlesner *et al.*, 2001).

The predominant isoprenoid quinone of strain BH163^T was menaquinone-7 (MK-7) and the G + C content of genomic DNA was about 41 mol%. The cellular fatty acid profile of strain BH163^T was characterized as containing saturated branched fatty acids such as anteiso-C_{15:0} (~39.7%), iso-C_{15:0} (~30.9%), anteiso-C_{17:0} (~11.8%) and iso-C_{16:0} (~8.9%) on MA containing 10% (w/v) NaCl, which was similar to that of [*B.*] *haloalkaliphilus* (Fritze, 1996). The fatty acid profiles on MA with 5 or 10% (w/v) NaCl were

similar (see Supplementary Table S1 in IJSEM Online). The cell-wall peptidoglycan, fatty acid profiles, major liponone and DNA G + C content of strain BH163^T were similar to those of [*Bacillus*] *haloalkaliphilus* and clearly distinguishable from those of *F. milosensis* within the group classically defined as the genus *Bacillus* (Fritze, 1996; Schlesner *et al.*, 2001). The typical phenotypic and chemotaxonomic properties of strain BH163^T are summarized and compared with those of phylogenetically related type relatives in Table 1.

Comparative analysis of 16S rRNA gene sequences showed that strain BH163^T was a member of the phyletic group classically defined as the genus *Bacillus* and was associated with 'Bacillus Group 1' (Ash *et al.*, 1991; Nielsen *et al.*, 1994; Schlesner *et al.*, 2001; Stackebrandt & Liesack, 1993). The phylogenetic analysis showed that the isolate formed a phyletic group with [*Bacillus*] *haloalkaliphilus* DSM 5271^T in the neighbour-joining analysis (Fig. 1). The topologies of the phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to that

Table 1. Characteristics of strain BH163^T and related species

Strains: 1, strain BH163^T; 2, *Alkalibacillus haloalkaliphilus* DSM 5271^T; 3, *Filobacillus milosensis* DSM 13259^T; 4, *Tenuibacillus multivorans* NBRC 100370^T; 5, *Bacillus halophilus* DSM 4771^T; 6, *Marinococcus albus* DSM 20748^T; 7, *Halobacillus halophilus* DSM 2266^T. Data from Fritze (1996); Schlesner *et al.* (2001); Ren & Zhou (2005); Hao *et al.* (1984); Ventosa *et al.* (1989) and Spring *et al.* (1996). s, Spherical; E, ellipsoidal; T, terminal; C, central; ST, subterminal; *m*-DAP, meso-diaminopimelic acid; +, positive; –, negative; w, weak; ND, no data.

Characteristics	1	2	3	4	5	6	7
Morphology	Rod	Rod	Rod	Rod	Rod	Coccoid	Coccoid
Size (µm)	0.4–0.5 × 1.6–3.0	0.3–0.5 × 3.0–8.0	0.3–0.5 × 3.0–7.0	0.3–0.5 × 2.0–6.0	0.5–1.0 × 2.5–9.0	1.0–1.2	ND
Pigmentation	Cream	Cream white	White	Translucent	None	None	Orange
Gram reaction	+	–	–	+	+	+	+
Spore shape	s	s	s	s	E	None	s
Spore position	T	T	T	T	C	None	C/T
NaCl range (% w/v)	5–20	0–25	2–23	1–20	3–30	5–20	0–25
Catalase	+	+	+	+	+	+	+
Oxidase	–	+	–	–	+	+	+
Nitrate reduction	+	–	–	–	–	+	–
Hydrolysis of:							
Aesculin	+	ND	–	+	+	–	–
Casein	–	+	–	+	–	–	+
Gelatin	–	+	–	+	–	–	+
Starch	–	w	–	–	–	–	+
Urea	–	–	ND	ND	+	+	–
Acid production from:							
D-Glucose	–	–	–	–	–	–	–
D-Fructose	w	ND	–	–	ND	–	–
D-Mannitol	–	ND	–	–	–	–	–
D-Xylose	–	–	–	–	+	–	–
Maltose	–	ND	–	–	–	–	–
D-Trehalose	–	–	–	–	+	–	–
Cell-wall type	<i>m</i> -DAP	<i>m</i> -DAP	Orn-D-Glu	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	Orn-D-Asp
G + C content (mol%)	41	37–38	35	36.5–37	51.5	44.9	40–41

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequences of strain BH163^T and other related taxa. Bootstrap values are shown in percentages of 1000 replicates, when more than 50%. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

of the tree constructed by the neighbour-joining analysis (data not shown). The closest relative of strain BH163^T was [*Bacillus*] *haloalkaliphilus* DSM 5271^T with 95.9% 16S rRNA gene sequence similarity, a level at which they can be considered to be in the same genus. However, strain BH163^T shared low 16S rRNA gene sequence similarity of 94.5 and 94.2% with two other close relatives, *F. milosensis* DSM 13259^T and *Tenuibacillus multivorans* NBRC 100370^T, respectively.

The chemotaxonomic and molecular characteristics described here show that strain BH163^T and [*Bacillus*] *haloalkaliphilus* should be described as members of the same genus and that they are distinguishable from other closely related genera such as *Filobacillus* and *Tenuibacillus* within the group classically defined as the genus *Bacillus*. Therefore, we propose the reclassification of [*Bacillus*] *haloalkaliphilus* DSM 5271^T to the genus *Alkalibacillus* as *Alkalibacillus haloalkaliphilus* DSM 5271^T gen. nov., comb. nov. In addition, strain BH163^T represents a novel species in the genus *Alkalibacillus*, for which the name *Alkalibacillus salilacus* sp. nov. is proposed.

Description of *Alkalibacillus* gen. nov.

Alkalibacillus (Al.ka.li.ba.cil'lus. N.L. n. *alkali* alkali; L. n. *bacillus* rod; N.L. masc. n. *Alkalibacillus* bacillus living under alkaline conditions).

Cells are Gram-variable, spore-forming rods. Catalase-positive. Urease-negative. Spherical endospores are formed terminally in swollen sporangia. Strictly aerobic and

moderately halophilic. Cells are motile by means of peritrichous flagella. Cell-wall peptidoglycan is of the A1 γ type with *meso*-DAP as the diagnostic diamino acid. Major isoprenoid quinone is MK-7. DNA G+C content is 38–41 mol% (HPLC). Predominant cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. Phylogenetically, the genus belongs to the family *Bacillaceae*. The type species of the genus is *Alkalibacillus haloalkaliphilus* (= DSM 5271^T).

Description of *Alkalibacillus haloalkaliphilus* (Fritze 1996) comb. nov.

Alkalibacillus haloalkaliphilus (hal.o.al.ka.li'phi.lus. Gr. n. *hals* salt; N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. masc. adj. *haloalkaliphilus* loving briny and alkaline media).

Basonym: *Bacillus haloalkaliphilus* Fritze 1996.

Gram reaction is negative. Catalase- and oxidase-positive. Nitrate not reduced to nitrite. Cells are 0.3–0.5 μ m wide and 3–8 μ m long. On alkaline nutrient agar supplemented with 5–10% (w/v) NaCl, colonies are cream white. In the presence of 20% (w/v) NaCl, colonies are slightly yellowish. The major cellular fatty acids are the saturated branched chain fatty acids iso-C_{15:0} (48%), anteiso-C_{15:0} (11%), anteiso-C_{17:0} (11%) and iso-C_{17:0} (6%). A small amount (19%) of unsaturated fatty acids is also present. Chemo-organotrophic. Does not grow (or grows only very poorly) in nutrient broth or on nutrient agar without NaCl. Mesophilic. Obligately alkaliphilic. No growth occurs at pH 7 and good growth occurs at pH 9.7. Hydrolysis of starch is weak and no, or only weak, hydrolysis of casein occurs. Gelatin

and hippurate are hydrolysed. Pullulan, Tween 20, Tween 80 and 4-methylumbelliferone glucuronide are not hydrolysed. Egg yolk lecithinase and urease tests are negative. The DNA G + C content is 38.0 mol% (HPLC).

The type strain, WN13^T (= DSM 5271^T = ATCC 700606^T = CIP 106702^T = JCM 12303^T = LMG 17943^T), was isolated from alkaline, highly saline mud from Wadi Natrun, Egypt.

Description of *Alkalibacillus salilacus* sp. nov.

Alkalibacillus salilacus (sa.li.lac'us. L. n. *sal* salt; L. n. *lacus* lake; N.L. gen. masc. n. *salilacus* of a salt lake).

Colonies are cream, smooth, low-convex and circular/slightly irregular. Cells are approximately 0.4–0.5 µm wide and 1.6–3.0 µm long. Strictly aerobic, spore-forming, motile rod. Gram-positive. KOH test negative. Catalase-positive and oxidase-negative. Nitrate is reduced to nitrite. Growth occurs at 15–40 °C (optimum 30 °C), pH 7.0–9.0 (optimum pH 8.0) and 5–20% (w/v) NaCl (optimum 10–12%). Aesculin is hydrolysed. Hydrolysis of casein, starch, Tween 80, L-tyrosine, hypoxanthine, xanthine, gelatin and urea is not observed. Acids are produced from L-arabinose, D-ribose, α-D-lactose and D-fructose, but not from D-glucose, maltose, glycerol, D-trehalose, D-xylose, L-rhamnose, adonitol, D-raffinose, D-mannitol, arbutin, D-salicin, D-melibiose or D-mannose. DNA G + C content is 41.0 mol% (HPLC). Predominant cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}.

The type strain, BH163^T (= KCTC 3916^T = DSM 16460^T), was isolated from a salt lake in the Xin-jiang province of China.

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