Aquisalibacillus elongatus gen. nov., sp. nov., a moderately halophilic bacterium of the family *Bacillaceae* isolated from a saline lake

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A novel moderately halophilic, non-motile, rod-shaped bacterium was isolated from a saline lake, Lake Shangmatala, in the Inner Mongolia Autonomous Region, China. This bacterium, designated SH4s^T, was strictly aerobic, catalase-positive and oxidase-negative. It grew at salinities of 3–20 % (w/v) NaCl, with an optimum at 10 % (w/v) NaCl. The cell-wall peptidoglycan was of the A4 β type, based on L-Orn–D-Asp, and the major quinone was a menaquinone with seven isoprene units (MK-7). The major fatty acids were iso-C_{16:0} and iso-C_{15:0}. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, a glycolipid and four different unidentified phospholipids. The DNA G + C content was 45.9 mol%. In a maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences, strain SH4s^T was found to belong to the family *Bacillaceae* and to be most closely related to members of the genera *Filobacillus* (95.9 % sequence similarity), *Piscibacillus* (95.7 %) and *Tenuibacillus* (95.4 %). DNA–DNA hybridization experiments revealed 10 % relatedness (12 %, reciprocally) between strain SH4s^T and *Filobacillus milosensis* DSM 13259^T, the sole species of the genus. All of these data show that strain SH4s^T represents a novel genus and species in the family *Bacillaceae*, for which the name *Aquisalibacillus elongatus* gen. nov., sp. nov. is proposed. The type strain of *Aquisalibacillus elongatus* is SH4s^T (=CCM 7366^T =CECT 7149^T =DSM 18090^T).

Moderately halophilic bacteria that grow optimally in media containing 3–15 % NaCl are widely distributed in different saline environments such as salt lakes, salterns and salty foods (Ventosa *et al.*, 1998). Moderately halophilic, aerobic, Gram-positive rods are taxonomically diverse and are represented in many genera within the family *Bacillaceae*. Three of these genera, namely *Filobacillus* (Schlesner *et al.*, 2001), *Tenuibacillus* (Ren & Zhou, 2005) and *Piscibacillus* (Tanasupawat *et al.*, 2007), form a

coherent phylogenetic cluster within rRNA group I of *Bacillus* (Ash *et al.*, 1991). To date, only a single species has been recognized for each of these genera, *Filobacillus milosensis*, *Tenuibacillus multivorans* and *Piscibacillus salipiscarius*. During an investigation of the presence of halophilic bacteria in saline lakes in China, we isolated a moderately halophilic organism, designated strain SH4s^T, that shared many physiological and biochemical characteristics with species of the genera mentioned above, but was distinctive in terms of its peptidoglycan composition and DNA G + C content. Furthermore, 16S rRNA gene sequence analysis showed that this organism represents a novel subline within the family *Bacillaceae*. In this study, morphological, cultural, physiological, biochemical, chemotaxonomic and phylogenetic characteristics of strain SH4s^T were determined.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain SH4s^T is AM911047.

Neighbour-joining and maximum-likelihood 16S rRNA gene sequence-based phylogenetic trees and a two-dimensional thin-layer chromatogram of the polar lipids of SH4s^T are available as supplementary material with the online version of this paper.

Strain SH4s^T was isolated in September 2003 from a water sample collected during an expedition to Lake Shangmatale (located at 43° 22' N 114° 01' E), a saline lake in Inner Mongolia, China. At the time of sampling, the temperature of the water was 21 °C, the pH was 8.5 and the salinity was 16.7 % (w/v). The isolation medium (HM medium) and the method used for isolation have been described previously (Ventosa *et al.*, 1982). The strain was routinely cultured on the maintenance medium described by Ventosa *et al.* (1982). *F. milosensis* DSM 13259^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and was cultivated according to its recommended procedures.

Genomic DNA from strain SH4s^T was prepared using the method described by Marmur (1961). The 16S rRNA gene was amplified by using a PCR with the forward primer 16F27 and the reverse primer 16R1488. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). 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 closely related bacteria. The alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule by using the alignment tool of the ARB software package. The phylogenetic trees were constructed using three different methods: maximum likelihood (Felsenstein, 1981), maximum parsimony (Fitch, 1971) and neighbour joining (Saitou & Nei, 1987), algorithms that are integrated into the ARB software for phylogenetic inference. The 16S rRNA gene sequences used for the phylogenetic comparisons were obtained from GenBank.

An almost-complete 16S rRNA gene sequence of strain SH4s^T (1484 nt) was obtained and then used for the phylogenetic analyses. Comparative analysis of the 16S rRNA gene sequence with those of representative strains from the family *Bacillaceae* and from related species,

performed using the maximum-parsimony algorithm, revealed that strain SH4s^T was part of a cluster that included the genera *Filobacillus*, *Piscibacillus* and *Tenuibacillus*. This cluster was also closely related to a cluster that included *Alkalibacillus* species (Fig. 1). Strain SH4s^T exhibited the highest sequence similarity with respect to *F. milosensis* SH 714^T (95.9%), *P. salipiscarius* RBU1-1^T (95.7%) and *T. multivorans* 28-1^T (95.4%). The values obtained for sequence similarity between strain SH4s^T and the type strains of *Alkalibacillus* species were in the range 93.5–94.9%. No other known bacteria shared more than 93% gene sequence similarity with strain SH4s^T. Similar topologies were obtained when other treeing methods (neighbour joining and maximum likelihood) were used (see Supplementary Figs S1 and S2, available in IJSEM Online). DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness of strain SH4s^T and *F. milosensis* DSM 13259^T by using the competition procedure of Johnson (1994), described in detail elsewhere (Márquez *et al.*, 2007). The reciprocal values for DNA–DNA hybridization between SH4s^T and *F. milosensis* DSM 13259^T were 10 and 12%, respectively.

To characterize strain SH4s^T phenotypically, standard phenotypic tests were performed. Vegetative cells of strain SH4s^T were Gram-stained using the method of Dussault (1955) and the Gram type was also determined using the KOH test (Powers, 1995). Cell morphology and motility were examined by using phase-contrast microscopy (BX41 microscope; Olympus) on an exponentially growing liquid culture. The morphology, size and pigmentation of colonies were observed under optimal growth conditions on agar medium after 2 days incubation at 37 °C. Growth at different concentrations of salts was determined on the maintenance medium, to which different salt concentrations had been added (0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30%, w/v). The pH range for growth was determined on the maintenance medium at pH 4.0–11.0, using the appropriate biological buffers [Na₂HPO₄/NaH₂PO₄ (below pH 8.0), Na₂CO₃/NaHCO₃ (pH 8.0–10.0) and Na₂HPO₄/NaOH (pH 11.0)], as described previously (Gomori, 1955). The pH was readjusted after sterilization and then growth was scored as the optical density at 600 nm. Growth at different temperatures (6, 10, 15, 20, 25, 30, 37, 40, 45, 50,

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between SH4s^T and related species. GenBank accession numbers are shown in parentheses. *Bacillus licheniformis* DSM 13^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Characteristics that serve to distinguish strain SH4s^T from its phylogenetic relatives

Taxa: 1, strain SH4s^T (data from this study); 2, *F. milosensis* (Schlesner *et al.*, 2001; Tanasupawat *et al.*, 2007); 3, *P. salipiscarius* (Tanasupawat *et al.*, 2007); 4, *T. multivorans* (Ren & Zhou, 2005). +, Positive; –, negative; ND, no data available; Orn, Ornithine; *meso*-DAP, *meso*-diaminopimelic acid.

Characteristic	1	2	3	4
Gram reaction	–	–	+	+
Spore formation	Not observed	+	+	+
Motility	–	+	+	+
NaCl concentration for growth (% w/v)				
Range	3–20	2–23	2–30	1–20
Optimum	10	8–14	10–20	5–8
Temperature range (°C)	6–55	ND–42	15–48	21–42
Oxidase activity	–	–	+	+
Nitrate reduction	+	–	–	–
Hydrolysis of:				
Aesculin	–	–	ND	+
Casein	–	–	+	+
Gelatin	–	–	+	+
Starch	–	–	+	–
DNA G + C content (mol%)	45.9	35.0	36.7	36.5–37.0
Peptidoglycan type/variant	A4β/Orn–D–Asp	A4β/Orn–D–Glu	A1γ/ <i>meso</i> -DAP	A1γ/ <i>meso</i> -DAP
Distinctive major fatty acids	iso-C _{16:0} (26 %), iso-C _{15:0} (24 %)	anteiso-C _{15:0} (41.5 %), iso-C _{15:0} (23.2 %)	iso-C _{15:0} (35.3 %), anteiso-C _{15:0} (30.8 %)	iso-C _{15:0} (64.7 %), anteiso-C _{15:0} (12.7 %)

55 and 60 °C) was determined on the maintenance medium. Anaerobic growth was determined on the maintenance medium, using an anaerobe jar and H₂ and CO₂ GasPaks (BBL) for generating anaerobic conditions. Catalase activity was determined by adding 3 % (v/v) H₂O₂ to culture plates. The oxidase reaction was performed on filter paper moistened with a 1 % (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Sporulation was tested on solid maintenance medium and on this medium supplemented with 5 mg MnSO₄ l^{–1} (Merck). Plates were inoculated with 0.3 ml aliquots of an overnight liquid culture and then incubated at 37 °C for up to 1 month to determine whether endospores were present (Claus & Berkeley, 1986; Tiago *et al.*, 2006). The heat resistance of the cells was determined using cultures on the maintenance medium. Aliquots (5 ml) of the cultures were collected at (approximately) the exponential (4 h), late-exponential (10 h), stationary (20 h) and late-stationary (48 h, 72 h and 10 days) phases and were heated at 80 °C for 10 min. Aliquots (0.3 ml) of the heated cultures were inoculated onto solid maintenance medium and incubated for 48 h at 37 °C. In addition, the viability of the cells at each growth stage was checked by subculturing them on the same medium before heating (Tiago *et al.*, 2006). The utilization of various substrates as sole carbon and energy sources was determined as described previously (Ventosa *et al.*, 1982). Carbohydrates were added at 0.2 % (w/v), organic acids at 0.1 % (w/v) and alcohols at 0.1 % (w/v). Other tests shown in Table 1 or included in the genus or species descriptions were carried out using methods

described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987). Unless indicated otherwise, the tests were carried out on the maintenance medium and incubated at 37 °C in sealed containers.

Strain SH4s^T was moderately halophilic, growing in the maintenance medium supplemented with 3–20 % (w/v) NaCl and optimally in media containing 10 % (w/v) NaCl. No growth was observed in the absence of NaCl. Cells of strain SH4s^T were non-motile, whereas those of *F. milosensis*, *P. salipiscarius* and *T. multivorans* have been described as motile. Strain SH4s^T was oxidase-negative, like *F. milosensis* but in contrast to *P. salipiscarius* and *T. multivorans*. The Gram reaction of very young cultures (9 h) and older ones (up to 7 days) was negative. However, the KOH reaction was indicative of a Gram-positive cell-wall type (Powers, 1995). Similar observations have been reported (and confirmed by our tests) for the closest phylogenetic relative of the isolate, *F. milosensis* (Schlesner *et al.*, 2001), and for *A. haloalkaliphilus* (Fritze, 1996). Despite all of our attempts to observe endospores in cells of strain SH4s^T, they were not detected; furthermore, the cells did not exhibit heat resistance when subjected to heating at 80 °C for 10 min, which might indicate that endospores were not formed under the conditions tested. The inability to produce endospores has been described for some *Bacillus* species, such as *Bacillus foraminis* (Tiago *et al.*, 2006) and *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995). In the case of *A. haloalkaliphilus*, sporulation was enhanced at NaCl concentrations close to 5 % and was delayed at higher concentrations (Fritze, 1996). Results for

other phenotypic features are included in the genus and species descriptions and in Table 1.

The G+C content of the genomic DNA was determined from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The DNA G+C content of strain SH4s^T was 45.9 mol%, which is much higher than values described for *F. milosensis* (35.0 mol%) (Schlesner *et al.*, 2001), *P. salipiscarius* (36.7 mol%) (Tanasupawat *et al.*, 2007) and *T. multivorans* (36.5–37.0 mol%) (Ren & Zhou, 2005).

Analysis of the cell-wall peptidoglycan, quinones and polar lipids of strain SH4s^T was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cell biomass for these analyses was obtained from cultivation on the maintenance medium at 37 °C. Strain SH4s^T had peptidoglycan type A4 β : L-Orn was the diamino acid at position 3 of the peptidoglycan and the dicarboxylic amino acid present in the cross-linkage was D-Asp. The peptidoglycan composition of the novel strain is clearly different from that described for members of phylogenetically related genera. *F. milosensis* also has peptidoglycan type A4 β , but with D-Glu instead D-Asp in the cross-linkage (Schlesner *et al.*, 2001); *P. salipiscarius* and *T. multivorans* have a direct cross-linkage between position 3 and 4 and *meso*-diaminopimelic acid as the diamino acid. The isoprenoid quinones detected in strain SH4s^T were MK-7 (91 %) and MK-8 (9 %). With respect to the polar lipid composition, strain SH4s^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine (traces), a glycolipid and four different phospholipids of unknown structure (Supplementary Fig. S3).

Fatty acid analysis was performed using the MIDI system (Microbial Identification System). Cells were cultured on the maintenance medium at 37 °C for 24 h. This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG Bacteria Collection (Ghent University, Belgium). The major fatty acids of strain SH4s^T were iso-C_{16:0} (26.1 %) and iso-C_{15:0} (24.2 %); the complete profile of the cellular fatty acids is given in detail in the species description. This cellular fatty acid profile is clearly different from that described for phylogenetically related genera. In *F. milosensis*, anteiso-C_{15:0} was most abundant, whereas, in *P. salipiscarius* and *T. multivorans*, iso-C_{15:0} was the predominant fatty acid (Schlesner *et al.*, 2001; Tanasupawat *et al.*, 2007; Ren & Zhou, 2005) (Table 1).

The data obtained show that strain SH4s^T belongs to the family *Bacillaceae*, forming part of a cluster comprising the monospecific genera *Filobacillus*, *Piscibacillus* and *Tenuibacillus*. Strain SH4s^T shares some phenotypic features with these three genera. However, in contrast to *P. salipiscarius* and *T. multivorans*, strain SH4s^T is negative for Gram-staining and oxidase activity. Furthermore, the isolate clearly differs from members of the three genera in

being non-motile, in being able to reduce nitrate to nitrite and in terms of its DNA G+C content, fatty acid profile and cell-wall peptidoglycan composition. Additional differences between strain SH4s^T and its phylogenetically close relatives are presented in Table 1. The values for 16S rRNA gene sequence similarity between strain SH4s^T and the type strains of the three aforementioned genera ranged from 95.4 to 95.9 %. These values are lower than those found between the genera *Tenuibacillus* and *Piscibacillus* and the most closely related genus, *Filobacillus* (97 and 96.9 % 16S rRNA gene sequence similarity, respectively). Therefore, on the basis of the data presented, it is proposed that strain SH4s^T represents a novel genus and species, for which the name *Aquisalibacillus elongatus* gen. nov., sp. nov. is proposed.

Description of *Aquisalibacillus* gen. nov.

Aquisalibacillus (A.qui.sa.li.ba.cil'lus. L. n. *aqua* water; L. n. *sal*, *salis* salt; L. n. *bacillus* rod; N.L. masc. n. *Aquisalibacillus* a rod living in salt water).

Cells are Gram-negative, non-motile rods. Endospores are not observed. Moderately halophilic and strictly aerobic. Catalase-positive and oxidase-negative. Nitrate and nitrite are reduced. The KOH test is negative. The cell wall contains peptidoglycan of the A4 β type, with L-Orn as the diamino acid and D-Asp as the dicarboxylic amino acid present in the cross-linkage. The major cellular fatty acids are iso-C_{16:0} and iso-C_{15:0}. The predominant menaquinone is MK-7. The genus belongs to the family *Bacillaceae* and is related phylogenetically to the genera *Filobacillus* and *Piscibacillus*. The type species is *Aquisalibacillus elongatus*.

Description of *Aquisalibacillus elongatus* sp. nov.

Aquisalibacillus elongatus (e.lon.ga'tus. L. masc. part. adj. *elongatus* elongated, stretched out).

Exhibits the following characteristics in addition to those described for the genus. Cells are 0.5 μ m wide and 2.0–10.0 μ m long. Colonies are circular and entire, 1 mm in diameter and cream-pigmented on the maintenance medium after 2 days cultivation. Grows with a wide range (3–20 %, w/v) of NaCl concentrations, with optimal growth at 10 % (w/v) NaCl. No growth occurs in the absence of NaCl. Grows at 20–55 °C (optimally at 37 °C) and at pH 7.0–10.0 (optimally at pH 7.5). Aesculin, casein, gelatin, Tween 80 and starch are not hydrolysed. Indole, Voges–Proskauer, phenylalanine deaminase, H₂S production and phosphatase tests are negative. Acids are not produced from arabinose, fructose, D-glucose, glycerol, lactose, maltose, D-mannitol, sucrose or trehalose. The following compounds are utilized as sole carbon and energy sources: acetate, butanol, ethanol, L-fucose, fumarate, methanol, raffinose, D-sorbitol and valerate. The following compounds are not utilized as sole carbon and energy sources: D-arabinose, benzoate, cellobiose, citrate,

formate, galactose, maltose, D-mannose, melezitose, melibiose, propanol, propionate, succinate, trehalose and D-xylose. The following compounds are utilized as sole carbon, nitrogen and energy sources: L-alanine, DL-arginine, cysteine, L-ornithine and L-serine. The following compounds are not utilized as sole carbon, nitrogen and energy sources: aspartic acid, glutamic acid, DL-lysine, methionine, phenylalanine, L-threonine and L-tryptophan. Menaquinones present are MK-7 (91 %) and MK-8 (9 %). The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, a glycolipid and four different unidentified phospholipids. The fatty acids comprise iso-C_{16:0} (26.1 %), iso-C_{15:0} (24.2 %), iso-C_{16:1}ω7c (14.0 %), anteiso-C_{15:0} (10.0 %), iso-C_{14:0} (8.0 %), iso-C_{17:0} (6.1 %), anteiso-C_{17:0} (4.0 %), iso-C_{17:1}ω10c (2.3 %), C_{16:1}ω11c (1.6 %), C_{16:0} (1.1 %), iso-C_{18:0} (0.9 %), C_{17:0} (0.6 %), C_{18:0} (0.5 %) and C_{14:0} (0.2 %). The DNA G + C content is 45.9 mol% (*T_m*).

The type strain, SH4s^T (=CCM 7366^T =CECT 7149^T =DSM 18090^T), was isolated from water from a saline lake (Lake Shangmatale) in Inner Mongolia, China.

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