Streptohalobacillus salinus gen. nov., sp. nov., a moderately halophilic, Gram-positive, facultative anaerobe isolated from subsurface saline soil

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A Gram-stain-positive, rod-shaped, non-sporulating, motile and moderately halophilic bacterium, designated strain H96B60^T, was isolated from a saline soil sample of the Qaidam basin, China. The strain was facultatively anaerobic. Major end products formed from glucose fermentation were acetate, ethanol and lactic acid. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The isoprenoid quinone component was menaquinone-6 (MK-6). The predominant cellular fatty acids were C_{16:0}, anteiso-C_{13:0} and anteiso-C_{15:0}. The genomic DNA G + C content of strain H96B60^T was 36.2 mol%. Phylogenetic analysis based on comparative 16S rRNA gene sequences indicated that strain H96B60^T represented a novel phyletic lineage within the family *Bacillaceae* and was related most closely to *Halolactibacillus* species (96.1–96.4 % similarity). Based on the phenotypic, chemotaxonomic and phylogenetic data presented, strain H96B60^T is considered to represent a novel species of a new genus, for which the name *Streptohalobacillus salinus* gen. nov., sp. nov. is proposed. The type strain of *Streptohalobacillus salinus* is H96B60^T (=DSM 22440^T =CGMCC 1.7733^T).

Isolation and polyphasic taxonomic studies have shown that Gram-positive, spore-forming, moderately halophilic or halotolerant, aerobic or facultatively anaerobic rods are highly heterogeneous and comprise a number of phylogenetically distinct lineages within rRNA 1 group of Ash *et al.* (1991) in the phyletic assemblage of bacteria classically defined as representing the genus *Bacillus* (Ash *et al.*, 1991; Priest, 1981; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994). They have been isolated mostly from saline or hypersaline environments such as salt lakes, salterns, seawater, saline soils, soda lakes and salted foods (Kushner, 1985; Ventosa *et al.*, 1998), and some of the species have been subjected to polyphasic taxonomic study and reclassified within several new genera such as *Halobacillus* (Spring *et al.*, 1996), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* (Wainø *et al.*, 1999), *Filobacillus* (Schlesner *et al.*, 2001), *Oceanobacillus* (Lu *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002), *Paraliobacillus* (Ishikawa *et al.*, 2002), *Halolactibacillus* (Ishikawa *et al.*, 2005), *Tenuibacillus* (Ren & Zhou, 2005), *Thalassobacillus* (García *et al.*, 2005), *Sediminibacillus* (Carrasco *et al.*, 2008) and *Natronobacillus* (Sorokin *et al.*, 2008).

During the course of a study of microbial diversity of subsurface saline soil at a scientific drilling site (37° 03' 50" N 94° 43' 41" E) in the Qaidam basin of China, a great variety of moderately halophilic strains were isolated. Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that most of the moderately halophilic isolates were related to the genera *Bacillus*, *Salinicoccus*, *Marinobacter*, *Halomonas*, *Idiomarina*, *Halolactibacillus*, *Halobacillus*, *Thalassobacillus* and *Virgibacillus*. Here, we describe the phenotypic and genotypic characteristics of one of these moderately halophilic strains, designated H96B60^T, which appeared to represent a novel species of a new genus.

Strain H96B60^T was isolated by the standard dilution-plating technique on SG agar. The SG medium (pH adjusted to 8.0 with 1 M NaOH before autoclaving at 121 °C for 20 min) contained the following (per litre): 7.5 g Casamino acids (Difco), 10.0 g yeast extract (Difco), 1.0 g sodium glutamate, 3.0 g trisodium citrate, 20.0 g MgSO₄ · 7H₂O, 2.0 g KCl, 80.0 g NaCl and trace amounts of FeSO₄ · 7H₂O and MnCl₂ · 4H₂O (Sehgal & Gibbons, 1960). Strain H96B60^T was cultured routinely on modified GM medium (pH 7.5) containing the following (per litre): 5.0 g Casamino acids (Difco), 5.0 g yeast extract (Difco), 70.0 g NaCl, 5.0 g MgSO₄ · 7H₂O, 2.0 g KCl and trace amounts of FeSO₄ · 7H₂O and MnCl₂ · 4H₂O (Wang *et al.*, 2009). Strain H96B60^T was maintained in this modified

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A supplementary figure and two supplementary tables are available with the online version of this paper.

medium supplemented with 30 % (v/v) glycerol at -80°C for long-term preservation.

The phenotypic characteristics of the novel strain were determined by using methods described previously (Wang *et al.*, 2009), including Gram-type (staining and non-staining methods), morphology, growth properties, susceptibility to antimicrobial agents, catalase and oxidase activities, methyl red reaction, H_2S production, indole production, Voges–Proskauer reaction, urease production, nitrate reduction and API 50 CH tests. For analysis of anaerobic fermentation, strain H96B60^T was cultivated in modified GYPF medium under a gaseous atmosphere of 100 % N_2 in anaerobic tubes. The GYPF medium (pH adjusted to 8.0 with 1 M NaOH before autoclaving at 121°C for 20 min) contained the following (per litre): 10.0 g glucose, 5.0 g yeast extract (Difco), 5.0 g polypeptone, 1.0 g K_2HPO_4 , 70.0 g NaCl, 5.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2.0 g KCl. Fermentation products from glucose were analysed by HPLC as described by Ishikawa *et al.* (2003). A Durham tube was placed in an anaerobic tube to detect gas production from glucose under anaerobic fermentation conditions. The production of extracellular hydrolases was detected on GM agar plates containing different enzyme substrates. The following enzyme activities were assayed as described previously: amylase and pullulanase (Ryan *et al.*, 2006), proteolysis and lipolysis (Sánchez-Porro *et al.*, 2003), β -glucosidase (Saqib & Whitney, 2006; Kwon *et al.*, 1994), DNase (Jeffries *et al.*, 1957), phosphatase (Quesada *et al.*, 1984) and gelatinase (Ventosa *et al.*, 1982).

Preparation of the cell wall and determination of peptidoglycan structure were performed by using the methods described by Schleifer & Kandler (1972) with the modification that TLC on cellulose sheets was used instead of paper chromatography. The amino acid composition of the cell-wall hydrolysate was determined by using one-dimensional descending film chromatography on cellulose sheets. Respiratory quinones were extracted according to the method of Collins *et al.* (1977) and were analysed by reversed-phase HPLC (Groth *et al.*, 1996). Fatty acids were extracted, methylated and analysed by GC by using the standard Sherlock MIDI system (Microbial Identification) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Cell mass for the above procedures was obtained by cultivating strain H96B60^T at 37°C on GM agar. The genomic DNA G+C content of the strain was determined by the thermal denaturation method according to Marmur & Doty (1962).

Genomic DNA of strain H96B60^T was extracted by using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR by using the universal primers described by Duckworth *et al.* (1996). The almost-complete nucleotide sequence was determined by direct sequencing and was compared with available 16S rRNA gene sequences in the GenBank database by using the BLAST program. Multiple alignments with sequences of closely related members of the family *Bacillaceae* were performed by

using the CLUSTAL X program (Thompson *et al.*, 1997). Ambiguous and unalignable bases were omitted manually and the phylogenetic tree was then reconstructed from the evolutionary distance matrix calculated by using the neighbour-joining, minimum-evolutionary and maximum-parsimony methods in the MEGA program (version 3.1; Kumar *et al.*, 2004). Robustness of the resultant tree topology was evaluated by bootstrap resampling analysis with 1000 replicates (Felsenstein, 1985).

The phenotypic characteristics of strain H96B60^T are presented in the genus and species descriptions. The cell-wall peptidoglycan of strain H96B60^T contained meso-diaminopimelic acid as the diagnostic diamino acid. The menaquinone component detected in cells of strain H96B60^T was menaquinone-6 (MK-6). Cells contained large amounts of straight-chain and branched fatty acids. The major components (>15 % of total fatty acids) were $\text{C}_{16:0}$, anteiso- $\text{C}_{13:0}$ and anteiso- $\text{C}_{15:0}$ (see Supplementary Table S1 in IJSEM Online). The genomic DNA G+C content of strain H96B60^T was 36.2 mol%. The 16S rRNA gene sequence of strain H96B60^T determined in this study comprised 1481 nt. Comparative 16S rRNA gene sequence analyses revealed that strain H96B60^T was phylogenetically related most closely to members of the family *Bacillaceae*. In the phylogenetic tree based on the neighbour-joining algorithm, strain H96B60^T formed a lineage independent of those of members of the family *Bacillaceae* (Fig. 1); it was related most closely to the type strains of *Halolactibacillus* species (96.1–96.4 % 16S rRNA gene sequence similarity). The topologies of phylogenetic trees constructed by using the minimum-evolutionary and maximum-parsimony algorithms were similar to that of the tree constructed with the neighbour-joining analysis (Supplementary Fig. S1). Strain H96B60^T shared less than 95.1 % 16S rRNA gene sequence similarity with other spore-forming, halophilic or halotolerant taxa in the family *Bacillaceae*, such as type strains of species of *Amphibacillus* (93.6–95.1 %), *Paraliobacillus* (95.1–95.3 %) and *Gracilibacillus* (94.2–94.4 %) and *Natronobacillus azotifigens* 24KS-1^T (the sole species of the genus *Natronobacillus*; 95.0 %).

Our phylogenetic analysis thus showed that strain H96B60^T represents a novel branch within the Gram-positive, spore-forming, moderately halophilic or halotolerant rod-shaped bacteria. Although strain H96B60^T exhibited closest phylogenetic affiliation to *Halolactibacillus* species, it could be clearly distinguished from members of the genus *Halolactibacillus* by differences in physiological and chemotaxonomic properties, in particular the requirement for glucose for growth under aerobic conditions, oxidase activity and respiratory quinone; strain H96B60^T did not require glucose for growth under aerobic conditions and may possess a respiratory chain, given that it was positive for respiratory quinone and oxidase, whereas *Halolactibacillus halophilus* requires glucose for growth under aerobic conditions and its cells lack cytochromes and quinone components. Thus, *H. halophilus* grows aerobically by using

Fig. 1. Phylogenetic tree showing the relationships between strain H96B60^T and some other related taxa based on 16S rRNA gene sequence analysis. Numbers at nodes represent percentage levels of bootstrap support, based on a neighbour-joining analysis of 1000 resampled datasets. Accession numbers of nucleotide sequences are given in parentheses. Bar, 1.0% sequence divergence.

an oxidative metabolic pathway that is not mediated by the respiratory chain. The menaquinone component detected in cells of strain H96B60^T was MK-6, whereas no menaquinone components have been detected in cells of some species of the genus *Halolactibacillus* (Ishikawa *et al.*, 2005). Cao *et al.* (2008) reported that *Halolactibacillus alkaliphilus* contained MK-9 (H₂) and MK-9 (H₄) as predominant isoprenoid quinone components. We did not detect any menaquinone components in cells of *H. alkaliphilus* NBRC 103919^T in parallel experiments. Regardless, the menaquinone component of strain H96B60^T differed significantly from that of members of the genus *Halolactibacillus*. Moreover, *H. halophilus* NBRC 103919^T produced lactate, formate, acetate and ethanol from glucose without gas production under anaerobic conditions; the molar ratio of formate, acetate and ethanol was approximately 2:1:1. However, strain H96B60^T did not produce formate as a fermentative metabolite from glucose. Under anaerobic conditions, strain H96B60^T produced lactate at yields of 65% of the amount of glucose consumed. The other fermentative metabolic end products from glucose were acetate and ethanol at a molar ratio of approximately 1:3. No gas was produced. Carbon recovery from glucose consumed was about 94% (Supplementary Table S2). These data indicated that the new isolate may have a different pathway of glucose fermentation from members of the genus *Halolactibacillus*. In addition, some phenotypic features also differentiate strain H96B60^T from species of the genus *Halolactibacillus* (Table 1). Furthermore, the DNA G+C content of strain H96B60^T (36.2 mol%) was lower than the range reported for *Halolactibacillus* species (38.3–40.7 mol%).

In conclusion, the phenotypic, chemotaxonomic and phylogenetic data presented suggest that strain H96B60^T represents a novel species of a new genus, for which the name *Streptohalobacillus salinus* gen. nov., sp. nov. is proposed.

Description of *Streptohalobacillus* gen. nov.

Streptohalobacillus [Strep'to.ha.lo.ba.cil'lus. Gr. adj. *streptos* pliant, bent; Gr. n. *hals* salt; L. masc. n. *bacillus* stick, a small rod; N.L. masc. n. *Streptohalobacillus* a pliant or bent, salt (-loving) rod].

Cells are Gram-stain-positive, facultatively anaerobic, non-sporulating rods, occurring singly, in pairs or in short or long chains. Cells are motile by means of peritrichous flagella. Growth occurs in the absence of sugars. Acetate, ethanol and lactic acid are major metabolic end products from glucose under anaerobic fermentation conditions. Oxidase-positive but catalase-negative. Nitrate is reduced to nitrite. The cell-wall peptidoglycan type is A1 γ with meso-diaminopimelic acid as the diagnostic diamino acid. The predominant cellular fatty acids are C_{16:0}, anteiso-C_{13:0} and anteiso-C_{15:0}. The major isoprenoid quinone is MK-6. Phylogenetically, the genus is a member of the family *Bacillaceae*. The type species is *Streptohalobacillus salinus*.

Description of *Streptohalobacillus salinus* sp. nov.

Streptohalobacillus salinus (sa.li'nus. N.L. masc. adj. *salinus* salted, salty).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.2–0.4 × 2–8 μ m, occurring as single short rods in liquid GM medium under aerobic conditions, but as long chains in exponential growth phase in liquid GM medium under anaerobic conditions. After cultivation on GM agar at 37 °C for 4 days, colonies are circular, white, opaque, slightly convex and 0.2–0.4 mm in diameter. Grows at 20–40 °C (optimum 37 °C), at pH 6.5–9.0 (optimum pH 7.5) and with 0–20% (w/v) NaCl [optimum 7% (w/v) NaCl]. Positive in tests for methyl red reaction, amylase,

Table 1. Differential phenotypic characteristics between strain H96B60^T and closely related members of the family *Bacillaceae*

Taxa: 1, strain H96B60^T; 2, *Halolactibacillus*; 3, *Amphibacillus*; 4, *Natronobacillus*; 5, *Paraliobacillus*; 6, *Gracilibacillus*. Data are from Ishikawa *et al.* (2002, 2005), Cao *et al.* (2008), Niimura *et al.* (1990), An *et al.* (2007), Sorokin *et al.* (2008), Wainø *et al.* (1999), Zhilina *et al.* (2001), Jeon *et al.* (2008), Carrasco *et al.* (2006), Chen *et al.* (2008), Ahmed *et al.* (2007) and this study. v, Variable; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6
Spore formation	—	—	+	+	+	+
Anaerobic fermentation	+	+	+	+	+	—
Glucose required under aerobic conditions	—	+	+	ND	+	—
NaCl range (% w/v)	0–20	0–25.5	0–20.9	11–23	0–22	0.5–20
pH range	6.5–9.0	6–10	7.0–11.5	7.5–10.6	5.5–9.5	5–10
Temperature range (°C)	20–40	5–45	17–56	ND	10–47.5	4–50
Oxidase	+	—	ND	ND	ND	v
Nitrate reduction	+	—	ND	—	—	v
Starch hydrolysis	+	w	ND	+	+	v
Major end products from glucose under anaerobic conditions*	A, E, L	F, A, E, L	F, A, E	A, F, E	F, A, E, L	None
Major cellular fatty acids†	C _{16:0} , ai-C _{13:0} , ai-C _{15:0}	ai-C _{13:0} , C _{16:0}	ai-C _{15:0} , C _{16:0} , i-C _{16:0}	ai-C _{15:0} , i-C _{15:0} , ai-C _{13:0} , C _{16:0}	ND	ai-C _{15:0} , i-C _{15:0} , ai-C _{17:0}
Major isoprenoid quinone	MK-6	None‡	None	ND	MK-7	MK-7
DNA G + C content (mol%)	36.2	38.3–40.7	36.0–42.3	36.1–38.5	35.6	38–39
Isolation source(s)	Saline soil	Decaying marine algae, living sponge, sediment	Alkaline manure with grass and rice straw, sediment	Soda soils and lakes	Decomposing algae	Saline lake

*A, Acetate; E, ethanol; F, formate; L, lactate.

†ai, Anteiso-branched; i, iso-branched.

‡Cao *et al.* (2008) reported that *H. alkaliphilus* contained MK-9(H₂) and MK-9(H₄) as predominant isoprenoid quinone components. However, menaquinone components were not detected in cells of *H. alkaliphilus* NBRC 103919^T in our experiments.

pullulanase and β -glucosidase, but negative in tests for H₂S production, indole production, Voges–Proskauer reaction, urease production, protease, lipase, DNase, phosphatase and gelatinase. Acid is produced from glycerol, D-xylose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-arabitol and potassium 5-ketogluconate, but not from L-arabinose, D-galactose, L-sorbose, L-rhamnose, arbutin, melibiose, D-sorbitol, erythritol, D-ribose, D-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, dulcitol, inositol, methyl α -D-glucopyranoside, inulin, lactose, lyxose, D-tagatose, D- and L-fucose, L-arabitol, potassium gluconate, 2-ketogluconate or methyl α -D-mannopyranoside. Susceptible to ampicillin (10 μ g), penicillin G (10 U), cefazolin (30 μ g), gentamicin (10 μ g), streptomycin (10 μ g), tetracycline (30 μ g), chloramphenicol (30 μ g), clindamycin (2 μ g), erythromycin (15 μ g), norfloxacin (10 μ g), vancomycin (30 μ g), ciprofloxacin (5 μ g), kanamycin (30 μ g), neomycin (30 μ g), polymyxin B (300 U), rifampicin (5 μ g), spectinomycin (100 μ g), josamycin (15 μ g), medicamycin (15 μ g), bacitracin (0.04 U) and novobiocin (5 μ g). The genomic DNA

G + C content of the type strain is 36.2 mol% (T_m method).

The type strain, H96B60^T (=DSM 22440^T =CGMCC 1.7733^T), was isolated from a saline soil sample of the Qaidam basin, China.

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