Virgibacillus subterraneus sp. nov., a moderately halophilic Gram-positive bacterium isolated from subsurface saline soil

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A Gram reaction-positive, moderately halophilic bacterium, designated H57B72^T, was isolated from subsurface saline soil of Qaidam basin in the Qinghai province, China. Cells were rod-shaped, strictly aerobic, spore-forming and motile. The isolate grew optimally at 9% (w/v) NaCl, pH 7.5 and 30 °C. The cell-wall peptidoglycan of strain H57B72^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant isoprenoid quinone was MK-7. The major cellular fatty acids were anteiso-C_{15:0} (59.97%) and anteiso-C_{17:0} (17.14%). Phosphatidylglycerol, diphosphatidylglycerol and a glycolipid were found to be the predominant polar lipids. The genomic DNA G+C content of strain H57B72^T was 37.1 mol%. 16S rRNA gene sequence analysis showed that strain H57B72^T was a member of the genus *Virgibacillus* and was most closely related to *Virgibacillus salinus* DSM 21756^T (98.3% gene sequence similarity). The level of DNA–DNA relatedness between strain H57B72^T and *V. salinus* DSM 21756^T was 8.5%. Based on the phenotypic, genotypic and phylogenetic data presented, strain H57B72^T represents a novel species, for which the name *Virgibacillus subterraneus* sp. nov. is proposed. The type strain is H57B72^T (=DSM 22441^T =CGMCC 1.7734^T).

The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) with the transfer of *Bacillus pantothenicus* to *Virgibacillus pantothenicus* based on data from polyphasic studies; its description was later emended by Heyrman *et al.* (2003). At the time of writing, a total of 18 *Virgibacillus* species have been described: the mesophile *V. pantothenicus*, isolated from soil (type species of the genus) (Heyndrickx *et al.*, 1998), *V. proomii* from a water supply (Heyndrickx *et al.*, 1999), *V. carmonensis* and *V. necropolis* from a deteriorated mural painting (Heyrman *et al.*, 2003), *V. marismortui* from Dead Sea water (Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003), *V. salexigens* from a saltern and from hypersaline soils (Garabito *et al.*, 1997; Heyrman *et al.*, 2003), *V. halodenitrificans* from a saltern (Yoon *et al.*, 2004), *V. dokdonensis* from seawater (Yoon *et al.*, 2005), *V. koreensis* from a salt field (Lee *et al.*, 2006), *V. olivae* from the wastewater of green olive processing (Quesada *et al.*, 2007), *V. halophilus* from field soil (An *et al.*, 2007), *V. chiguensis* from a disused salt field (Wang *et al.*, 2008), *V.*

salarius from a salt lake (Hua *et al.*, 2008), *V. kekensis* from saline mud (Chen *et al.*, 2008), *V. sediminis* from a salt lake (Chen *et al.*, 2009), *V. arcticus* from permafrost in the Canadian high Arctic (Niederberger *et al.*, 2009), *V. byunsanensis* from a marine solar saltern (Yoon *et al.*, 2010) and *V. salinus* from the sediment of a salt lake (Carrasco *et al.*, 2009).

During the course of a study on the microbial diversity of subsurface saline soil samples taken at a scientific drilling site (37° 03' 50" N 94° 43' 41" E) in the Qaidam basin, China, large numbers of halophilic bacteria were isolated. Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that most of the moderately halophilic isolates were found to be related to the genera *Bacillus*, *Salinicoccus*, *Marinobacter*, *Halomonas*, *Idiomarina*, *Halolactibacillus*, *Halobacillus*, *Thalassobacillus* and *Virgibacillus*. In this study, we present the taxonomic characterization of a *Virgibacillus*-like strain, H57B72^T.

Strain H57B72^T was isolated from a subsurface saline soil sample by using the standard dilution-plating technique on SG agar (Sehgal & Gibbons, 1960), containing the following (g l⁻¹): Casamino acids (Difco), 7.5; yeast extract (Difco), 10; sodium glutamate, 1; trisodium citrate, 3; MgSO₄·7H₂O, 20; KCl, 2; NaCl, 80; FeSO₄·7H₂O, 0.036; MnCl₂·4H₂O, 0.00036. The medium was adjusted to pH 8.0 with 1 M

Abbreviation: RAPD, random amplification of polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H57B72^T is FJ746573.

A phase-contrast micrograph of cells of strain H57B72^T and RAPD fingerprints of strains H57B72^T and *V. salinus* DSM 21756^T are available as supplementary data with the online version of this paper.

NaOH before autoclaving and was solidified by the addition of 2.0% (w/v) agar. Strain H57B72^T was maintained in liquid SG medium supplemented with 30% (v/v) glycerol at -80 °C for long-term preservation. Strain H57B72^T was routinely cultured on growth medium (GM, pH 7.5) containing the following (g l⁻¹): Casamino acids (Difco), 5; yeast extract (Difco), 5; NaCl, 90; MgSO₄·7H₂O, 2 and KCl, 2 (Wang *et al.*, 2009).

To characterize strain H57B72^T, standard phenotypic tests were performed. *Virgibacillus salinus* DSM 21756^T was kindly provided by Professor A. Ventosa (University of Seville, Spain) and used as a reference strain for comparative phenotypic studies. The morphology, pigmentation and size of colonies were observed under optimal growth conditions on GM agar after 48 h of incubation at 30 °C. Cell morphology and motility were examined by phase-contrast and transmission electron microscopy. Gram-type was determined by the staining method (Dussault, 1955) and the KOH lysis method (Gregersen, 1978). The range of NaCl concentration for growth was determined in modified liquid GM medium with 0, 1, 3, 5, 7, 9, 12, 16, 20, 22, 25 and 30% (w/v) NaCl. The pH range for growth was tested at intervals of 0.5 pH units in liquid GM medium buffered with 50 mM MES (pH 5.0–6.5), PIPES (pH 6.5–7.5), HEPES (pH 7.0–8.0), Tricine (pH 7.5–9.0) and CHES (pH 9.0–10.0). Growth at 4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C was determined in liquid GM medium. The ability of the strain to grow anaerobically was evaluated by streaking on plates of GM agar or inoculation of liquid GM medium supplemented with nitrate (5 g l⁻¹) and incubation in a gaseous atmosphere of 100% N₂ in anaerobic chambers. The level of growth was checked after 5, 10 and 15 days of incubation. Sporulation ability was assessed on GM agar supplemented with 5 mg MnSO₄ l⁻¹. Plates were inoculated and incubated at 30 °C for up to 2 weeks to determine the presence of endospores. API 50CH test strips (Analytab Products, bioMérieux) were used to examine the assimilation of carbohydrates and the production of acid as recommended by the manufacturer, but with a modification that the suspension medium supplied by bioMérieux and used to resuspend cells of strain H57B72^T was supplemented with 9% (w/v) NaCl. Tests for other biochemical characteristics, listed in Table 1 or included in the species description, were performed according to previously described methods (Smibert & Krieg, 1981; Ventosa *et al.*, 1982). Unless otherwise indicated, all tests were carried out in triplicate and under optimal growth conditions. Growth in liquid medium was monitored at OD₆₀₀. Susceptibility to antimicrobial agents was tested by spreading exponential-phase cultures on GM agar medium plates with absorbent paper discs impregnated with antimicrobial agents. Zones of inhibition were determined after incubation at 30 °C for 2 days.

Cells of strain H57B72^T were Gram reaction-positive, rod-shaped, motile and endospore-forming (Supplementary Fig. S1, available in IJSEM Online). The strain displayed phenotypic characteristics typical of the genus *Virgibacillus*,

Table 1. Characteristics that distinguish strain H57B72^T from related species of the genus *Virgibacillus*

Taxa: 1, strain H57B72^T (data from this study); 2, *V. salinus* (Carrasco *et al.*, 2009); 3, *V. carmonensis* (Heyrman *et al.*, 2003); 4, *V. necropolis* (Heyrman *et al.*, 2003); 5, *V. byunsanensis* (Yoon *et al.*, 2010); 6, *V. arcticus* (Niederberger *et al.*, 2009). All species are rod-shaped, motile, strictly aerobic and positive for catalase activity. w, Weakly positive; ND, no data available.

Characteristics	1	2	3	4	5	6
Spore shape*	E/S	E/S	E/S	E	S/O	E
Spore position†	T	s/T	S	C/T/S	T	C/S
Temperature range (°C)	10–50	15–40	10–40	10–40	4–40	0–30
Growth in:						
0.5% (w/v) NaCl	+	–	–	W	+	+
25% (w/v) NaCl	+	–	–	–	–	–
Oxidase	+	–	+	ND	+	–
H ₂ S production	+	–	–	–	–	–
Hydrolysis of:						
Starch	–	–	–	–	+	ND
Casein	–	–	+	+	–	+
Gelatin	–	–	–	W	–	+
Aesculin	+	+	W	–	–	ND
Tween 80	–	+	–	W	–	ND
Acid production from:						
Glycerol	+	–	–	W	–	ND
D-Galactose	+	+	–	–	+	ND
D-Glucose	+	+	–	W	–	W
D-Fructose	+	+	–	W	W	W
Trehalose	–	+	–	W	–	+
DNA G + C content (mol%)	37.1	38.8	38.9	37.4	37.6	38.2

*E, Ellipsoidal; s, spherical; o, oval.

†C, Central; s, subterminal; T, terminal.

details of which are presented in the species description, but there are some differences between the phenotypic characteristics of strain H57B72^T and those of other recognized *Virgibacillus* species (Table 1). In particular, strain H57B72^T is clearly distinct from *V. salinus* DSM 21756^T, its phylogenetically closest neighbour, differing in the range of NaCl concentrations and temperatures suitable for growth, Tween 80 hydrolysis, H₂S production, oxidase activities, acid production from some substrates, some of the substrates used as sole carbon and energy sources and susceptibility to some antimicrobial agents.

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. Respiratory quinones were extracted according to the method of Collins *et al.* (1977) and analysed by reversed-phase HPLC (Groth *et al.*, 1996). Polar lipids were extracted and identified by

one-dimensional TLC followed by spraying with appropriate detection reagents (Torreblanca *et al.*, 1986). Fatty acids were extracted, methylated and analysed by GC using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The genomic DNA G+C content was determined by the thermal denaturation method according to Marmur & Doty (1962). Cell mass used for procedures mentioned above was obtained by cultivating strain H57B72^T and reference strain *V. salinus* DSM 21756^T at 30 °C on GM agar.

Strain H57B72^T possessed peptidoglycan type A1 γ with *meso*-diaminopimelic acid as the diagnostic diamino acid. The isoprenoid quinone detected in strain H57B72^T was menaquinone-7 (MK-7), as in other members of the genus *Virgibacillus* (Heyndrickx *et al.*, 1998; Heyrman *et al.*, 2003). The cellular polar lipids were diphosphatidylglycerol, phosphatidylglycerol and a glycolipid. Comparative analysis results based on one-dimensional TLC indicated that the profile of cellular polar lipid components of strain H57B72^T was the same as that of *V. salinus* DSM 21756^T. The major fatty acids in cells of strain H57B72^T were branched fatty acids anteiso-C_{15:0} (59.97 %), anteiso-C_{17:0} (17.14 %), iso-C_{16:0} (7.69 %) and iso-C_{15:0} (6.01 %), similar to those of the type strains of other recognized species of the genus *Virgibacillus*. These chemotaxonomic data are in accordance with those described for the genus *Virgibacillus* (Heyrman *et al.*, 2003; Heyndrickx *et al.*, 1998).

Genomic DNA of strain H57B72^T was extracted using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR using the universal bacterial primers 8F and 1492R as described previously (Duckworth *et al.*, 1996). The almost-complete nucleotide sequence (1481 bp) was determined by direct sequencing and compared with available 16S rRNA gene sequences in the GenBank database by using the BLAST program. Multiple alignments with closely related species were performed by using the program CLUSTAL_X (Thompson *et al.*, 1997). Ambiguous and unalignable bases were manually omitted and then the phylogenetic tree was constructed from the evolutionary distance matrix calculated by using the neighbour-joining, minimum-evolutionary and maximum-parsimony methods in the program MEGA version 3.1 (Kumar *et al.*, 2004). The robustness of the resultant tree topology was evaluated by bootstrap resampling analysis with 1000 replicates. This phylogenetic analysis revealed that strain H57B72^T was most closely related to *V. salinus* DSM 21756^T, with a 16S rRNA gene sequence similarity value of 98.3 %, and had less than 95.2 % similarity to other recognized species of the genus *Virgibacillus*. The phylogenetic tree based on the neighbour-joining method (Fig. 1) showed that strain H57B72^T clustered with *V. salinus* DSM 21756^T. Topologies of phylogenetic trees constructed using the minimum-evolutionary and maximum-parsimony algorithms were similar to that of the tree constructed using neighbour-joining analysis (not shown).

Fig. 1. Phylogenetic tree showing the relationships between strain H57B72^T and related strains based on 16S rRNA gene sequences. Numbers at nodes represent levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. Accession numbers are given in parentheses. Bar, 1 % sequence divergence.

Random amplification of polymorphic DNA (RAPD) analysis was used to compare the genomic profile of strains H57B72^T and *V. salinus* DSM 21756^T as described by Chao *et al.* (2008). RAPD analysis of strain H57B72^T showed a significantly different gel pattern from that of *V. salinus* DSM 21756^T (Supplementary Fig. S2), indicating that the genomic profile of strain H57B72^T was different from that of *V. salinus* DSM 21756^T. DNA–DNA hybridization was performed by the spectrophotometric renaturation rate method (Huß *et al.*, 1983; De Ley *et al.*, 1970); the level of genomic DNA–DNA relatedness between strain H57B72^T and *V. salinus* DSM 21756^T was 8.5 % (mean of three independent experiments, which did not differ by more than 1.1 %). This value was far below the threshold value of 70 % recommended for assigning strains to the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Overall, based on the evidence above, strain H57B72^T represents a novel species of the genus *Virgibacillus* for which the name *Virgibacillus subterraneus* sp. nov. is proposed.

Description of *Virgibacillus subterraneus* sp. nov.

Virgibacillus subterraneus (sub.ter.ra'ne.us. L. masc. adj. *subterraneus* underground, subterranean, indicating the source of isolation of the type strain).

Cells are Gram reaction-positive rods (0.1–0.3 × 1.0–3.0 µm), strictly aerobic, spore-forming and motile by means of peritrichous flagella. Cells occur singly, in pairs or as short chains. Ellipsoidal or spherical endospores form terminally in swollen sporangia. After cultivation on GM agar at 30 °C for 2 days, colonies are circular with slightly irregular margins, cream, opaque, slightly convex, 2.0–3.0 mm in diameter. After 3 days, the colonies turn slightly light-yellow. Grows at 10–50 °C (optimum 30 °C) and at pH 6.0–9.0 (optimum pH 7.5). Grows at NaCl concentrations between 0 and 25 % (w/v) with optimum growth at 9 % (w/v) NaCl. Positive for oxidase and catalase activities, methyl red reaction, H₂S production and gelatinase activity, but is negative for Voges–Proskauer reaction, indole production and urease, protease, pullulanase, amylase, lipase, DNase, phosphatase and β-glucosidase activities. Cells are able to reduce nitrate to nitrite aerobically but growth does not occur under anaerobic conditions in liquid GM medium supplemented with nitrate. Utilizes glucose by oxidative metabolism. Produces acids from glycerol, D-xylose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, aesculin, cellobiose, maltose, sucrose, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-arabitol, potassium 5-ketogluconate, D- and L-arabinose, D-galactose, L-sorbose, L-rhamnose, arbutin, melibiose, D-sorbitol, erythritol, D-ribose, 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 and methyl α-D-mannopyranoside, but not from D-mannitol,

salicin, trehalose, 2-ketogluconate, potassium gluconate and starch. The following compounds are utilized as sole carbon and energy sources: glycerol, erythritol, D- and L-arabinose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, *myo*-inositol, D-sorbitol, cellobiose, maltose, lactose, melibiose, sucrose, inulin, raffinose and glycogen. The following compounds are not utilized as sole carbon and energy sources: trehalose, D-mannitol, salicin and potassium gluconate. Cells are susceptible to (µg unless specified otherwise): ampicillin (10), penicillin G (10 U), cefazolin (30), gentamicin (10), streptomycin (10), tetracycline (30), chloramphenicol (30), clindamycin (2), erythromycin (15), norfloxacin (10), vancomycin (30), ciprofloxacin (5), kanamycin (30), neomycin (30), polymyxin B (300 U), rifampicin (5), spectinomycin (100), josamycin (15), medicamycin (15), bacitracin (0.04 U) and novobiocin (5). Cell-wall peptidoglycan type A1γ with *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The predominant isoprenoid quinone is menaquinone-7 (MK-7). The genomic DNA G + C content of the type strain is 37.1 mol% (*T*_m).

The type strain, H57B72^T (=DSM 22441^T =CGMCC 1.7734^T), was isolated from a subsurface saline soil sample of the Qaidam basin, China.

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