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 H57B72T, 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 H57B72T contained meso-diaminopimelic acid as the diagnostic diamino acid. The predominant isoprenoid quinone was MK-7. The major cellular fatty acids were anteiso-C15 : 0 (59.97 %) and anteiso-C17 : 0 (17.14 %). Phosphatidylglycerol, diphosphatidylglycerol and a glycolipid were found to be the predominant polar lipids. The genomic DNA G + C content of strain H57B72T was 37.1 mol%. 16S rRNA gene sequence analysis showed that strain H57B72T was a member of the genus Virgibacillus and was most closely related to Virgibacillus salinus DSM 21756T (98.3 % gene sequence similarity). The level of DNA–DNA relatedness between strain H57B72T and V. salinus DSM 21756T was 8.5 %. Based on the phenotypic, genotypic and phylogenetic data presented, strain H57B72T represents a novel species, for which the name Virgibacillus subterraneus sp. nov. is proposed. The type strain is H57B72T (=DSM 22441T =CGMCC 1.7734T).

The genus Virgibacillus was first proposed by Heyndrickx et al. (1998) with the transfer of Bacillus pantothenticus to Virgibacillus pantothenticus 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. pantothenticus, 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. salarius from Dead Sea water (Arahel 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, H57B72T.

Strain H57B72T was isolated from a subsurface saline soil sample by using the standard dilution-plateing 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 H57B72T is FJ746573.

A phase-contrast micrograph of cells of strain H57B72T and RAPD fingerprints of strains H57B72T and V. salinus DSM 21756T are available as supplementary data with the online version of this paper.

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Sporulation ability was assessed on GM agar supplemented with 2.0% (w/v) agar. Strain H57B72\(^T\) was maintained in NaOH before autoclaving and was solidified by the addition of 5% (w/v) yeast extract (Difco), 5% NaCl, 90 g MgSO\(_4\).7H\(_2\)O, 2 and KCl (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% N\(_2\) 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 MnSO\(_4\) 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 OD\(_{600}\). 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*, 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, H\(_2\)S 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

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>Spore shape*</td>
<td>E/S</td>
<td>E/S</td>
<td>E/S</td>
<td>E</td>
<td>S/O</td>
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<td>Spore position†</td>
<td>T</td>
<td>S/T</td>
<td>S</td>
<td>C/T/S</td>
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<td>C/S</td>
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<td>Temperature range (°C)</td>
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<td>15–40</td>
<td>10–40</td>
<td>10–40</td>
<td>4–40</td>
<td>0–30</td>
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<td>Growth in:</td>
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<tr>
<td>0.5% (w/v) NaCl</td>
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<td>–</td>
<td>–</td>
<td>W</td>
<td>+</td>
<td>+</td>
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<td>25% (w/v) NaCl</td>
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<td>–</td>
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<td>–</td>
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<td>Oxidase</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>–</td>
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<td>H(_2)S production</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>Hydrolysis of:</td>
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<td>Starch</td>
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<td>Casein</td>
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<td>Gelatin</td>
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<td>+</td>
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<tr>
<td>Aesculin</td>
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<td>+</td>
<td>W</td>
<td>–</td>
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<td>+</td>
<td>–</td>
<td>W</td>
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<td>Acid production from:</td>
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<tr>
<td>Glycerol</td>
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<td>–</td>
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<td>W</td>
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<td>D-Galactose</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>W</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Fructose</td>
<td>+</td>
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<td>–</td>
<td>W</td>
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<td>Trehalose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>W</td>
<td>+</td>
<td>–</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>37.1</td>
<td>38.8</td>
<td>38.9</td>
<td>37.4</td>
<td>37.6</td>
<td>38.2</td>
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*E, Ellipsoidal; s, spherical; o, oval.
†C, Central; s, subterminal; t, terminal.
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 H57B72T and reference strain V. salinus DSM 21756T at 30 °C on GM agar.

Strain H57B72T possessed peptidoglycan type A1γ with meso-diaminopimelic acid as the diagnostic diamino acid. The isoprenoid quinone detected in strain H57B72T 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 H57B72T was the same as that of V. salinus DSM 21756T. The major fatty acids in cells of strain H57B72T were branched fatty acids anteiso-C15 : 0 (59.97 %), anteiso-C17 : 0 (17.14 %), iso-C16 : 0 (7.69 %) and iso-C15 : 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 H57B72T 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 H57B72T was most closely related to V. salinus DSM 21756T, 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 H57B72T clustered with V. salinus DSM 21756T. 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 H57B72T 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 H57B72T and V. salinus DSM 21756T as described by Chao et al. (2008). RAPD analysis of strain H57B72T showed a significantly different gel pattern from that of V. salinus DSM 21756T (Supplementary Fig. S2), indicating that the genomic profile of strain H57B72T was different from that of V. salinus DSM 21756T. DNA–DNA hybridization was performed by the spectrophotometric renaturation rate method (Huelsenbeck et al., 1983; De Ley et al., 1970); the level of genomic DNA–DNA relatedness between strain H57B72T and V. salinus DSM 21756T 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 H57B72T 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 × 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, amyrase, 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-xyllose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, aesculin, cellobiose, maltose, sucrose, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-arabinose, potassium 5-ketogluconate, D- and L-arabinose, D-galactose, D-sorbitose, L-rhamnose, arbutin, melibiose, D-sorbitol, erythritol, D-ribose, L-xyllose, D-adenosinet, methyl β-D-xylopyranoside, dulcitol, inositol, methyl α-D-glycopyranoside, and 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-xyllose, D-adenosinet, 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), 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-C15:0 and anteiso-C17:0. The predominant isoprenoid quinone is menaquinone-7 (MK-7). The genomic DNA G+C content of the type strain is 37.1 mol% (Tm).

The type strain, H57B72T (=DSM 22441T =CGMCC 1.7734T), was isolated from a subsurface saline soil sample of the Qaidam basin, China.

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**References**


Oceanobacillus picturae


