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Virgibacillus xinjiangensis sp. nov., Isolated from a Salt Lake of Xin-jiang Province in China

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A strictly aerobic Gram-positive, moderately halophilic spore forming bacterium, designated strain SL6-1^T, was isolated from a salt lake in Xin-jiang province, China. Growth of strain SL6-1^T was observed at NaCl concentrations of 0~20% (w/v) (the optimum being 5~7%, w/v). The peptidoglycan type of strain SL6-1^T was A1γ-meso-diaminopimelic acid and its major cellular fatty acids were iso-$C_{14:0}$ and iso-$C_{16:0}$ and ante-iso-$C_{15:0}$. The major respiratory isoprenoid quinone was MK-7 and the G+C content of the genomic DNA was 44.5 mol%. The major cellular phospholipids were phosphatidylglycerol and diphosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain SL6-1^T formed a phylogenetic lineage within the genus *Virgibacillus*. Based on 16S rRNA gene sequence similarity, the strain was most closely related to *Virgibacillus olivae* $E_{30}8^T$, *Virgibacillus kekensis* YIM kkny16^T, *Virgibacillus marismortui* DSM 12325^T with 97.1%, 97.1%, and 97.0% gene sequence similarities, respectively and the sequence similarities to other related taxa were less than 96.7%. The DNA relatedness values between strain SL6-1^T and *V. olivae* $E_{30}8^T$, *V. kekensis* YIM kkny16^T, *V. marismortui* DSM 12325^T were 16.7%, 51.0%, and 22.8%, respectively. On the basis of physiological, biochemical and phylogenetic properties, strain SL6-1^T represents a novel species, for which the name *Virgibacillus xinjiangensis* sp. nov. is proposed. The type strain is SL6-1^T (=KCTC 13128^T =DSM 19031^T).

***Keywords*:** *Virgibacillus xinjiangensis* sp. nov., halotolerant, Gram-positive

Since the genus *Virgibacillus*, which forms a phylogenetic lineage within the group classically defined as the genus *Bacillus* (Ash *et al.*, 1993; Nielsen *et al.*, 1994), was first proposed by Heyndrickx *et al*. (1998), many *Virgibacillus* species have been isolated from marine and related habitats. At the time of writing, the genus *Virgibacillus* comprises recognized 15 species. The members of *Virgibacillus* are motile, Gram-positive rods bearing spherical to ellipsoidal endospores and have DNA G+C contents ranging from 36~43 mol%. In the course of screening of halophilic bacteria, an aerobic Gram-positive, moderately halophilic bacterium belonging to the genus *Virgibacillus*, designated strain SL6-1^T, was isolated from soil sediment of a salt lake. Here, we classified strain SL6-1^T as a new species using a polyphasic approach, for which the name *Virgibacillus xinjiangensis* sp. nov. is proposed.

Materials and Methods

Collection of microorganisms

Strain SL6-1^T was isolated from a salt lake in Xin-jiang province, China by serial dilution plating method with marine agar 2216 (MA; Difco, USA) supplemented with 10% (w/v) NaCl [final concentration: 11.94% NaCl (w/v)] at 35°C for 3 days. The isolate was routinely grown aerobically on MA agar with the addition of 5% NaCl (w/v) for 2 days at 32°C except where indicated otherwise and the strain was stored at -80°C in marine broth (MB; Difco) supplemented with 10% (v/v) glycerol for preservation.

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Phylogenetic analysis

Amplification and sequencing of the 16S rRNA gene of strain H7^T was carried out as described by Kim *et al*. (2008). Briefly, a single colony of strain H7^T grown on R2A agar was resuspended in 100 μl of 5% (w/v) Chelex-100 solution (Bio-Rad, USA) and boiled for 10 min to prepare crude genomic DNA lysates. PCR amplification of 16S rRNA genes from the crude lysates was performed using the universal primer F1; 5'-AGAGTTTGATCMTGGCTCAG-3' and R13; 5'-TA CGGYTACCTTGTTACGACTT-3' as described previously (Lu *et al*., 2006). The resultant 16S rRNA gene sequence of strain SL6-1^T was compared with available sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to determine an approximate phylogenetic affiliation and was aligned with those of closely related members using the CLUSTAL W software (Thompson *et al*., 1994). Phylogenetic trees were constructed using three different methods, neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) algorithms, which are available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Resulting tree topologies were evaluated by bootstrap analysis based on 1,000 resamplings. Sequence similarity

values between the isolate and related bacteria were evaluated using the EzTaxon Server (http://www.eztaxon.org; Chun *et al*., 2007).

Phenotypic characterization

Requirement and tolerance of NaCl were determined at 0.5% intervals in MH medium (Carrasco *et al.*, 2006) [0∼25% (w/v) NaCl, 0.7 g $MgCl_2$, 0.96 g $MgSO_4$, 0.036 g $CaCl_2$, 0.2 g KCl, 0.006 g $NaHCO_3$, 0.0026 g NaBr, 1 g yeast extract (Difco), 0.5 g proteose peptone no. 3 (Difco) and 0.1 g glucose per liter]. Gram staining was performed using the bioMerieux Gram Stain kit according to the instructions of the manufacturer. Cell morphology and motility were observed using a transmission electron microscope (TEM, JEM-1010; JEOL) and a light microscope (Nikon E600) by according to the methods described previously Jeon *et al*. (2005). Endospore formation was tested according to the method of Schaeffer-Fulton (Smibert and Krieg, 1981). Growth was tested at different temperatures (4∼55°C) on MA with the addition of 5% NaCl (w/v) and at different pH values in marine broth (MB) with the addition of 5% NaCl (w/v). MB media with different pH values were prepared as described previously (Gomori, 1955). Oxidase activity was tested using Bactident Oxidase strip (Merck, USA) and catalase activity was evaluated by production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution. Nitrate reduction and the hydrolysis of compounds were checked according to the methods described previously (Lanyi, 1987; Smibert and Krieg, 1994) and acid production from carbohydrates were evaluated as described by Leifson (1963). The utilization or oxidation tests of carbon sources were performed in parallel on strain N7^T and on its closest relatives *Virgibacillus olivae* $E_{30}8^T$, *Virgibacillus kekensis* YIM kkny16^T, *Virgibacillus marismortui* DSM 12325^T using Biolog GN2 Microplates (Biolog, USA) at 32°C for 3 days in accordance with the manufacturer's instructions. Anaerobic growth was checked under anaerobic (with 4∼10% CO_2) condition using GasPak Plus system (BBL) at 32°C for 15 days on MA with the addition of 5% NaCl (w/v).

Chemotaxonomy

Whole cell fatty acids of strain SL6-1^T and the three reference type strains were analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.) after cultivation on MA with the addition of 5% NaCl (w/v) for 2 days at 32°C. Preparation of cell walls from strain SL6-1^T and analysis of peptidoglycan were carried out by DSMZ (Germany) using the method described by Schleifer and Kandler (1972). Analyses of isoprenoid quinones and polar lipids were carried out as described by Komagata and Suzuki (1987). The genomic DNA G+C content of strain SL6-1^T was determined using a HPLC fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE, GROM) according to the method of Tamaoka and Komagata (1984).

DNA-DNA hybridization

DNA-DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain SL6-1^T and *Virgibacillus kekensis* YIM kkny16^T using the fluorometric microplate method (Ezaki *et al*., 1989). Fluorometric data recorded after 30 min reaction of the substrates were used for the calculation of DNA-DNA hybridization values. The signals produced by self-hybridization were inferred as 100% and percentages of DNA relatedness were calculated from the mean values of five replications.

Results and Discussion

Phylogenetic analysis

Phylogenetic analysis using a nearly complete 16S rRNA gene sequence (1455 nucleotide) of strain SL6-1^T indicated that the isolate formed a distinct phylogenetic line within the genus *Virgibacillus* (Fig. 1). The topologies of phylogenetic trees built using the ML and MP algorithms also supported the notion that the isolate belongs to the genus *Virgibacillus* and can be differentiated from the other species of the genus *Virgibacillus* (Fig. 1). Based on 16S rRNA gene sequence similarities, strain SL6-1^T was most closely related to *Virgibacillus olivae* $E_{30}8^T$, *Virgibacillus kekensis* YIM kkny16^T, *Virgibacillus marismortui* DSM 12325^T with 97.1%, 97.1%, and 97.0% gene sequence similarities, respectively and the sequence similarities to other related taxa used in the phylogentic tree were less than 96.7%.

Phenotypic characteristics

Strain SL6-1^T formed cream, circular/slightly irregular and slightly convex colonies on MA supplemented with 5% NaCl (w/v) when grown at 32°C for 2 days. The strain grew optimally on MH medium supplemented with 5∼7% (w/v) NaCl,

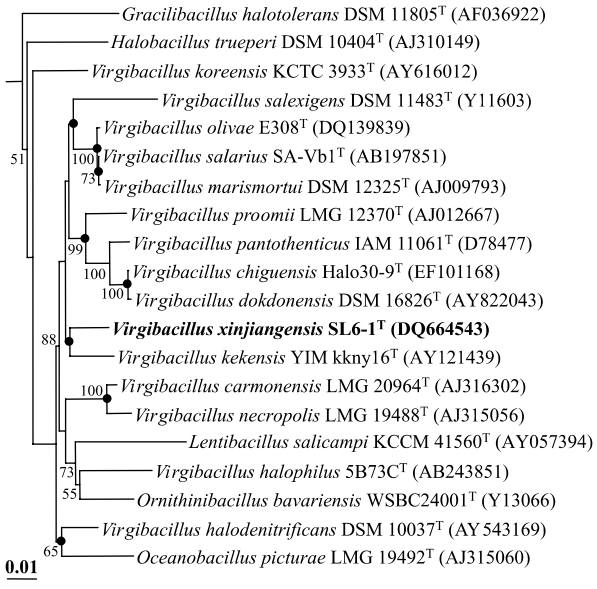

Fig. 1. Neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequence of strain SL6-1^T. Bootstrap values are shown in percentages of 1,000 replicates, when greater than 50%. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum parsimony algorithms. *Brevibacillus brevis* JCM 2503P^{TP} was used as an outgroup (not shown). The scale bar equals 0.01 changes per nucleotide position.

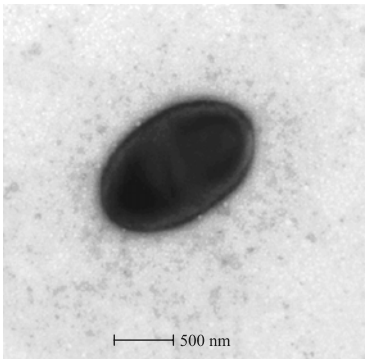

Fig. 2. Transmission electron micrograph showing general morphology of negatively stained cells of strain SL6-1^T after growth for 2 days at 32°C on MA supplemented with 5% (w/v) NaCl.

but did not grow on MH medium with the addition of 21% (w/v) NaCl or more. Growth occurred at temperatures between 8 and 52°C (optimum: 32∼35°C) and a pH of 6.5∼9.5 (optimum: pH 7.5∼8.0). Cells of strain SL6-1^T were non-motile, endospore forming rods without flagella (0.8∼1.2 μm wide and 1.4∼2.4 μm long) (Fig. 2). The absence of flagella can differentiate strain SL6-1^T from other *Virgibacillus* type strains. Anaerobic growth was not observed under the anaerobic condition over 15 days at 32°C on MA with the addition of 5% (w/v) NaCl.

Chemotaxonomy

The major isoprenoid quinone of strain SL6-1^T was menaquinone-7 (MK-7). Strain SL6-1^T contained anteiso-$C_{15:0}$ (44.5%), anteiso-$C_{17:0}$ (16.5%), $C_{16:0}$ (16.4%), iso-$C_{16:0}$ (7.4%), iso-$C_{14:0}$ (5.4%), iso-$C_{15:0}$ (4.1%), $C_{14:0}$ (2.0%), $C_{15:0}$ (1.8%), $C_{18:0}$ (1.0%), and iso-$C_{17:0}$ (0.7%) as the cellular fatty acids. Although the overall fatty acid profile of strain SL6-1^T was similar to those of phylogenetically related *Virgibacillus* species, significant differences in the respective proportions of several components clearly distinguished strain SL6-1^T from the related species (Table 2). Polar lipids were dominated by a large amount of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), but a small amount of an unknown

Table 1. Characteristics of strain SL6-1^T and some other related type strains
Species: 1, SL6-1^T; 2, *Virgibacillus olivae* $E_{30}8^T$ (Quesada *et al*., 2007); 3, *Virgibacillus kekensis* YIM kkny16^T (Chen *et al*., 2008); 4, *Virgibacillus marismortui* DSM 12325^T (Arahal *et al*., 1999); 5, *Virgibacillus pantothenticus* IAM 11061^T (Heyndrickx *et al*., 1999). +, positive; –, negative; w, weakly positive; v, variable; E, ellipsoidal; S, spherical; ST, subterminal; T, terminal.

Characteristic	1[a]	2	3	4
Spore shape	E/S	E/S	E/S	E
Spore position	T/ST	T/ST	T	T/ST
Motility	–	+	+	+
Anaerobic growth	–	–	–	–
Nitrate reduction	+	+	+	+
NaCl range	0-20	0-20	0-25	5-25
Growth range (°C)	8-52	20-45	10-50	15-50
Growth at pH 10	–	–	+	–
Hydrolysis of:				
Aesculin	–	+	–	+
Casein	+	+	–	+
Gelatin	–	+	–	+
Starch	–	+	+	–
Biolog utilization of[a]:				
L-Arabinose	–	+	+	–
D-Mannose	–	+	+	+
D-Melibiose	–	w	+	+
Maltose	+	–	–	–
D-Cellobiose	+	+	–	–
α-D-Lactose	–	+	+	–
D-Trehalose	+	–	–	+
D-Arabitol	+	–	–	–
Acid production from:				
D-Galactose	–	–	–	–
D-Glucose	+	–	+	+
Glycerol[a]	–	–	–	w
D-Fructose	+	+	–	+
Sucrose	+	–	–	–
Major fatty acids[a]	anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, $C_{16:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	anteiso-$C_{15:0}$, iso-$C_{14:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$
G+C content (mol%)	44.5	33.4	41.8	40.7

[a] Results from this study

Table 2. Whole-cell fatty acid profiles of strain SL6-1^T and other related *Virgibacillus* type strains grown on MA with the addition of 5% NaCl (w/v) for 2 days at 32°C
Strains: 1, SL6-1^T; 2, *V. olivae* E$_{30}$8^T; 3, *V. kekensis* YIM kkny16^T; 4, *V. marismortui* DSM 12325^T. All data from this study. Fatty acids amounting to less than 1.0% in all strains are not shown. tr, trace amount (< 1.0%); –, not detected.

Fatty acid	1	2	3	4
Straight chain				
$C_{14:0}$	2.1	tr	-	-
$C_{15:0}$	1.8	-	tr	-
$C_{16:0}$	16.4	1.0	-	1.3
$C_{18:0}$	1.0	-	-	-
Branched				
iso-$C_{14:0}$	5.4	5.7	19.0	4.4
iso-$C_{15:0}$	4.1	42.9	3.4	29.2
iso-$C_{16:0}$	7.4	3.7	2.5	4.2
iso-$C_{17:0}$	tr	3.7	-	3.0
anteiso-$C_{13:0}$	-	tr	1.2	-
anteiso-$C_{15:0}$	44.5	30.7	63.0	28.5
anteiso-$C_{17:0}$	16.5	6.3	2.3	6.4
Unsaturated				
$C_{16:1}$ *ω7c* alcohol	-	1.8	5.6	tr
Summed feature 4[a]	-	1.1	1.9	-
Summed feature 7[a]	-	-	-	21.8

[a] Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 4 contains $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH and summed feature 7 contains $C_{18:1}$ *ω7c*, $C_{18:1}$ *ω9t* and/or $C_{18:1}$ *ω12t*.

phospholipid was also present. The analysis of cell wall peptidoglycan showed that strain SL6-1^T possessed A1γ-meso-diaminopimelic acid as the diagnostic diamino acid. The genomic DNA G+C content of strain SL6-1^T was 44.5 mol%, which is a little higher than those other *Virgibacillus* species.

DNA-DNA relatedness

The DNA-DNA relatedness values obtained from hybridization experiments of strain SL6-1^T with *V. olivae* E$_{30}$8^T, *V. kekensis* YIM kkny16^T, *V. marismortui* DSM 12325^T were 16.7%, 51.0%, and 22.8%, respectively, which are below the 70% threshold generally accepted for species delineation (Rossello-Mora and Amann, 2001).

The major lipoquinone, major fatty acids, and G+C content were in line with those of members of the genus *Virgibacillus* (Heyndrickx *et al*., 1998; Heyrman *et al*., 2003; Quesada *et al*., 2007; Chen *et al*., 2008; Hua *et al*., 2008; Wang *et al*., 2008). Typical phenotypes of strain SL6-1^T are summarized and compared with those of the type strains of closely related taxa (Table 1). DNA-DNA relatedness values, DNA G+C content, and the absence of flagellum showed that strain SL6-1^T could be differentiated from other *Virgibacillus* species. In conclusion, the physiological, biochemical and phylogenetic properties of strain SL6-1^T support its description as a novel species within the genus *Virgibacillus*, for which the name *Virgibacillus xinjiangensis* sp. nov. is proposed.

Description of *Virgibacillus xinjiangensis* sp. nov.

Virgibacillus xinjiangensis (xin.ji.ang.e'nsis. N.L. masc. adj. *xinjiangensis* pertaining to Xinjiang in China).

Colonies are cream, circular/slightly irregular, and slightly convex. Cells are strictly aerobic, Gram-positive with approximately 0.8∼1.2 μm wide and 1.4∼2.4 μm long. Catalase-positive and oxidase-negative. Cells are non-motile and endospore forming rods without flagellar. Grows between 8 and 52°C (optimum: 32∼35°C) and from pH 6.5 to 9.5 (optimum: pH 7.5∼8.0). Nitrate is reduced to nitrite. Cells grow at salinities of 0∼20% (w/v) NaCl [optimum: 5∼7% (w/v)]. Hydrolyzes casein, but not aesculin, DNA, hypoxanthine, L-tyrosine, starch, Tween 80, xanthine, and urea. Acid is produced from D-glucose, D-fructose, maltose, and sucrose, but not from L-arabinose, D-mannitol, α-D-lactose, D-raffinose, glycerol, inositol, D-melibiose, and D-mannose. Positive for Biolog GN2 MicroPlate system substrates dextrin, D-arabitol, D-fructose, α-D-glucose, maltose, D-trehlose, acetic acid, β-hydroxybutyric acid, α-keto butyric acid, α-keto valeric acid, D-saccharic acid, glycyl-L-aspartic acid, uridine, and thymidine. Other organic substrates included in Biolog GN2 microplates are not oxidized. Major isoprenoid quinone is MK-7. Cell wall contains A1γ-meso-diaminopimelic acid as the diagnostic diamino acid. Predominant polar lipids are phosphatidylglycerol and diphosphatidylglycerol. The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, and $C_{16:0}$. The G+C content of the genomic DNA is 44.5 mol% (HPLC).

The type strain is SL6-1^T (=KCTC 13128^T =DSM 19031^T), isolated from a salt lake in Xin-jiang province, China.

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