Virgibacillus salinus sp. nov., a moderately halophilic bacterium from sediment of a saline lake

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A novel, moderately halophilic, Gram-positive bacterium, designated strain XH-22^T, was isolated from sediment of a saline lake located near Xilinhote, Inner Mongolia Autonomous Region, China. Cells were rod-shaped, endospore-forming and motile. The isolate was able to grow in the presence of 3–20% (w/v) total salts (optimum, 10%, w/v), and at 15–40 °C (optimum, 37 °C) and pH 6.0–10.0 (optimum, pH 7.5). Strain XH-22^T had diaminopimelic acid in the cell-wall peptidoglycan, MK-7 as the predominant menaquinone, and anteiso-C_{15:0}, C_{16:0} and iso-C_{14:0} as major fatty acids. The polar lipids comprised diphosphatidylglycerol, phosphatidylglycerol, a glycolipid and two unidentified phospholipids. The DNA G+C content of strain XH-22^T was 38.8 mol%. 16S rRNA gene sequence analysis revealed that the novel strain was affiliated with the genus *Virgibacillus*. Levels of 16S rRNA gene sequence similarity between strain XH-22^T and the type strains of recognized *Virgibacillus* species ranged from 97.6% (with *Virgibacillus carmonensis*) to 94.9% (with *Virgibacillus koreensis*). Levels of DNA–DNA relatedness between strain XH-22^T and *V. carmonensis* DSM 14868^T and *Virgibacillus necropolis* DSM 14866^T were 32 and 28%, respectively. Strain XH-22^T could be differentiated from recognized *Virgibacillus* species based on phenotypic characteristics, chemotaxonomic differences, phylogenetic analysis and genotypic features. On the basis of these results, strain XH-22^T is considered to represent a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus salinus* sp. nov. is proposed. The type strain is XH-22^T (=CCM 7562^T=CECT 7439^T=DSM 21756^T).

The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) based on data from amplified rRNA gene restriction analysis, fatty acid profiles, SDS-PAGE patterns of whole-cell proteins and phenotypic characterization; its description was later emended by Heyrman *et al.* (2003). At the time of writing, the genus comprised 14 recognized species, namely *Virgibacillus pantothenicus* (type species of the genus) (Heyndrickx *et al.*, 1998), *V. proomii* (Heyndrickx *et al.*, 1999), *V. carmonensis*, *V. necropolis*, *V. marismortui*, *V. salexigens* (Heyrman *et al.*, 2003), *V. halodenitrificans* (Yoon *et al.*, 2004), *V. dokdonensis* (Yoon *et al.*, 2005), *V. koreensis* (Lee *et al.*, 2006), *V. olivae* (Quesada *et al.*, 2007), *V. halophilus* (An *et al.*, 2007), *V. chiguensis* (Wang *et al.*, 2008), *V. kekensis* (Chen *et al.*, 2008) and *V. salarius* (Hua *et al.*, 2008). Members of the genus are motile, Gram-positive or Gram-variable rods, endospore-forming, catalase-positive, have a cell wall containing peptidoglycan of the *meso*-diaminopimelic acid type and DNA G+C contents ranging from 30.7 to 42.8 mol% (Wang *et al.*, 2008; Chen *et al.*, 2008). During the course of a study of halophilic bacteria from a saline lake in China, a moderately halophilic, Gram-positive

bacterium, designated strain XH-22^T, was isolated. Phenotypic and chemotaxonomic characterization, together with DNA–DNA hybridization data and phylogenetic analysis based on 16S rRNA gene sequences, showed that strain XH-22^T was related to members of the genus *Virgibacillus* but represented a novel species.

Strain XH-22^T was isolated from a sediment sample from a saline lake located near Xilinhote, Inner Mongolia Autonomous Region, China (43° 55' N 115° 37' E), according to the methodology described by Carrasco *et al.* (2007). No other strains with characteristics similar to that of the new isolate were found in this habitat. At the time of sampling, the water in the lake had a conductivity of 44.9 mS cm⁻¹, a temperature of 18 °C and a pH of 7.5. The medium used for isolation was HM medium (Ventosa *et al.*, 1982). The composition of this medium was (w/v) 17.8% NaCl, 0.1% MgSO₄·7H₂O, 0.036% CaCl₂·2H₂O, 0.2% KCl, 0.006% NaHCO₃, 0.023% NaBr, trace FeCl₃·6H₂O, 1.0% yeast extract (Difco), 0.5% proteose peptone no. 3 (Difco) and 0.1% glucose. The pH was adjusted to 7.2 with 1 M KOH. When necessary, solid media were prepared by adding 2.0% (w/v) Bacto-agar (Difco). Strain XH-22^T was routinely cultured on the maintenance medium described by Ventosa *et al.* (1982).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain XH-22^T is FM205010.

supplemented with 10 % (w/v) marine salts. The composition of this medium was (w/v) 8.1 % NaCl, 0.7 % MgCl₂, 0.96 % MgSO₄, 0.036 % CaCl₂, 0.2 % KCl, 0.006 % NaHCO₃, 0.026 % NaBr, 1 % yeast extract, 0.5 % proteose peptone no. 3 and 0.1 % glucose; this was solidified with 2.0 % (w/v) Bacto-agar. The pH was adjusted to 7.2 with 1 M KOH. *V. carmonensis* DSM 14868^T and *V. necropolis* DSM 14866^T were obtained from the DSMZ, Braunschweig, Germany, and were cultivated on maintenance medium at 28 °C.

In order to characterize strain XH-22^T phenotypically, standard tests were performed. The Gram stain reaction was carried out by using the method described by Dussault (1955). For the determination of cellular morphology and motility, a culture of cells at exponential growth phase in liquid medium was examined by using phase-contrast light microscopy (BX41 microscope; Olympus). The morphology of colonies, their pigmentation and size were observed under optimal growth conditions on maintenance medium after 24 h incubation at 37 °C. Growth at different concentrations of salts was determined on maintenance medium at salt concentrations of 0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v). Growth at different pH (5, 6, 7, 8, 9, 10 and 11) and temperatures (10, 14, 15, 20, 25, 30, 37, 40, 41, 45 and 50 °C) was determined on maintenance medium. Anaerobic growth was determined on maintenance medium by using an anaerobe jar and H₂ and CO₂ GasPaks (BBL) to generate anaerobic conditions. Catalase was assayed by addition of 3 % (v/v) H₂O₂ to culture plates. The oxidase reaction was performed on filter paper moistened with 1 % (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenyldiamine. *V. carmonensis* DSM 14868^T was employed in these tests as a control. Sporulation was tested on solid maintenance medium, and on this medium without proteose peptone or glucose, and supplemented with 1 g yeast extract l⁻¹ (Difco) and 5 mg MnSO₄ l⁻¹ (Merck) (Mn-medium) by using different temperatures for cultivation. Utilization of various substrates as sole carbon and energy, or carbon, nitrogen and energy sources was determined as described by 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). Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966) on maintenance medium. *V. carmonensis* DSM 14868^T and *V. necropolis* DSM 14866^T were employed in these tests as reference strains. Other tests detailed in Table 1 or included in the species description were carried out by using the methods of Ventosa *et al.* (1982), Quesada *et al.* (1984) and García *et al.* (1987). Unless indicated otherwise, the tests were carried out on maintenance medium at 37 °C in sealed containers.

Cells of strain XH-22^T were Gram-positive, approximately 0.9 × 1.5–6.0 µm, occurring singly, in pairs or in short chains. Cells were motile and strictly aerobic. When grown for 24 h at 37 °C on maintenance medium, colonies were circular with entire margins, cream-pigmented and 1 mm

in diameter. Spherical to ellipsoidal endospores were formed at subterminal or terminal positions in swollen sporangia after 4 days cultivation on Mn-medium at 28 °C. The isolate was moderately halophilic, being able to grow in media containing 3–20 % (w/v) salts and optimally in media containing 10 % (w/v) salts. No growth was observed in the absence of NaCl. Growth was observed at 15–40 °C and at pH 6.0–10.0. Optimal growth conditions in maintenance medium were at 37 °C and pH 7.5. Other phenotypic features are included in the species description.

Genomic DNA from strain XH-22^T was prepared according to the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with forward primer 16F27 and reverse primer 16R1488. Direct sequence determination of the PCR-amplified DNA was carried out by using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity values were achieved by using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig *et al.*, 2004). The 16S rRNA gene sequence of strain XH-22^T was aligned with 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. Phylogenetic trees were constructed by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms integrated within the ARB software for phylogenetic inference. Confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

An almost-complete 16S rRNA gene sequence of strain XH-22^T (1472 nt) was obtained and used for initial BLAST and EzTaxon searches in GenBank and for phylogenetic analysis. Comparative 16S rRNA gene sequence analysis revealed that strain XH-22^T was affiliated most closely with the genus *Virgibacillus*. In the phylogenetic tree based on the neighbour-joining algorithm, strain XH-22^T fell within the radiation of a cluster comprising *Virgibacillus* species, forming a cluster with the type strains of *V. carmonensis* and *V. necropolis* with a high level of bootstrap support (88 %) (Fig. 1). The relationships among strain XH-22^T and the type strains of *V. carmonensis* and *V. necropolis* were also maintained in trees based on the maximum-likelihood and maximum-parsimony algorithms (data not shown). Strain XH-22^T exhibited levels of 16S rRNA gene sequence similarity of 97.6 and 97.5 % with *V. carmonensis* LMG 20964^T and *V. necropolis* LMG 19488^T, respectively, and values of between 96.9 and 94.9 % with the type strains of other recognized *Virgibacillus* species. DNA–DNA

Table 1. Differential characteristics between strain XH-22^T and related *Virgibacillus* species

Taxa: 1, strain XH-22^T (*Virgibacillus salinus* sp. nov.); 2, *V. carmonensis* (data from Heyrman *et al.*, 2003); 3, *V. necropolis* (Heyrman *et al.*, 2003); 4, *V. pantothenicus* (Heyndrickx *et al.*, 1998); 5, *V. proomii* (Heyndrickx *et al.*, 1998); 6, *V. salexigens* (Garabito *et al.*, 1997); 7, *V. marismortui* (Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003); 8, *V. halodenitrificans* (Yoon *et al.*, 2004); 9, *V. olivae* (Quesada *et al.*, 2007); 10, *V. dokdonensis* (Yoon *et al.*, 2005); 11, *V. koreensis* (Lee *et al.*, 2006); 12, *V. halophilus* (An *et al.*, 2007); 13, *V. kekensis* (Chen *et al.*, 2008); 14, *V. chiguensis* (Wang *et al.*, 2008); 15, *V. salarius* (Hua *et al.*, 2008). +, Positive; –, negative; w, weakly positive; ND, no data available; v, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Oxidase	–	+	+	ND	ND	+	+	+	+	+	+	+	+	ND	+
Colony pigmentation	Cream	Pink	–	–	–	–	–	–	Yellow	–	–	Yellow	–	–	White
Anaerobic growth	–	–	–	+	+	–	–	+	–	+	+	–	–	+	–
Temperature range (°C)	10–40	10–40	10–40	15–50	15–50	15–45	15–50	10–45	20–45	15–50	10–45	5–45	10–50	ND	10–50
Optimum temperature (°C)	37	25–30	25–35	37	37	37	37	38	30	ND	25	ND	37	ND	30–35
Nitrate reduced to nitrite	+	+	+	v	–	–	+	+	+	–	–	+	+	+	–
Hydrolysis of:															
Aesculin	+	–*	–	+	+	+	+	v	v	+	+	ND	–	+	+
Casein	–	–*	–*	+	+	+	+	–	v	+	ND	ND	–	+	+
Gelatin	–	+	+	+	v	+	+	+	+	+	–	ND	–	+	w
Acid production from:															
D-Galactose	+	–	–	–	+	–	–	+	–	+	–	w	–	+	–
D-Glucose	+	–	+	–	+	+	+	+	–	+	w	+	+	+	+
D-Fructose	+	–	+	–	+	+	+	v	+	+	+	+	–	+	+
D-Mannitol	–	–	–	–	–	+	–	+	–	–	–	+	w	w	w
D-Rhamnose	+	–	–	+	–	–	–	–	–	–	ND	–	ND	–	–
Trehalose	+	–	w	+	+	–	–	+	ND	–	w	+	w	–	–
DNA G+C content (mol%)	38.8	38.9	37.3	36.9–38.3	36.8–37.0	36.3–39.5	39.0–42.8	38.0–39.0	30.7–33.4	36.7	41.0	42.6	41.8	37.3	37.3

*Data from the present study.

hybridization was carried out to evaluate levels of genomic DNA–DNA relatedness between strain XH-22^T and *V. carmonensis* DSM 14868^T and *V. necropolis* DSM 14866^T, following the competition procedure of Johnson (1994) as described in detail by Márquez *et al.* (2007). The hybridization temperature used was 45.2 °C, which was within the limit of validity for the filter method (De Ley & Tijtgat, 1970) and the level of DNA–DNA relatedness was calculated according to Johnson (1994). Experiments were carried out in triplicate. Levels of DNA–DNA relatedness between strain XH-22^T and *V. carmonensis* DSM 14868^T and *V. necropolis* DSM 14866^T were 32 and 28 %, respectively. These values were clearly lower than the 70 % cut-off generally accepted for species delineation and support the placement of strain XH-22^T as representing a genotypically distinct species within the genus *Virgibacillus* (Wayne *et al.*, 1987).

The G+C content of the genomic DNA was determined from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979). The G+C content of the DNA of

strain XH-22^T was 38.8 mol%. This is within the range of values described for species of the genus *Virgibacillus*, and is similar to those described for the type strains of *V. carmonensis* (38.9 mol%; Heyrman *et al.*, 2003) and *V. necropolis* (37.3 mol%; Heyrman *et al.*, 2003) (Table 1).

Analysis of the cell-wall peptidoglycan, quinones and polar lipids of strain XH-22^T was carried out by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Cell biomass for these analyses was obtained by cultivation on maintenance medium at 37 °C. Strain XH-22^T contained a cell-wall peptidoglycan based on *meso*-diaminopimelic acid. MK-7 (88 %) was the predominant menaquinone, as reported for recognized species of the genus *Virgibacillus*; however, strain XH-22^T had MK-6 (3 %), MK-5 (3 %) and an unidentified respiratory quinone (about 6 %) present in minor amounts, whereas *V. carmonensis* DSM 14868^T and *V. necropolis* DSM 14866^T had MK-6 and MK-8 as minor components (Heyrman *et al.*, 2003). The polar lipid profile of strain XH-22^T comprised diphosphatidylglycerol, phosphatidylglycerol, a glycolipid and two phospholipids of

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence comparisons, showing the relationship between strain XH-22^T and related species. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup. Bootstrap values of $\geq 50\%$ are shown at nodes. Bar, 0.02 substitutions per nucleotide position.

unknown structure. The presence of diphosphatidylglycerol and phosphatidylglycerol as major polar lipids is a common characteristic of *Virgibacillus* species (Heyrman *et al.*, 2003; Wang *et al.*, 2008) but the presence of a glycolipid has not previously been reported for members of the genus.

Fatty acid analysis was performed by using the MIDI system (Microbial Identification System) and was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG bacterial collection. Cells were cultured on maintenance medium at 37 °C for 24 h. The predominant fatty acids of strain XH-22^T were anteiso-C_{15:0} (32.0 %), C_{16:0} (20.5 %) and iso-C_{14:0} (17.1 %). The detailed fatty acid profile was similar to those of recognized species of the genus *Virgibacillus*, although there were differences in the proportion of some fatty acids that might have been caused by different cultivation conditions (Table 2).

Strain XH-22^T showed some differences in phenotypic characteristics from recognized *Virgibacillus* species (Table 1). In particular, strain XH-22^T can be distinguished clearly from *V. carmonensis*, its nearest phylogenetic neighbour, based on differences in the oxidase reaction, colony pigmentation, optimum temperature for growth and acid production from various substrates (Table 1). The phylogenetic distinctiveness and DNA–DNA relatedness data clearly suggest that strain XH-22^T represents a species that is distinct from recognized *Virgibacillus* species

(Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain XH-22^T should be classified in the genus *Virgibacillus* as representing a novel species, for which the name *Virgibacillus salinus* sp. nov. is proposed.

Table 2. Cellular fatty acid profiles of strain XH-22^T and the type strains of *V. carmonensis* and *V. necropolis*

Strains: 1, XH-22^T (*Virgibacillus salinus* sp. nov.); 2, *V. carmonensis* DSM 14868^T (data from Heyrman *et al.*, 2003); 3, *V. necropolis* DSM 14866^T (Heyrman *et al.*, 2003). Values are percentages of total fatty acids; values <1 % are not shown.

Fatty acid	1	2	3
Straight chain			
C _{14:0}	4.2	—	—
C _{16:0}	20.5	1.1	1.3
Branched			
iso-C _{14:0}	17.1	3.5	2.9
iso-C _{15:0}	9.4	4.5	4.2
anteiso-C _{15:0}	32.0	65.5	71.5
iso-C _{16:0}	3.6	3.6	3.6
anteiso-C _{17:0}	1.7	9.4	9.3
Unsaturated			
C _{16:1} ω7c alcohol	1.4	5.0	2.4
C _{16:1} ω11c	6.7	1.0	1.0

Description of *Virgibacillus salinus* sp. nov.

Virgibacillus salinus (sa.li'nus. L. masc. adj. *salinus* of or belonging to salt).

Cells are Gram-positive, $0.9 \times 1.5\text{--}6.0\ \mu\text{m}$, occurring singly, in pairs or in short chains. Cells are motile. Spherical to ellipsoidal endospores are produced at subterminal or terminal positions in swollen sporangia after 4 days cultivation on Mn-medium at $28\ ^\circ\text{C}$. Colonies are circular, opaque, entire, 1 mm in diameter and cream-pigmented on HM medium supplemented with 10% salts after 24 h cultivation at $37\ ^\circ\text{C}$. Moderately halophilic; is able to grow over a wide range of salt concentrations (3–20%, w/v), with optimal growth at 10% (w/v) salts. No growth occurs in the absence of NaCl. Grows at $15\text{--}40\ ^\circ\text{C}$ (optimum, $37\ ^\circ\text{C}$) and pH 6.0–10.0 (optimum, pH 7.5). Strictly aerobic. Catalase-positive and oxidase-negative. Nitrate is reduced to nitrite. Acid is produced from arabinose, D-fructose, galactose, D-glucose, D-lactose, maltose, sucrose, trehalose and D-xylose, but not from glycerol or D-mannitol. Casein, gelatin and starch are not hydrolysed, but Tween 80 and aesculin are hydrolysed. H_2S is not produced. Indole, methyl red, Simmons citrate, Voges–Proskauer, phosphatase, arginine dihydrolase, and lysine and ornithine decarboxylase tests are negative. The following compounds are utilized as sole carbon and energy sources: D-arabinose, D-galactose, D-lactose, maltose, sucrose, D-xylose, ethanol, D-mannitol, xylitol, citrate, formate and propionate. The following compounds are not utilized: starch, cellobiose, D-fructose, L-fucose, D-glucose, D-mannose, melibiose, raffinose, D-ribose, trehalose, butanol, dulcitol, glycerol, *myo*-inositol, propanol, D-sorbitol, methanol, acetate, benzoate, fumarate, succinate and valerate. The following compounds are used as sole carbon, nitrogen and energy sources: L-alanine, L-arginine, L-methionine, L-serine, L-threonine and tryptophan. The following compounds are not used: L-cysteine, phenylalanine, glutamic acid and L-ornithine. Susceptible to ampicillin (10 μg), bacitracin (10 U), chloramphenicol (30 μg), erythromycin (15 μg) and novobiocin (30 μg). Resistant to gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (10 μg), penicillin (10 U) and rifampicin (30 μg). The peptidoglycan type is A1 γ , with *meso*-diaminopimelic acid as the diagnostic diamino acid. MK-7 is the predominant menaquinone, with MK-6, MK-5 and an unidentified respiratory quinone present in minor amounts. The predominant cellular fatty acids are anteiso-C_{15:0}, C_{16:0} and iso-C_{14:0}; iso-C_{15:0}, C_{16:1} ω 11c, C_{14:0}, iso-C_{16:0}, anteiso-C_{17:0}, C_{18:0}, C_{16:1} ω 7c alcohol, iso-C_{17:0}, anteiso-C_{13:0} and iso-C_{13:0} are present as minor components. Polar lipids are diphosphatidylglycerol, phosphatidylglycerol, a glycolipid and two unidentified phospholipids. The DNA G+C content of the type strain is 38.8 mol% (T_m). The description of this species is based upon study of a single strain.

The type strain, XH-22^T (=CCM 7562^T=CECT 7439^T=DSM 21756^T), was isolated from sediment of a salt lake near Xilinhot in Inner Mongolia, China.

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References

- An, S.-Y., Asahara, M., Goto, K., Kasai, H. & Yokota, A. (2007). *Virgibacillus halophilus* sp. nov., spore-forming bacteria isolated from soil in Japan. *Int J Syst Evol Microbiol* **57**, 1607–1611.
- Arahal, D. R., Márquez, M. C., Volcani, B. E., Schleifer, K. H. & Ventosa, A. (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. *Int J Syst Bacteriol* **49**, 1501–1503.
- Arahal, D. R., Márquez, M. C., Volcani, B. E., Schleifer, K. H. & Ventosa, A. (2000). Reclassification of *Bacillus marismortui* as *Salibacillus marismortui* comb. nov. *Int J Syst Evol Microbiol* **50**, 1501–1503.
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**, 493–496.
- Carrasco, I. J., Márquez, M. C., Xue, Y., Ma, Y., Cowan, D. A., Jones, B. J., Grant, W. D. & Ventosa, A. (2007). *Salsuginibacillus kocurii* gen. nov., sp. nov., a moderately halophilic bacterium from soda-lake sediment. *Int J Syst Evol Microbiol* **57**, 2381–2386.
- Chen, Y.-G., Cui, X.-L., Fritze, D., Chai, L.-H., Schumann, P., Wen, M.-L., Wang, Y.-X., Xu, L.-H. & Jiang, C.-L. (2008). *Virgibacillus kekensis* sp. nov., a moderately halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **58**, 647–653.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- De Ley, J. & Tijtgat, R. (1970). Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie van Leeuwenhoek* **36**, 461–474.
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Garabito, M. J., Arahal, D. R., Mellado, E., Márquez, M.-C. & Ventosa, A. (1997). *Bacillus salexigens* sp. nov., a new moderately halophilic *Bacillus* species. *Int J Syst Bacteriol* **47**, 735–741.
- García, M. T., Ventosa, A., Ruiz-Berraquero, F. & Kocur, M. (1987). Taxonomic study and amended description of *Vibrio costicola*. *Int J Syst Bacteriol* **37**, 251–256.
- Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N. A. (1998). *Virgibacillus*: a new genus to accommodate *Bacillus pantothenicus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenicus*. *Int J Syst Bacteriol* **48**, 99–106.
- Heyndrickx, M., Lebbe, L., Kersters, K., Hoste, B., De Wachter, R., De Vos, P., Forsyth, G. & Logan, N. A. (1999). Proposal of *Virgibacillus promii* sp. nov. and emended description of *Virgibacillus pantothenicus* (Proom and Knight 1950) Heyndrickx et al. 1998. *Int J Syst Bacteriol* **49**, 1083–1090.
- Heyrman, J., Logan, N. A., Busse, H.-J., Balcaen, A., Lebbe, L., Rodríguez-Díaz, M., Swings, J. & De Vos, P. (2003). *Virgibacillus*

- carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. *Int J Syst Evol Microbiol* 53, 501–511.
- Hua, N.-P., Hamza-Chaffai, A., Vreeland, R. H., Isoda, H. & Naganumal, T. (2008). *Virgibacillus salarius* sp. nov., a halophilic bacterium isolated from a Saharan salt lake. *Int J Syst Evol Microbiol* 58, 2409–2414.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–681. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Lee, J.-S., Lim, J.-M., Lee, K. C., Lee, J.-C., Park, Y.-H. & Kim, C.-J. (2006). *Virgibacillus koreensis* sp. nov., a novel bacterium from a salt field, and transfer of *Virgibacillus picturae* to the genus *Oceanobacillus* as *Oceanobacillus picturae* comb. nov. with emended descriptions. *Int J Syst Evol Microbiol* 56, 251–257.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadukumar, X., Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* 32, 1363–1371.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5, 109–118.
- Márquez, M. C., Carrasco, I. J., Xue, Y., Ma, Y., Cowan, D. A., Jones, B. J., Grant, W. D. & Ventosa, A. (2007). *Aquisalimonas asiatica* gen. nov., sp. nov., a moderately halophilic bacterium isolated from an alkaline, saline lake in Inner Mongolia, China. *Int J Syst Evol Microbiol* 57, 1137–1142.
- Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists* (Society for Applied Bacteriology Technical Series no. 14), 2nd edn, pp. 277–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.
- Quesada, E., Ventosa, A., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1984). *Deleya halophila*, a new species of moderately halophilic bacteria. *Int J Syst Bacteriol* 34, 287–292.
- Quesada, T., Aguilera, M., Morillo, J. A., Ramos-Cormenzana, A. & Monteoliva-Sánchez, M. (2007). *Virgibacillus olivae* sp. nov., isolated from waste wash-water from processing of Spanish-style green olives. *Int J Syst Evol Microbiol* 57, 906–910.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44, 846–849.
- Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* 128, 1959–1968.
- Wang, C.-Y., Chang, C.-C., Ng, C. C., Chen, T.-W. & Shyu, Y.-T. (2008). *Virgibacillus chiguensis* sp. nov., a novel halophilic bacterium isolated from Chigu, a previously commercial saltern located in southern Taiwan. *Int J Syst Evol Microbiol* 58, 341–345.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37, 463–464.
- Yoon, J.-H., Oh, T. K. & Park, Y.-H. (2004). Transfer of *Bacillus halodenitrificans* Denariáz et al. 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov. *Int J Syst Evol Microbiol* 54, 2163–2167.
- Yoon, J.-H., Kang, S. J., Lee, S. Y., Lee, M. H. & Oh, T. K. (2005). *Virgibacillus dokdonensis* sp. nov., isolated from a Korean island, Dokdo, located at the edge of the East Sea in Korea. *Int J Syst Evol Microbiol* 55, 1833–1837.