Virgibacillus salarius sp. nov., a halophilic bacterium isolated from a Saharan salt lake

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A Gram-positive, endospore-forming, rod-shaped and moderately halophilic bacterium was isolated from a salt-crust sample collected from Gharsa salt lake (Chott el Gharsa), Tunisia. The newly isolated bacterium, designated SA-Vb1^T, was identified based on polyphasic taxonomy including genotypic, phenotypic and chemotaxonomic characterization. Strain SA-Vb1^T was closely related to the type strains of *Virgibacillus marismortui* and *Virgibacillus olivae*, with 16S rRNA gene sequence similarities of 99.7 and 99.4 %, respectively. However, strain SA-Vb1^T was distinguished from these two type strains on the basis of phenotypic characteristics and DNA–DNA relatedness (29.4 and 5.1 %, respectively). The genetic relationship between strain SA-Vb1^T and *Virgibacillus pantothenicus* IAM 11061^T (the type strain of the type species) and other type strains of the genus was 96–98 % based on 16S rRNA gene sequence similarity and 18.3–22.3 % based on DNA–DNA hybridization. Biochemical analysis resulted in determination of major fatty acids iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0} (33.3, 29.2 and 9.8 %, respectively); phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine were the main polar lipids and MK-7 was the predominant menaquinone (~100 %). The distinct characteristics demonstrated by strain SA-Vb1^T represent properties of a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus salarius* sp. nov. is proposed. The type strain is SA-Vb1^T (=JCM 12946^T =DSM 18441^T).

Chott el Gharsa (also known as Chott Gharsa or Chott el Rharsa) is one of several dry salt lakes located in the Tunisian Sahara. The lake is 10–25 m below sea level (Swezey *et al.*, 1999) and represents an important local source of salt. The salt composition is similar to that of concentrated ocean water, making these lakes thalassohaline even though they are located in a desert (Kbir-Arigoib *et al.*, 2001). Other than early work on *Halobacterium* species (Pfeifer *et al.*, 1981) and on microbial communities in evaporites such as gypsum (Barbieri *et al.*, 2006;

Stivaletta *et al.*, 2006), few microbiological studies have been conducted in the Tunisian chotts. Recently, from samples collected from Tunisian salt lakes, we isolated several halophilic and halotolerant bacteria including strains tentatively affiliated with the genus *Virgibacillus* Heyndrickx *et al.* 1998, with type species *Virgibacillus pantothenicus*, which has 13 species at the time of writing. Here we report the taxonomic description of a moderately halophilic, Gram-positive, rod-shaped bacterium, strain SA-Vb1^T, which represents a novel species of the genus *Virgibacillus*.

Strain SA-Vb1^T was originally isolated from a mixture of dry sediment and salt crusts collected directly from Chott el Gharsa. The sample was inoculated into ATCC medium 925 HP 101 [containing 1 % (w/v) peptone, 0.1 % yeast extract, 10 % NaCl, 0.43 % MgSO₄·7H₂O, 0.2 % NaNO₃, pH 7.2] and incubated at 30 °C. Pure cultures were

Abbreviation: CZE, capillary zone electrophoresis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SA-Vb1^T is AB197851.

Fatty acid profiles of strain SA-Vb1^T and related type strains and detailed 16S rRNA gene sequence similarity and DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

obtained by three successive single colony isolations. The following strains showing 16S rRNA gene sequence similarity of approximately $\geq 97\%$ to the novel isolate were used in DNA–DNA hybridization and complementary tests: *Virgibacillus marismortui* DSM 12325^T (Arahal *et al.*, 1999; Heyrman *et al.*, 2003), *Virgibacillus olivae* DSM 18098^T (Quesada *et al.*, 2007), *Oceanobacillus picturae* DSM 14867^T (Heyrman *et al.*, 2003; Lee *et al.*, 2006), *Virgibacillus carmonensis* DSM 14868^T, *Virgibacillus necropolis* DSM 14866^T (Heyrman *et al.*, 2003), *Virgibacillus halodenitrificans* DSM 10037^T (Denariáz *et al.*, 1989) and *Virgibacillus proomii* DSM 13055^T (Heyndrickx *et al.*, 1999).

The strains were characterized phenotypically using several common tests. ATCC medium 925 HP 101 with addition of NaCl up to 30% (w/v) was applied to determine the optimal NaCl concentration for growth. Other tests that were conducted using conventional methods included Gram reaction, acid-fast staining, motility, endospore observation (Doetsch, 1981), anaerobic growth, nitrate reduction, enzyme activities (catalase, urease, phenylalanine deaminase, oxidase) and hydrolysis of starch, aesculin, gelatin and casein (Smibert & Krieg, 1981). The ability of the bacteria to use single carbon sources was tested using Biolog GP microplates following the instructions of the manufacturer (Garland & Mills, 1991). Acid production from carbohydrates, nitrate reduction and hydrolysis of some polymers were determined using API 50CH and API 20 CE kits (bioMérieux). In all these tests at 30 °C, strains SA-Vb1^T and DSM 12325^T were cultured optimally in media supplemented with 10% NaCl; similarly, strains DSM 10037^T, DSM 14866^T, DSM 14867^T and DSM 14868^T were cultured with 7% NaCl and strain DSM 13055^T was cultured in DSM medium 1 without NaCl (0.5% peptone, 0.3% meat extract, trace MnSO₄·H₂O, pH 7.0; Heyndrickx *et al.*, 1999).

Phenotypic characteristics of strain SA-Vb1^T are shown in the species description. Several physiological and biochemical properties are compared with related species in Table 1. Strain SA-Vb1^T was distinguished from the closely related strain *V. olivae* DSM 18098^T in its halophilism and NaCl range for growth (0.5–25% compared with 0–20%), in colony morphology and in the range of temperature for growth (10–50 °C compared with 20–45 °C) and from both *V. olivae* DSM 18098^T and *V. marismortui* DSM 12325^T in its inability to reduce nitrate to nitrite and its hydrolysis of Tween 80.

Cellular fatty acids of strain SA-Vb1^T were determined using the Microbial Identification System (MIDI) using an HP6890 GC (Hewlett Packard) (Sasser, 2001). The major fatty acids detected in strain SA-Vb1^T were iso-C_{14:0} (4.8%), iso-C_{15:0} (33.3%), anteiso-C_{15:0} (29.2%), C_{16:0} (3.9%), iso-C_{16:0} (5.9%), C_{16:1 ω 11c} (1.8%), C_{16:1 ω 7c} alcohol (1.9%), iso-C_{17:0} (6.6%) and anteiso-C_{17:0} (9.8%). Acids C_{15:0}, C_{18:0} and C_{18:1 ω 9c} found in strain *V. olivae* E₃₀₈^T (Quesada *et al.*, 2007) were not detected in

strain SA-Vb1^T. The fatty acid profile of isolate SA-Vb1^T was also qualitatively and quantitatively different from that of strain *V. marismortui* DSM 12325^T (Vreeland *et al.*, 2006) (see Supplementary Table S1 available in IJSEM Online).

In order to determine other chemotaxonomic properties of strain SA-Vb1^T, cell-wall peptidoglycan was determined by HPTLC and HPLC (Komagata & Suzuki, 1987), quinone analysis was done by HPLC according to Nishijima *et al.* (1997) and polar lipids were examined by TLC (Komagata & Suzuki, 1987). Strain SA-Vb1^T possessed *meso*-diaminopimelic acid in the cell-wall peptidoglycan. The predominant quinone detected was MK-7 (~100%). The major polar lipids were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine; two unknown phospholipids were present as minor components (Table 1). These chemotaxonomic properties are commonly found in other members of genus *Virgibacillus* (Heyndrickx *et al.*, 1998, 1999; Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003). The genomic DNA G + C content of strain SA-Vb1^T was determined by capillary zone electrophoresis (CZE) (Fraga *et al.*, 2002; Hua & Naganuma, 2007). Briefly, highly purified genomic DNAs were enzymically hydrolysed into nucleosides (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). Nucleosides were then eluted in an alkaline phosphate buffer system, separated and quantitatively detected by a CAPI-3300 multichannel CZE (Otsuka Electronics). The analysis resulted in a G + C content of 37.4 mol% for strain SA-Vb1^T (Hua & Naganuma, 2007) compared with 40.7 and 33.4 mol%, respectively, for *V. marismortui* DSM 12325^T and *V. olivae* DSM 18098^T (Arahal *et al.*, 1999; Quesada *et al.*, 2007) (Table 1).

Genomic DNA was extracted from stationary phase cultures according to the protocol of Wilson (1995). The 16S rRNA gene was amplified by the protocol of DeLong (1992). A nearly full-length sequence (1564 bp) of the 16S rRNA gene was obtained using a BigDye Terminator ready reaction cycle sequencing kit. Separation of bases was carried out on a 3730xl DNA analyser (Applied Biosystems). Related sequences were identified by normal BLAST search on public databases (GenBank\EMBL\DDBJ) using the FASTA program (Pearson & Lipman, 1988). All sequences were aligned with CLUSTAL_X software v. 1.83 (Thompson *et al.*, 1997) and checked manually. Phylogeny was inferred using treeing programs constructed with the neighbour-joining, minimum evolution and maximum-parsimony methods in MEGA 3.1 (Kumar *et al.*, 2004) and compared with those inferred by using the SEQBOOT, DNADIST, DNAMLK and CONSENSE programs of the PHYLIP package v. 3.61 (Felsenstein, 2004) through 100–1000 replications. The neighbour-joining tree (Fig. 1) is displayed since it showed a topology similar to all other trees and had high bootstrap values (Page, 1996). The 16S rRNA gene sequence of strain SA-Vb1^T was very similar to those of *V. marismortui* 123^T and *V. olivae* E₃₀₈^T (99.7 and 99.4% similarity, respectively; Supplementary Table S2),

Table 1. Differential characteristics of the novel isolate and type strains of related species

Strains: 1, strain SA-Vb1^T (data from this study); 2, *V. olivae* E₃₀8^T (data from Quesada *et al.*, 2007); 3, *V. marismortui* DSM 12325^T (unless indicated, data from Arahal *et al.*, 1999; Heyrman *et al.*, 2003); 4, *V. proomii* DSM 13055^T (Heyndrickx *et al.*, 1999); 5, *V. carmonensis* DSM 14868^T (Heyrman *et al.*, 2003); 6, *V. halodenitrificans* DSM 10037^T (Denariáz *et al.*, 1989; Yoon *et al.*, 2004); 7, *O. picturae* DSM 14867^T (Heyrman *et al.*, 2003; Lee *et al.*, 2006); 8, *V. necropolis* DSM 14866^T (Heyrman *et al.*, 2003); 9, *V. dokdonensis* DSW-10^T (Yoon *et al.*, 2005); 10, *V. chiguensis* NTU-101^T (Wang *et al.*, 2008). +, Positive; –, negative; w, weak; v, variable; ND, no data available. All strains are Gram-positive, rod-shaped, motile and spore-forming and are positive for catalase, oxidase and hydrolysis of casein.

Characteristic	1	2	3	4	5	6	7	8	9	10
Spore morphology*										
Shape	E(s)	SE	E	ES	ES	E	ES	E	SE	SE
Position	ST	TS	TS	TS	S	TS	T	CTS	TS	TS
Temperature range for growth (°C)	10–50	20–45	11–50†	15–50	10–40	10–45	5–40	10–40	15–50	15–55
Growth in:										
0.5 % NaCl	+	+	+†	+†	–	–	w	w	+	+
25 % NaCl	+	–	–	–†	–	+	–	–	–	+
Anaerobic growth	–	–	–	+	–	+	–	–	+	+
Nitrate reduction	–	+	+	–	+	+	+	+	–	+
Hydrolysis of:										
Aesculin	+	+	+	+	w	–	w	–	+	+
Gelatin	w	+	+	+	–	+	v	w	+	+
Starch	–	+	–	v	–†	–	–†	–†	+	+
Tween 80	+	–	–	–†	–†	–	–†	–†	+	+
Urease	–	ND	+	–†	–	–	–	w	–	ND
H ₂ S production	–	ND	+	–	–	–	–	–	ND	–
Acid production from:										
N-Acetylglucosamine	+	ND	+	+	–	+†	w	w	ND	ND
D-Arabinose	–	ND	–	–	–	+†	–	–	–	ND
D-Galactose	–	–	–	+	–	+	w	–	+	+
D-Glucose	+	–	+	+	–	+	w	w	+	+
D-Fructose	+	+	+	+	–	+	w	w	+	+
L-Fucose	–	ND	–	–	–	–†	–	–	ND	ND
D-Mannose	+	–	+	v	–	+	w	–	+	+
L-Rhamnose	–	–	–	v	–	–	–	–	–	ND
Trehalose	–	ND	–	v	–	+	v	w	–	–
Turanose	–	ND	–	–	–	+†	v	–	ND	ND
Glycerol	+	ND	w	–	–	+†	w	w	ND	ND
DNA G + C content (mol%)	37.3	33.4	40.7	36.8	38.9	38.0	39.5	37.4	36.7	37.3
Major polar lipids‡	DPG, PG, PE	ND	DPG, PG, PE	DPG, PG, PE	DPG, PG	DPG, PG	DPG, PG	DPG, PG	DPG, PG, PE	DPG, PG, PE

*Shape: E, ellipsoidal; S, spherical. Position: C, central; S, subterminal; T, terminal.

†Data obtained in this study.

‡DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

which formed a lineage distinct from all other species (96–98 % sequence similarity; Supplementary Table S2) in phylogenetic trees constructed by different algorithms. The data indicate that strain SA-Vb1^T should be classified as a member of the genus *Virgibacillus* (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

DNA–DNA hybridization between strain SA-Vb1^T and phylogenetically related strains was done by applying the DIG non-radioactive nucleic acid labelling and detection

system (Roche Molecular Biochemicals). DIG-11–dUTP-labelled ssDNAs of the target strain were hybridized with reference ssDNAs immobilized on positively charged nylon filter membranes (Brown, 1995) using the DIG-High Prime DNA Labelling, Detection Starter kit II and DIG Wash and block buffer set (Roche Molecular Biochemicals) following the manufacturer's instructions. Chemiluminescent density of hybrids was detected using the VersaDoc Imaging System model 5000 and analysed with Quantity One software v. 4.4 (Bio-Rad). Hybridizations were performed

Fig. 1. Neighbour-joining phylogenetic tree showing positions of strain SA-Vb1^T and species of the genus *Virgibacillus* and other related bacteria based on 16S rRNA gene sequences. Bootstrap values (percentages of 1000 replications) >50% are shown at nodes. Bar, 0.01 nucleotide substitutions per site.

at least three times and means were calculated. The DNA–DNA relatedness of strain SA-Vb1^T to strains *V. marismortui* DSM 12325^T and *V. olivae* DSM 18098^T was 39.9 and 5.1%, respectively; hybridization values with other related strains *O. picturae* DSM 14867^T (22.3%), *V. carmonensis* DSM 14868^T (23.1%), *V. halodenitrificans* DSM 10037^T (21.1%), *V. necropolis* DSM 14866^T (18.3%) and *V. proomii* DSM 13055^T (21.6%) were also lower than the level conventionally accepted for species delineation (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002) (Supplementary Table S2).

Strain SA-Vb1^T shares the common phenotypic, chemotaxonomic and genotypic characteristics of species of the genus *Virgibacillus* but it is different from existing species. Therefore it is proposed to represent a novel species of the genus, named *Virgibacillus salarius* sp. nov.

Description of *Virgibacillus salarius* sp. nov.

Virgibacillus salarius (sa.la'ri.us. L. masc. adj. *salarius* of or belonging to salt).

Rod-shaped, Gram-positive, motile cells, 0.6–0.9 × 1.8–3.5 µm (Fig. 2). Cells occur singly, in pairs or as short chains. Endospores are spherical or ellipsoidal and are located in a subterminal or terminal position of swollen sporangia. Colonies are circular and convex with erose or slightly filamentous margins, opaque and white in colour, 2.0–2.5 mm in diameter after 48 h at 30–35 °C on solid medium containing 10% NaCl. Halophilic; grows weakly at 0.5% NaCl and does not grow in medium without NaCl. Growth occurs optimally at 30–35 °C, 7–10% (w/v) NaCl and around pH 7.5. Ranges for growth are 10–50 °C, 0.5–25% NaCl and pH 5.5–10. Growth does not occur under anaerobic conditions and nitrate is not reduced to nitrite. Tests for catalase, oxidase and gelatinase are positive. Tests for acid-fast staining, phenylalanine deaminase, tryptophan deaminase, arginine dihydrolase and urease are negative. Tweens 40 and 80, casein and aesculin are hydrolysed. Starch is not hydrolysed. H₂S and indole are not produced. Acid is produced from D-glucose, D-fructose, D-mannose,

N-acetylglucosamine, arbutin, maltose, D-tagatose, glycerol, salicin and cellobiose. Acid is not produced from DL-arabinose, DL-xylose, D-galactose, L-rhamnose, inositol, D-mannitol, sucrose, trehalose or melibiose. The following substrates are used as single carbon sources: Tweens 40 and 80, N-acetyl-D-glucosamine, D-fructose, D-mannose, gentiobiose, α-ketobutyric acid, α-ketoglutaric acid, uridine and thymidine. The major menaquinone is MK-7. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. Phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two other unknown phospholipids are cellular polar lipids. Fatty acids are iso-C_{15:0} and anteiso-C_{15:0} (major components) and iso-C_{14:0}, C_{16:0}, iso-C_{16:0}, C_{16:1ω11c}, C_{16:1ω7c} alcohol, iso-C_{17:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 37.3 mol% (determined by CZE).

The type strain, SA-Vb1^T (=JCM 12946^T =DSM 18441^T), was isolated from a salt crust collected from Chott el Gharsa, Tunisia.

Fig. 2. Phase-contrast micrograph of cells of strain SA-Vb1^T. Bar, 5 µm.

Acknowledgements

We thank the Alliance for Research on North Africa, University of Tsukuba, Japan, for their help in sample collection. Part of this work was supported by a Grant-in-Aid for Scientific Research (17657032) from the Japan Society for the Promotion of Science.

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