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

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

Summary

A Gram-positive, endospore-forming, rod-shaped and moderately halophilic bacterium was isolated from a salt crust sample collected in 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 *Virgibacillus marismortui* and *V. olivae* with 16S rRNA gene sequence similarities of 99.7% and 99.4%, respectively. However, strain SA-Vb1^T was distinguished from these two species on phenotypic characteristics and on the basis of DNA-DNA relatedness (29.4% and 5.1%, respectively). Genetic relationships between strain SA-Vb1^T and the type species *V. pantothenticus* IAM 11061^T and other type strains of the genus were 96-98% based on 16S rRNA gene 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); phosphatidyl glycerol, diphosphatidyl glycerol, and phosphatidyl ethanolamine were the main polar lipids; and MK-7 the predominant menaquinone (~100%). The distinct characteristics demonstrated by these data show that strain SA-Vb1^T represents properties of a novel species of the genus *Virgibacillus*. Accordingly, this isolate is proposed as *Virgibacillus salarius* sp. nov. 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, with the Tunisian word *Chott* meaning lake) is one of several dry salt lakes located in the Tunisian end of Grand Erg Oriental, the Sahara. The salt 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 concentrated ocean water making these lakes thallassohaline even though they are located in a desert (Kbir-Ariguib *et al.*, 2001). Other than early work on *Halobacterium* spp. (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 in Tunisian salt lakes, we isolated several halophilic and halotolerant bacteria including those tentatively affiliated with genus *Virgibacillus* which was described by Heyndrickx *et al*. since 1998 with type species *V. pantothenticus* that has thirteen species so far. Here we report the taxonomic description of one moderately halophilic, Gram-positive, rod-shaped bacterium, strain SA-Vb1^T as a novel species of genus *Virgibacillus*.

Strain SA-Vb1^T was originally isolated from a mixture of dry sediment and salt crusts collected directly in Chott el Gharsa, Tunisia. The sample was inoculated into ATCC medium 925 HP 101 (containing 1% (w/v) peptone, 0·1% yeast extract, 10% NaCl, 0·43% $MgSO_4 \cdot 7H_2O$, 0·2% $NaNO_3$, pH 7·2) and incubated at 30 °C. Pure cultures were obtained by three successive single colony isolations. The strains of following species whose 16S rDNA sequence similarity of approximately ≥ 97% to the novel isolate were used in DNA-DNA hybridization and complementary tests: *V. marismortui* DSM 12325^T (Arahal *et al.* 1999; Heyrman *et al.* 2003), *V. olivae* DSM 18098^T (Quesada *et al.*, 2007), *Oceanobacillus picturae* DSM 14867^T (Heyrman *et al.,* 2003; Lee *et al.,* 2006), *V. carmonensis* DSM

14868^T, *V. necropolis* DSM 14866^T (Heyrman *et al.,* 2003), *V. halodenitrificans* DSM 10037^T (Denariaz *et al*., 1989), and *V. 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 from 0% (w/v) to 30% was applied to determine 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, enzymatic activity (catalase, urease, phenylalanine deaminase, oxidase), hydrolysis of starch, aesculin, gelatine, and casein (Smibert & Krieg, 1981). The ability of the bacteria to uptake single carbon sources was tested using Biolog GP microplates (Biolog) following 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 (Biomerieux). In all these tests at 30 ºC, 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 with 7% NaCl; strain DSM 13055^T in DSM medium 1 without NaCl (0·5% peptone, 0·3% meat extract, trace $MnSO_4$ x H_2O, 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 of this bacterium are compared with related species in the Table 1. Strain SA-Vb1^T was distinguished from closely related strain *V. olivae* DSM 18098^T in halophilism and NaCl range for growth (0·5-25% versus 0-20%), in colony morphology and in the range of temperature for growth (10-50 ºC versus 20-45ºC); and from both strains *V. olivae* DSM 18098^T and *V. marismortui* DSM 12325^T in its unability to reduce nitrate to nitrite and its hydrolysis of Tween 80.

Cellular fatty acids of strain SA-Vb1^T were determined following the Microbial Identification System (MIDI) using an HP6890 (Hewlett-Packard) GC (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%), $C_{16:1}\alpha 11c$ (1·8%), $C_{16:1}\alpha 7c$ alcohol (1·9%), iso-$C_{17:0}$ (6·6%) and anteiso-$C_{17:0}$ (9·8%). Acids $C_{15:0}$, $C_{18:0}$ and $C_{18:1}\omega 9c$ those found on strain *V. olivae* DSM 18098^T (Quesada *et al.*, 2007) were not detected in strain SA-Vb1^T. Percentages and the composition of fatty acids of isolate SA-Vb1^T were also different from those of strain *V. marismortui* (Vreeland *et al.*, 2006; Supplementary Table 1).

For other chemotaxonomic properties of strain SA-Vb1^T, cell wall peptidoglycan was determined with HPTLC and HPLC (Komagata & Suzuki, 1987); quinones analysis was done with HPLC according to Nishijima *et al.* (1997); polar lipids with TLC (Komagata & Suzuki, 1987). Strain SA-Vb1^T possessed *meso*-diaminopimelic acid in the cell wall peptidoglycan. The predominant quinone detected in this bacterium was MK-7 (~100%). The major polar lipids were phosphatidyl glycerol, diphosphatidyl glycerol, and phosphatidyl ethanolamine and minor presence of two unknown phospholipids (Table 1). These chemotaxonomic properties are commonly found in other members of genus *Virgibacillus* (Heyndrickx *et al.,* 1998 & 1999; Arahal *et al.*, 1999a & b; Heyrman *et al.,* 2003). The genomic DNA G+C content of the strain SA-Vb1^T was determined by capillary zone electrophoresis (CZE) (Fraga *et al.,* 2002; Hua & Naganuma, 2007). Briefly, highly purified genomic DNAs were enzymatically hydrolysed into nucleosides (Tamaoka & Komagata, 1984; Mesbah *et al.,* 1989). Then, nucleosides were eluted in an alkaline phosphate buffer system, separated and quantitatively detected by a CAPI-3300 multichannel CZE (Otsuka Electronics). The analysis resulted in 37·4 mol% for strain SA-Vb1^T (Hua & Naganuma, 2007) compared to 40·7 and 33·4 mol% of *V. marismortui* DSM

116 12325^T and *V. olivae* DSM 18098^T, respectively (Arahal *et al.*, 1999a; Quesada *et al.*,
117 2007; Table 1).

118 Genomic DNA was extracted from stationary phase cultures according to the protocol of
119 Wilson (1995). The 16S rRNA gene was amplified by the protocol of DeLong (1992). The
120 nearly full length (1564 bp) of amplified 16S rDNA were sequenced using the BigDye^{TM}
121 Terminator Ready Reaction Cycle Sequencing Kit. The separation of the bases was carried
122 out on a 3730xl DNA analyzer (Applied Biosystems). Homologous sequences were
123 identified by normal BLAST search on public databases (GenBank\EMBL\DDBJ) using
124 FASTA program (Pearson & Lipman, 1988). All sequences were aligned with CLUSTAL
125 X software ver. 1.83 (Thompson *et al.,* 1997) and checked manually. Phylogeny was
126 inferred using treeing programs constructed with the neighbour-joining, minimum
127 evolution, and maximum parsimony methods in the Molecular Evolutionary Genetics
128 Analysis software (MEGA 3.1) (Kumar *et al.,* 2004) and compared with those inferred by
129 using SEQBOOT, DNADIST, DNAMLK, and CONSENSE programs of the PHYLIP
130 package ver. 3.61 (Felsenstein, 2004) through 100-1000 replications. The neighbour
131 joining tree shown in Figure 1 was chosen since it showed a topology similar to all other
132 trees and had high bootstrap values (Page, 1996). The 16S rRNA gene sequence of strain
133 SA-Vb1^T was very similar to those of *V. marismortui* DSM 12325^T and *V. olivae* DSM
134 18098^T (99.7% and 99.4%, respectively; Supplementary Table S2) that formed a distinct a
135 lineage to all others species (96-98% sequence similarity, Supplementary Table S2) in
136 phylogenetic trees constructed by different algorithms. The data indicate that strain SA-
137 V1^T should certainly be classified as members of the genus *Virgibacillus* (Stackebrandt &
138 Goebel, 1994; Stackebrandt *et al*., 2002).

139 DNA-DNA hybridization between the present bacterium and phylogenetically related
140 strains was done applying digoxigenin (DIG) nonradioactive nucleic acid labelling and

detection system (Roche Molecular Biochemicals). DIG-11-dUTP-labeled ssDNAs of the targeted strain were hybridized with reference ssDNAs immobilized on positively charged nylon filter membrane (Brown, 1995) using 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 VersaDoc Imaging System model 5000 and analyzed with Quantity One software ver. 4.4 (BIO-RAD Laboratories). Hybridizations were performed in at least triplicate and the results were averaged. The DNA-DNA relatedness of strain SA-Vb1^T to strains *V. marismortui* DSM 12325^T and *V. olivae* DSM 18098^T were 39.9% and 5.1%, respectively and other related species (Supplementary Table S2), *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 lower than species level conventionally accepted (Wayne *et al*., 1987; Stackebrandt *et al*., 2002).

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

Description of *Virgibacillus salarius* sp. nov.

Virgibacillus salarius (sala'rius. L. masc. adj. *salarius,* of or belonging to salt). The bacterium is rod-shaped, Gram-positive, and motile cells 0.6-0.9 μm by 1.8-3.5 μm. Cells occur singly, in pairs or as short chains. Endospores are spherical or ellipsoidal located at subterminal or terminal positions of swollen sporangia. Colonies are circular, convex with erose or slightly filamentous margins, in opaque and white colour; 2.0-2.5 mm in diameter after 48 hours at 30-35 ^oC on solid media containing 10% NaCl. Strain SA-Vb1^T is

166 halophilic and grows weakly at 0.5% NaCl or does not grow in media without NaCl.
167 Grows occurs optimally at 30-35 ºC, 7-10% (w/v) NaCl and pH around 7·5. Ranges for
168 growth of temperature is 10-50 ºC, of NaCl concentration is 0·5-25%, and of pH is 5·5-10.
169 Growth does not occur in anaerobic conditions and nitrate is not reduced to nitrite. Catalase,
170 oxidase, and gelatinase are positive. Acidfast, phenylalanine deaminase, tryptophan
171 diaminase, arginine dihydrolase, and urease are negative. Tween 40, Tween 80, casein, and
172 aesculin are hydrolysed. Starch is not hydrolysed. H_2S and indole are not produced. Acid is
173 produced from D-glucose, D-fructose, D-mannose, N-acetylglucosamine, arbutin, D-
174 maltose, D-tagatose, glycerol, salicin, and cellobiose. Acid is not produced from D,L-
175 arabinose, D,L-xylose, D-galactose, L-rhamnose, D-inositol, D-mannitol, D-sucrose, D-
176 trehalose, and D-melibiose. Following substrates are up taken as single carbon sources:
177 Tween 40 and 80, *N*-acetyl-D-glucosamine, D-fructose, D-mannose, gentiobiose, α-
178 ketobutyric acid, α-glutaric acid, uridin, and thymidin. The major menaquinone is MK-7.
179 Cell wall peptidoglycan contains *meso*-diaminopimelic acid. Phosphatidyl glycerol,
180 diphosphatidyl glycerol, phosphatidyl ethanolamine and two other unknown phospholipids
181 are cellular polar lipids. Fatty acids are iso-$C_{14:0}$ (4·77%), iso-$C_{15:0}$ (33·3%), anteiso-$C_{15:0}$
182 (29·2%), $C_{16:0}$ (3·9%), iso-$C_{16:0}$ (5·9%), $C_{16:1}$ ω11*c* (1·8%), $C_{16:1}$ ω7*c* alcohol (1·9%), iso-
183 $C_{17:0}$ (6·6%), and anteiso-$C_{17:0}$ (9·8%). The DNA G+C content is 37·3 mol% (determined by
184 capillary zone electrophoresis). Isolated from salt crust collected in Chott el Gharsa,
185 Tunisia. The type strain is SA-Vb1^T (=JCM 12946^T =DSM 18441^T).
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339 **List of Figure Legends**

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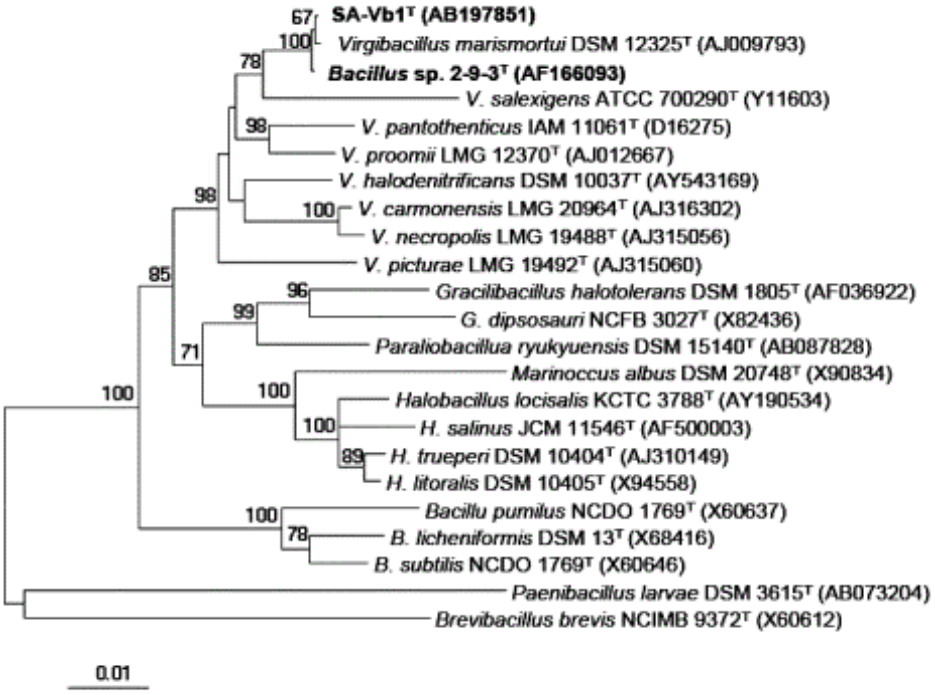

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342 **Fig. 1**. Neighbor-joining phylogenetic tree showing positions of strain SA-Vb1^T to
343 species of genus *Virgibacillus* and other related bacteria based on 16S rRNA gene
344 sequences. Bootstrap values (percentage of 1000 replications) greater than 50 are
345 shown horizontally to nodes. Scale bar indicates 0.01 nucleotide substitutions per
346 site

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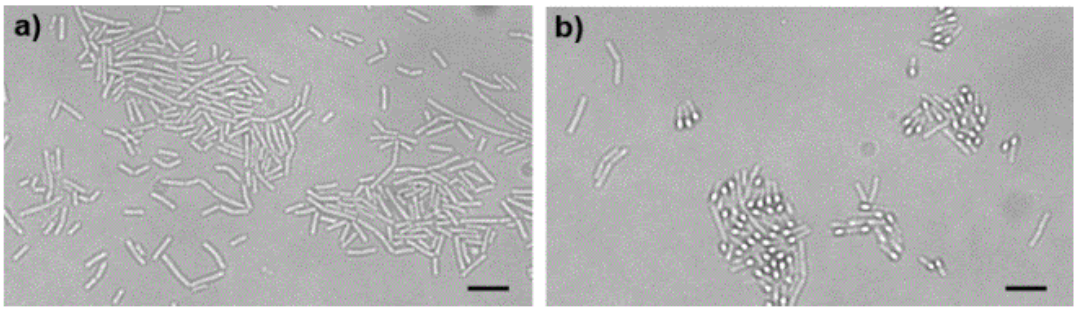

Figure 2. Phase-constrast micrographs of strain SA-Vb1^{T} . Scale bar, 5 μm.

Table 1. Differential characteristics of novel isolate and related species

Species: 1, *Virgibacillus salarius* sp. nov. (this study); 2, *V. olivae* DSM 18098^T (Quesada *et al.,* 2007) ; 3, *V. marismortui* DSM 12325^T (Arahal *et al.,* 1999a and 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 (Denariaz *et al*, 1989 and Yoon *et al.*, 2004); 7, *O. picturae* DSM 14867^T (Heyrman *et al.*, 2003 and Lee *et al.,* 2006); 8, *V. necropolis* DSM 14866^T (Heyrman *et al.*, 2003); 9, *V. dokdonensis* (Yoon *et al.*, 2005); 10, *V. chiguensis* DSM ^{T} (Wang *et al.*, 2008). Symbols: +, positive; -, negative; w, weak; v, variable; ND, no data available. All species are Gram-positive rod-shaped, motile, spore-forming, and positive for catalase, oxidase, hydrolysis of casein.

Characteristics	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
Growth at/in:										
Temperature range (°C)	10-50	20-45	11-50†	15-50	10-40	10-45	5-40	10-40	15-50	15-55
0·5% NaCl	+	+	+†	+†	-	-	w	w	+	+
25% NaCl	+	-	-	-†	-	+	-	-	-	+
anaerobic conditions	-	-	-	+	-	+	-	-	+	+
Nitrate reduction	-	+	+	-	+	+	+	+	-	+
Hydrolysis of:										
Aesculin	+	+	+	+	w	-	w	-	+	+
Gelatin	w	+	+	+	-	+	v	w	+	+
Starch	-	+	-	v	-†	-	-†	-†	+	+
Tween 80	+	-	-	-†	-†	-	-†	-†	+	+
Urease	-	ND	+	-†	-	-	-	w	-	ND
H_2S 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
D-Trehalose	-	ND	-	v	-	+	v	w	-	-
D-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

*Spore shape: E, ellipsoidal; S, spherical. Spore position: C, central; T, terminal; S, subterminal.
†Data obtained from tests in this study.
‡ DPG, diphosphatidyl glycerol; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine.

Supplementary Table S1. Compositions (% of total) of cellular fatty acids of the novel isolate and related species.

Species: 1, *Virgibacillus salarius* sp. nov. (this study); 2, *V. olivae* DSM 18098^T (Quesada *et al.,* 2007) ; 3, *V. marismortui* DSM 12325^T (Vreeland *et al.,* 2006); 4, *V. proomii* DSM 13055^T (Heyndrickx *et al.*, 1999); 5, *V. halodenitrificans* DSM 10037^T (Lee *et al.*, 2006); 6, *V. carmonensis* DSM 14868^T (Heyrman *et al.*, 2003); 7, *O. picturae* DSM 14867^T (Heyrman *et al.*, 2003); 8, *V. necropolis* DSM 14866^T (Heyrman *et al.*, 2003); 9, *V. dokdonensis* (Yoon *et al.*, 2005); 10, *V. chiguensis* DSM ^{T} (Wang *et al.*, 2008). ND, not detected.

	1	2	3	4	5	6	7	8	9	10
iso-$C_{14:0}$	4·8	2·1	3·01	6·0	7·4	3·5	10·7	2·95	4·7	1·8
$C_{15:0}$	ND	1·1	ND	ND	trace	ND	ND	ND	ND	ND
iso-$C_{15:0}$	33·3	33·7	30·0	33·5	2·4	4·6	2·9	4·18	19·4	12·1
anteiso-$C_{15:0}$	29·2	28·4	39·6	33·0	51·8	65·5	59·2	71·5	34·4	52·9
$C_{16:0}$	3.9	3·1	ND	7·6	1·1	1·1	<1·0	1·3	2·4	ND
iso-$C_{16:0}$	5·9	3·9	4·9	4·9	11·8	3·7	7·0	3·63	12·3	4·2
$C_{16:1}$ *ω11c*	1·8	1·7	1·0	ND	0·4	1·0	<1·0	1·09	ND	ND
$C_{16:1}$ *ω7c* alcohol	1·9	1·1	1·4	ND	3·1	5·0	4·2	2·45	ND	ND
iso-$C_{17:0}$	6·6	10·1	4·2	4·3	trace	ND	ND	ND	7·2	1·5
anteiso-$C_{17:0}$	9·8	9·2	11·5	5·8	19·5	9·43	11·9	9·30	15·4	15·9
$C_{18:0}$	ND	1·2	ND	ND	ND	ND	ND	ND	ND	ND
$C_{18:1}$ *ω9c*	ND	1·2	ND	ND	ND	ND	ND	ND	ND	ND

Supplementary Table S2. Similarity (%) of 16S rRNA gene sequence (upper) and DNA-DNA relatedness (lower) of the novel isolate and phylogenetically related species

Species: 1, *Virgibacillus salarius* sp. nov.; 2, *V. olivae* DSM 18098^T; 3, *V. marismortui* DSM 12325^T; 4, *V. halodenitrificans* DSM 10037^T; 5, *V. proomii* DSM 13055^T; 6, *V. carmonensis* DSM 14868^T; 7, *V. necropolis* DSM 14866^T; 8, *O. picturae* DSM 14867^T .

Strain/Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
SA-Vb1^T		99·4	99·7	97·6	97·0	96·6	96·3	96·5
		5·1	39·9	21·1	21·6	23·1	18·3	22·3