Virgibacillus campisalis sp. nov., from a marine solar saltern

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A Gram-staining-variable, motile, endospore-forming and rod-shaped bacterial strain, IDS-20^T, was isolated from a marine solar saltern in Korea and subjected to a polyphasic taxonomic investigation. Strain IDS-20^T grew optimally at 37 °C, at pH 7.5–8.0 and in the presence of 4–5 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain IDS-20^T belongs to the genus *Virgibacillus*. Strain IDS-20^T exhibited 93.4–96.6 % 16S rRNA gene sequence similarity to the type strains of species of the genus *Virgibacillus*. Strain IDS-20^T had MK-7 as the predominant menaquinone and a cell-wall peptidoglycan based on meso-diaminopimelic acid. The major fatty acids were anteiso-C_{15:0} and anteiso-C_{17:0} and major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids. The DNA G + C content was 39.5 mol%. The phylogenetic distinctiveness and differential phenotypic characteristics of strain IDS-20^T demonstrated that this strain can be distinguished from recognized species of the genus *Virgibacillus*. On the basis of the data presented, strain IDS-20^T represents a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus campisalis* sp. nov. is proposed. The type strain is IDS-20^T (=KCTC 13727^T =CCUG 59308^T).

The genus *Virgibacillus* was created by Heyndrickx *et al.* (1998) through the reclassification of *Bacillus pantothenicus*. At the time of writing, the genus comprises at least 21 species with validly published names, including the recently described species *Virgibacillus chiguensis* (Wang *et al.*, 2008), *V. kekensis* (Chen *et al.*, 2008), *V. salarius* (Hua *et al.*, 2008), *V. sediminis* (Chen *et al.*, 2009), *V. arcticus* (Niederberger *et al.*, 2009), *V. salinus* (Carrasco *et al.*, 2009), *V. byunsanensis* (Yoon *et al.*, 2010), *V. xinjiangensis* (Jeon *et al.*, 2009, 2010), *V. subterraneus* (Wang *et al.*, 2010) and *V. soli* (Kämpfer *et al.*, 2011). Members of the genus *Virgibacillus* were characterized chemotaxonomically by having meso-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan, MK-7 as the predominant menaquinone and anteiso-C_{15:0} as the common major fatty acid (Chen *et al.*, 2008, 2009; Hua *et al.*, 2008; Kämpfer *et al.*, 2011). However, a species of the genus *Virgibacillus*, *V. arcticus*, having L-lysine as the diagnostic diamino acid has been described (Niederberger *et al.*, 2009). In this study, a bacterial strain, IDS-20^T, which was isolated from a marine solar saltern on the west coast of Korea, is described. Comparative 16S rRNA gene sequence analysis

indicated that strain IDS-20^T should be considered to be a member of the genus *Virgibacillus*. The aim of the present work was to determine the exact taxonomic position of strain IDS-20^T by using a polyphasic characterization that included the determination of phenotypic properties, a detailed phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

A sediment sample collected from a marine solar saltern of Byunsan, Korea, was suspended in a 0.85 % (w/v) NaCl solution. The suspension was diluted serially, spread on modified S-G medium (Sehgal & Gibbons, 1960) solidified with 1.5 % (w/v) agar and incubated at 25 °C. This medium contained the following components (l⁻¹ distilled water): 100 g NaCl, 20 g MgSO₄·7H₂O, 2 g KCl, 3 g trisodium citrate, 10 g yeast extract and 7.5 g Casamino acids (pH 7.2). The morphological, physiological and biochemical properties of strain IDS-20^T were investigated using routine cultivation at 37 °C on another modified S-G (MSG) medium which contained (l⁻¹ distilled water) 50 g NaCl, 20 g MgSO₄·7H₂O, 2 g KCl, 3 g trisodium citrate, 10 g yeast extract and 7.5 g Casamino acids (pH 7.2). Cell morphology was determined by light microscopy (Nikon E600) and transmission electron microscopy (Philips CM-20). The presence of flagella was investigated by using a transmission electron microscope with cells from exponentially growing

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IDS-20^T is GU586225.

Three supplementary figures are available with the online version of this paper.

cultures. For this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was performed by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MSG agar and on MSG agar supplemented with 0.1% (w/v) potassium nitrate, both of which had been prepared anaerobically under nitrogen atmosphere. Growth at various temperatures (4, 10, 15, 20, 25, 30, 37, 40 and 45 °C) was measured on MSG agar. The pH range for growth and optimal pH were determined in MSG broth that was adjusted to pH 4.5–10.5 (at intervals of 0.5 pH units) by using sodium acetate/acetic acid and Na₂CO₃ buffers. Growth in the absence of NaCl and growth at various NaCl concentrations (0.5% and 1.0–30.0%, w/v,

in increments of 1.0%) was investigated by using liquid media prepared according to the formula of the MSG medium except that NaCl was excluded. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, hypoxanthine, starch and xanthine was tested on MSG agar by using the substrate concentrations described by Cowan & Steel (1965). Nitrate reduction and hydrolysis of aesculin, gelatin, urea and Tweens 20, 40, 60 and 80 were tested as described by Lányi (1987) by using artificial seawater (Bruns *et al.*, 2001) for preparation of media. The artificial seawater contained (l⁻¹ distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). Acid production from various carbohydrates was investigated according to the method of Leifson (1963). Utilization of various substrates was tested

Table 1. Differential phenotypic characteristics of *Virgibacillus campisalis* IDS-20^T and seven phylogenetically related species of the genus *Virgibacillus*

Taxa: 1, *V. campisalis* sp. nov. IDS-20^T; 2, *V. arcticus* (data from Niederberger *et al.*, 2009); 3, *V. byunsanensis* (Yoon *et al.*, 2010); 4, *V. carmonensis* (Heyrman *et al.*, 2003); 5, *V. halodenitrificans* (Yoon *et al.*, 2004); 6, *V. necropolis* (Heyrman *et al.*, 2003); 7, *V. salinus* (Carrasco *et al.*, 2009); 8, *V. subterraneus* (Wang *et al.*, 2010). +, Positive reaction; –, negative reaction; w, weakly positive reaction; v, variable reaction; ND, not determined. Data in parentheses are for the type strain. All taxa are positive for catalase and nitrate reduction.

Characteristic	1	2	3	4	5	6	7	8
Spore shape*	S	E	S or O	S or E	E	E	E or S	E or S
Spore position†	T	C or ST	T	ST	T or ST	C, T or ST	T or ST	T
Anaerobic growth	+	+	–	–	+	–	–	–
Gram-staining	v	+	v	+	v	+	+	+
Pigmentation	–	Light pink	–	Pink	–	–	Cream	Cream
Growth without NaCl	–	+	+	–	–	w	–	+
Hydrolysis of:								
Aesculin	–	ND	–	w	v(–)	–	+	+
Casein	–	+	–	+	+	+	–	–
Gelatin	–	+	–	–	+	w	–	–
Acid production from:								
D-Glucose	–	w	–	–	+	w	+	+
D-Fructose	–	w	w	–	+	w	+	+
D-Mannitol	–	+	–	–	v(+)	–	–	–
D-Mannose	–	w	–	–	+	w	ND	+
Melibiose	–	+	–	–	–	–	ND	+
Trehalose	–	+	–	–	+	w	+	–
Utilization of:								
D-Glucose	+	+	–	–	+	+	–	+
D-Fructose	–	ND	–	–	v(+)	–	–	+
Sucrose	–	+	–	+	+	+	+	+
D-Xylose	–	ND	–	–	–	–	+	+
Major polar lipids‡	DPG, PG, PLs	DPG, PG, PLs	DPG, PG, PLs	DPG, PG, PLs	PG, DPG, PLs	DPG, PG, PLs	DPG, PG, GL, PLs	DPG, PG, GL
Cell-wall type§	<i>m</i> -DAP	<i>L</i> -Lys-direct	<i>m</i> -DAP	ND	<i>m</i> -DAP	ND	<i>m</i> -DAP	<i>m</i> -DAP
DNA G + C content (mol%)	39.5	38.2	37.6	38.9	38–39	37.3	38.8	37.1

*S, Spherical; O, oval; E, ellipsoidal.

†T, Terminal; ST, subterminal; C, central.

‡PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol; GL, glycolipid; PLs, unidentified phospholipids.

§*m*, meso.

according to the method of Baumann & Baumann (1981) using supplementation with 3 % (w/v) NaCl, 0.01 % (w/v) yeast extract, 1 % (v/v) vitamin solution (Staley, 1968) and 2 % (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957). Susceptibility to antibiotics was investigated on MSG agar plates by using antibiotic discs with the following concentrations: ampicillin, 10 µg; carbenicillin, 100 µg; cephalothin, 30 µg; chloramphenicol, 100 µg; gentamicin, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; neomycin, 30 µg; novobiocin, 5 µg; oleandomycin, 15 µg; penicillin G, 20 U; polymyxin B, 100 U; streptomycin, 50 µg; and tetracycline, 30 µg. Enzyme activities and other physiological properties were determined by using API ZYM and API 20E systems (bioMérieux). Morphological, cultural, physiological and biochemical characteristics of strain IDS-20^T are shown in Supplementary Fig. S1 (available in IJSEM Online) and given in the species description (see below) and in Table 1.

Cell biomass for DNA extraction and for the analyses of cell wall, isoprenoid quinones and polar lipids was obtained from cultures grown for 3 days in MSG broth at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with

RNase A to minimize the contamination by RNA. The 16S rRNA gene was amplified by PCR as described by Yoon *et al.* (1998) using two universal primers, 9F (5'-GAGTTTGATC-CTGGCTCAG-3') and 1542R (5'-AGAAAGGAGGTGAT-CCAGCC-3'), and purified using a QIAquick PCR purification kit (Qiagen). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed according to the method of Yoon *et al.* (2003). The near-complete 16S rRNA gene sequence of strain IDS-20^T determined in this study comprised 1523 nt. In the phylogenetic trees reconstructed using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms, strain IDS-20^T fell within the clade comprising species of the genus *Virgibacillus* (Fig. 1; Supplementary Figs S2 and S3). Strain IDS-20^T exhibited the highest 16S rRNA gene sequence similarity (96.6 %) to *Virgibacillus byunsanensis* ISL-24^T and 93.4–96.4 % 16S rRNA gene similarity to the type strains of the other species of the genus *Virgibacillus*. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The DNA G+C content of strain IDS-20^T was 39.5 mol%.

Fig. 1. Neighbour-joining phylogenetic tree (using Jukes–Cantor distance model) based on 16S rRNA gene sequences showing the positions of strain IDS-20^T, species of the genus *Virgibacillus* and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branching points. *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

The isomer type of the diamino acid in the cell wall peptidoglycan was analysed by using TLC according to the method described by Komagata & Suzuki (1987). Strain IDS-20^T contained *meso*-diaminopimelic acid as diagnostic diamino acid in the cell-wall peptidoglycan. Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The predominant isoprenoid quinone detected in strain IDS-20^T was menaquinone-7 (MK-7) at a peak area ratio of approximately 91 %. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Major polar lipids detected in strain IDS-20^T were diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids. For cellular fatty acid analysis, cell mass of strain IDS-20^T was harvested from marine agar 2216 (Difco) plates after cultivation for 3 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The cellular fatty acid profile of strain IDS-20^T is shown in Table 2, together with those of *Virgibacillus pantothenticus*, the type species of the genus, and some phylogenetically related species of the genus *Virgibacillus*, which were obtained by using the same conditions. The major fatty acids (>10 % of total fatty acids) found in strain IDS-20^T were anteiso-C_{15:0} and anteiso-C_{17:0}. These chemotaxonomic properties, which are similar to those of species of the genus *Virgibacillus*, are sufficient to support the result of phylogenetic analysis, i.e. that strain IDS-20^T belongs to the genus *Virgibacillus*.

Strain IDS-20^T was distinguishable from some phylogenetically related species of the genus *Virgibacillus* by differences in several phenotypic characteristics as listed in Table 1. Therefore, phylogenetic distinctiveness and differential phenotypic properties of strain IDS-20^T are sufficient to demonstrate that this strain is separate from the recognized species of the genus *Virgibacillus*. On the basis of the phenotypic and phylogenetic data, strain IDS-20^T is considered to represent a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus campisalis* sp. nov. is proposed.

Description of *Virgibacillus campisalis* sp. nov.

Virgibacillus campisalis (cam.pi.sa'lis. L. n. *campus* field; L. gen. n. *salis* of salt; N.L. gen. n. *campisalis* of the field of salt).

Cells are Gram-staining-variable, facultatively anaerobic and rod-shaped, 0.5–1.0 µm in diameter and 1.0–4.0 µm in length. Terminal spherical endospores are observed in swollen sporangia. Motile by means of a single polar flagellum. Colonies on MSG agar are circular, smooth, slightly convex, greyish-yellow and 0.5–1.5 mm in diameter after incubation for 5 days at 37 °C. Optimal growth temperature is 37 °C; growth occurs at 15 and 40 °C, but

Table 2. Cellular fatty acid compositions (%) of strain IDS-20^T and the type strains of several species of the genus *Virgibacillus*

Strains: 1, *V. campisalis* sp. nov. IDS-20^T; 2, *V. pantothenticus* KCTC 3539^T; 3, *V. byunsanensis* ISL-24^T; 4, *V. carmonensis* DSM 14868^T; 5, *V. necropolis* DSM 14866^T. Data for reference strains from Yoon *et al.* (2010). Fatty acids that represented <0.5 % in all strains were omitted. tr, Trace (<0.5 %). –, Not detected.

Fatty acid	1	2	3	4	5
Straight-chain					
C _{14:0}	1.2	tr	tr	tr	tr
C _{15:0}	–	tr	tr	0.9	tr
C _{16:0}	4.9	0.9	0.9	1.3	1.7
Unsaturated					
C _{16:1ω7c} alcohol	–	–	tr	6.1	1.0
Branched					
anteiso-C _{13:0}	–	tr	0.7	tr	tr
iso-C _{14:0}	1.9	1.1	7.6	8.9	3.3
iso-C _{15:0}	2.1	4.8	1.2	2.6	2.2
anteiso-C _{15:0}	59.0	50.4	71.7	58.7	73.0
iso-C _{16:0}	4.8	5.5	5.2	11.7	4.9
iso-C _{17:0}	–	2.1	–	tr	tr
anteiso-C _{17:0}	26.1	34.5	10.3	6.8	11.0
Summed feature 4*	–	–	1.1	1.6	0.9

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 4 contained iso-C_{17:1} and/or anteiso-C_{17:1}.

not at 10 or 45 °C. Optimal pH for growth is between 7.5 and 8.0; growth occurs at pH 6.0–9.0. Growth occurs optimally in the presence of 4–5 % (w/v) NaCl; growth occurs in the presence of 0.5–20 % (w/v) NaCl. Anaerobic growth occurs on MSG and on MSG supplemented with nitrate. Oxidase- and catalase-positive. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities are absent. Tweens 20, 40, 60 and 80 are hydrolysed, but aesculin, casein, gelatin, hypoxanthine, L-tyrosine, urea and xanthine are not. D-Glucose, acetate, citrate, L-malate, pyruvate and succinate are utilized as sole carbon and energy sources, but L-arabinose, cellobiose, D-fructose, D-galactose, maltose, D-mannose, sucrose, trehalose, D-xylose, salicin, benzoate, formate and L-glutamate are not. Acid is not produced from L-arabinose, cellobiose, D-galactose, D-glucose, D-fructose, *myo*-inositol, lactose, maltose, D-mannose, D-mannitol, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose or D-xylose. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, lincomycin, novobiocin, oleandomycin, penicillin G and tetracycline. Tolerant to gentamicin, kanamycin, neomycin, polymyxin B and streptomycin. In assays with the API ZYM system, esterase (C4) and esterase lipase (C8) are present, but alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase,

β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosamine, α -mannosidase and α -fucosidase are absent. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major cellular fatty acids (>10 % total fatty acids) are anteiso-*C*_{15:0} and anteiso-*C*_{17:0}. The predominant menaquinone is MK-7. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids. The DNA G + C content of the type strain is 39.5 mol% (determined by HPLC).

The type strain, IDS-20^T (=KCTC 13727^T =CCUG 59308^T), was isolated from a marine solar saltern on the west coast of Korea.

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