Virgibacillus dokdonensis sp. nov., isolated from a Korean island, Dokdo, located at the edge of the East Sea in Korea

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A Gram-variable, motile, endospore-forming, slightly halophilic bacterial strain, DSW-10^T, was isolated from Dokdo, an island located at the edge of the East Sea, Korea, and was characterized taxonomically by using a polyphasic approach. This isolate grew optimally at 37 °C and in the presence of 4–5 % NaCl. Strain DSW-10^T had cell-wall peptidoglycan based on meso-diaminopimelic acid, MK-7 as the predominant menaquinone, and anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as major fatty acids. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids. The DNA G + C content was 36.7 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DSW-10^T is phylogenetically affiliated to the genus *Virgibacillus*, and exhibited sequence similarity of 95.3–98.7 % to the type strains of *Virgibacillus* species. DNA–DNA relatedness levels between strain DSW-10^T and the type strains of some phylogenetically related *Virgibacillus* species were in the range 8.4–17.5 %. On the basis of phenotypic properties and phylogenetic and genetic distinctiveness, strain DSW-10^T (=KCTC 3933^T = DSM 16826^T) was classified as the type strain of a novel *Virgibacillus* species, for which the name *Virgibacillus dokdonensis* sp. nov. is proposed.

The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) with the transfer of *Bacillus pantothenicus* to *Virgibacillus pantothenicus*. Subsequently, four further species, *Virgibacillus proomii* (Heyndrickx *et al.*, 1999) and *Virgibacillus carmonensis*, *Virgibacillus necropolis* and *Virgibacillus picturae* (Heyrman *et al.*, 2003), were described. Two *Salibacillus* species (Wainø *et al.*, 1999; Arahall *et al.*, 2000) have been reclassified in the genus as *Virgibacillus marismortui* and *Virgibacillus salexigens* (Heyrman *et al.*, 2003). Recently, *Bacillus halodenitrificans* was transferred to the genus as *Virgibacillus halodenitrificans* (Yoon *et al.*, 2004). The genus *Virgibacillus* currently comprises eight recognized species. Here we report on the taxonomic characterization of a slightly halophilic *Virgibacillus*-like bacterial strain, DSW-10^T, which was isolated from sea water at Dokdo, an island in the East Sea, Korea.

Sea water collected at Dokdo provided the source for

isolation of the bacterial strains. A single isolate, DSW-10^T, was obtained by the standard dilution plating technique on marine agar 2216 (MA; Difco) at 30 °C. *V. pantothenicus* DSM 26^T, *V. proomii* DSM 13055^T and *V. marismortui* DSM 12325^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and cultivated according to recommended procedures of the DSMZ. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). For the latter, cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air-drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). Presence of flagella was examined by TEM using cells from exponentially growing cultures. The Gram-reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA and MA supplemented with nitrate, both of which had been prepared anaerobically. The pH range for growth was determined in marine broth 2216 (MB; Difco) supplemented with 3 % (w/v) NaCl that was adjusted to various pH values (initial pH 4.0–12.0 at intervals of 0.5 pH units). The pH (below

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Abbreviation: TEM, transmission electron microscopy.

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10.5) was adjusted prior to sterilization to various levels by the addition of HCl or Na₂CO₃. Growth at various NaCl concentrations was investigated in MB or trypticase soy broth (TSB; Difco). Growth in the absence of NaCl was investigated in TSB lacking NaCl. Growth at various temperatures (4–55 °C) was measured on MA supplemented with 3 % (w/v) NaCl. Oxidase and catalase activities and hydrolysis of casein, starch, urea and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and gelatin and nitrate reduction were determined as described by Lanyi (1987) with a modification that artificial sea water was used for preparation of media. The artificial sea water contained (per litre 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). H₂S production was tested as described by Bruns *et al.* (2001). Hydrolysis of hypoxanthine, tyrosine and xanthine was performed on MA supplemented with 3 % NaCl using the substrate concentrations given by Cowan & Steel (1965). Acid production from carbohydrates was determined as described by Leifson (1963), and utilization of various substrates as sole carbon and energy sources was tested according to the method of Baumann & Baumann (1981) using supplementation with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957), 1 % (v/v) vitamin solution (Staley, 1968) and 0.01 % (w/v) yeast extract. Enzyme activity and other physiological properties were determined using the API ZYM and API 20E systems (bioMérieux) with a modification that suspension media supplemented with 5 % (w/v) NaCl were used.

Cell mass for analyses of the cell wall, menaquinones and polar lipids and for DNA extraction was produced in MB supplemented with 3 % NaCl at 37 °C. The isomer type of diaminopimelic acid in the peptidoglycan was determined by the method described by Komagata & Suzuki (1987). Menaquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. Polar lipids were extracted using the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). For fatty acid methyl ester analysis, cell mass of strain DSW-10^T was harvested from

agar plates after cultivation for 2 days at 37 °C on MA supplemented with 3 % NaCl. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). 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. The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The 16S rRNA gene was amplified by PCR using two universal primers as described by Yoon *et al.* (1998). Sequencing of the 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values.

The 16S rRNA gene sequence of strain DSW-10^T determined in this study comprised 1520 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analyses showed that strain DSW-10^T is most closely related to *Virgibacillus* species (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain DSW-10^T joined *V. pantothenicus* at a bootstrap confidence level of 99.3 % (Fig. 1). Although there were differences in topologies between the phylogenetic tree based on the neighbour-joining algorithm and those based on the maximum-likelihood and maximum-parsimony algorithms, the relationships among strain DSW-10^T and some *Virgibacillus* species were also recovered in the trees based on the latter two algorithms (Fig. 1). Strain DSW-10^T exhibited 16S rRNA gene sequence similarity values of 98.7 % to *V. pantothenicus* IAM 11061^T and 95.3–97.0 % to the type strains of the other *Virgibacillus* species (Fig. 1). Sequence similarities to all other species included in the phylogenetic analysis were lower than 94.3 % (Fig. 1). DNA–DNA

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequence data showing the phylogenetic positions of strain DSW-10^T, *Virgibacillus* species and other related taxa. Bootstrap values of 50 % or greater (1000 replications) are shown as percentages at each node. Bar, 0.01 substitutions per nucleotide position. Dots indicate that the corresponding nodes are also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms.

hybridization was performed to determine the genetic relatedness between strain DSW-10^T and the type strains of three *Virgibacillus* species that showed 16S rRNA gene sequence similarity values of >97.0% to strain DSW-10^T. Strain DSW-10^T exhibited DNA–DNA relatedness levels of 17.5, 8.4 and 9.2% to *V. pantothenicus* DSM 26^T, *V. proomii* DSM 13055^T and *V. marismortui* DSM 12325^T, respectively.

Morphological, cultural, physiological and biochemical characteristics of strain DSW-10^T are shown in Table 1 or are given in the species description below. Strain DSW-10^T contained *meso*-diaminopimelic acid as the diagnostic di-amino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone found in strain DSW-10^T was unsaturated menaquinone with seven isoprene units (MK-7), and a minor amount of MK-6 was also detected. Major

Table 1. Differential phenotypic characteristics of *Virgibacillus dokdonensis* sp. nov. and other *Virgibacillus* species

Species: 1, *V. dokdonensis* sp. nov.; 2, *V. pantothenicus* (data from Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003); 3, *V. proomii* (Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003); 4, *V. salexigens* (Wainø *et al.*, 1999; Heyrman *et al.*, 2003); 5, *V. marismortui* (Arahal *et al.*, 1999, 2000; Heyrman *et al.*, 2003); 6, *V. carmonensis* (Heyrman *et al.*, 2003); 7, *V. necropolis* (Heyrman *et al.*, 2003); 8, *V. picturae* (Heyrman *et al.*, 2003); 9, *V. halodenitrificans* (Yoon *et al.*, 2004). +, Positive; –, negative; W, weakly positive; ND, not determined; V, variable; E, ellipsoidal; S, spherical; C, central; ST, subterminal; T, terminal; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PLS, unidentified phospholipids; APL, unknown aminophospholipid; tr, trace. Data in parentheses are for the type strain. Cells of all species are rod-shaped, motile, positive for catalase and negative for growth on D-arabinose and D-xylose.

Character	1	2	3	4	5	6	7	8	9
Spore shape	S or E	S or E	S or E	E	E	E or S	E	E or S	E
Spore position	T or ST	T or ST	T or ST	C, ST or T	T or ST	ST	C, ST or T	T	T or ST
Gram stain	V	+	+	+	+	+	+	+	V
Pigmentation	–	–	–	–	–	Pink	–	–	–
Anaerobic growth	+	+	+	–	–	–	–	–	+
Growth in the presence of:									
0.5% NaCl	+	W	W	–	–	–	W	W	V (–)
25% NaCl	–	–	–	W*	–	–	–	–	V (+)
Temperature range (°C)	15–50	15–50	15–50	15–45	15–50	10–40	10–40	5–40	10–45
Nitrate reduction	–	V	–	–	+	+	+	+	+
H ₂ S production	–	V	–	+	+	–	–	–	–
Hydrolysis of:									
Aesculin	+	+	+	+	+	W	–	W	V (–)
Casein	+	+	+	+	+	+	+	W	+
Gelatin	+	+	V	+	+	–	W	V	+
Growth on:									
D-Glucose	+	W	W	W	–	–	+	V	+
D-Fructose	+	–	–	–	–	–	–	–	V (+)
Sucrose	+	–	+	+	+	+	+	V	+
Acid production from:†									
D-Galactose	+	–	+	W	–	–	–	W	+
D-Glucose	+	–	+	W	+	–	W	W	+
D-Fructose	+	–	+	W	+	–	W	W	+
D-Mannose	+	–	+	W	+	–	W	W	+
D-Melibiose	–	–	–	–	–	–	–	W	–
L-Rhamnose	–	+	V	–	–	–	–	–	–
D-Trehalose	–	+	+	–	–	–	W	V	+
D-Mannitol	–	–	–	–	–	–	–	W	V (+)
Major polar lipids	PG, DPG, PE, PLS	PG, DPG, PE, PLS	PG, DPG, PE, PLS	PG, DPG, PLS	PG, DPG, PE, PLS	PG, DPG, PLS	PG, DPG, PLS	PG, DPG, PLS	PG, DPG, PLS
Presence of APL	–	+	tr	–	tr	–	–	–	ND
DNA G+C content (mol%)	36.7	36.9–38.3	36.8–37.0	36.3–39.5	39.0–42.8	38.9	37.3	39.5–40.0	38–39

*Data from Heyrman *et al.* (2003); a different result was obtained by Wainø *et al.* (1999).

†Data for *V. dokdonensis* were obtained using the methods of Leifson (1963); data for the other *Virgibacillus* species were obtained using the API 50CHB system (bioMérieux) by Heyrman *et al.* (2003).

components of the fatty acids (>1%) detected in strain DSW-10^T were branched fatty acids anteiso-C_{15:0} (34.4%), iso-C_{15:0} (19.4%), anteiso-C_{17:0} (15.4%), iso-C_{16:0} (12.3%), iso-C_{17:0} (7.2%), iso-C_{14:0} (4.7%) and iso-C_{13:0} (1.1%) and straight-chain fatty acid C_{16:0} (2.4%). This fatty acid profile was similar to those of *Virgibacillus* species, although there were differences in the proportions of some fatty acids that might have been caused by different cultivation conditions (Heyndrickx *et al.*, 1999; Wainø *et al.*, 1999; Heyrman *et al.*, 2003). Major polar lipids detected in strain DSW-10^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids. An unknown aminophospholipid that was ninhydrin-positive was not detected. The DNA G+C content of strain DSW-10^T was 36.7 mol%. The results obtained from chemotaxonomic analyses, showing the properties that are shared by *Virgibacillus* species, were in agreement with the result of phylogenetic classification based on 16S rRNA gene sequence analysis.

There are some differences in the phenotypic characteristics of strain DSW-10^T and recognized *Virgibacillus* species (Table 1). In particular, strain DSW-10^T is clearly distinguished from *V. pantothenticus*, its nearest phylogenetic neighbour, by differences in acid production from some substrates (Table 1). The phylogenetic distinctiveness and DNA–DNA relatedness data are sufficient to categorize strain DSW-10^T as representing 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 DSW-10^T should be classified in the genus *Virgibacillus* as a member of a novel species, for which the name *Virgibacillus dokdonensis* sp. nov. is proposed.

Description of *Virgibacillus dokdonensis* sp. nov.

Virgibacillus dokdonensis (dok.do.nen'sis. N.L. masc. adj. *dokdonensis* of Dokdo, a Korean island located at the edge of the East Sea in Korea from where the type strain was isolated).

Cells are rod-shaped, 0.6–0.8 × 2.5–5.0 µm, Gram-variable and motile by means of peritrichous flagella. Terminal or subterminal ellipsoidal or spherical endospores are observed in swollen sporangia. Colonies are irregular, flat, translucent, milky white in colour and 3–5 mm in diameter after 2 days of incubation at 37 °C on MA supplemented with 3% (w/v) NaCl. Optimal growth temperature is 37 °C; growth occurs at 15 and 50 °C, but not at 10 or 55 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 4–5% (w/v) NaCl; growth occurs without NaCl and in the presence of 23% (w/v) NaCl, but not in the presence of >24% (w/v) NaCl. Growth occurs under anaerobic conditions on MA and MA supplemented with nitrate. Oxidase-positive. Urease-negative. Starch and Tweens 20, 40, 60 and 80 are hydrolysed. Hypoxanthine, xanthines and

tyrosine are not hydrolysed. Voges–Proskauer reaction is negative. Indole is not produced. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and α-glucosidase are present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Acid is produced from D-cellobiose, lactose, maltose, D-ribose, sucrose, *myo*-inositol and D-sorbitol. Acid is not produced from L-arabinose, D-melezitose, D-raffinose or D-xylose. The following substrates are utilized: D-cellobiose, D-mannose, maltose, acetate, citrate, pyruvate and salicin. D-Galactose, D-trehalose, benzoate, succinate, L-malate, formate and L-glutamate are not utilized. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids. The major fatty acids are anteiso-C_{15:0} (34.4%), iso-C_{15:0} (19.4%), anteiso-C_{17:0} (15.4%) and iso-C_{16:0} (12.3%). The DNA G+C content is 36.7 mol%.

The type strain, DSW-10^T (= KCTC 3933^T = DSM 16826^T), was isolated from Dokdo, an island located at the edge of the East Sea, Korea.

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