Virgibacillus halophilus sp. nov., spore-forming bacteria isolated from soil in Japan

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Two Gram-positive, round-spore-forming, rod-shaped, halophilic bacterial strains, 5B73C^T and 5B133E, were isolated from field soil in Kakegawa, Shizuoka, Japan, and were characterized taxonomically using a polyphasic approach. These two strains were found to comprise strictly aerobic, motile rods that formed subterminal endospores. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains 5B73C^T and 5B133E are phylogenetically affiliated to the genus *Virgibacillus*, exhibiting sequence similarities of 94.1–96.4% with respect to the type strains of *Virgibacillus* species. The DNA G + C contents of strains 5B73C^T and 5B133E were 42.6 and 42.3 mol%, respectively. The cell-wall peptidoglycan type (*meso*-diaminopimelic acid), the major cellular fatty acids (*anteiso*-C_{15:0}, *iso*-C_{15:0}, *anteiso*-C_{17:0} and *iso*-C_{16:0}) and the quinone type (MK-7) of the isolates support their affiliation to the genus *Virgibacillus*. On the basis of their genotypic and phenotypic characteristics, the isolates represent a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus halophilus* sp. nov. is proposed. The type strain is 5B73C^T (=IAM 15308^T=KCTC 13935^T).

The genus *Virgibacillus* was emended from *Bacillus pantothenicus* on the basis of amplified DNA restriction analysis results, fatty acid profiles, SDS-PAGE patterns of whole-cell proteins and phenotypic characterization (Heyndrickx *et al.*, 1998). The genus *Virgibacillus* currently consists of 10 recognized species (Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003; Lee *et al.*, 2006; Yoon *et al.*, 2004, 2005), with *Virgibacillus pantothenicus* as the type species. The members of the genus *Virgibacillus* are motile, Gram-positive rods that bear oval to ellipsoidal endospores. They have DNA G + C contents ranging from 36 to 43 mol%, their cell walls contain peptidoglycan of the *meso*-diaminopimelic type and they possess *anteiso*-C_{15:0} as the major cellular fatty acid (Heyrman *et al.*, 2003). Here, we report two novel strains, 5B73C^T and 5B133E, isolated from field soil in Kakegawa, Shizuoka, Japan, in the course of an environmental investigation and characterized phenotypically, chemotaxonomically and in terms of their 16S rRNA gene sequences.

Strains 5B73C^T and 5B133E were isolated by suspending soil samples in a 0.9% NaCl solution and heating the suspension

at 80 °C for 10 min. The suspension was diluted serially, spread on plate count agar (Merck) and incubated at 35 °C. Purified colonies were selected, and all cultivations and phenotypic tests were carried out in media containing 50% Herbst's artificial seawater and incubated at 30 °C. Herbst's artificial seawater contains the following (per litre distilled water): NaCl, 30 g; KCl, 0.7 g; MgSO₄·7H₂O, 5.3 g; CaSO₄·2H₂O, 1.3 g; and MgCl₂·6H₂O, 10.8 g. Cell morphology and motility were examined by using phase-contrast microscopy (BX60 microscope; Olympus). Growth under anaerobic conditions was determined after 1 week incubation in an AnaeroPack (Mitsubishi Gas Chemical). Catalase was determined with 3% H₂O₂, the production of bubbles representing a positive reaction. Oxidase was determined using cytochrome oxidase paper (Nissui Pharmaceutical). API 20E and API 50 CH microtest galleries (bioMérieux) were used to determine physiological and biochemical characteristics. The API tests were read after 48 h. The isolates were Gram-positive and strictly aerobic, and the cells were motile and rod-shaped. Morphological and physiological characteristics of the isolates are given in the species description. The isolates were similar to *Virgibacillus* species in terms of morphological and some physiological characteristics, but were distinct regarding anaerobic growth, growth temperature, nitrate reduction and H₂S production (Table 1).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 5B73C^T and 5B133E are AB243851 and AB243853, respectively.

Table 1. Differential characteristics of strain 5B73C^T and related *Virgibacillus* species

Taxa: 1, strain 5B73C^T; 2, *V. pantothenticus* (Heyndrickx *et al.*, 1999); 3, *V. proomii* (Heyndrickx *et al.*, 1999); 4, *Virgibacillus dokdonensis* (Yoon *et al.*, 2005; acid production from carbohydrates determined in this study); 5, *Virgibacillus salexigens* (Heyrman *et al.*, 2003); 6, *V. marismortui* (Heyrman *et al.*, 2003); 7, *V. carmonensis* (Heyrman *et al.*, 2003); 8, *Virgibacillus necropolis* (Heyrman *et al.*, 2003); 9, *V. halodenitrificans* (Yoon *et al.*, 2004; Lee *et al.*, 2006); 10, *Virgibacillus koreensis* (Lee *et al.*, 2006). +, Positive; –, negative; v, variable; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10
Spore shape*	E	E, S	E, S	E, S	E	E	E, S	E	E	E
Spore position†	S	T, S	T, S	T, S	C, S, T	T, S	S	C, S, T	T, S	T
Anaerobic growth	–	+	+	+	–	–	–	–	+	+
Temperature for growth (°C)	5–45	15–50	15–50	15–50	15–45	15–50	10–40	10–40	10–45	10–45
Nitrate reduction	+	–	–	–	–	+	+	+	+	–
H ₂ S production	–	+	–	–	+	–	–	–	–	–
Acid production from:										
N-Acetylglucosamine	+	+	+	+	w	+	–	w	+	–
Amygdalin	–	+	–	–	w	–	–	–	–	+
D-Arabinose	–	+	–	–	–	–	–	–	–	–
Galactose	w	–	+	+	w	–	–	–	+	–
D-Glucose	+	–	+	+	w	+	–	w	+	w
D-Fructose	+	–	+	+	w	+	–	w	+	+
L-Fucose	–	+	–	–	–	–	–	–	–	–
Glycerol	w	+	–	+	–	w	–	w	w	–
myo-Inositol	–	–	+	+	–	–	–	–	–	–
D-Mannose	+	–	+	+	w	+	–	w	+	–
L-Rhamnose	–	+	v	–	–	–	–	–	–	–
D-Trehalose	+	+	+	–	–	–	–	w	+	w
D-Turanose	–	+	–	–	–	–	–	–	–	–
DNA G + C content (mol%)	42.6	36.9–38.3	36.8–37.0	36.7	36.3–39.5	39.0–42.8	38.9	37.3	38–39	41

*E, Ellipsoidal; S, spherical.

†C, Central; S, subterminal; T, terminal.

On the basis of analyses of partial 16S rRNA gene sequences (Goto *et al.*, 2000, 2002), the strains were grouped within the *Virgibacillus* cluster. However, they were found to be distinct from previously described species of the genus *Virgibacillus*. 16S rRNA gene sequences were determined using an Applied Biosystems 16S rRNA gene kit, according to the instructions of the manufacturer. The 16S rRNA gene sequences of strains 5B73C^T and 5B133E were compared with sequences obtained from GenBank. The sequences were aligned with the CLUSTAL W software package (Thompson *et al.*, 1994), and evolutionary distances and K_{nuc} values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not taken into consideration. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), and the topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985), based on 1000 replicates. Similarity values were calculated using MEGA3 (Kumar *et al.*, 2004). Almost-complete 16S rRNA gene sequences of strains 5B73C^T and 5B133E were determined and subjected to a comparative analysis. The 16S rRNA gene sequences of the two isolates shared 100 % similarity and were on the same

phylogenetic branch. Strain 5B73C^T showed the highest level of 16S rRNA gene sequence similarity with *Virgibacillus marismortui* (96.4 %), followed by *Virgibacillus carmonensis* (96.2 %), *Virgibacillus halodenitrificans* (96.2 %) and *Virgibacillus proomii* (95.9 %). On the other hand, the strains showed lower levels of sequence similarity (< 95 %) with respect to other recognized low-G + C, Gram-positive species, including *Lentibacillus salarius* (94.9 %), *Lentibacillus salicampi* (94.0 %) and *Oceanobacillus iheyensis* (94.0 %). Generally, around 95 % would be a practicable border zone for genus definition (Ludwig *et al.*, 1998). The phylogenetic tree shown in Fig. 1 indicates that strains 5B73C^T and 5B133E are closely related to the genus *Virgibacillus* but form a separate clade within that genus.

The 16S rRNA gene sequence similarity of type strains of *Virgibacillus* species with respect to 5B73C^T lies between 94.1 and 96.4 %. The overall genomic DNA relatedness of 5B73C^T with respect to these species may not be high because, as Stackebrandt & Goebel (1994) observed, strains with less than 97 % 16S rRNA gene sequence similarity have DNA–DNA relatedness values below 70 %. Thus, in the

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strains 5B73C^T, 5B133E and related taxa. Numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped trees; only values greater than 70 % are shown. Bar, 1 substitution per 100 nt.

absence of a close relative showing significant 16S rRNA gene sequence similarity, strains 5B73C^T and 5B133E can be considered as representing a novel species.

Cellular fatty acids from strains 5B73C^T and 5B133E grown on trypticase soy agar (BD BBL) for 48 h at 30 °C were prepared, separated and identified with the Microbial Identification System (MIDI). The major fatty acids of strains 5B73C^T and 5B133E were anteiso-C_{15:0} (35.8–40.9 %), iso-C_{15:0} (21.2–20.6 %), anteiso-C_{17:0} (16.1–19.9 %) and iso-C_{16:0} (10.3–6.5 %). This fatty acid profile is quite similar to those of species of the genus *Virgibacillus* (Table 2).

Genomic DNA was prepared according to the method of Marmur (1961). The G + C content of the total DNA was measured by HPLC according to the method described by Mesbah *et al.* (1989). The DNA G + C contents of 5B73C^T and 5B133E were 42.6 and 42.6 mol%, which is a little higher than those for known species of the genus *Virgibacillus* (Table 1). DNA–DNA hybridization was performed by using the photobiotin-labelling method of Ezaki *et al.* (1989) with a multi-well plate reader (CytoFluoR; PerSeptive Biosystems). The DNA–DNA hybridization values for strains 5B73C^T and 5B133E and for 5B133E and 5B73C^T were 95.4 and 101.5 %, respectively. The two strains should therefore be considered as representing a single species (Stackebrandt *et al.*, 2002).

Analysis of the cell-wall peptidoglycan of strain 5B73C^T (selected as the representative strain for the two isolates) was carried out by using the methods of Schleifer & Kandler (1972). Strain 5B73C^T possessed the *meso*-diaminopimelic-type cell wall. Analysis of the respiratory quinones of strain

5B73C^T was performed as described by Collins & Jones (1981): the major isoprenoid quinone was MK-7.

On the basis of phenotypic, chemotaxonomic and phylogenetic data, we conclude that strains 5B73C^T and 5B133E represent a novel species of the genus *Virgibacillus*, for which we propose the name *Virgibacillus halophilus* sp. nov.

Description of *Virgibacillus halophilus* sp. nov.

Virgibacillus halophilus (ha.lo.phi'lus. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are Gram-positive, strictly aerobic, motile rods (0.5 × 1.75 µm). Ellipsoidal spores are formed subterminally. Colonies grown on trypticase soy agar containing 50 % Herbst's artificial seawater are circular, convex and pale yellow. The growth temperature and pH are 5–45 °C and 5.0–10.0, respectively. Growth occurs both in the absence of NaCl and in the presence of 18 % NaCl (w/v). Catalase and oxidase activities are positive. H₂S and indole are not produced. Nitrate is reduced to nitrite whereas nitrite is not reduced. Acetoin is produced. Urease, gelatinase and β-galactosidase are hydrolysed. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and citrate utilization. Acid is produced from glucose, fructose, mannose, mannitol, *N*-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, lactose, sucrose and trehalose, but not from erythritol, D-arabinose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, maltose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose,

Table 2. Fatty acid compositions of strains 5B73C^T and 5B133E and related *Virgibacillus* species

Strains: 1, 5B73C^T; 2, 5B133E; 3, *V. pantothenicus* KCTC 3539^T; 4, *V. proomii* KCTC 3822^T; 5, *V. dokdonensis* KCTC 3933^T; 6, *V. salexigens* KCTC 3844^T; 7, *V. marismortui* KCTC 3845^T; 8, *V. carmonensis* KCTC 3819^T; 9, *V. necropolis* KCTC 3820^T; 10, *V. halodenitrificans* KCTC 3790^T; 11, *V. koreensis* KCTC 3823^T. Data for strains 5B73C^T, 5B133E and *V. dokdonensis* KCTC 3933^T are from this study; all other data are from Lee *et al.* (2006). Data for fatty acids representing less than 0.5% are not shown.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
C _{14:0}	2.1	1.5			2.1						1.8
C _{15:0}	1.4	0.7							0.9		5.1
C _{16:0}	5.3	4.4	1.3	4.0	12.7	5.1	1.8	1.0	1.6	1.1	5.9
C _{16:1} ω7c						0.9	1.1	6.5	2.4	3.1	3.4
alcohol											
C _{16:1} ω11c						0.6	0.6	0.6	1.0		2.2
iso-C _{13:0}					1.0						
anteiso-					1.8						
C _{13:0}											
iso-C _{14:0}	5.6	2.9	2.3	2.4	2.1	4.2	2.9	3.3	2.2	7.4	8.3
iso-C _{15:0}	21.2	20.6	7.1	15.1	15.5	34.0	39.0	5.7	3.8	2.4	6.1
anteiso-	35.8	40.9	55.3	38.5	47.0	30.5	33.9	58.3	67.9	51.8	43.4
C _{15:0}											
iso-C _{16:0}	10.3	6.5	7.2	7.3	3.7	8.4	4.3	6.4	4.2	11.8	14.4
iso-C _{17:0}	1.8	1.6	2.0	6.3	2.4	5.6	5.6	0.9	0.9		
anteiso-	16.1	19.9	24.0	24.9	11.5	9.3	8.6	11.1	12.2	19.5	7.0
C _{17:0}											
Summed								5.0	2.1		
feature 3*											
Summed							0.7			1.6	
feature 4*											

*Summed features: 3, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 4, iso-C_{15:0} 2-OH and/or C_{16:1}ω7t.

D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid production from glycerol, L-arabinose, ribose, D-xylose, galactose and gentiobiose is weak. The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type. The major isoprenoid quinone system is MK-7. The major cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The genomic DNA G+C content of the type strain is 42.6 mol%.

The type strain, 5B73C^T (=IAM 15308^T=KCTC 13935^T), was isolated from field soil in Kakegawa, Shizuoka, Japan.

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