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, 5B73C7 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 5B73C7 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 5B73C7 and 5B133E were 42.6 and 42.3 mol%, respectively. The cell-wall peptidoglycan type (meso-diaminopimelic acid), the major cellular fatty acids (anteiso-C15:0, iso-C15:0, anteiso-C17:0 and iso-C16:0) 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 5B73C7 (=IAM 15308T =KCTC 13935T).

The genus Virgibacillus was emended from Bacillus pantothenticus on the basis of amplified DNA restriction analysis results, fatty acid profiles, SDS-PAGE patterns of whole-cell proteins and phenotypic characterization (Heyrman et al., 1998). The genus Virgibacillus currently consists of 10 recognized species (Heyrman et al., 1999; Heyrman et al., 2003; Lee et al., 2006; Yoon et al., 2004, 2005), with Virgibacillus pantothenticus 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-C15:0 as the major cellular fatty acid (Heyrman et al., 2003). Here, we report two novel strains, 5B73C7 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 5B73C7 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 5B73C7 and 5B133E are AB243851 and AB243853, respectively.
Table 1. Differential characteristics of strain 5B73C<sup>T</sup> and related Virgibacillus species

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<td>DNA G + C content (mol%)</td>
<td>42.6</td>
<td>36.9–38.3</td>
<td>36.8–37.0</td>
<td>36.7</td>
<td>36.3–39.5</td>
<td>39.0–42.8</td>
<td>38.9</td>
<td>37.3</td>
<td>38–39</td>
<td>41</td>
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*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<sup>T</sup> 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<sub>nuc</sub> 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> and 5B133E are closely related to the genus Virgibacillus but form a separate clade within that genus.
absence of a close relative showing significant 16S rRNA
gene sequence similarity, strains 5B73CT and 5B133E can be
considered as representing a novel species.

Cellular fatty acids from strains 5B73CT 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 5B73CT and 5B133E were anteiso-C\textsubscript{15}:0 (35.8–
40.9 %), iso-C\textsubscript{15}:0 (21.2–20.6 %), anteiso-C\textsubscript{17}:0 (16.1–
19.9 %) and iso-C\textsubscript{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 5B73CT
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-labeling method of
Ezaki et al. (1989) with a multi-well plate reader
(CytoFluoR; PerSeptive Biosystems). The DNA–DNA
hybridization values for strains 5B73CT and 5B133E and
for 5B133E and 5B73CT 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 5B73CT
(selected as the representative strain for the two isolates) was
conducted by using the methods of Schleifer & Kandler
(1972). Strain 5B73CT possessed the meso-diaminopimelic-
type cell wall. Analysis of the respiratory quinones of strain
5B73CT was performed as described by Collins & Jones
(1981): the major isoprenoid quinone was MK-7.

On the basis of phenotypic, chemotaxonomic and phylo-
genetic data, we conclude that strains 5B73CT 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. hal’s 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 subtermin-
ally. 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. H\textsubscript{2}S 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-xylene, adonitol, methyl β-D-xyloside, sorbose,
rhamnose, dulcitol, inositol, sorbitol, methyl α-D-manno-
side, methyl α-D-glucoside, amygdalin, maltose, melibiose,
inulin, melezitose, raffinose, starch, xylodex, xylitol,
D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose,
Table 2. Fatty acid compositions of strains 5B73C<sup>T</sup> and 5B133E and related Virgibacillus species

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D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid production from glycerol, L-arabinose, ribose, D-xylene, 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<sub>15</sub>:0, iso-C<sub>15</sub>:0, anteiso-C<sub>17</sub>:0, and iso-C<sub>16</sub>:0. The genomic DNA G+C content of the type strain is 42.6 mol%.

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

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