**Virgibacillus alimentarius** sp. nov., isolated from a traditional Korean food

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A novel, Gram-positive, rod-shaped, motile, endospore-forming, halophilic bacterial strain, J18ᵀ, was isolated from a traditional salt-fermented seafood made of gizzard shad in Korea. Colonies were convex, cream-coloured and 1.0–2.0 mm in diameter after incubation for 3 days on marine agar. Growth occurred at pH 7.0–1.10 (optimum, pH 10.0), at 4–40 °C (optimum, 37 °C) and in the presence of 0–30 % NaCl (optimum, 9–10 %). On the basis of 16S rRNA gene sequence analysis, strain J18ᵀ was related most closely to *Virgibacillus byunsanensis* ISL-24ᵀ (96.3 % similarity), *Virgibacillus carmonensis* LMG 20964ᵀ (96.2 %), *Virgibacillus halodenitrificans* DSM 10038ᵀ (96.0 %), *Virgibacillus arcticus* Hal 1ᵀ (95.5 %) and *Virgibacillus necropolis* LMG 19488ᵀ (95.5 %). The major fatty acids were anteiso-C₁₅ : 0 and anteiso-C₁₇ : 0. The DNA G+C content of strain J18ᵀ was 37.0 mol%. The cell-wall peptidoglycan was of the *meso*-diaminopimelic acid type. The major quinone was menaquinone 7 (MK-7). Based on phenotypic, chemotaxonomic and phylogenetic data, strain J18ᵀ is considered to represent a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus alimentarius* sp. nov. is proposed. The type strain is J18ᵀ (=KACC 14624ᵀ =JCM 16994ᵀ).

The genus *Virgibacillus* was originally described by Heyndrickx et al. (1998) and, at the time of writing, comprises 21 recognized species: *V. pantothenticus* (Heyndrickx et al., 1998), *V. proomii* (Heyndrickx et al., 1999), *V. marismortui* (Arahali et al., 2000), *V. carmonensis*, *V. necropolis* (Heyman et al., 2003), *V. salexigens* (Heyman et al., 2003), *V. halodenitrificans* (Yoon et al., 2004), *V. dokdonensis* (Yoon et al., 2005), *V. koreensis* (Lee et al., 2006), *V. halophilus* (An et al., 2007), *V. olivae* (Quesada et al., 2007), *V. chiguisiensis* (Wang et al., 2008), *V. kokenesi* (Chen et al., 2008), *V. salarius* (Hua et al., 2008), *V. arcticus* (Niederberger et al., 2009), *V. byunsanensis* (Yoon et al., 2010), *V. salinus* (Carrasco et al., 2009), *V. sediminis* (Chen et al., 2009), *V. xinjiangensis* (Jeon et al., 2009), *V. soli* (Kämpfer et al., 2011; Kazimirov et al., 1998) and *V. subterraneus* (Wang et al., 2010). Members of the genus are Gram-positive, motile, flagellated, rod-shaped, endospore-forming halophiles. They have DNA G+C contents ranging from 33.4 to 42.6 mol%, their cell-wall peptidoglycan is of the *meso*-diaminopimelic acid type, except for *V. arcticus*, which has peptidoglycan type A1₂, and menaquinone 7 (MK-7) is the predominant isoprenoid quinone. In this study, we describe a novel strain, J18ᵀ, and show that this represents a novel species of the genus *Virgibacillus* based on phenotypic and chemotaxonomic characteristics as well as on phylogenetic analysis of 16S rRNA gene sequences.

Strain J18ᵀ was isolated from jeotgal, a traditional salt-fermented seafood made by mixing fresh gizzard shad with rock salt and leaving it to ferment (Suh & Yoon, 1987). Isolation was performed with the dilution-plating method on marine agar (MA; BBL) at 25 °C and on trypticase soy agar (TSA; BBL) plates. Growth at pH 3–12 was examined by using trypticase soy broth (TSB; BBL). TSB adjusted to pH 10.0 was used to determine NaCl requirements and tolerance ranges (0–30 %), and growth at 0, 4, 10, 15, 25, 30, 37, 40 and 45 °C. Strain J18ᵀ grew at pH 7.0–11.0, at 4–40 °C and in the presence of 0–30 % NaCl, with optimal growth occurring at pH 10.0, at 37 °C and with 9–10 % NaCl. Anaerobic growth was determined following incubation at 37 °C on TSA in an anaerobic chamber (BACLITE-2; Sheldon Manufacturing) with an atmosphere of N₂/CO₂/H₂ (18 : 1 : 1, by volume). Cell morphology was inspected under a light microscope (model ECLIPSE 50; Nikon) and a transmission electron microscope (model JEM-1010; JEOL). The Gram reaction was determined by using the 3 % KOH method (Buck, 1982). Motility was examined...
according to the method of Tittsler & Sandholzer (1936) with semi-solid agar. Endospore formation was examined with the spore-staining method (Schaeffer & Fulton, 1933). Catalase and oxidase tests were performed by using 3% H$_2$O$_2$ and an oxidase reagent (bioMérieux). Hydrolysis of starch, casein, and Tweens 20, 40, 60 and 80 was determined as described by Smibert & Kreig (1994). Physiological and biochemical characteristics were determined by using API 20E and API 50CH test strips according to the manufacturer’s instructions (bioMérieux). The CHB suspension medium for the API 50CH test strips was supplemented with 7% NaCl. The API tests were read after 48 h. Colonies were circular, convex, cream-coloured and 1.0–2.0 mm in diameter after incubation for 3 days on MA. Cells were strictly aerobic, motile, flagellated, endospore-forming, Gram-positive rods, approximately 0.5 μm wide and 1.2 μm long. Strain J18$^T$ was catalase-negative and oxidase-positive. It was positive for hydrolysis of Tweens 20, 40 and 60, but negative for hydrolysis of casein, starch and Tween 80. A detailed description is presented in the species description below, and Table 1 provides a phenotypic comparison of strain J18$^T$ and the type strains of four related species of the genus Virgibacillus.

The 16S rRNA gene sequence of strain J18$^T$ was amplified by PCR by using PCR Pre-mix (Solgent) and two universal primers: forward primer 8F (5′-AGAGTTTGATCCTGCGG-TCAG-3′) and reverse primer 1492R (5′-GAGTTACCTGACTT-TACGACTT-3′). The PCR product was purified with a QIAquick PCR Purification kit (Qiagen). After purification, the PCR product was sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as previously described (Roh et al., 2008). 16S rRNA gene sequences were assembled by using the SeqMan software (DNASTAR). Sequence similarities were determined via the EzTaxon server (Chun et al., 2007) to identify the closest phylogenetic neighbours of strain J18$^T$. The nearly full-length 16S rRNA gene sequence of strain J18$^T$ (1458 bp) and reference 16S rRNA gene sequences collected from GenBank were aligned by using the CLUSTAL X multiple sequence alignment program (Thompson et al., 1997). Phylogenetic distances from representative species of the genus Virgibacillus were determined with the MEGA 4 software (Tamura et al., 2007). Phylogenetic trees were generated by using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Kluge & Farris, 1969) methods. A bootstrap test with 1000 replicates was used to determine the

### Table 1. Differential characteristics between strain J18$^T$ and the type strains of closely related species of the genus Virgibacillus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>Pigmentation</td>
<td>Cream</td>
<td>Yellow</td>
<td>Pink</td>
<td>Cream</td>
<td>Yellow</td>
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<tr>
<td>Growth temperature (°C)</td>
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<td>10–45</td>
<td>10–40</td>
<td>10–40</td>
<td>5–45</td>
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<td>Spore shape*</td>
<td>E</td>
<td>E</td>
<td>E or S</td>
<td>E</td>
<td>E</td>
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<tr>
<td>Spore position†</td>
<td>T or ST</td>
<td>T or ST</td>
<td>ST</td>
<td>C or ST or T</td>
<td>ST</td>
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<td>Anaerobic growth</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Acid production from:</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>w</td>
<td>–</td>
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<tr>
<td>D-Ribose</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>w</td>
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<td>w</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Potassium 5-ketogluconate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>w</td>
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<td>DNA G + C content (mol%)</td>
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<td>38.0–39.0</td>
<td>38.9</td>
<td>37.3</td>
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</tr>
</tbody>
</table>

*E, ellipsoidal; S, spherical.
†C, central; ST, subterminal; T, terminal.
confidence of the branching patterns of the trees created (Felsenstein, 1985). Strain J18T was most closely related to V. byunsanensis ISL-24T (96.3 % 16S rRNA gene sequence similarity), V. carmonensis LMG 20964T (96.2 %), V. halodenitrificans DSM 10037T (96.0 %), V. arcticus Hal 1T (95.5 %) and V. necropolis LMG 19488T (95.5 %). These values are all below 97.0 %, indicating that strain J18T represents a novel species. The phylogenetic relationships between strain J18T and the type strains of recognized species of the genus Virgibacillus are shown in Fig. 1.

Analysis of cellular fatty acids was conducted by using cells grown on MA for 3 days at 37 °C. Cellular fatty acids were extracted, analysed by GC (Hewlett Packard 6890) and identified by using the Sherlock Microbial Identification System (MIDI). The main cellular fatty acids of strain J18T were anteiso-C15 : 0 (52.1 %), anteiso-C17 : 0 (32.0 %), iso-C16 : 0 (4.5 %), iso-C17 : 1 and/or anteiso-C17 : 1 (4.0 %), C16 : 0 (3.1 %), iso-C15 : 0 (2.4 %) and C16 : 1 ω11c (2.0 %). The amino acid composition of cell-wall hydrolysates was determined by using one-dimensional TLC on cellulose sheets (Bousfield et al., 1985). The diagnostic amino acid of the cell wall was meso-diaminopimelic acid. The major polar lipids were extracted according to Xin et al. (2000), separated by two-dimensional TLC on a Merck silica gel 60 F254 glass-backed plate with chloroform/methanol/water (65:25:4, by volume) as the first solvent and chloroform/acetone/methanol/water (80:15:12:4, by volume) as the second solvent, and detected by spraying the plate with specific reagents, as described by Tindall (1990). The designations of all spots were as referred to by Kämpfer et al. (2011). The polar lipids present were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylyethanolamine and two unknown lipids (see Supplementary Fig. S1 available in IJSEM Online).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain J18T among related taxa. Filled circles and open circles indicate generic branches that were also recovered by using the minimum-evolution and maximum-parsimony algorithms, and the minimum-evolution algorithm, respectively. Numbers at nodes are bootstrap values (expressed as percentages of 1000 replications) as calculated based on neighbour-joining/minimum-evolution/maximum-parsimony probabilities; only values greater than 70 % are shown. Bar, 0.005 accumulated changes per nucleotide.
Quinone extraction was performed following the method of Komagata & Suzuki (1987). MK-7 was the predominant menaquinone in strain J18T.

Genomic DNA was extracted by using a G-spin DNA extraction kit (iNtRON Biotechnology). The genomic G+C content was estimated with a fluorimetric method by using SYBR Green I and real-time PCR (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from the completely sequenced Bacteroides finegoldii DSM 17565T and Escherichia coli K-12 was used for calibration. The DNA G+C content of strain J18T was 37.0 mol%. This result is consistent with values reported for recognized members of the genus Virgibacillus, further supporting the affiliation of the isolate to this genus.

Phenotypic (major fatty acid profile, predominant isoprenoid quinone, cell-wall peptidoglycan type) and genotypic characteristics (genomic DNA G+C content) support the inclusion of strain J18T in the genus Virgibacillus. However, morphological, cultural, physiological and biochemical characteristics, as well as low levels of 16S rRNA gene sequence similarity between strain J18T and the type strains of recognized species of the genus Virgibacillus, support its recognition as a representative of a novel species, for which we propose the name Virgibacillus alimentarius sp. nov.

**Description of Virgibacillus alimentarius sp. nov.**

*Virgibacillus alimentarius* (a.li.men.ta’ri.uis. L. masc. adj. alimentarius pertaining to food).

Colonies are circular, convex, cream-coloured and 1.0–2.0 mm in diameter after incubation for 3 days on MA. Cells are aerobic, Gram-positive, motile rods (0.5 × 1.2 μm) which form chains. Oxidase-positive but catalase-negative. Spores are formed in terminal or subterminal position. Growth occurs at pH 7.0–11.0 (optimum, pH 10.0), at 4–40 °C (optimum, 37 °C) and with 0–30 % NaCl (optimum, 9–10 %). Hydrolyses Tweens 20, 40 and 60. Produces gas from glycerol, D-ribose, D-adonitol, sulphide, urease, tryptophan deaminase, indole or gelatine-decarboxylase, ornithine decarboxylase, citrate, hydrogen acetoin, but not ONPG, arginine dihydrolase, lysine (9–10 %). Hydrolyses Tweens 20, 40 and 60. Produces 37.0 mol%. The cell-wall peptidoglycan is of the meso-diaminopimelic acid type. The predominant menaquinone is MK-7.

The type strain, J18T (=KACC 14624T =JCM 16994T), was isolated from a traditional salt-fermented seafood made from gizzard shad in Korea.

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**References**


