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

Jandi Kim,[1] Mi-Ja Jung,[1] Seong Woon Roh,[1] Young-Do Nam,[1] Kee-Sun Shin[2] and Jin-Woo Bae[1]

Correspondence
Jin-Woo Bae
baejw@khu.ac.kr

[1]Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

[2]Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Republic of Korea

A novel, Gram-positive, rod-shaped, motile, endospore-forming, halophilic bacterial strain, J18^T, 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 7.0–11.0 (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 J18^T was related most closely to *Virgibacillus byunsanensis* ISL-24^T (96.3 % similarity), *Virgibacillus carmonensis* LMG 20964^T (96.2 %), *Virgibacillus halodenitrificans* DSM 10037^T (96.0 %), *Virgibacillus arcticus* Hal 1^T (95.5 %) and *Virgibacillus necropolis* LMG 19488^T (95.5 %). The major fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The DNA G+C content of strain J18^T 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 J18^T 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 J18^T (=KACC 14624^T =JCM 16994^T).

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* (Arahal *et al.*, 2000), *V. carmonensis*, *V. necropolis* (Heyrman *et al.*, 2003), *V. salexigens* (*Heyrman 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. chiguensis* (Wang *et al.*, 2008), *V. kekensis* (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 A1α, and menaquinone 7 (MK-7) is the predominant isoprenoid quinone. In this study, we describe a novel strain, J18^T, 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 J18^T 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 J18^T 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 $N_2/CO_2/H_2$ (18 : 1 : 1, by volume). Cell morphology was inspected under a light microscope (model ECLIPSE 50*i*; 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain J18^T is GU202420.

One supplementary figure is available with the online version of this paper.

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_2O_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′-AGAGTTTGATCCTGGC-TCAG-3′) and reverse primer 1492R (5′-GGYTACCTT-GTTACGACTT-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*

Strains: 1, J18^T; 2, *V. halodenitrificans* DSM 10037^T (data from Yoon *et al.*, 2004); 3, *V. carmonensis* LMG 20964^T (Heyrman *et al.*, 2003); 4, *V. necropolis* LMG 19488^T (Heyrman *et al.*, 2003); 5, *V. halophilus* IAM 15308^T (An *et al.*, 2007). W, Weakly positive.

Characteristic	1	2	3	4	5
Pigmentation	Cream	Yellow	Pink	Cream	Yellow
Growth temperature (°C)	4–40	10–45	10–40	10–40	5–45
Spore shape*	E	E	E or S	E	E
Spore position†	T or ST	T or ST	ST	C or ST or T	ST
Anaerobic growth	−	+	−	−	−
Acid production from:					
Glycerol	+	+	−	W	−
D-Ribose	−	−	−	−	+
D-Xylose	−	−	−	−	+
D-Galactose	−	+	−	−	−
D-Glucose	−	W	−	−	+
D-Fructose	−	+	−	+	+
D-Mannose	−	+	−	−	+
L-Rhamnose	−	−	−	W	−
N-Acetylglucosamine	+	+	W	W	+
Aesculin	+	−	+	−	+
Melibiose	−	−	−	W	−
Sucrose	−	+	−	−	+
Trehalose	−	+	−	−	+
Potassium 5-ketogluconate	+	+	+	W	−
DNA G+C content (mol%)	37.0	38.0–39.0	38.9	37.3	42.6

*E, ellipsoidal; S, spherical.

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

confidence of the branching patterns of the trees created (Felsenstein, 1985). Strain J18^T was most closely related to *V. byunsanensis* ISL-24^T (96.3 % 16S rRNA gene sequence similarity), *V. carmonensis* LMG 20964^T (96.2 %), *V. halodenitrificans* DSM 10037^T (96.0 %), *V. arcticus* Hal 1^T (95.5 %) and *V. necropolis* LMG 19488^T (95.5 %). These values are all below 97.0 %, indicating that strain J18^T represents a novel species. The phylogenetic relationships between strain J18^T 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 J18^T were anteiso-$C_{15:0}$ (52.1 %), anteiso-$C_{17:0}$ (32.0 %), iso-$C_{16:0}$ (4.5 %), iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$ (4.0 %), $C_{16:0}$ (3.1 %), iso-$C_{15:0}$ (2.4 %) and $C_{16:1}\omega11c$ (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/acetic acid/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, phosphatidylethanolamine and two unknown lipids (see Supplementary Fig. S1 available in IJSEM Online).

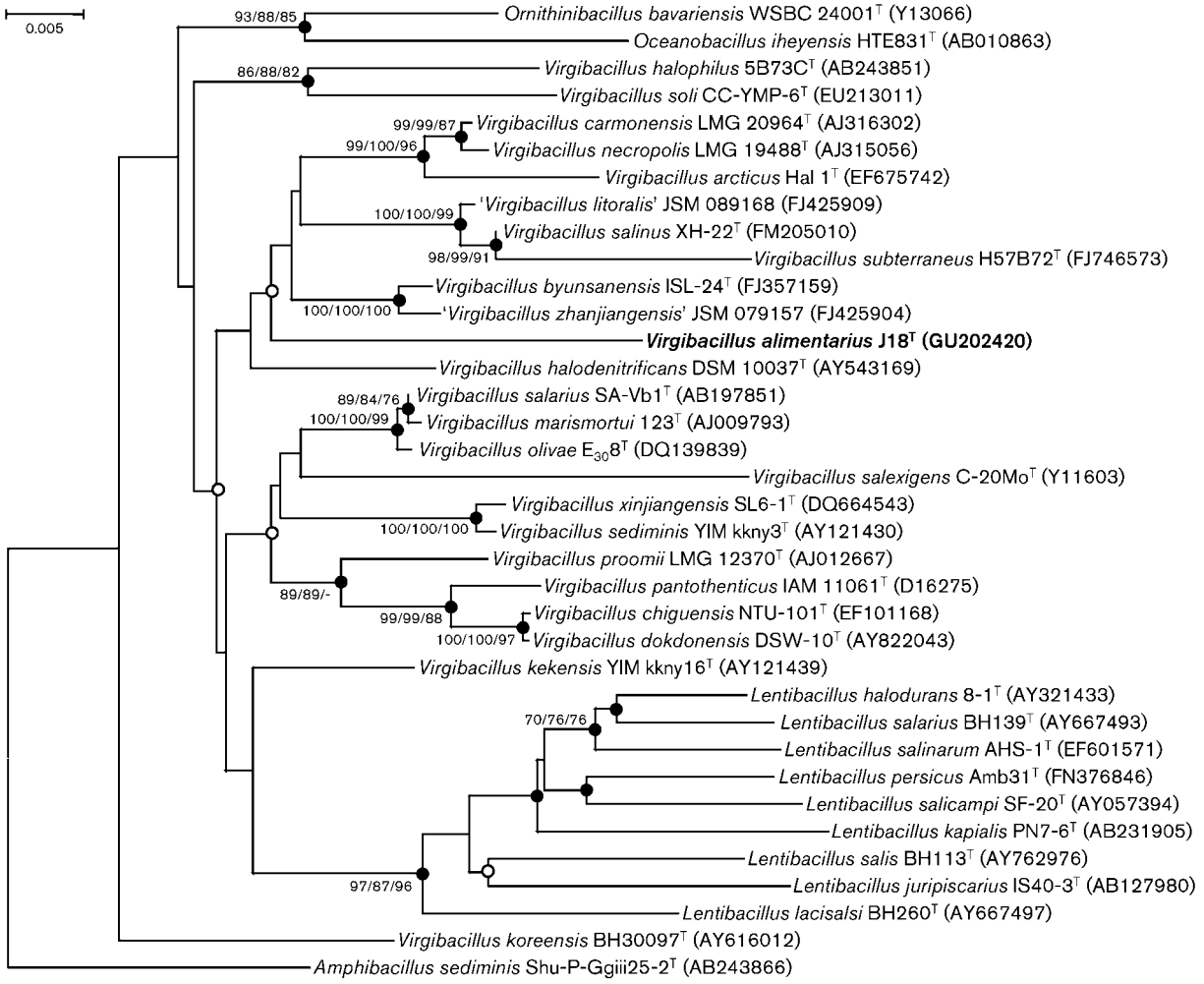

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain J18^T among related taxa. Filled circles and open circles indicate generic branches that were also recovered by using the minimum-evolution and maximum-parsimony algorithms, and the minimum-evolution 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 J18^T.

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 17565^T and *Escherichia coli* K-12 was used for calibration. The DNA G+C content of strain J18^T 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 J18^T in the genus *Virgibacillus*. However, morphological, cultural, physiological and biochemical characteristics, as well as low levels of 16S rRNA gene sequence similarity between strain J18^T 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.us. 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 acetoin, but not ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate, hydrogen sulphide, urease, tryptophan deaminase, indole or gelatinase. Acid is produced from glycerol, D-ribose, D-adonitol, D-glucose, *N*-acetylglucosamine, aesculin, D-tagatose, potassium 2-ketogluconate and potassium 5-ketogluconate, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol or potassium gluconate. The predominant fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$, iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$, $C_{16:0}$, iso-$C_{15:0}$ and $C_{16:1}\omega11c$. The polar lipids present are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and two unknown lipids. The genomic DNA G+C content of the type strain is 37.0 mol%. The cell-wall peptidoglycan is of the *meso*-diaminopimelic acid type. The predominant menaquinone is MK-7.

The type strain, J18^T (=KACC 14624^T =JCM 16994^T), was isolated from a traditional salt-fermented seafood made from gizzard shad in Korea.

Acknowledgements

We thank J. P. Euzéby (Ecole Nationale Vétérinaire, France) for etymological advice. This study was supported by the TDPAF (Technology Development Program for Agriculture and Forestry).

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