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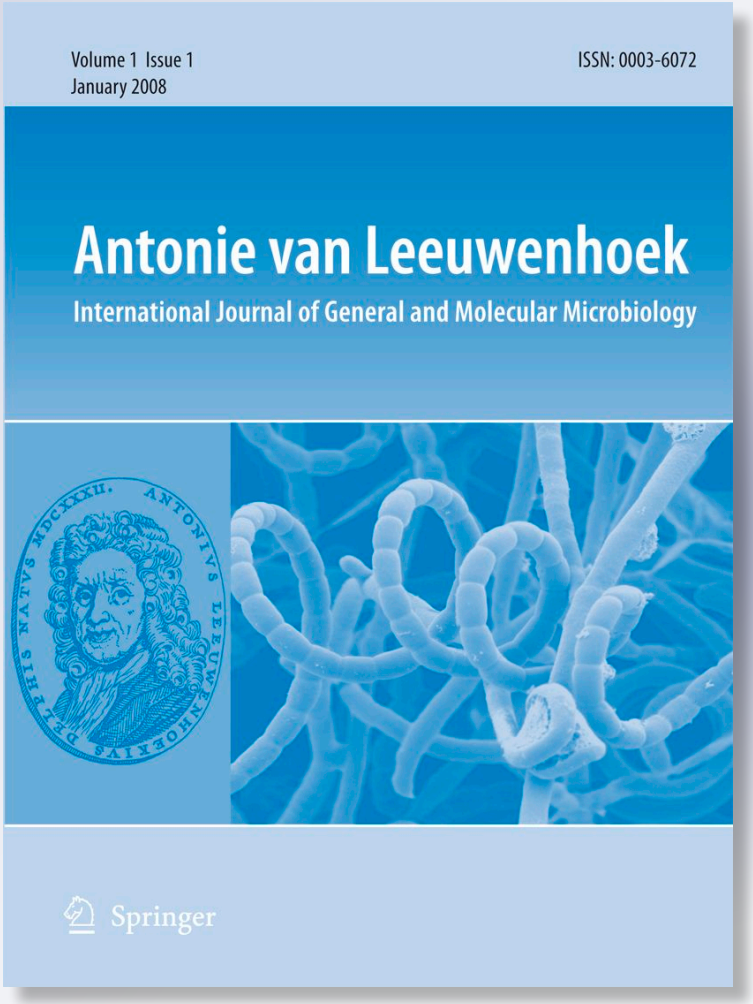

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ORIGINAL PAPER

Ornithinibacillus scapharcae sp. nov., isolated from a dead ark clam

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Abstract A novel Gram-positive, aerobic, motile, hemolytic, endospore-forming and rod-shaped bacterium TW25^T was isolated from a dead ark clam during a mass mortality event on the South coast of Korea. The strain grew optimally at 30°C, at pH 8–9, and with 1% (w/v) NaCl. The 16S rRNA gene sequence analysis indicated that strain TW25^T was associated with the genus *Ornithinibacillus* and that it was most closely related to the type strain of *Ornithinibacillus californiensis* (98.5% similarity). The dominant cellular fatty acids were iso-C15:0, anteiso-C15:0 and C16:0. The peptidoglycan amino acid type was A4β, containing L-ornithine and D-aspartic acid. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, four unidentified phospholipids, two unidentified aminolipids and two unidentified lipids. The major respiratory quinone was menaquinone-7 (MK-7). The G + C content of genomic DNA was 36.7 mol%. DNA–DNA hybridization experiments with related strains revealed lower than 11 ± 3% relatedness. Based on this polyphasic taxonomic study, strain TW25^T represents a novel species in the genus *Ornithinibacillus*, for which the name *Ornithinibacillus scapharcae* sp. nov. is proposed. The type strain is TW25^T (=KACC 15116^T = JCM 17314^T).

Keywords Ornithinibacillus scapharacae · Polyphasic taxonomy · Ark clam · A4β type peptidoglycan

Na-Ri Shin and Tae Woong Whon contributed equally to this work.

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Introduction

Two species of the genus *Ornithinibacillus* were first introduced by Mayr et al. (2006) and the genus now contains three validated species: *Ornithinibacillus bavariensis*, *Ornithinibacillus californiensis* (Mayr et al. 2006) and *Ornithinibacillus contaminans* (Kämpfer et al. 2010). Species in this genus are distinguished from other genera in the family *Bacillaceae* by their L-ornithine-containing (A4β) peptidoglycan type, their major fatty acid components (iso-C15:0 and anteiso-C15:0) and a 36–41 mol% G + C content (Mayr et al. 2006). Members of the genus *Ornithinibacillus* have been isolated from various environments, such as

pasteurized milk, coastal surface sediments (Mayr et al. 2006) and human blood (Kämpfer et al. 2010). The current study reports a novel bacterial strain that putatively belongs to the genus *Ornithinibacillus*, which was identified during an attempt to isolate and characterize tentative pathogenic bacteria following a mass ark clam mortality event. The current study describes the taxonomic characterization of the novel bacterial strain, TW25^T, and it is suggested that this strain represents a novel species of the genus *Ornithinibacillus*.

Materials and methods

Bacterial strain and culture conditions

Strain TW25^T was isolated from a dead ark clam collected at Gang-jin Bay in the Southern coastal region of the Korean peninsula. Bacteria were isolated by homogenizing the sample with sterilized phosphate buffered saline followed by disruption with sonication for 30 s. After serial dilution, the diluted sample was spread on tryptic soy agar (TSA; Bacto) plates and incubated at 20°C. The isolate was repeatedly transferred to obtain a pure culture. After primary purification, the isolate was preserved at −80°C in tryptic soy broth (TSB; Bacto) containing 40% (v/v) glycerol. Type strains of three closely related species were obtained from the German Culture Collection (DSMZ) and used as reference species, i.e., *O. californiensis* MB-9^T, *O. bavariensis* WSBC 24001^T and *O. contaminans* CCUG 53201^T.

Morphological, physiological, and biochemical characterization

Physiological and biochemical tests were conducted with strain TW25^T after cultivation for 48 h at 30°C in tryptic soy broth (TSB; Bacto) or TSA medium. Gram-staining was performed using a Gram staining kit (bioMérieux), according to the manufacturer's instructions. Endospore formation was determined using the spore-staining method with malachite green, as described by Schaeffer and Fulton (1933). Cell morphology and Gram- and spore-staining of the novel isolate were observed by light microscopy and phase contrast microscopy (Eclipse 50*i*, Nikon). Bacterial hemolysis was determined by green colony formation, followed by the reduction of hemoglobin on Columbia agar (BBL) medium supplemented with 5% horse blood. Motility was determined using semi-solid agar (Tittsler and Sandholzer 1936) and the presence of flagella was determined with an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss). Catalase and oxidase activities were tested with 3% (v/v) hydrogen peroxide solution and 1% (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. Growth ranges and optimal conditions for strain TW25^T were determined by culture in TSB medium at different temperatures (4, 10, 15, 25, 30, 37, 45 and 55°C), in different pHs (pH 4–11, at intervals of 1 pH unit) and in the presence of various concentrations of NaCl (0, 1, 2, 3, 4, 5, 8, 10, 12, 15 and 20%, w/v). pH values were adjusted using 10 mM MES (for pH 4, 5 and 6), 10 mM TAPS (for pH 7, 8 and 9), or 10 mM Na_2HPO_4 (for pH 10 and 11). The turbidity of each culture was measured as the optical density at 600 nm (OD_{600}) using a spectrophotometer (SYNERGY MX, BioTek) after 24 h, 48 h and 7 days of incubation. Anaerobic growth was assessed in cultures grown on TSA plates incubated in an anaerobic chamber (N_2:H_2:CO_2, 90:5:5) at 37°C for 7 days. Hydrolysis of starch and casein were tested by incubating the novel isolate on TSA medium supplemented with 0.5% soluble starch (JUNSEI) and 5% skim milk, respectively. Gelatin hydrolysis was assessed using API 20NE test strips (bioMérieux), according to the manufacturer's instructions. Citrate utilization was tested by observing changes in the medium color on Simmons citrate agar (BBL). The ability to assimilate specific sole carbon sources was determined for strain TW25^T and the reference species using GP2 MicroPlates (Biolog) and inoculating fluid (Biolog), according to the manufacturer's instructions with modification. The inoculating fluid was supplemented with 1% (w/v) NaCl for strain TW25^T, *O. californiensis* MB-9^T and *O. bavariensis* WSBC 24001^T. Acid production from carbohydrates was tested using API 50CH test strips (bioMérieux) with 50 CHB/E medium (bioMérieux) and enzyme activity was tested with API ZYM test strips (bioMérieux), according to the manufacturer's instructions.

Chemotaxonomic characterization

Cells of strain TW25^T and the reference species were cultured on TSA or TSB medium at 30°C for 72 h and then harvested for chemotaxonomic analyses.

Peptidoglycan amino acids, polar lipids and isoprenoid quinones were determined using freeze-dried cells. Fatty acids were extracted as described for the Sherlock Microbial Identification System (MIDI, 1999), analyzed by gas chromatography (Hewlett Packard 6890) and identified using the Microbial Identification software package (Sasser, 1990) based on the TSBA40 database. Cell-wall peptidoglycan amino acids were extracted (6 N HCl, 121°C, 15 min) and identified by one-dimensional thin layer chromatography (TLC) on a cellulose TLC plate (Merck) with standard amino acids, according to the method of Bousfield et al. (1985). Polar lipids were extracted in accordance with the method described by Xin et al. (2000) and separated by two-dimensional TLC on a silica TLC plate (Merck). After separation, each component was identified by spraying individual plates with appropriate detection reagents (Tindall 1990), i.e., molybdatophosphoric acid for total lipids, ninhydrin reagent for amino-containing lipids, Zinzadze reagent for phospholipids and alpha-naphthol reagent for glycolipids. Isoprenoid quinones were extracted with chloroform–methanol (2:1, v/v) as described by Collins and Jones (1981a), purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and then identified by reverse-phase high performance liquid chromatography (HPLC) (Collins and Jones 1981b) using a Thermo ODS Hypersil (250 × 4.6 mm) column.

16S rRNA gene sequence determination and phylogenetic analysis

Phylogenetic analysis was conducted by amplifying the 16S rRNA gene sequence of strain TW25^T using colony PCR with PCR pre-mix (iNtRon Biotechnology) and two universal bacteria-specific primers, 8F and 1492R (Baker et al. 2003). The purified PCR product (QIAquick® PCR Purification Kit) was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's instructions. The reaction mixtures were analyzed using an automated DNA analyzer system (PRISM 3730XL DNA analyzer, Applied Biosystems). Sequence fragments were assembled using SeqMan software (DNASTAR) to obtain a near full-length 16S rRNA gene sequence. The 16S rRNA gene sequence of strain TW25^T was then compared with other type strains in the EzTaxon server (Chun et al. 2007). The quality of the 16S rRNA gene sequences was confirmed manually using Bioedit software (Hall 1999). The confirmed 16S rRNA gene sequences of strain TW25^T and those of the most closely related species were aligned using the multiple alignment program CLUSTAL W (Thompson et al. 1994). Phylogenetic consensus trees based on the aligned 16S rRNA gene sequences were constructed using the neighbor-joining (Saitou and Nei 1987), maximum-parsimony (Kluge and Farris 1969) and maximum-likelihood (Felsenstein 1981) methods, with 1000 randomly selected bootstrap replicates using MEGA version 5 (Tamura et al. 2011).

DNA G + C content determination and DNA–DNA hybridization (DDH)

The genomic DNA of strain TW25^T and reference species was extracted using the method described by Rochelle et al. (1992) for genotypic analysis. The DNA G + C content of strain TW25^T was determined by whole genome sequencing using Roche 454 GS (FLX Titanium) pyrosequencing. DDH was performed using genome-probing microarrays (Bae et al. 2005; Chang et al. 2008) with reciprocal analysis to determine the genetic relatedness between the novel isolate and the reference species. DDH values were calculated based on the signal-to-noise ratio (SNR) of each probe using a previously reported formula (Loy et al. 2005).

Results and discussion

Cells of strain TW25^T were Gram-positive, rod-shaped (1.0–1.3 μm in length and 0.3–0.5 μm in width), hemolytic, endospore-forming in a single terminal or both terminal positions with swollen sporangia, and the cells possessed peritrichous flagella for motility (Supplementary Fig. S1). Endospore formation is a general characteristic of members of the family *Bacillaceae* (Logan et al. 2009). Hemolysis was observed in strain TW25^T and the reference species. The novel isolate was catalase- and oxidase-positive. Colonies were brownish, circular, with a diameter of 2.0–3.0 mm, translucent and crateriform after incubation on TSA medium at 30°C for 48 h. Colonies of the isolate were stuck on TSA medium after 24 h. After 5 days incubation, colonies were

aggregated and formed an irregular yellow colored mass. Growth of strain TW25^T was observed at 10–37°C and pH 7–10, with optimum growth at 30°C and pH 8–9. Strain TW25^T grew at NaCl concentrations of 0–5% (w/v), with optimal growth at 1% (w/v) NaCl. Growth under anaerobic conditions was not observed.

Starch was hydrolyzed, but casein and gelatin were not hydrolyzed. Citrate was not utilized. Strain TW25^T assimilated only α-ketovaleric acid on GP2 MicroPlates. This assimilation inactivity was shared by other species of the genus *Ornithinibacillus* (Kämpfer et al. 2010). The novel isolate produced acid from the following on API 50CHB/E test strips: glycerol, D-ribose, D-xylose, D-glucose, D-mannitol, methyl-α-D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, sucrose, D-trehalose, starch, glycogen, xylitol and gentiobiose. Strain TW25^T was positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase on API ZYM test strips. Biochemical characteristics that distinguished the novel isolate from the reference species are presented in Table 1.

The major cellular fatty acids (>10% of total fatty acids) of strain TW25^T were iso-C15:0 (56.1%), anteiso-C15:0 (15.2%) and C16:0 (11.8%). The novel isolate was distinguished from other species of the genus *Ornithinibacillus* based on differences in the relative proportions of the fatty acids, such as C16:0, C14:0, iso-C15:0 and anteiso-C17:0 (Table 2). The purified cell-wall peptidoglycan amino acids of strain TW25^T contained L-ornithine, L-alanine, D-aspartic acid and D-glutamic acid. Based on the amino acid composition, the isolate had peptidoglycan type A4β, which matched the reference species. The polar lipids (Supplementary Fig. S2) consisted of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), four unidentified phospholipids (PL1–PL4), two unidentified aminolipids (AL1–AL2) and two unidentified lipids (L1–L2). The presence of DPG and PG was shared with the reference species. The predominant menaquinone detected in strain TW25^T was MK-7. This quinone type is a common feature of the genus *Ornithinibacillus* (Mayr et al. 2006).

The 16S rRNA gene sequence analysis showed that strain TW25^T had the highest level of similarity to *O. californiensis* MB-9^T (98.5% similarity), *O. bavariensis* WSBC 24001^T (98.3%) and *O. contaminans* CCUG 53201^T (98.0%). This indicated that the novel isolate was affiliated with the genus *Ornithinibacillus*. The phylogenetic tree showed that strain TW25^T was clustered within a branch containing the other species of the genus *Ornithinibacillus* (Fig. 1). The type species of the genus *Ornithinibacillus* were related to the genus *Oceanobacillus* but differences between the two genera based on cell-wall peptidoglycan amino acids (A4β type), the predominant fatty acids (iso-C15:0 and anteiso-C15:0), and the amount of PG suggested that they should be classified as a separate genus (Mayr et al. 2006).

The DNA G + C content of strain TW25^T was 36.7 mol% (Whon et al. 2011), which was in the range of other species in the genus *Ornithinibacillus*. The DDH values for the novel isolate and the reference species were as follows: 11 ± 3% (reciprocal 22 ± 3%) for *O. californiensis* MB-9^T; 10 ± 1% (reciprocal 37 ± 2%) for *O. bavariensis* WSBC 24001^T; and 11 ± 2% (reciprocal 27 ± 4%) for *O. contaminans* CCUG 53201^T. The results of the reciprocal analysis are given in Table 3. According to earlier research by Chang et al. (2008), the DDH method using a genome-probing microarray provides improved results in terms of accuracy and precision compared with other traditional methods using microplates (Ezaki et al. 1989) or nylon membranes (Kafatos et al. 1979). Strain TW25^T was considered a distinct genospecies, because a DDH value of less than 70% indicates species distinctness (Wayne et al. 1987). Additionally, the major features revealed from genome sequencing are that strain TW25^T has the genes putatively involved in inclusion of L-ornithine and pathogenesis such as adhesion, bile hydrolysis and multidrug resistance (Whon et al. 2011).

Based on its different phylogenetic, phenotypic and genotypic properties, strain TW25^T represents a novel species of the genus *Ornithinibacillus*, for which the name *Ornithinibacillus scapharcae* sp. nov. is proposed.

Description of *Ornithinibacillus scapharcae* sp. nov

Ornithinibacillus scapharcae (sca.phar'ca.e. N.L. n. *Scapharca*, a scientific zoological generic name; scapharcae, of Scapharca, isolated from the ark clam, *Scapharca inaequivalvis*).

Based upon a single isolate, the strain is Gram-positive, strictly aerobic, motile with peritrichous

Table 1 Physiological and biochemical characteristics of strain TW25^T and phylogenetic relatives in the genus *Ornithinibacillus*

Characteristic	1	2	3	4
Temperature range	10–37	10–37[a]	15–45[a]	20–45[b]
Optimal temperature	30	30[a]	42[a]	30[b]
Salinity range (%)	0–5	0.5–12[a]	0–10[a]	0–6[b]
Optimal salinity (%)	1	0.5–8[a]	0.5–4[a]	0[b]
Motility	+	+[a]	+[a]	−[b]
Assimilation of				
α-D-Glucose	−	−	−	+
D-Mannose	−	−	−	+
Salicin	−	+	−	−
Sucrose	−	w	−	−
D-Trehalose	−	+	−	w
L-Malic acid	−	w	−	−
Pyruvatic acid methyl ester	−	−	w	−
Acid production from				
Glycerol	+	+	+	w
D-Arabinose	−	−	−	w
D-Ribose	+	w	+	−
D-Xylose	w	−	−	+
D-Fructose	−	−	+	w
D-Mannose	−	−	−	+
D-Mannitol	+	−	+	w
D-Sorbitol	−	−	w	−
Methyl-α-D-glucoside	w	−	−	+
N-Acetylglucosamine	+	−	+	+
Amygdalin	+	−	−	w
Arbutin	+	−	−	w
Salicin	+	+	−	+
D-Cellobiose	+	−	w	+
D-Maltose	+	+	−	+
Sucrose	+	+	−	−
D-Trehalose	+	+	w	+
Starch	+	w	−	+
Glycogen	+	−	−	−
Xylitol	w	−	w	w
Gentiobiose	w	−	−	w
D-Arabitol	−	−	w	+
5-Ketogluconate	−	w	w	w
Enzyme activity				
Alkaline phosphatase	+	−	+	−
Esterase (C4)	+	+	w	+
Esterase lipase (C8)	w	+	w	w
Leucine arylamidase	w	w	+	w
Acid phosphatase	−	−	+	−
Naphthol-AS-BI-phosphohydrolase	w	w	+	w
α-Glucosidase	+	+	−	+
β-Glucosidase	w	−	−	−

Strains: 1, strain TW25^T; 2, *O. californiensis* MB-9^T; 3, *O. bavariensis* WSBC 24001^T; 4, *O. contaminans* CCUG 53201^T. All data were obtained from current study, unless stated otherwise. All strains were positive for α-ketovaleric acid on GP2 MicroPlates and D-glucose on API 50CHB/E test strips. *Symbol*: + positive, − negative, *w* weakly positive

[a] Data from Mayr et al. (2006)

[b] Data from Kämpfer et al. (2010)

Table 2 Cellular fatty acid profiles (%) of strain TW25^T and the reference species

Fatty acid	1	2	3	4
Saturated acids				
C10:0	tr	tr	tr	0.5
C12:0	–	–	–	0.6
C14:0	4.4	0.5	0.6	1.1
C15:0	0.6	tr	tr	–
C16:0	11.8	1.8	3.0	1.8
C18:0	–	–	tr	–
Unsaturated acids				
C16:1 ω11*c*	tr	3.2	1.3	–
C16:1 ω7*c* alcohol	–	3.1	0.8	–
C18:1 ω7*c*	–	–	tr	–
C18:1 ω9*c*	–	–	tr	–
Branched acids				
iso-C11:0	–	–	tr	–
iso-C13:0	1.0	tr	tr	0.7
iso-C14:0	0.9	1.1	1.0	1.5
iso-C15:0	56.1	43.4	46.6	46.6
iso-C15:0 3OH	tr	–	–	–
anteiso-C15:0	15.2	22.2	17.5	17.4
iso-C16:0	1.2	1.4	2.9	4.8
iso-C17:0	5.1	5.9	9.8	13.0
anteiso-C17:0	4.1	8.8	10.8	12.1
iso-C17:1 ω10*c*	–	3.1	2.7	–
Summed features*				
3	tr	–	–	–
4	–	4.8	1.2	–

Strains: 1, strain TW25^T; 2, *O. californiensis* MB-9^T; 3, *O. bavariensis* WSBC 24001^T; 4, *O. contaminans* CCUG 53201^T. All data were obtained from the current study. Values are percentages of total fatty acids. *tr* trace (<0.5%); – not detected

* Summed features represent two or three fatty acids that cannot be separated using the Microbial Identification System. Summed feature 3 comprises C16:1 ω7*c*/iso-C15 2OH. Summed feature 4 comprises iso-C17:1 I/antei B

flagella, and rod-shaped (1.0–1.3 μm × 0.3–0.5 μm). The strain has hemolytic activity and forms endospore. The spores are spherical and they are located at one terminal or both terminal positions in swollen sporangia. Colonies on TSA medium are circular, 2.0–3.0 mm in diameter, brownish in color, translucent and crateriform. It is catalase- and oxidase-positive. Growth occurs under a range of conditions: 10–37°C; 0–5% (w/v) NaCl; and pH 7–10. Optimal growth occurs at 30°C in the presence of 1% (w/v) NaCl and at pH 8–9. Growth occurs under aerobic, but not anaerobic conditions. The strain hydrolyzes starch, but not casein or gelatin. The test for citrate utilization is negative. Only α-ketovaleric acid is assimilated on GP2 MicroPlates. Acid is produced from glycerol, D-ribose, D-xylose, D-glucose, D-mannitol, methyl-α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, sucrose, D-trehalose, starch, glycogen, xylitol and gentiobiose on API 50CHB/E test strips. The API ZYM test strips revealed positive results for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. The major cellular fatty acids are iso-C15:0, anteiso-C15:0 and C16:0. The cell-wall peptidoglycan contains ornithine as a diamino acid (A4β type). The polar lipids comprise DPG, PG, PL1–PL4, AL1–AL2 and L1–L2. The predominant isoprenoid quinone is

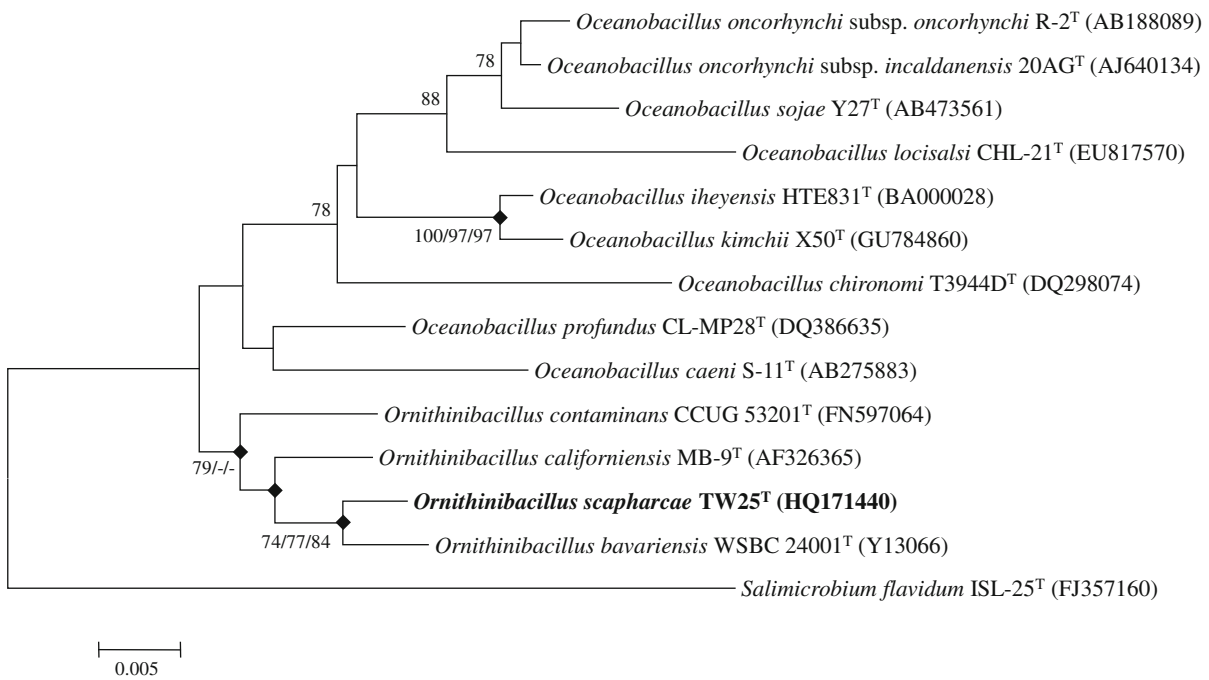

Fig. 1 Phylogenetic consensus tree based on 16S rRNA gene sequences, showing the taxonomic position of strain TW25^T with respect to other species in the genus *Ornithinibacillus*. Bootstrap values (>70%, *dashes* indicate values below 70%) are shown at branch nodes, based on 1000 replicates and calculated using the neighbor-joining, maximum-parsimony and maximum-likelihood methods. *Filled diamonds* at branch nodes indicate identical branches generated using the three different methods. *Salimicrobium flavidum* ISL-25^T served as an outgroup. *Bar* 0.005 substitutions per nucleotide position

Table 3 DNA–DNA hybridization values for strain TW25^T and the reference species (from reciprocal analysis)

Species	DNA-DNA hybridization (%) with			
	TW25^T	MB-9^T	WSBC 24001^T	CCUG 53201^T
O. scapharcae TW25^T	100 ± 0	22 ± 3	37 ± 2	27 ± 4
O. californiensis MB-9^T	11 ± 3	100 ± 0	17 ± 0	14 ± 1
O. bavariensis WSBC 24001^T	10 ± 1	7 ± 1	100 ± 0	6 ± 2
O. contaminans CCUG 53201^T	11 ± 2	8 ± 1	12 ± 0	100 ± 0

menaquinone-7 (MK-7). The DNA G + C content is 36.7 mol%.

The type strain, TW25^T (=KACC 15116^T = JCM 17314^T), was isolated from a dead ark clam following a mass mortality event on the Southern coastal region of the Korean peninsula.

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