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ORIGINAL PAPER

Lysobacter caseinilyticus, sp. nov., a casein hydrolyzing bacterium isolated from sea water

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Abstract A novel bacterial strain, designated KVB24^T, was isolated from sea-water of Busan Harbour in South Korea. Cells of strain KVB24^T were Gram-stain negative, aerobic, rod shaped and non-motile. Strain KVB24^T grew optimally at 25–28 °C and pH 6.5–7.0. Based on 16S rRNA gene sequence analysis, strain KVB24^T was shown to belong to the genus *Lysobacter* within the class *Gammaproteobacteria* and to be closely related to *Lysobacter dokdonensis* DS-58^T, *Lysobacter hankyongensis* KTce-2^T and *Lysobacter niastensis* GH41-7^T. DNA–DNA relatedness between strain KVB24^T and its current closest relative was below 70%. The predominant fatty acids of strain KVB24^T were iso-$C_{11:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ and summed feature 9 comprising (iso-$C_{17:1}$ ω9c and/or 10 methyl $C_{16:0}$); the prominent isoprenoid was Q-8 and the major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The G + C content of genomic DNA from strain KVB24^T was determined to be 67.5 mol%. Based on the phenotypic, genotypic and chemotaxonomic analyses, strain KVB24^T represents a novel species of the genus *Lysobacter*, for which the name *Lysobacter caseinilyticus* sp. nov. is proposed. The type strain is KVB24^T (= KACC19816^T = JCM32879^T).

Keywords *Lysobacter caseinilyticus* sp. nov · Polyphasic analysis · 16S rRNA gene · Phenotypic characteristics · Sea water · Casein hydrolysis

Introduction

The genus *Lysobacter,* which belongs to the family *Xanthomonadaceae* in *Gammaproteobacteria* was proposed by Christensen and Cook (1978) with *Lysobacter enzymogenes* as the type species. There are currently 49 species with valid names in the genus *Lysobacter* (https://www.bactero,net), which were isolated from diverse environmental habitats, especially soil (Luo et al. 2012; Zhang et al. 2011; Liu et al. 2011; Siddiqi et al. 2016), sludge (Ye et al. 2015; Siddiqi et al. 2016), freshwater (Fukuda et al. 2013) and freshwater sediments (Siddiqi et al. 2016). Members of the genus are Gram-stain negative, aerobic, rod-shaped, have high DNA G + C content (61.7–70.7 mol%) and contain ubiquinone 8 (Q-8) as

The GenBank accession number for the 16S rRNA gene sequence of strain KVB24^T is MK177529.

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the major respiratory quinone (Christensen and Cook 1978; Wei et al. 2012; Woen et al. 2006). Polar lipids consist mainly of diphosphatidyglycerol, phosphatidyglycerol, and phosphatidylethanolamine (Luo et al. 2019; Zhang et al. 2011; Wang et al. 2011). Most of the members of the genus are reported to lack flagella (Siddiqi et al. 2016; Wei et al. 2012) except *Lysobacter terricola* (Kim et al. 2015), *Lysobacter arseniciresistens* (Luo et al. 2012) and *Lysobacter mobilis* (Yang et al. 2015). Some species, such as *Lysobacter silvisoli*, *Lysobacter korlensis* and *Lysobacter bugurensis* (Zhang et al. 2011, 2018) exhibit gliding motility. A few members of *Lysobacter* produce flexirubin-type pigments, *Lysobacter novalis* (Singh et al. 2015), *Lysobacter terrae* (Ngo et al. 2015) and *Lysobacter lycopersici* (Lin et al. 2014). All species of the genus *Lysobacter* with validly published names show negative results for urease activity and indole production (Zhang et al. 2011). Moreover, *Lysobacter* species have become a new source of bioactive natural products and new antibiotics due to their fascinating secondary metabolites (Xie et al. 2012; Panthee et al. 2016). Here, we report the polyphasic characterization of a Gram-negative, aerobic bacterium, designated KVB24^T, isolated from sea water. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KVB24^T belongs to the genus *Lysobacter* and represents a novel species of this genus.

Materials and methods

Isolation of bacterial strain and culture conditions

To study the bacterial community and explore the novel strains, a sea water sample was collected from Busan (35°18′85′72.1″N, 129°22′32′19.2″E), a large port city in South Korea, using the standard dilution plating technique. Isolation was achieved using R2A agar (Difco) at 28 °C for 1 week. A single colony chosen on the plates was purified by transferring to new R2A plates. Purified colonies were sent to Bionics (Daejeon, Republic of Korea) for 16S rRNA gene analysis. Among the purified bacteria, a novel strain was identified to be a member of *Lysobacter* and designated as KVB24^T. For routine work, cells were stored in R2A broth containing 50% (v/v) glycerol at − 80 °C. Strain KVB24^T was deposited in the Korean Agricultural Culture Collection (KACC 19816^T) and the Japan Collection of Microorganisms (JCM 32879^T). *Lysobacter dokdonensis* KACC 18711^T (purchased from KACC, South Korea) was used as a reference strain and evaluated together with strain KVB24^T under identical experimental conditions.

16S rRNA gene sequencing and phylogenetic analysis

16S rRNA gene sequencing and phylogenetic analysis were performed as described previously (Chhetri et al. 2018a). The EzTaxone database (Kim et al. 2012) and NCBI BLAST search were used to compare the 16S rRNA gene sequence with those of other taxa. Multiple sequences were aligned using MEGA 7 software (Kumar et al. 2016) and analyzed using ClustalX 2.1 (Thompson et al. 1997) and related taxa and gaps were edited with the BioEdit programme (Hall 1997). Phylogenetic trees were constructed using MEGA 7 software (Kumar et al. 2016). Evolutionary distances were calculated using the Kimura 2-paramaeter model (Kimura 1980) in the neighbour-joining (NJ) algorithm (Saitou and Nei 1987) and maximum-likelihood analysis. Minimum-evolution tree was also constructed using the MEGA 7 software in order to estimate the confidence of tree topologies (Rzhetsky and Nei 1992). MEGA 7 software was used to construct a phylogenetic tree by bootstrap analysis with 1000 replications (Felsenstein 1985). The taxonomic relationships between strain KVB24^T and its close phylogenetic neighbors were examined using the DNA–DNA hybridization method as previously described (De Ley et al. 1970; Gillis et al. 1970) and an optimized procedure described previously (Loveland-Curtze et al. 2011). The sequence of *Rhodanobacter lindaniclasticus* RP5557^T (AF039167) was used as an out-group.

Morphological, physiological, and biochemical characterization

Cell morphology of strain KVB24^T was visualized using a transmission electron microscope (TEM) (LIBRA 120, Carl Zeiss, Germany), using cells grown in R2A at 28 °C. The non-staining KOH method (3% KOH; Buck 1982) was adopted to determine the Gram-stain reaction. Catalase activity was assessed by

detecting oxygen bubble production using 3% (v/v) aqueous hydrogen peroxide solution and oxidase activity via oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux). Motility was evaluated in R2A medium containing 0.4% agar. Gliding motility was tested using the hanging-drop method after growing the cells in R2A broth (Difco) at 28 °C for 48 h (Bernardt et al. 2002). Growth under anaerobic conditions was observed via incubation in a GasPak jar (BBL, Cockeysville, MD, USA) at 30 °C for 10 days. Growth on nutrient agar (Difco), R2A agar (MB cell), tryptic soy agar (Difco), marine agar (Difco) and Luria–Bertani agar was assessed with incubation at 28 °C for one week. The pH range for growth was determined by cultivation at 30 °C in R2A broth adjusted to pH 4–10 (at pH 1 unit intervals) before sterilization with citrate/NaH_2PO_4 buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 9.0–10.0) (Breznak and Costilow 2007). Tolerance of various NaCl concentrations was tested in R2A broth containing 0–3% NaCl (w/v, at 0.5% intervals). Growth at various temperatures (9, 10, 15, 20, 25, 28, 30, 37, 38 and 40 °C) was measured in R2A agar. The presence of flexirubin-type pigments was investigated with 20% (w/v) KOH solution (Fautz and Reichenbach 1980). Hydrolysis of chitin, CM cellulose, starch, and casein was determined as previously described (Chhetri et al. 2018b). Hydrolysis of Tween 80, Tween 60 and Tween 40 was examined as described by Smibert and Krieg (1994). Additional biochemical tests for strain KVB24^T and its reference strain were performed using API ZYM and API 20 NE kits according to the methods outlined by the manufacturer (bioMérieux) under the equivalent test conditions.

DNA base composition and DNA–DNA hybridization

For calculation of DNA G + C mol%, genomic DNA was extracted and purified according to the standard cetyl trymethylammonium bromide/NaCl protocol. The G + C content of the chromosomal DNA for the strain KVB24^T was measured based on a simple fluorimetric method (Gonzalez and Saiz-Jimenez 2002) using SYBR Green 1 (Life Technologies, Waltham, USA) and a real-time PCR thermocycler (Rotor-Gene Q, Qiagen, Hilden, Germany). The genomic DNA of *Lactococcus lactis* subsp. *lactis* KACC 13877^T, *Bacillus subtilis* subsp. *subtilis* KACC 17796^T, *Bacillus licheniformis* KACC 10476^T, *Escherichia coli* KACC 14818^T, *Corynebacterium glutamicum* KACC 20786^T, *Pseudomonas aeruginosa* ATCC 15442^T, and *Micrococcus luteus* KACC 13377^T were used as references for calibration (Ausubel et al. 1995).

DNA–DNA hybridization was performed to analyse the relationships of the novel isolate with related taxa based on the thermal denaturation principles and equations (De Ley et al. 1970, Gillis et al. 1970) and an optimized procedure described previously (Loveland-Curtze et al. 2011). For genomic characterization of strain KVB24^T, we performed DNA–DNA hybridization analyses between the strain KVB24^T and *L. dokdonensis* DS-58^T using the fluorimetric method. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness value.

Chemotaxonomic analysis

For chemotaxonomic analyses, cells of strain KVB24^T and the reference strain were harvested at the late exponential growth phase after cultivation for 4 days at 28 °C. Cellular fatty acids of strain KVB24^T and its reference strain *L. dokdonensis* DS-58^T were obtained by saponification, methylation and extraction, as reported previously (Kuykendall et al. 1988). The Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc., Newark, DE, USA) was used to identify the extract

Respiratory quinone was extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum, re-extracted with acetone and analyzed using high-performance lipid chromatography (HPLC) according to previous reports (Hirashi et al. 1996; Collins and Jones 1981).

Polar lipids were extracted and separated by two-dimensional thin layer chromatography (TLC) (Minninkin et al. 1984; Komagata and Suzuki 1987). The plate dotted with sample was subjected to two-dimensional development, with the first solvent of chloroform:methanol:water (65:25:4, by vol.) For the presence of all lipids, TLC plates were sprayed with 5% molybdophosphoric acid, then charred at 120 °C for 5 min. Amino lipids were detected by spraying

with 0.2% ninhydrin on a new plate followed by incubation in an oven at 120 °C for 2 min. Phospholipids were detected by spraying with molybdenum blue reagent at room temperature and glycolipids were visualised with 2.5% α-napthol-sulfuric acid, followed by charring at 120 °C for 5 min.

Results and discussion

Phylogenetic analysis

For strain KVB24^T, the 16S rRNA gene sequence (1485 nucleotides) has been deposited in GenBank under accession number MK177529. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain KVB24^T belongs to the genus *Lysobacter*. Strain KVB24^T formed a monophyletic cluster with *L. dokdonensis* DS-58^T and *L. lycopersici* CC-Bw-6^T (Fig. 1). The overall topologies of the maximum-likelihood (Fig S2) and maximum-parsimony (not shown) trees were similar. Sequence identity calculations (over 1400 bp) indicated that strain KVB24^T is closely related to members of the genus *Lysobacter* (95.4–98.8% sequence identity), and has high sequence identity with *L. dokdonensis* DS-58^T (98.8%), followed by *L. hankyongensis* KTCe-2^T (96.5%), *L. niastensis* GH 41-7^T (96.4%) and *L.brunescens* KCTC 12130^T (96.2%), while lower similarities (95.4-96%) were observed between KVB24^T and other members of the genus *Lysobacter* listed in Fig. 1. In agreement with these observations, phylogenetic analysis showed that strain KVB24^T was located in the genus *Lysobacter* and grouped together with these strains (Fig. 1).

Phenotypic characteristics

Cells of strain KVB24^T were observed to be Gram negative, aerobic, non-motile, non-spore-forming, rod-shaped, 0.4–0.8 μm wide and 1.4–2.3 μm long (Supplementary Fig. S1). For API ZYM kit, positive reactions are observed for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase and Napthol-AS-BI-phosphohydrolase are

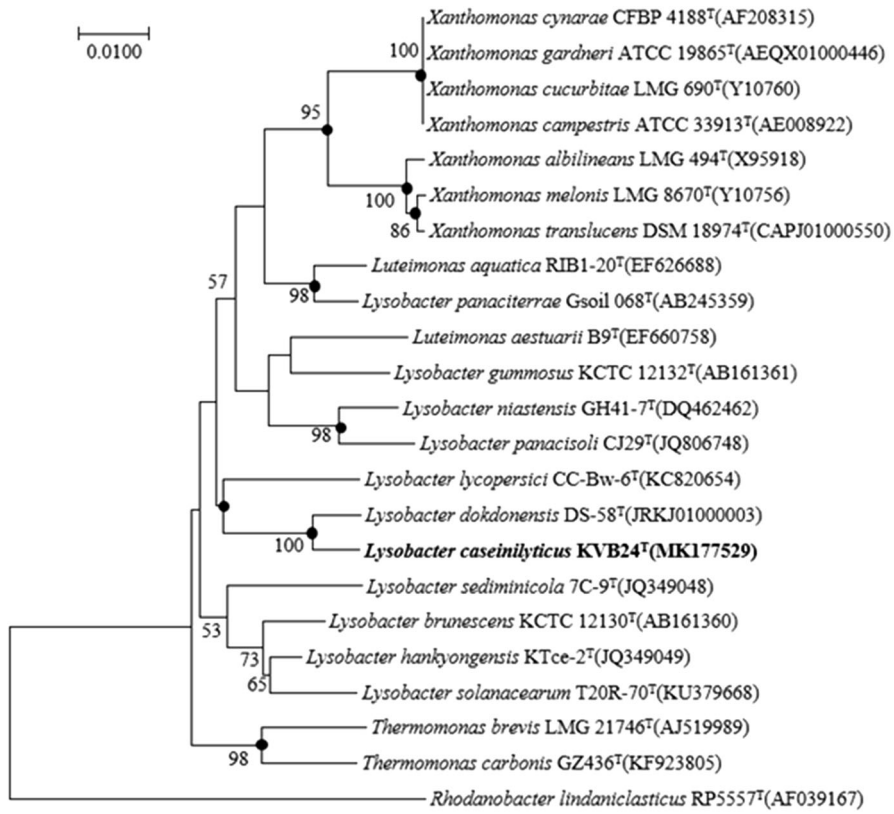

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences, reconstructed with the neighbor-joining method, showing the position of strain KVB24T among related taxa within the genus Lysobacter. Numbers at nodes are levels of bootstrap support (> 50%) based on 1000 resamplings. Filled circles indicate that the node branches were in consensus with the maximum-likelihood and maximum-parsimony algorithms. *Rhodanobacter lindaniclasticus* RP5557^T (AF039167) was used as outgroups. Bar, 0.0100 substitutions per nucleotide position

strong while those for trypsin, α-chymotrypsin, α-glucosidase and β-glucosidase are weak. No activity for α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase is detected. Nitrate reduction was found to be positive.

Chemotaxonomic characteristics

The major cellular fatty acids detected in strain KVB24^T were iso-$C_{15:0}$ (23.8%), iso-$C_{16:0}$ (21.3%), iso-$C_{14:0}$ (10%), summed feature 9 comprising (iso-$C_{17:1}$ ω9c and/or 10 methyl $C_{16:0}$), iso-$C_{11:0}$ 3-OH (8.9%), anteiso-$C_{15:0}$ (7.3%) and iso-$C_{11:0}$ (5.5%) and the minor traces of fatty acids shown in Table 1. The cellular fatty acid analyses indicate that the novel isolate shares similar major fatty acids with members of the genus *Lysobacter*, although there were differences in the proportions of some fatty acids, particularly iso-$C_{16:1}$ H. Futhermore, a minor proportion of summed feature 1($C_{13:0}$ 3OH/$C_{15:1}$ i H) was detected only in strain KVB24^T but not in other strains. Strain KVB24^T contained ubiquinone Q-8 as the major respiratory quinone. Our results are in good agreement with those of other members of the genus *Lysobacter*. The major polar lipids of strain KVB24^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) which is the same as the reported major polar lipids of the genus *Lysobacter*. However, the presence of unidentified aminophospholipids, unidentified phospholipids, unidentified glycolipid and moderate amount of unidentified lipids differentiates the strain KVB24^T from closely related strains. Some phenotypic differences were found between strain KVB24^T and phylogenetically related *Lysobacter* type strains (Table 2). Phenotypic examination revealed several common traits between strain KVB24^T and its four close relatives. However, strain KVB24^T could be clearly distinguished from these phylogenetic relatives by differences in phenotypic characteristics, including ability to reduce nitrate, hydrolyze casein, starch and aesculin and the inability to hydrolyze gelatin. Besides, there was a difference in the temperature range for growth, and the ability of strain KVB24^T to produce some enzymes and assimilate a few substrates. Detailed results from the phenotypic and biochemical analyses of strain KVB24^T are provided in Table 2 and in the species description. The results obtained from the phenotypic and phylogenetic characterizations indicated that strain KVB24^T belongs to the genus *Lysobacter*. The phylogenetic distinctiveness and low value of DNA–DNA hybridization confirmed that this isolate represent a species that is distinct from recognized species of the genus *Lysobacter*.

Table 1 Cellular fatty acid compositions (%) of strain KVB24^T and reference strains of the closely related species

Fatty acids	1	2	3	4
iso-$C_{10:0}$	TR	1	TR	1
iso-$C_{11:0}$	5.5	8.2	5.3	6.4
iso-$C_{11:0}$ 3-OH	8.6	10.1	7.4	9.3
iso-$C_{14:0}$	10	5.4	13.8	8.7
iso-$C_{15:0}$	23.8	26.7	13	12.7
anteiso-$C_{15:0}$	7.3	3.2	3.9	5.9
$C_{16:0}$	TR	1.6	TR	1.1
iso-$C_{16:0}$	21.3	19.7	18.1	17.4
iso-$C_{17:0}$	TR	1.5	TR	1.6
iso-$C_{16:1}$ H	3.4	TR	1.4	1
iso-$C_{12:0}$ 3-OH	–	TR	1	TR
Summed features*				
1	1.9	TR	TR	TR
3	2.3	2.6	19	5.2
9	8.6	13.6	4.3	10.9

Taxa: 1. KVB24^T (data from this study); 2, *Lysobacter dokdonensis* KACC 18711^T (data from this study); 3, *Lysobacter hankyongensis* KTce-2^T (data from Siddiqi and Im 2016); 4, *Lysobacter nisatensis* GH41-7^T (data from Woen et al. 2007). Some fatty acids that account for less than 1% of the total fatty acids are not shown, therefore the percentages do not add up to 100%. Bold type: some fatty acids that account for more than 5% of the total fatty acids. Values are percentages of fatty acids. TR, Trace (< 1%); –, not detected

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 comprised $C_{13:0}$ 3-OH/$C_{15:1}$ i H; summed feature 3 comprised $C_{16:1}$ ω7c/$C_{16:1}$ ω6c; summed feature 9 comprised iso-$C_{17:1}$ ω9c/$C_{16:0}$ 10-methyl

DNA G + C content and DNA–DNA hybridization

The level of DNA–DNA relatedness of KVB24^T with *L. dokdonensis* DS-58^T was 57.9 ± 2.1%, which is below the threshold of 70% that has been suggested for species delineation by Wayne et al. (1987) and indicate that strain KVB24^T represents a separate

Table 2 Physiological and biochemical characteristics of strain KVB24^T and closely related type species of the genus *Lysobacter*

Characteristics	1	2	3	4
Isolation source	Sea water	Soil	Sludge	Green house soil
Cell size (μm)	0.4–0.8 × 1.4–2.3	0.4–0.8 × 1.0–5.0	0.3–0.4 × 1.0–1.5	0.5–0.6 × 2.0–4.0
Colony color	Yellow–brown	Yellow	Yellow–orange	Light beige
Temperature range for growth (°C)	10–37	4–38	10–30	10–40
Salinity range for growth (%)	0–0.5	0–0.5	0–0.5	0–1
Catalase/oxidase	+/+	+/+	±	+/+
Motility	−	−	−	+
Hydrolysis of				
Casein	+	+	−	+
Aesculin	+	−	−	+
Urease	−	−	−	+
Starch	+	−	+	+
Gelatin	−	+	+	+
Nitrate reduction	+	−	−	−
β-galactosidase	+	+	−	+
Assimilation of				
D-glucose	−	−	+	−
D-maltose	−	W +	+	+
D-mannitol	−	−	+	−
Enzyme activity				
Esterase (C4)	+	+	−	+
Esterase lipase (C8)	+	+	−	+
Valine arylamidase	+	+	−	−
Trypsin	W +	−	+	−
α-chymotrypsin	W +	W +	+	+
Acid phosphatase	+	+	−	+
Napthol-AS-BI-phosphohydrolase	+	+	−	+
α-glucosidase	W +	W +	+	+
DNA G + C content (mol%)	67.8	(68.1)	(68.6)	(66.6)

Strains: 1, KVB24^T; 2, *Lysobacter dokdonensis* KACC 18711^T (Oh et al. 2011); 3, *Lysobacter hankyongensis* KTCe-2^T (Siddiqi and Im 2015); 4, *Lysobacter niastensis* GH41-7^T (Woen et al. 2007). All data are from the present study except indicated in the parenthesis. + positive, W + weakly positive, −negative

species. Therefore, the phylogenetic distinctiveness and low value of DNA–DNA hybridization confirmed that this isolate represents a species that is distinct from recognized species of the genus *Lysobacter* and thus can be classified as a novel species, for which the name *Lysobacter caseinilyticus* sp. nov. is proposed. The DPD Taxonumber of strain KVB24^T is TA00818.

Description of *Lysobacter caseinilyticus* sp. nov.

Lysobacter caseinilyticus (ca.se.i.ni.ly'ti.cus. N.L. n. *caseinum*, casein; Gr. adj. *lytikos*, dissolving; N.L. masc. adj. *caseinilyticus*, casein-dissolving)

Cells are Gram-stain negative, aerobic, non-motile, non-spore forming, rod shaped (0.4–0.8 μm in diameter and 1.4–2.3 μm in length), catalase and oxidase positive. Colonies grown on R2A agar for 4 days at 28 °C are yellow–brown, smooth, transparent, shiny and circular. Grows well on R2A, weakly on Luria–

Bertani and Nutrient agar, but not on Marine and tryptic soy agar. Growth temperature is between 10 and 37 °C (optimum, 25–30 °C), at pH 6.0–8.0 (optimum, 7.0) and at 0–0.5% NaCl (optimum 0%). No growth is observed at 9 and 38 °C, and in 1% NaCl. Cells are positive for nitrate reduction, β-galactosidase, hydrolysis of casein, starch, Tween 80 and esculin but negative for indole and flexirubin-type pigment production, hydrolysis of Tween 20, DNase, gelatin, urease and L-arginine. Does not ferment glucose. The polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phosphoglycolipid, an unidentified glycolipid, three unidentified aminophospholipids, five unidentified phospholipids and two unidentified lipids. The sole respiratory quinone is ubiquinone-8 (Q-8) and the major fatty acids are iso-$C_{14:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and summed feature 9 comprising iso-$C_{17:1}$ ω9c and/or 10 methyl $C_{16:0}$).

The type strain is KVB24^T (= KACC19816^T = JCM32879^T), isolated from sea water in Busan, Republic of South Korea (GPS location: 35°18′85′72.1″N, 129°22′32′19.2″E). The GenBank accession numbers for the 16S rRNA gene sequence of strain KVB24^T is MK177529.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical standards This study does not describe any experimental work related to human.

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