**Microbulbifer okhotskensis** sp. nov., isolated from a deep bottom sediment of the Okhotsk Sea

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**Abstract**
A Gram-negative, aerobic, non-motile bacterium KMM 9862T was isolated from a deep bottom sediment sample obtained from the Okhotsk Sea, Russia. Based on the 16S rRNA gene and whole genome sequences analyses the novel strain KMM 9862T fell into the genus *Microbulbifer* (class *Gammaproteobacteria*) sharing the highest 16S rRNA gene sequence similarities of 97.4% to *Microbulbifer echini* AM134T and *Microbulbifer epialgicus* F-104T, 97.3% to *Microbulbifer pacificus* SPO729T, 97.1% to *Microbulbifer variabilis* ATCC 700307T, and similarity values of < 97.1% to other recognized *Microbulbifer* species. The average nucleotide identity and digital DNA–DNA hybridization values between strain KMM 9862T and *M. variabilis* ATCC 700307T and *M. thermotolerans* DSM 19189T were 80.34 and 77.72%, and 20.2 and 19.0%, respectively. Strain KMM 9862T contained Q-8 as the predominant ubiquinone and C₁₆:₀, C₁₆:₁ω₇c, C₁₂:₀, and C₁₀:₀ 3-OH as the major fatty acids. The polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, an unidentified aminophospholipid, an unidentified aminolipid, two unidentified phospholipids, phosphatidic acid, and an unidentified lipid. The DNA G+C content of 49.8% was calculated from the genome sequence. On the basis of the phylogenetic evidence and distinctive phenotypic characteristics, the marine bacterium KMM 9862T is proposed to be classified as a novel species *Microbulbifer okhotskensis* sp. nov. The type strain of the species is strain KMM 9862T (= KACC 22804T).

**Keywords** *Microbulbifer okhotskensis* sp. nov · *Gammaproteobacteria* · Marine bacteria · Bottom sediments

**Introduction**

The genus *Microbulbifer* was created by Gonzalez et al. (1997) with the description of the type species *Microbulbifer hydrolyticus* and emended by Tang et al. (2008). The genus *Microbulbifer* belongs to the family *Microbulbiferaceae* (Spring et al. 2015) and contains currently 25 species with validly published names as listed at https://lpsn.dsmz.de/genus/microbulbifer. Bacteria of the genus *Microbulbifer* are widely distributed in marine or saline environments being recovered from diverse sources, including solar salt-tern (Yoon et al. 2007), deep-sea sediment (Miyazaki et al. 2008), saline soil (Tang et al. 2008), Pacific marine algae (Nishijima et al. 2009), mangroves (Baba et al. 2011; Vashist et al. 2013), coastal soil (Kämpfer et al. 2012), marine sediment (Zhang et al. 2012; Xiong et al. 2019), intertidal sediment and marine sponge specimen (Jeong et al. 2013), rhizosphere of a halophytic plant (Camacho et al. 2016), a purple sea urchin (Lee et al. 2017), coastal sand (Huang et al. 2020). Several *Microbulbifer* members have been reported to be polysaccharide-degrading bacteria (Miyazaki et al. 2008; Baba et al. 2011; Vashist et al. 2013; Huang et al. 2020). In the present study the taxonomic position of a Gram-negative, aerobic, non-motile bacterium KMM 9862T, isolated from a deep bottom sediment sampled from the Okhotsk Sea, Russia, was characterized. On the basis of combined phylogenetic analyses and phenotypic properties, a novel species, *Microbulbifer okhotskensis* sp. nov., is described.
Materials and methods

Bacterial strains

Strain KMM 9862T was isolated from a deep bottom sediment sample obtained at a depth of 46.2 m from the Okhotsk Sea, Russia, during the expedition of R/V Academician Oparin, in September 2020. The novel bacterium was cultivated aerobically on marine agar 2216 (MA) or in marine broth (MB) 2216 (BD Difco) at 28 °C and stored at −70 °C in MB 2216 supplemented with 30% (v/v) glycerol. The type strains of Microbulbifer echini KACC 18258T was kindly provided by the Korean Agricultural Culture Collection (KACC), Agricultural Microbiology Division, National Academy of Agricultural Science, Korea, and Microbulbifer thermotolerans DSM 19189T was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany, to be used in the phenotypic and lipids analyses.

Phenotypic characterization

Gram-staining, oxidase and catalase reactions, and motility (the hanging drop method) were determined as described by Gerhardt et al. (1994). The morphology of cells grown in MB and negatively stained with a 1% phosphotungstic acid on carbon-coated 200-mesh copper grids was examined by electronic transmission microscopy [Libra 120 FE (Carl Zeiss)], provided by the Far Eastern Centre of electronic microscopy, Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences]. Hydrolysis of starch, casein, gelatin, Tween 80, DNA, L-tyrosine, chitin, nitrate reduction (sulfanilic acid/α-naphthylamine test), and growth at different salinities (0–12% NaCl), temperatures (5–45 °C), and pH values (4.0–10.5) were carried out using artificial sea water (ASW) as described in a previous paper (Romanenko et al. 2013). The artificial sea water (ASW) contained (per liter of distilled water): 30 g NaCl, 4.9 g MgCl2, 2.0 g MgSO4, 0.5 g CaCl2, 1.0 g KCl, 0.01 g FeSO4. Biochemical tests were performed using API 20E, API 20NE, API ID32 GN, and API ZYM test kits (bioMérieux, France) as described by the manufacturer except the cultures were suspended in ASW.

16S rRNA gene sequence and phylogenetic analysis

Genomic DNA of the strain KMM 9862T was extracted using a commercial genomic DNA extraction kit (Fermentas, EU) following the manufacturer’s instruction. The universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTC AG-3′) and 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989) were used for amplification of the 16S rRNA gene. The 16S rRNA gene was PCR-amplified and sequenced as described in a previous paper (Romanenko et al. 2019). The 16S rRNA gene sequence of the strain KMM 9862T was compared with those of the closest relatives using the BLAST (http://www.ncbi.nlm.nih.gov/blast/) and EzBioCloud service (Yoon et al. 2017). Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al. 2018). Phylogenetic trees were constructed by the neighbor-joining and the maximum-likelihood methods and the distances were calculated according to the Kimura two-parameter model (Kimura 1980). The robustness of phylogenetic trees was estimated by the bootstrap analysis of 1000 replicates.

Whole-genome sequencing and genome-based phylogenetic analysis

The genomic DNA was obtained from the strain KMM 9862T using the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland). The quantity and quality of the genomic DNA was measured using DNA gel electrophoresis and the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA). Preparation of the DNA sequencing library was carried out using Nextera DNA Flex kits (Illumina, San Diego,
CA, USA) and whole-genome sequencing was performed subsequently using paired-end runs on an Illumina MiSeq platform with a 150-bp read length. The reads were trimmed using Trimmomatic version 0.39 (Bolger et al. 2014) and their quality assessed using FastQC version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Filtered reads were assembled into contigs with SPAdes version 3.15.3 (Bankevich et al. 2012) and genome metrics were calculated with the help of QUAST version 5.0.2 (Gurevich et al. 2013). The draft genome assembly was annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016). Comparisons of the Average Nucleotide Identity (ANI) and in silico DNA-DNA hybridization (dDDH) values of the strain KMM 9862^T and its closest neighbors were performed with the online server ANI-AAI-Matrix (Rodriguez-R and Konstantinidis 2016), and TYGS platform (Meier-Kolthoff and Göker 2019), respectively. The phylogenomic analysis was performed using PhyloPhlAn 3.0 software based on a set of 400 conserved bacterial protein sequences (Asnicar et al. 2020).

**Results and discussion**

**Genomic and phylogenetic characteristics**

The draft genome of strain KMM 9862^T was de novo assembled into 626 contigs and determined to be 4,996,206 bp long with a G+C ratio of 49.8% and coverage of 62 X. The N50 and L50 values were 35,948 bp and 41, respectively. Within the genome, 4699 genes were identified, including 3 rRNA genes, 47 tRNA genes and 61 pseudogenes. On the basis of 16S rRNA gene sequence comparative analysis strain KMM 9862^T was affiliated with the genus *Microbulbifer* sharing the highest sequence similarity of 97.4% to *Microbulbifer echini* KACC 18258^T and *Microbulbifer epialgicus* F-104^T each, 97.3% to *Microbulbifer pacificus* SPO729^T, 97.1% to *Microbulbifer variabilis* ATCC 700307^T, and similarity values of < 97.1% to the remaining *Microbulbifer* species. The phylogenetic trees generated by two different algorithms showed that the novel strain KMM 9862^T placed in the cluster comprising *M. echini* KACC 18258^T, *M. epialgicus* F-104^T, and *M. variabilis* ATCC 700307^T as an individual line (Fig. 1). The 16S rRNA gene sequence similarities were not exceeding the threshold value of 98.7%–98.6% recommended by Stackebrandt and Ebers (2006) and Kim et al. (2014) for the species discrimination.

![Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences available from the GenBank/EMBL/DDBJ databases (accession numbers are given in parentheses) showing relationships of the novel strain KMM 9862^T and *Microbulbifer* species. Filled circles indicate the corresponding nodes that were observed in the maximum-likelihood tree. Numbers indicate bootstrap values as percentage greater than 60. These values are based on 1000 replicates. Bar, 0.010 substitutions per nucleotide position](https://www.springer.com)
confirming that the novel strain KMM 9862T could represent a novel species of the genus Microbulbifer. The phylogenetic tree based on concatenated 400 protein sequences from whole genome sequence positioned strain KMM 9862T in the genus Microbulbifer where it clustered with \textit{M. thermotolerans} DSM 19189\textsuperscript{T} and \textit{M. variabilis} ATCC 700307\textsuperscript{T} (Fig. 2). The ANI between strain KMM 9862T and \textit{M. variabilis} ATCC 700307\textsuperscript{T} and \textit{M. thermotolerans} DSM 19189\textsuperscript{T} were 80.34 and 77.72\%, respectively, which are below the threshold ANI values of 95–96\% for delineating bacterial species (Richter and Rossello-Mora 2009). The estimated dDDH values between strain KMM 9862T and \textit{M. variabilis} ATCC 700307\textsuperscript{T} and \textit{M. thermotolerans} DSM 19189\textsuperscript{T} were 20.2 and 19.0\%, respectively, which are lower than the dDDH value of 70\% accepted as the threshold value for bacterial species discrimination (Goris et al. 2007; Chun et al. 2018). The genomic and phylogenetic analyses data evidence strain KMM 9862T could be classified as an individual species of the genus Microbulbifer.

**Morphological, physiological, and chemotaxonomic characteristics**

Morphological, physiological, biochemical, and chemotaxonomic characteristics of strain KMM 9862T are given in Table 1, Table S1, Table S2, Supplementary Figure S1, Supplementary Figure S2, and in the species description. Strain KMM 9862\textsuperscript{T} was rod-shaped bacteria capable of producing extracellular material (Supplementary Figure S1). The novel bacterium KMM 9862\textsuperscript{T} was able to grow in the narrow salinity (1–4\%) and temperature (7–35 °C) ranges and not able to assimilate carbon sources in the 32ID GN tests (Table S1). Strain KMM 9862\textsuperscript{T} contained ubiquinone Q-8 as the major respiratory quinone and \textit{C}_{16:0}, \textit{C}_{16:1\omega7c}, \textit{C}_{12:0}, \textit{C}_{10:0} 3-OH as the major fatty acids (Table S2). Strain KMM 9862\textsuperscript{T} was close in its fatty acid profile to those of related type strains \textit{M. echini} KACC 18258\textsuperscript{T} and \textit{M. variabilis} ATCC 700307\textsuperscript{T} (Nishijima et al. 2009) although strain KMM 9862\textsuperscript{T} differed in content of \textit{C}_{12:0} and \textit{C}_{10:0} 3-OH. 

\textit{M. thermotolerans} DSM 19189\textsuperscript{T} contained significant amounts of iso-\textit{C}_{15:0} and iso-\textit{C}_{17:1} and lesser amounts of \textit{C}_{16:0} and \textit{C}_{16:1\omega7c} (Table S2). The polar lipids of the strain KMM 9862\textsuperscript{T} comprised phosphatidylglycerol (PG), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), an unidentified aminophospholipid (APL), an unidentified aminolipid (AL), two unidentified phospholipids (PL1, PL2), phosphatidic acid (PA), and an unidentified lipid (L) (Fig. S2). The polar lipid profile of strain KMM 9862\textsuperscript{T} was most similar to that of related type strain \textit{M. thermotolerans} DSM 19189\textsuperscript{T}, but one unidentified aminolipid

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**Fig. 2** Maximum-likelihood tree based on concatenated 400 protein sequences from genome sequences showing phylogenetic position of the novel strain KMM 9862\textsuperscript{T} among \textit{Microbulbifer} species and related taxa. Bootstrap values are based on 100 replicates. Bar, 0.05 substitutions per amino acid position.
Enzyme activity (API ZYM):

| Characteristic | 1   | 2   | 3   | 4   |
|----------------|-----|-----|-----|-----|
| DNA G+C content (%)b | 49.8 | 56.5 | 56.1 | 48.8 |
| Motility        | −   | +   | −   | −   |
| Growth in/at: 7% NaCl | −   | +   | +   | −   |
| 37 °C           | −   | +   | −   | +   |
| 42 °C           | −   | +   | −   | +   |
| Hydrolysis of:  |
| Aesculin        | +   | +   | −   | +   |
| Casein          | −   | +   | −   | +   |
| Agar            | −   | +   | −   | +   |
| Chitin          | −   | +   | −   | +   |
| DNA             | −   | +   | −   | +   |
| Enzyme activity (API ZYM): |
| Lipase C 14    | −   | −   | −   | +   |
| Valine arylamidase | −   | (+) | −   | +   |
| Cystine arylamidase | −   | (+) | −   | +   |
| Trypsin         | −   | −   | −   | +   |
| Acid phosphatase | +   | +   | −   | +   |
| α-Galactosidase | −   | (+) | −   | −   |
| α-Glucosidase   | −   | −   | −   | +   |
| N-acetyl-β-glucosaminidase | −   | +   | −   | +   |

**Strains:** 1. KMM 9862T, 2. Microbulbifer thermotolerans DSM 19189T, 3. Microbulbifer echini KACC 18258T (data were obtained from present study unless otherwise indicated); 4. Microbulbifer variabilis ATCC 700307T (data from Nishijima et al. 2009). +, Positive; −, negative; (+), weak reaction. All strains were positive for catalase, oxidase, sodium ions requirement, nitrate reduction, hydrolysis of gelatin, starch, Tween 80, API ZYM tests of alkaline phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and negative for α-chymotrypsin, β-galactosidase, β-glucuronidase, β-glucosidase α-mannosidase, and α-fucosidase.

*a* Data were taken from Lee et al. (2017)

*b* The DNA G+C contents of the strain KMM 9862T, and M. thermotolerans DSM 19189T, and M. variabilis ATCC 700307T were derived from the QUAST and GenBank, respectively.

and one unidentified lipid was found only in strain KMM 9862T. *M. echini* KACC 18258T did not contain aminophospholipids, aminolipids and lipids (Fig. S2). The DNA G+C content of 49.8% was calculated from the genome sequence of the strain KMM 9862T. The value obtained for the strain KMM 9862T was close to those of 48.1–49.7% as reported for *M. variabilis* and *M. epilagicus* strains (Nishijima et al. 2009), whereas the DNA G+C means found for other recognized *Microbulbifer* species were significantly higher and ranged from 55 up to 64% (Miyazaki et al. 2008; Tang et al. 2008). Chemotaxonomic characteristics found for the strain KMM 9862T, including ubiquinone Q-8, the predominance of C16:0, C16:1ω7c, C12:0 and C10:0 3-OH, the major polar lipid components of PE, PG, DPG, APL, and the DNA G+C content, are corroborated with those previously described for some of *Microbulbifer* species (Nishijima et al. 2009; Kämpfer et al. 2012; Zhang et al. 2012; Lee et al. 2017) and supported its assignment to this genus.

The phylogenetic and genetic distinctions obtained were supported by phenotypic differences of the novel isolate KMM 9862T in its growth temperature and salinity ranges, enzyme activity and substrate hydrolysis. Differential phenotypic characteristics are indicated in Table 1. Based on the combined phylogenetic evidence, phenotypic, and biochemical characteristics, it is proposed to classify strain KMM 9862T as a novel species, *Microbulbifer okhotskensis* sp. nov.

**Description of Microbulbifer okhotskensis** sp. nov.

*Microbulbifer okhotskensis* (ok.hotsk.en’sis. N. L. masc./fem. adj. okhotskensis, from the Okhotsk Sea, pertaining to the place where the bacterium was isolated).

Gram-negative, aerobic, oxidase-positive, catalase-positive, rod-shaped non-motile cells, 0.5–0.8 μm in width and 4–10 μm in length. Grows in MB 2216 or MA 2216. On MA 2216 produces hemi-transparent lightly yellowish-pigmented, shiny smooth colonies with the regular edges of 2–4 mm. Requires NaCl for growth; growth occurs between 1 and 4% (w/v) NaCl with an optimum of 3% NaCl. The temperature range for growth is 7–35 °C with an optimum of 25–28 °C. The pH range for growth is 5.5–9.5 (optimal pH 7.5–8.0). Positive for hydrolysis of gelatin, starch, Tween 80, and nitrate reduction, and negative for hydrolysis of DNA, casein, tyrosine, chitin, and agar. In the API 20E tests weakly positive for gelatin hydrolysis. According to the API 20NE, positive for nitrate reduction, gelatin and aesculin hydrolysis. Positive API ZYM test results are obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase. The major isoprenoid quinone is ubiquinone Q-8. Major fatty acids are C16:0, C16:1ω7c, C12:0, and C10:0 3-OH. The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, an unidentified aminophospholipid, an unidentified aminolipid, two unidentified phospholipids, phosphatidic acid, and an unidentified lipid. The DNA G+C content of 49.8% is calculated from the genome sequence.

The type strain of the species is strain KMM 9862T (= KACC 22804T), isolated from a bottom sediments sample, collected from the Okhotsk Sea, Russia.

The strain KMM 9862T has been deposited in the Collection of Marine Microorganisms (KMM), G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia, and in the Korean Agricultural Culture Collection (KACC),
Agricultural Microbiology Division, National Academy of Agricultural Science, Korea, as KACC 22804T.

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Author contributions L.R. and V.K. designed the study, isolated and tested bacteria, and wrote the first draft of the manuscript. N.O. and M.I. performed sequencing and phylogenetic analyses; P.V. did lipid analyses, M.I. and V.M. managed the study and analyzed the results. All authors edited and agreed to the published version of the manuscript.

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Declarations

Conflict of interest All the authors have declared that they have no conflict of interest.

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