Microbulbifer donghaiensis sp. nov., isolated from marine sediment of the East China Sea

Chun-Sheng Wang,[1,2] Yu Wang,[3] Xue-Wei Xu,[1,2] Dong-Sheng Zhang,[1,2] Yue-Hong Wu[3] and Min Wu[3]

Correspondence
Chun-Sheng Wang
wangsio@sio.org.cn

[1]Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Hangzhou 310012, PR China

[2]Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, PR China

[3]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

A Gram-negative, aerobic, neutrophilic, rod-shaped bacterium, designated strain CN85^T, was isolated from a sediment sample collected from the East China Sea and was subjected to a polyphasic taxonomic characterization. This isolate grew in the presence of 0.5–6.0 % (w/v) NaCl and at 20–40 °C; optimum growth was observed with 3 % (w/v) NaCl and at 35 °C. Chemotaxonomic analysis showed that Q-8 was the predominant respiratory quinone and that iso-$C_{15:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$, iso-$C_{11:0}$ and $C_{16:0}$ were the major fatty acids. The G+C content of the genomic DNA was 57.8 mol%. On the basis of 16S rRNA gene sequence analysis, the isolate was affiliated to the genus *Microbulbifer*. Strain CN85^T exhibited most phylogenetic affinity with respect to the type strain of *Microbulbifer maritimus* (97.0 % sequence similarity) and showed less than 97 % sequence similarity with respect to other described *Microbulbifer* species with known 16S rRNA gene sequences. The DNA–DNA hybridization between strain CN85^T and *M. maritimus* JCM 12187^T was 44 %. On the basis of phenotypic and genotypic data, strain CN85^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer donghaiensis* sp. nov. is proposed. The type strain is CN85^T (=CGMCC 1.7063^T =JCM 15145^T).

The genus *Microbulbifer* was first proposed in 1997 to accommodate a Gram-negative, rod-shaped, strictly aerobic gammaproteobacterium capable of utilizing a variety of hydrocarbons (González *et al.*, 1997). The type species, *Microbulbifer hydrolyticus*, was isolated from enrichment cultures from a marine pulp-mill effluent associated with paper manufacture. Subsequently, four additional species, *Microbulbifer salipaludis* (Yoon *et al.*, 2003a), *Microbulbifer maritimus* (Yoon *et al.*, 2004), *Microbulbifer agarilyticus* and *Microbulbifer thermotolerans* (Miyazaki *et al.*, 2008), have been described. *Pseudomonas elongata* was reclassified within the genus as *Microbulbifer elongatus* (Yoon *et al.*, 2003b). Here, we present the results of a polyphasic study of a novel *Microbulbifer* strain isolated from sediment from the East China Sea.

The sediment sample was collected using a multicorer from the East China Sea (27° 19′ 57″ N 120° 34′ 29″ E). A subsample (approx. 100 mg) was suspended in 3 ml sterile seawater and vortexed for 15 min. The dispersed sediment suspension was diluted and subsequently added to modified ZoBell medium (ZoBell, 1941). The modified ZoBell medium contained the following (l^{-1} distilled water): NaCl, 19.45 g; $MgCl_2$, 8.8 g; Na_2SO_4, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; ferric citrate, 0.1 g; KBr, 0.08 g; $CsCl_2$, 34 mg; H_3BO_3, 22 mg; Na_2SiO_3, 4.0 mg; NaF, 2.4 mg; NH_4NO_3, 1.6 mg; Na_3PO_4, 8.0 mg; peptone (Difco), 0.5 g; yeast extract (Difco), 0.1 g; pH 7.4. After 3 days incubation aerobically at 37 °C, one colony, designated CN85^T, was picked. The novel strain was purified by repeated restreaking and was maintained on marine agar 2216 (Difco).

The optimal conditions for growth were determined in ZoBell medium and trypticase soy yeast extract medium (DSMZ medium 92) with different NaCl concentrations (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 15 and 20 %, w/v). The pH range for growth was determined in marine broth 2216 and marine salts basal medium according to González *et al.* (1997). The marine salts basal medium (Mikhailov *et al.*, 2006) contained the following (l^{-1} distilled water): NH_4Cl, 1.0 g; K_2HPO_4, 0.044 g; $FeSO_4.7H_2O$, 0.028 g; artificial seawater, 500 ml; Tris/HCl (1 M, pH 7.5), 100 ml. The artificial seawater contained the following (l^{-1} distilled

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CN85^T is EU365694.

Fatty acid compositions and phenotypic characteristics of strain CN85^T and related strains are provided in supplementary tables available with the online version of this paper.

water): NaCl, 23.4 g; $MgSO_4 . 7H_2O$, 24.6 g; KCl, 1.5 g; $CaCl_2$, 2.9 g. The temperature range for growth was determined by using incubation temperatures ranging from 4 to 48 °C. Cell morphology and motility were examined by means of optical microscopy (BX40; Olympus) and electron microscopy (S260; Cambridge; JEM-1230, JEOL).

Oxidase activity was determined by assessing the oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by assessing bubble production in a 3 % (v/v) H_2O_2 solution. Tests to determine the assimilation of single carbon sources were performed using marine salts basal medium. The corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %), organic acid (0.1 %) or amino acid (0.1 %) was added into liquid medium. Acid production was tested using modified MOF medium supplemented with 0.5 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Biochemical and nutritional tests were performed in marine agar 2216, as described by Mata *et al.* (2002). API 20E, API 20NE and API ZYM tests (all from bioMérieux) were also used to determine physiological and biochemical characteristics.

Fatty acid methyl esters obtained from cells grown in marine agar 2216 for 3 days at 37 °C were analysed using GC/MS (Kuykendall *et al.*, 1988). Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform/methanol (2 : 1) and were analysed using reversed-phase HPLC. Purified DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with calf intestine alkaline phosphatase (Mesbah & Whitman, 1989). The G+C content of the resulting deoxyribonucleosides was determined using reversed-phase HPLC and was calculated from the ratio of deoxyguanosine and thymidine (Mesbah & Whitman, 1989).

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). The sequence was compared with closely related sequences of reference organisms from FASTA and the EzTaxon service (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971) (with the MEGA3 package; Kumar *et al.*, 2004) and the maximum-likelihood method (Felsenstein, 1981) (with TreePuzzle, version 5.2). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method.

The cells of strain CN85^T were long rods, 0.3–0.4 μm wide and 2.5–4.0 μm long (Fig. 1). An electron micrograph of negatively stained cells did not reveal flagella. Strain CN85^T was oxidase- and catalase-positive and could hydrolyse aesculin, casein, gelatin, starch and Tween 20. Detailed results from the phenotypic and biochemical tests are given in the species description or shown in Table 1.

The almost-complete 16S rRNA gene sequence (1416 nt) of strain CN85^T was obtained. Strain CN85^T showed the highest sequence similarity with respect to *M. maritimus* TF-17^T (97.0 %) and showed <97 % sequence similarity with respect to other described *Microbulbifer* species. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CN85^T formed a coherent cluster with types strain of *M. maritimus* and *M. thermotolerans*, with a high level of bootstrap support (100 % with the neighbour-joining method) (Fig. 2).

Comparisons of phenotypic properties (Table 1) and cellular fatty acid compositions (Supplementary Table S1, available in IJSEM Online) also indicated differences between strain CN85^T and known *Microbulbifer* species. Growth of strain CN85^T was not observed at temperatures higher than 42 °C, which is lower than the optimal growth temperature for *M. thermotolerans* (43–49 °C) (Miyazaki *et al.*, 2008). The 16 rRNA gene sequence divergence values between strain CN85^T and the type strain of *M. thermotolerans* exceeded 3 %, an accepted value for the distinction of different genomospecies (Stackebrandt & Goebel, 1994).

DNA–DNA hybridizations were performed using the thermal denaturation and renaturation method of De Ley *et al.* (1970), as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer. The hybridization temperature used was 75 °C and the experiments were carried out in triplicate. The DNA relatedness between strain CN85^T and *M. maritimus* JCM 12187^T was 44.1 %. In addition, a comparison of phenotypic properties (Table 1 and Supplementary Table S2) also indicated differences

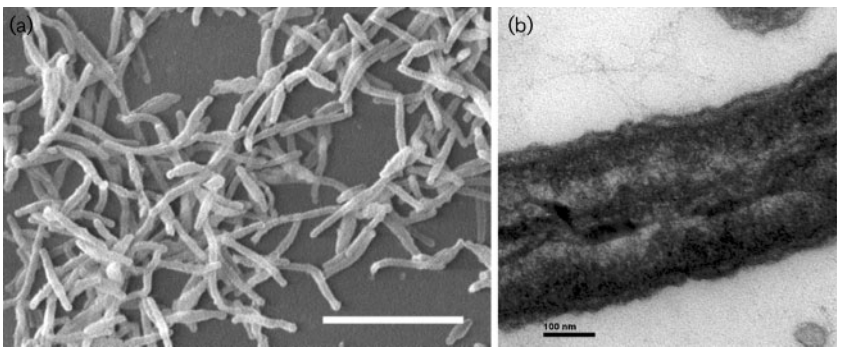

Fig. 1. Scanning electron micrograph (a) and transmission electron micrograph of an ultrathin section (b) of exponentially grown cells of strain CN85^T. Bars, 5 μm (a) and 100 nm (b).

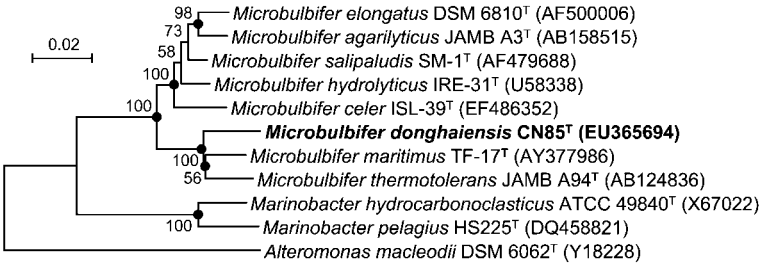

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain CN85^T and related taxa. Bootstrap percentages (based on 1000 replicates) are given at branch points; only values >50 % are shown. Filled circles indicate that nodes were recovered with bootstrap percentages >50 % in both maximum-likelihood and maximum-parsimony trees. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics of strain CN85^T and the type strains of related *Microbulbifer* species

Strains: 1, strain CN85^T; 2, *M. maritimus* JCM 12187^T; 3, *M. thermotolerans* JAMB A94^T (Miyazaki *et al.*, 2008); 4, *M. hydrolyticus* IRE-31^T (González *et al.*, 1997; Yoon *et al.*, 2003a, 2007); 5, *M. salipaludis* SM-1^T (Yoon *et al.*, 2003a, 2007); 6, *M. elongatus* DSM 6810^T (Yoon *et al.*, 2003b, 2007); 7, *M. celer* ISL-39^T (Yoon *et al.*, 2007); 8, *M. agarilyticus* JAMB A3^T (Miyazaki *et al.*, 2008). All strains are Gram-negative and catalase-positive. Results for *M. maritimus* JCM 12187^T were determined in this study in parallel with tests for strain CN85^T unless indicated. +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Cell shape	Rods	Rods	Cocci or rods	Rods	Rods	Cocci or rods	Rods	Rods
Colony colour*	LY	YB	B	C	GY	YB	GY	C
Growth at/with:								
10 % NaCl	−	+†	−	−	+	ND	+	−
pH 5.0	−	+†	−	−	W	ND	+	−
45 °C	−	+†	+	−	+	ND	+	−
Nitrate reduction	+	−†	+	−	+	−	−	+
N-Acetyl-β-glucosaminidase	+	−†	ND	+	+	+	+	ND
ONPG (β-galactosidase)	−	−	−	ND	ND	ND	ND	+
Hydrolysis of:								
Aesculin	+	−†	+	+	+	+	+	−
Agar	−	−	+	−	W	+	−	+
DNA	−	−	+	ND	ND	ND	ND	+
Gelatin	+	+	+	+	−	W	−	+
Tyrosine	−	−	ND	ND	W	ND	−	ND
Acid production from:								
Arabinose	−	−	−	+	−	+	+	−
Cellobiose	+	−	+	+	+	+	+	+
D-Glucose	+	−	+	+	+	−	−	+
Rhamnose	−	−	−	−	+	ND	−	−
Sucrose	−	−	−	−	−	+	−	−
Trehalose	−	+	+	−	−	−	+	−
Xylose	−	−	+	+	−	+	+	+
Sensitivity to:								
Ampicillin	−	−	+	+	ND	ND	−	+
Tetracycline	−	−	ND	+	ND	ND	−	−
Enzyme activities (API ZYM)								
α-Fucosidase	+	−	ND	−	−	−	−	ND
N-Acetyl-β-glucosaminidase	+	−	ND	−	+	+	+	ND
DNA G+C content (mol%)	57.8	59.9†	56.2	57.7	59	58.2	57.7	55.2

*B, Brown; C, cream; GY, greyish yellow; LY, light yellow; YB, yellowish brown.
†Data from Yoon *et al.* (2004).

between strain CN85^T and *M. maritimus* JCM 12187^T, such as salt or temperature range for growth, nitrate reduction, glucose oxidation, hydrolysis of substrates, acid production from sugars and sensitivity to antibiotics.

On the basis of phylogenetic, genotypic, chemotaxonomic and phenotypic data, therefore, strain CN85^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer donghaiensis* sp. nov. is proposed.

Description of *Microbulbifer donghaiensis* sp. nov.

Microbulbifer donghaiensis (dong.hai.en′sis. N.L. masc. adj. *donghaiensis* pertaining to Donghai, the Chinese name for the East China Sea).

Cells are Gram-negative and rod-shaped (0.3–0.4 × 2.5–4.0 μm). Colonies on marine agar 2216 are 1–2 mm in diameter, circular, convex and light yellow in colour after 24 h at 35 °C. No growth occurs in the absence of salt. Growth occurs at NaCl concentrations of 0.5–6.0 % (w/v), with optimum growth at 3.0 %. The optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 6.0, but not at pH 5.5. The temperature range for growth is 20–40 °C; optimum growth occurs at 35–37 °C. Oxidase- and catalase-positive. Nitrate is reduced. Aesculin, casein, gelatin, Tween 80 and starch are hydrolysed. DNA and tyrosine are not hydrolysed. Positive for *N*-acetyl-β-glucosaminidase. Negative for gluconate oxidation, indole production, lysine decarboxylase, ONPG (*o*-nitrophenyl β-D-galactopyranoside) hydrolysis, ornithine decarboxylase and urease. H_2S is not produced from thiosulfate. The following substrates are utilized for growth: acetate, L-alanine, L-arginine, cellobiose, glucose, L-histidine, isoleucine, malate, maltose, propionate, succinate, trehalose and L-valine. The following compounds are not utilized as sole carbon sources: L-arabinose, L-aspartate, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, gluconate, L-glutamate, glycerol, glycine, *myo*-inositol, lactate, lactose, malonate, mannitol, D-mannose, melibiose, L-methionine, L-ornithine, raffinose, rhamnose, ribose, L-serine, sorbitol, sorbose, sucrose and D-xylose. Acid is produced from cellobiose, glucose and maltose. Acid is not produced from L-arabinose, D-fructose, D-galactose, lactose, mannitol, D-mannose, raffinose, rhamnose, sorbitol, sorbose, sucrose, trehalose or D-xylose. Susceptible to cefotaxime (30 μg), chloramphenicol (30 μg), kanamycin (30 μg), cefoxitin (30 μg), nitrofurantoin (300 μg) and novobiocin (30 μg), but not to ampicillin (10 μg), bacitracin (0.04 IU), cefalexin (30 μg), erythromycin (15 μg), penicillin (10 μg), streptomycin (10 μg) or tetracycline (30 μg). In the API ZYM system, acid and alkaline phosphatases, *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present, whereas α-fucosidase, α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase and trypsin activities are absent. Q-8 is the major respiratory quinone. The major fatty acids (>5 %) are iso-$C_{15:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{17:1}\omega 9c$, iso-$C_{17:0}$, iso-$C_{11:0}$ and $C_{16:0}$. The DNA G+C content of the type strain is 57.8 mol% (HPLC).

The type strain, CN85^T (=CGMCC 1.7063^T =JCM 15145^T), was isolated from a sample of marine sediment from the East China Sea.

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References

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

González, J. M., Mayer, F., Moran, M. A., Hodson, R. E. & Whitman, W. B. (1997). *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* **47**, 369–376.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.

Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Béjar, V. (2002). A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* **25**, 360–375.

Mesbah, M. & Whitman, W. B. (1989). Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. *J Chromatogr* **479**, 297–306.

Mikhailov, V. V., Romanenko, L. A. & Ivanova, E. P. (2006). The genus *Alteromonas* and related proteobacteria. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 6, pp. 597–645.

Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

Miyazaki, M., Nogi, Y., Ohta, Y., Hatada, Y., Fujiwara, Y., Ito, S. & Horikoshi, K. (2008). *Microbulbifer agarilyticus* sp. nov. and *Microbulbifer thermotolerans* sp. nov., agar-degrading bacteria isolated from deep-sea sediment. *Int J Syst Evol Microbiol* **58**, 1128–1133.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Xu, X.-W., Wu, Y.-H., Zhou, Z., Wang, C.-S., Zhou, Y.-G., Zhang, H.-B., Wang, Y. & Wu, M. (2007). *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea* sp. nov., halophilic bacteria isolated from hypersaline environments of China. *Int J Syst Evol Microbiol* **57**, 1619–1624.

Xu, X.-W., Wu, Y.-H., Wang, C.-S., Yang, J.-Y., Oren, A. & Wu, M. (2008). *Marinobacter pelagius* sp. nov., a moderately halophilic bacterium. *Int J Syst Evol Microbiol* **58**, 637–640.

Yoon, J. H., Kim, I. G., Shin, D. Y., Kang, K. H. & Park, Y. H. (2003a). *Microbulbifer salipaludis* sp. nov., a moderate halophile isolated from a Korean salt marsh. *Int J Syst Evol Microbiol* **53**, 53–57.

Yoon, J. H., Kim, H., Kang, K. H., Oh, T. K. & Park, Y. H. (2003b). Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. *Int J Syst Evol Microbiol* **53**, 1357–1361.

Yoon, J. H., Kim, I. G., Oh, T. K. & Park, Y. H. (2004). *Microbulbifer maritimus* sp. nov., isolated from an intertidal sediment from the Yellow Sea, Korea. *Int J Syst Evol Microbiol* **54**, 1111–1116.

Yoon, J. H., Jung, S. Y., Kang, S. J. & Oh, T. K. (2007). *Microbulbifer celer* sp. nov., isolated from a marine solar saltern of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **57**, 2365–2369.

ZoBell, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**, 42–75.