Microbulbifer maritimus sp. nov., isolated from an intertidal sediment from the Yellow Sea, Korea

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A Gram-negative, rod-shaped, non-spore-forming, slightly halophilic bacterium (strain TF-17^T) was isolated from an intertidal sediment from the Yellow Sea, Korea. Pigment of strain TF-17^T was similar to that of *Microbulbifer elongatus*, but different from those of *Microbulbifer hydrolyticus* and *Microbulbifer salipaludis*. Strain TF-17^T was distinguishable from *M. elongatus* by some phenotypic properties, including motility, optimal growth temperature and others. Phylogenetic analysis based on 16S rDNA sequences showed that strain TF-17^T clustered with the type strains of the three *Microbulbifer* species with validly published names. Strain TF-17^T exhibited 16S rDNA sequence similarity levels of 95.1–95.7% to the type strains of the three *Microbulbifer* species. The predominant respiratory lipoquinone found in strain TF-17^T was ubiquinone-8. The major fatty acid was iso-C_{15:0} and significant amounts of iso-C_{11:0} 3-OH and iso-C_{17:1}ω9c were also present. The DNA G + C content of strain TF-17^T was 59.9 mol%. Levels of DNA–DNA relatedness between strain TF-17^T and the type strains of the three *Microbulbifer* species were in the range 10.0–13.0%. On the basis of phenotypic and phylogenetic data and genotypic distinctiveness, strain TF-17^T (=KCCM 41774^T = JCM 12187^T) is proposed as the type strain of a novel species of the genus *Microbulbifer*, *Microbulbifer maritimus* sp. nov.

The genus *Microbulbifer* was proposed by González *et al.* (1997) for a novel rod-shaped and strictly aerobic marine bacterium that belonged to the *Proteobacteria*. A single species, *Microbulbifer hydrolyticus*, was included within this genus. The second species of the genus *Microbulbifer* was *Microbulbifer salipaludis*, which was proposed recently by Yoon *et al.* (2003b). Phylogenetic analyses based on 16S rDNA sequences have shown that the two *Microbulbifer* species fall within the γ -subclass of the *Proteobacteria* (González *et al.*, 1997; Solano & Sanchez-Amat, 1999; Anzai *et al.*, 2000; Yoon *et al.*, 2003b). Chemical markers of this genus include the presence of a major amount of iso-C_{15:0} and ubiquinone with eight isoprene units (Q-8) (González *et al.*, 1997; Yoon *et al.*, 2003b). The DNA G + C content of the genus is 58–59 mol% (González *et al.*, 1997; Yoon *et al.*, 2003b). Pigmentation of the colonies of these two *Microbulbifer* species is cream or greyish-yellow. Recently, *Pseudomonas elongata* was transferred to the genus *Microbulbifer* as *Microbulbifer elongatus* (Yoon *et al.*,

2003a). The colour of *M. elongatus* was yellowish-brown, unlike the first two *Microbulbifer* species that were described. In the course of screening taxonomically useful micro-organisms that were present in an intertidal sediment from the Yellow Sea (Korea), a yellowish brown-coloured, slightly halophilic bacterial strain (TF-17^T) was isolated and characterized taxonomically. The result of 16S rDNA sequence comparison indicated that this isolate is related phylogenetically to members of the genus *Microbulbifer*. Accordingly, the aim of this work was to establish the exact taxonomic position of strain TF-17^T by polyphasic characterization, which included phenotypic properties, detailed phylogenetic analysis based on 16S rDNA sequences and genomic relatedness.

Strain TF-17^T was isolated by the dilution-plating technique on marine agar 2216 (MA; Difco). Cell biomass of strain TF-17^T for respiratory lipoquinone analysis and for DNA extraction was obtained from cultures in marine broth 2216 (MB; Difco) at 37 °C. For fatty acid methyl ester analysis, cell mass of strain TF-17^T was obtained from agar plates after incubation for 3 days at 37 °C on MA. Cell morphology was examined by light microscopy and transmission electron microscopy (TEM). Flagellum type was examined by TEM using cells from an exponentially growing culture. Gram-reaction was determined by using a Gram Stain kit

Published online ahead of print on 23 January 2004 as DOI 10.1099/ijs.0.02985-0.

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain TF-17^T is AY377986.

(bioMérieux) according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with anaerobically prepared MA. Growth in the absence of NaCl was investigated in trypticase soy broth to which NaCl was not added. Growth at various NaCl concentrations (0.5–15%) was investigated in MB or in trypticase soy broth. Growth at various temperatures (4–55 °C) was measured on MA. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolysis of gelatin and aesculin and nitrate reduction were determined as described by Lanyi (1987), with the modification that artificial sea water was used. Artificial sea water contained [(1 distilled water)⁻¹]: 23.6 g NaCl; 0.64 g KCl; 4.53 g MgCl₂·6H₂O; 5.94 g MgSO₄·7H₂O; 1.3 g CaCl₂·2H₂O (Levring, 1946). Hydrolysis of casein and starch was determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine, xanthine and Tween 80 was tested on MA plates by using substrate concentrations that were described by Cowan & Steel (1965). Hydrolysis of chitin (Sigma) and birchwood xylan (Sigma) was determined on solid and in liquid marine salts basal medium (Baumann & Baumann, 1981) that contained 0.5% chitin or xylan as the sole carbon source, respectively. *M. hydrolyticus* DSM 11525^T was used as positive control for hydrolysis tests of chitin and xylan. Additional enzyme activity was determined by using the API ZYM system (bioMérieux). Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994).

Chromosomal DNA was isolated and purified according to a method described previously (Yoon *et al.*, 1996), except that ribonuclease T1 was used together with ribonuclease A. Respiratory lipoquinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and fatty acid methyl esters were prepared and identified by following the instructions of the Microbial Identification system (MIDI). DNA G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

16S rDNA was amplified by PCR using two universal primers (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rDNA was performed by using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically on an Applied Biosystems model 377 automatic DNA sequencer. Alignment of sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the

alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods, implemented within the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated according to Jukes & Cantor (1969) by using the program DNADIST in the PHYLIP package. Stability of relationships was assessed by a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset, using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with five replications for each sample; the highest and lowest values obtained for each sample were excluded and the remaining three values were used to calculate similarity values. DNA–DNA relatedness values quoted are the means of these three values.

Cells of strain TF-17^T were rods that measured approximately 0.3–0.5 µm in width and 3.0–6.0 µm in length after cultivation for 3 days at 37 °C on MA. Strain TF-17^T had no flagella. Colonies on MA were yellowish-brown in colour, smooth, irregular, slightly raised and 2.0–4.0 mm in diameter after 3 days incubation at 37 °C on MA. Strain TF-17^T grew optimally at 37 °C, pH 6.5–7.5 and in the presence of 2–4% (w/v) NaCl. Strain TF-17^T did not grow under anaerobic conditions on MA. Phenotypic properties of strain TF-17^T are shown in Table 1 or are given in the species description (see below). As shown in Table 1, there are differences between TF-17^T and the other *Microbulbifer* species in some phenotypic characteristics.

The predominant respiratory lipoquinone found in strain TF-17^T was unsaturated ubiquinone with eight isoprene units (Q-8), at a peak area ratio of approximately 95%. Strain TF-17^T had a cellular fatty acid profile that contained major amounts of straight-chain, branched and hydroxy fatty acids; it contained iso-C_{15:0} as the major fatty acid and also significant amounts of iso-C_{11:0} 3-OH and iso-C_{17:1}ω9c (Table 2). This profile was similar to those of the type strains of *M. hydrolyticus*, *M. salipaludis* and *M. elongatus*, although there are differences in the proportions of some fatty acids, such as iso-C_{11:0}, iso-C_{11:0} 3-OH and C_{18:1}ω7c. The DNA G+C content of strain TF-17^T was 59.9 mol%.

An almost-complete 16S rDNA sequence of 1490 nt (approximately 96% of the *Escherichia coli* 16S rRNA gene sequence) was determined for strain TF-17^T. Strain TF-17^T was found to have highest 16S rDNA sequence similarity to members of the γ-subclass of the *Proteobacteria* and, in particular, to the genus *Microbulbifer*. In the phylogenetic tree based on the neighbour-joining algorithm, strain TF-17^T clustered with the type strains of *M. hydrolyticus*, *M. salipaludis* and *M. elongatus* (Fig. 1). The

Table 1. Differential phenotypic characteristics of strain TF-17^T, *M. hydrolyticus*, *M. salipaludis* and *M. elongatus*

Taxa: 1, strain TF-17^T; 2, *M. hydrolyticus* [data from González *et al.* (1997)]; 3, *M. salipaludis* [data from Yoon *et al.* (2003b)]; 4, *M. elongatus* [data from Humm (1946), Palleroni (1984) and Yoon *et al.* (2003a)]. +, Positive reaction; –, negative reaction; W, weakly positive reaction; ND, not determined. All species were positive for hydrolysis of starch, catalase and acid production from D-cellobiose and maltose. All species were negative for spore formation, Gram-staining, acid production from D-fructose, D-galactose, lactose and D-mannose and growth at 0 % NaCl.

Characteristic	1	2	3	4
Cell morphology	Rods	Rods	Rods	Cocci or rods
Colour of colonies	Yellowish-brown	Cream	Greyish-yellow	Yellowish-brown
Motility	–	–	–	+
Oxidase	+	+	+	ND
Nitrate reduction to nitrite	–	ND	ND	–
Hydrolysis of:				
Aesculin	–	+	–	ND
Agar	–	–	W+	+
Casein	+	+	W+	ND
Chitin	–	+	–	+
Gelatin	+	+	ND	+
Tween 80	+	+	+	ND
N-Acetyl-β-glucosaminidase	–	–	+	+
Acid production from:				
L-Arabinose	–	+	–	+
D-Glucose	–	+	+	–
Sucrose	–	–	–	+
D-Trehalose	+	–	–	ND
D-Xylose	–	+	–	+
Growth at pH 6.0	+	–	+	ND
Growth at 10 % NaCl	+	–	+	ND
Growth temperature (°C):				
Maximum	48	41	45	ND
Optimum	37	37	37	25–30
DNA G+C content (mol%)	59.9	58	59	ND

relationship between strain TF-17^T and the clade comprising the type strains of the three *Microbulbifer* species was supported by bootstrap analysis at a confidence level of 100 %. Similar tree topologies were generated by the maximum-parsimony method (data not shown). Strain TF-17^T showed 16S rDNA sequence similarity levels of 95.1, 95.7 and 95.3 % to *M. hydrolyticus* ATCC 700072^T, *M. salipaludis* KCCM 41586^T and *M. elongatus* DSM 6810^T, respectively. Sequence similarities to all other taxa included in the phylogenetic analysis were <90.5 % (Fig. 1). DNA–DNA hybridization was performed to determine genotypic relatedness between strain TF-17^T and the type strains of the three *Microbulbifer* species. Strain TF-17^T exhibited DNA–DNA relatedness levels of 11.5, 10.0 and 13.0 % to *M. hydrolyticus* DSM 11525^T, *M. salipaludis* KCCM 41586^T and *M. elongatus* DSM 6810^T, respectively.

The results of 16S rDNA sequence analysis revealed that strain TF-17^T has the closest phylogenetic affiliation to the genus *Microbulbifer*, confirmed by a high bootstrap

resampling value. Colonies of strain TF-17^T are similar to those of *M. elongatus* in colour, whereas they are distinguishable from colonies of *M. hydrolyticus* and *M. salipaludis* in colour. The results obtained in chemotaxonomic analyses are most similar to the chemotaxonomic properties that are characteristic of the genus *Microbulbifer* (Yoon *et al.*, 2003a, b). In particular, the predominant respiratory lipoquinone type and cellular fatty acid profile are useful for distinguishing strain TF-17^T and other members of the genus *Microbulbifer* from some other related genera. Strain TF-17^T and other members of the genus *Microbulbifer* contain Q-8 as the predominant respiratory lipoquinone (Yoon *et al.*, 2003a, b), whereas members of the genus *Marinobacter* and authentic pseudomonads have been known to contain Q-9 as the predominant respiratory lipoquinone (Oyaizu & Komagata, 1983; Yumoto *et al.*, 2001; Yoon *et al.*, 2003a). Strain TF-17^T can be distinguished from members of the genus *Alcanivorax* by cellular fatty acid profiles: strain TF-17^T and other members of the genus *Microbulbifer* contain iso-C_{15:0} as the major fatty

Table 2. Percentage cellular fatty acid composition of strain TF-17^T, *M. hydrolyticus* DSM 11525^T, *M. salipaludis* KCCM 41586^T and *M. elongatus* DSM 6810^T

Taxa: 1, strain TF-17^T; 2, *M. hydrolyticus* DSM 11525^T; 3, *M. salipaludis* KCCM 41586^T; 4, *M. elongatus* DSM 6810^T. –, Not detected.

Fatty acid*	1	2	3	4
Straight-chain:				
C _{10:0}	1.3	1.7	2.4	1.6
C _{14:0}	1.0	1.2	2.6	0.7
C _{15:0}	1.6	1.5	1.7	0.9
C _{16:0}	8.7	11.4	16.3	7.1
C _{17:0}	1.3	2.9	2.2	2.5
C _{17:0} cyclo	2.3	5.7	–	–
C _{18:0}	–	1.6	1.4	1.2
C _{19:0} ω8c cyclo	1.4	1.0	–	–
Branched:				
iso-C _{11:0}	10.0	5.7	4.8	6.5
iso-C _{15:0}	25.9	24.4	19.4	20.7
iso-C _{15:1} F†	0.8	1.0	0.7	1.0
iso-C _{16:0}	–	–	–	0.5
iso-C _{17:0}	6.9	10.4	5.5	9.9
anteiso-C _{17:0}	–	–	–	0.8
iso-C _{17:1} ω9c	12.6	10.1	9.5	11.3
Unsaturated:				
C _{17:1} ω8c	–	0.5	1.0	1.8
C _{18:1} ω5c	–	–	0.7	–
C _{18:1} ω7c	5.6	8.9	11.8	16.3
Hydroxy:				
C _{10:0} 3-OH	1.7	1.0	1.2	1.6
C _{16:0} 2-OH	–	–	0.9	–
iso-C _{11:0} 3-OH	14.2	6.2	5.7	7.7
iso-C _{17:0} 3-OH	–	–	0.9	–
Summed feature:‡				
3	2.2	2.7	7.1	6.0
4	–	–	1.7	–

*Fatty acids that represented <0.5% in all strains are omitted.

†The double-bond position indicated by a capital letter is unknown.

‡Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of the following fatty acids: iso-C_{15:0} 2-OH and/or C_{16:1}ω7c. Summed feature 4 contained one or more of the following fatty acids: anteiso-C_{17:1} B and/or iso-C_{17:1} I.

acid, whereas members of the genus *Alcanivorax* contain C_{18:1}ω7c and C_{16:0} as the major fatty acids (Fernández-Martínez *et al.*, 2003). Therefore, both phylogenetic and chemotaxonomic results indicate clearly that strain TF-17^T belongs to the genus *Microbulbifer*.

Although strain TF-17^T is similar to *M. elongatus* in its morphological properties, it is differentiated from *M. elongatus* in some physiological properties (Table 1). There are some noteworthy differences between strain TF-17^T and the other two *Microbulbifer* species in

morphological, physiological and biochemical properties, including colour of colonies, maximum growth temperature, ability to hydrolyse some substrates and acid production from carbohydrates (Table 1). Levels of 16S rDNA similarity between strain TF-17^T and the type strains of *Microbulbifer* species (95.1–95.7%) are low enough to exclude the possibility of assigning strain TF-17^T to a previously described *Microbulbifer* species (Stackebrandt & Goebel, 1994). In addition, DNA–DNA relatedness levels justify that strain TF-17^T is separate from the three *Microbulbifer* species with validly published names (Wayne *et al.*, 1987). Therefore, on the basis of phenotypic, chemotaxonomic and phylogenetic data and genotypic distinctiveness, strain TF-17^T should be placed in the genus *Microbulbifer* as the type strain of a novel species, for which we propose the name *Microbulbifer maritimus* sp. nov.

Description of *Microbulbifer maritimus* sp. nov.

Microbulbifer maritimus (ma.ri.ti' mus. L. masc. adj. *maritimus* living near the sea).

Cells are rods, 0.3–0.5 µm wide and 3.0–6.0 µm long. Gram-negative. Colonies on MA are yellowish-brown in colour, smooth, irregular, slightly raised and 2.0–4.0 mm in diameter after 3 days incubation at 37 °C. Optimal growth temperature is 37 °C. Growth occurs at 15 and 48 °C, but not at 10 °C or above 49 °C. Optimal growth pH is 6.5–7.5. Growth occurs weakly at pH 5.0, but not at pH 4.5. Optimal growth occurs in the presence of 2–4% NaCl. Growth occurs in the presence of 10% NaCl, but not without NaCl or in the presence of >11% NaCl. No growth occurs under anaerobic conditions on MA. Predominant respiratory lipoquinone is Q-8. Major fatty acid is iso-C_{15:0} and significant amounts of iso-C_{11:0} 3-OH and iso-C_{17:1}ω9c are also present. DNA G+C content is 59.9 mol% (determined by HPLC). Tyrosine is hydrolysed, but hypoxanthine, xanthine and xylan are not. Acid is not produced from adonitol, *myo*-inositol, D-mannitol, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol or stachyose. D-Glucose, D-cellobiose, L-arginine, L-serine, acetate, butyrate, DL-malate, pyruvate and succinate are utilized for growth. D-Fructose, lactose, mannose, sucrose, hexadecane, mannitol, ethanol, methanol, benzoate, citrate, formate, DL-glutamate and lactate are not utilized. The following enzymes are present when assayed with the API ZYM system: alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The following enzymes are absent when assayed with the API ZYM system: lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Other phenotypic properties are given in Table 1.

The type strain is TF-17^T (= KCCM 41774^T = JCM 12187^T), which was isolated from an intertidal sediment from the Yellow Sea, Korea.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain TF-17^T and representatives of the γ -subclass of the Proteobacteria, based on 16S rDNA sequences. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at branch-points. Bar, 0.01 substitutions per nucleotide position.

Acknowledgements

This work was supported by the 21C Frontier program of Microbial Genomics and Applications (grant MG02-0401-001-1-0-0) and NRL research programme (grant M10104000294-01J0 00012811) from the Ministry of Science and Technology (MOST) of the Republic of Korea.

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