Microbulbifer halophilus sp. nov., a moderately halophilic bacterium from north-west China

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A Gram-negative, rod-shaped, moderately halophilic bacterium, designated strain YIM 91118^T, motile with a single polar flagellum, was isolated from Xinjiang province in north-west China and subjected to a polyphasic taxonomic study. Strain YIM 91118^T grew optimally at 37 °C, pH 7.0–8.0 and 10 % NaCl (w/v). Its major cellular fatty acids were iso-C_{15:0}, iso-C_{17:0}, C_{16:0} and iso-C_{17:1}ω9c. The predominant lipoquinone was Q-8. The DNA G+C content was 63.2 mol%. All of these chemotaxonomic data supported the assignment of the new isolate to the genus *Microbulbifer*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 91118^T belongs to the genus *Microbulbifer* and it formed a distinct subclade with *Microbulbifer maritimus* KCCM 41774^T. The levels of 16S rRNA gene sequence similarity to the type strains of *Microbulbifer* species were in the range 93.5–95.0 %. The mean level of DNA–DNA relatedness between strain YIM 91118^T and *M. maritimus* KCCM 41774^T was 45.8 %. On the basis of phenotypic properties, phylogeny and genomic data, strain YIM 91118^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer halophilus* sp. nov. is proposed. The type strain is YIM 91118^T (=CCTCC AB 206094^T =KCTC 12848^T). Furthermore, it is necessary to emend the description of the genus *Microbulbifer*.

The genus *Microbulbifer* was established by González *et al.* (1997) and analysis of 16S rRNA gene sequences showed that the genus belongs to *Gammaproteobacteria*. At the time of writing, the genus *Microbulbifer* comprises five species with validly published names, *Microbulbifer celer*, *Microbulbifer elongatus*, *Microbulbifer hydrolyticus*, *Microbulbifer maritimus* and *Microbulbifer salipaludis*. With the exception of *M. hydrolyticus* (González *et al.*, 1997), these species were proposed by Yoon *et al.* (2003a, b, 2004, 2007). Members of the genus *Microbulbifer* are described as Gram-negative, non-motile rods, catalase-positive with the predominant respiration lipoquinone Q-8 (González *et al.*, 1997; Yoon *et al.*, 2003a, b, 2004). During

research into the diversity of culturable halophilic bacteria at Ganjiahu Natural Reserve in Xinjiang province, north-west China, a moderately halophilic, Gram-negative bacterium, designated strain YIM 91118^T, was characterized. Accordingly, the aim of this work was to determine the exact taxonomic status of this organism.

Strain YIM 91118^T was isolated from a saline soil by the conventional dilution-plating method at 37 °C. The isolation medium was modified ISP 5 and its composition was as follows (in 1 l distilled water, final pH 7.5): 1 g L-asparagine, 10 g glycerol, 5 g yeast extract, 1 g K₂HPO₄, 5 g KNO₃, 100 g NaCl and 15 g agar. NaCl was sterilized separately before being added to the medium. The strain was maintained on modified ISP 5 slants containing 10 % (w/v) NaCl at 4 °C and as glycerol suspensions (20 %, v/v) at –20 °C. Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 150 r.p.m.) using modified ISP medium 5 [10 % (w/v) NaCl, pH 7.5] at

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 91118^T is EF674853.

A transmission electron micrograph of a cell of strain YIM 91118^T is available as supplementary material with the online version of this paper.

37 °C for 1 week. *Microbulbifer maritimus* KCCM 41774^T was used as a reference strain for DNA–DNA hybridization and other phenotypic tests and was obtained from the Korea Culture Center of Microorganisms.

Cell morphology was determined on cultures grown for 6, 12, 16, 24 and 48 h on modified ISP 5 agar medium supplemented with 10 % (w/v) NaCl at 37 °C. Gram staining was carried out by the standard Gram reaction and was confirmed by using the KOH lysis test method (Cerny, 1978). Cell motility was confirmed by the presence of turbidity throughout the tube including semisolid medium (Leifson, 1960). Morphological characteristics of the strain were observed by light microscopy (model BH 2; Olympus) and by transmission electron microscopy (model H-800; Hitachi). Growth was tested at 0, 4, 10, 15, 20, 28, 37, 45 and 55 °C on modified ISP medium 5 containing 10 % (w/v) NaCl. For determination of NaCl tolerance, modified ISP medium 5 was used as the basal medium with 0, 1, 3, 5, 7, 10, 15, 20 and 25 % NaCl (w/v). The pH range for growth was investigated between pH 4.0 and 10.0 at intervals of 1 pH unit using the buffer systems 0.1 M citric acid/0.1 M sodium citrate (pH 4.0 and 5.0), 0.1 M KH₂PO₄/0.1 M NaOH (pH 6.0–8.0) and 0.1 M NaHCO₃/0.1 M Na₂CO₃ (pH 9.0 and 10.0). Catalase activity was determined by production of bubbles after the addition of a drop of 3 % H₂O₂. Oxidase activity was observed by oxidation of tetramethyl-*p*-phenylenediamine. Hydrolysis of aesculin, casein, gelatin, starch and Tweens 40 and 80 was determined as described by Cowan & Steel (1965). Enzyme activity and acid production from carbohydrates were determined by using the API ZYM and API 50CH systems (bioMérieux) according to the manufacturer's instructions.

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultivation in modified ISP 5 medium containing 10 % NaCl at 37 °C. The lipoquinone was investigated as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis, cell biomass was harvested from TSB medium containing 10 % NaCl after cultivation for 2 days at 37 °C. Whole-cell fatty acid analysis of strain YIM 91118^T was performed as described by Sasser (1990) using the Microbial Identification System (MIDI).

Genomic DNA of strain YIM 91118^T for the determination of G+C content and DNA–DNA relatedness experiments was prepared according to the method of Marmur (1961). The G+C content of the DNA was determined by reversed-phase HPLC (Mesbah *et al.*, 1989). DNA–DNA hybridization was carried out according to methods described previously (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Li *et al.* (2007). Multiple alignments with sequences of the most closely related strains and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson

et al., 1997). Phylogenetic analyses were performed using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was constructed using the neighbour-joining method from *K*_{nuc} values (Kimura, 1980) using MEGA version 2.1 (Kumar *et al.*, 2001). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Cells of strain YIM 91118^T were rods, approximately 0.3–0.5 µm wide and 1.3–1.5 µm long after cultivation for 3 days at 37 °C on modified ISP 5 agar containing 10 % NaCl. Strain YIM 91118^T was motile with a single polar flagellum (Supplementary Fig. S1, available in IJSEM Online). Colonies were yellowish-brown in colour, smooth, opaque, mucoid and 2.0–4.0 mm in diameter after 3 days incubation at 37 °C on modified ISP 5 agar containing 10 % NaCl. Strain YIM 91118^T grew at 10–45 °C, pH 6–9 and in the presence of 1–20 % (w/v) NaCl, but showed no growth without any salts. Phenotypic properties of strain YIM 91118^T are described in Table 1 and in the species description.

The predominant respiratory lipoquinone found in strain YIM 91118^T was Q-8. Strain YIM 91118^T had a cellular fatty acid profile that contained large amounts of branched and straight-chain fatty acids, and the major components (>10 % of total fatty acids) were iso-C_{15:0}, iso-C_{17:0}, C_{16:0} and iso-C_{17:1ω9c} (Table 2). The genomic DNA G+C content of strain YIM 91118^T was 63.2 mol%.

An almost-complete 16S rRNA gene sequence (1473 bp) was obtained for strain YIM 91118^T and the result of the alignment showed that strain YIM 91118^T had highest 16S rRNA sequence similarity to members of the genus *Microbulbifer*. A phylogenetic tree was constructed with sequences of representative strains of the genus *Microbulbifer*. In the phylogenetic tree based on the neighbour-joining algorithm, strain YIM 91118^T clustered with the type strains of the genus *Microbulbifer* (Fig. 1). The 16S rRNA gene sequence of YIM 91118^T showed 95.0, 94.6, 94.1, 94.1 and 93.5 % sequence similarity with those of the type strains of *M. celer*, *M. maritimus*, *M. hydrolyticus*, *M. salipaludis* and *M. elongatus*, respectively. In addition, strain YIM 91118^T and *M. maritimus* KCCM 41774^T shared the same branch, with a high bootstrap value of 93 %, which confirmed that the new isolate had much closer relationship with the latter species within the genus *Microbulbifer*. Topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the neighbour-joining tree (not shown).

The chemotaxonomic properties of strain YIM 91118^T (iso-C_{15:0} as the major fatty acid and Q-8 as predominant respiratory lipoquinone) indicated an affiliation to the genus *Microbulbifer* (González *et al.*, 1997; Yoon *et al.*, 2003a, b, 2004, 2007). The results of 16S rRNA gene sequence analysis revealed that strain YIM 91118^T had the

Table 1. Differential phenotypic characteristics of strain YIM 91118^T and *M. maritimus* KCCM 41774^T

Data for *M. maritimus* KCCM 41774^T are from this study unless indicated. Cells of both strains are rods. Both strains are positive for hydrolysis of gelatin, activities of catalase, oxidase, alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase and acid production from D-glucose, D-lactose, maltose, N-acetylglucosamine and turanose. Both strains are negative for Gram-staining, activities of acid phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase and acid production from aesculin, amygdalin, arbutin, dulcitol, D-adonitol, D-arabinose, D-arabitol, cellobiose, D-lyxose, raffinose, erythritol, gentiobiose, inositol, inulin, L-arabitol, L-fucose, L-sorbose, L-xylose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, methyl β -D-xylopyranoside, potassium gluconate, salicin, starch and xylitol.

Characteristic	YIM 91118 ^T	<i>M. maritimus</i> KCCM 41774 ^T
Flagellation	Single polar	—*
Motility	+	—*
Hydrolysis of casein and Tween 80	—	+*
Acid production (API 50CH) from:		
Glycerol	—	+
L-Arabinose	+	—
D-Xylose	+	—
D-Galactose	—	+
D-Fructose	—	+
D-Mannose	—	+
L-Rhamnose	+	—
D-Mannitol	—	+
D-Sorbitol	—	+
Sucrose	—	+
Trehalose	—	+
Glycogen	+	—
D-Tagatose	+	—
D-Fucose	+	—
Potassium 2-ketogluconate	+	—
Potassium 5-ketogluconate	+	—
Valine arylamidase (API ZYM)	+	—
Acid phosphatase (API ZYM)	—	+
NaCl range (% w/v)	1–20	0–10
Growth at 10 °C	+	—
Growth at pH 5.0	—	+
DNA G + C content (mol%)	63.2	59.9*

*Data from Yoon *et al.* (2004).

closest phylogenetic affiliation to the genus *Microbulbifer*, which was confirmed by a high bootstrap resampling value. Therefore, both the chemotaxonomic and phylogenetic results clearly indicate that strain YIM 91118^T belongs to the genus *Microbulbifer*. Although strain YIM 91118^T is similar to *M. maritimus* KCCM 41774^T in colony colour (yellowish brown) and shares the same branch in the phylogenetic tree, it can be differentiated from *M.*

Table 2. Comparison of the cellular fatty acid compositions of strain YIM 91118^T and *M. maritimus* KCCM 41774^T

Values are percentages of total fatty acids from this study. Fatty acids that represented <0.5% in both strains are omitted.

Fatty acid	YIM 91118 ^T	<i>M. maritimus</i> KCCM 41774 ^T
Straight-chain		
C _{10:0}	0.6	1.3
C _{14:0}	1.0	1.0
C _{15:0}	4.6	1.6
C _{16:0}	12.4	8.7
C _{17:0}	5.2	1.3
C _{18:0}	0.6	—
Branched		
iso-C _{11:0}	5.4	10.0
iso-C _{15:0}	17.4	25.9
iso-C _{15:0} F*	0.7	0.8
iso-C _{17:0}	15.5	6.9
anteiso-C _{17:0}	0.5	—
iso-C _{17:1} ω9c	11.2	12.6
Unsaturated		
C _{17:1} ω8c	0.7	—
C _{18:1} ω7c	5.2	5.6
Hydroxy		
C _{10:0} 3-OH	0.5	1.7
iso-C _{11:0} 3-OH	8.4	14.2
Cyclo		
C _{17:0} cyclo	4.2	2.3
C _{19:0} ω8c cyclo	1.8	1.4
Summed feature 3†	2.2	2.2

*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 iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

maritimus KCCM 41774^T in some morphological and physiological properties (Table 1); for example, strain YIM 91118^T is motile by means of a single polar flagellum, grows at 10 °C and does not grow at pH 5.0 or in the absence of NaCl, whereas *M. maritimus* KCCM 41774^T is non-motile and grows at pH 5.0 and in the absence of NaCl, but does not grow at 10 °C. In addition, strain YIM 91118^T and *M. maritimus* KCCM 41774^T differ in the presence of valine arylamidase and acid phosphatase and in acid production from various carbohydrates, as shown in Table 1. Additionally, the mean level of DNA–DNA relatedness between strain YIM 91118^T and the type strain of *M. maritimus* KCCM 41774^T was 45.8% (from three determinations), much lower than the value of 70% recommended for assignment of a strain to a species (Wayne *et al.*, 1987). Therefore, on the basis of phenotypic and genotypic distinctiveness, strain YIM 91118^T should be placed in the genus *Microbulbifer* as the type strain of a novel species, for which we propose the name *Microbulbifer halophilus* sp. nov. In addition, given that all five previously

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method (Saitou & Nei, 1987) showing the position of strain YIM 91118^T among members of the family Alteromonadaceae. The sequence of *Escherichia coli* ATCC 11775^T was used as the outgroup. Numbers at branching points refer to bootstrap values (percentages from 1000 resamplings; only values above 50 % are shown). Bar, 1 substitution per 100 nucleotide positions.

described species of the genus were non-motile, the description of the genus *Microbulbifer* González *et al.* 1997 needs to be emended.

Emended description of the genus *Microbulbifer* González *et al.* 1997

Aerobic, Gram-negative rods; motility is variable. All isolates are halophilic and the highest NaCl tolerance is up to 20 %. Contain iso-C_{15:0} as the major fatty acid and Q-8 as the predominant respiratory lipoquinone. The G + C content of the genomic DNA is 57–64 mol%. Phylogenetically, the genus is a member of the Gammaproteobacteria. The type species is *Microbulbifer hydrolyticus*.

Description of *Microbulbifer halophilus* sp. nov.

Microbulbifer halophilus (ha.lo.phi'lus. Gr. n. *hals*, *halos* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are aerobic, Gram-negative rods, motile with a single polar flagellum. Colonies are yellowish-brown, smooth, opaque and mucoid on modified ISP 5 medium. Moderately halophilic, growing in a wide range (1–20 % w/v) of salt concentrations, with optimal growth at 10 % NaCl (w/v). No growth in the absence of NaCl. Growth occurs at 10–45 °C and pH 6.0–9.0, with optimal growth at 37 °C and pH 7.0–8.0. Oxidase- and catalase-positive. Indole and H₂S are not produced. Nitrate is not reduced. Gelatin is hydrolysed, but aesculin, casein, starch and Tweens 40 and 80 are not hydrolysed. In the API 50CH system, acid is produced from *N*-acetylglucosamine, aesculin, L-arabinose, D-fucose, D-glucose, glycogen, D-lactose, maltose, L-rhamnose, turanose, D-tagatose, starch, D-xylose, potassium 2-ketogluconate and potassium 5-ketogluconate, but not from D-adonitol, amygdalin, D-arabinose, D- or L-arabitol, arbutin, cellobiose, dulcitol, erythritol, L-fucose, D-fructose, D-galactose, gentiobiose, glycerol, inositol, inulin, D-lyxose, D-mannitol, D-mannose, melezitose, melibiose, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, methyl β -D-xylopyranoside, raffinose, D-ribose, sucrose, salicin, D-sorbitol, L-sorbose, trehalose, xylitol, L-xylose or potassium gluconate. In the

API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase tests are positive. Lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, β -glucosidase, α -mannosidase and α -fucosidase tests are negative. Q-8 is the predominant lipoquinone, at a peak area ratio of approximately 91.5 %. Major fatty acids are iso-C_{15:0}, iso-C_{17:0}, C_{16:0} and iso-C_{17:1} ω 9c. The G + C content of the DNA of the type strain is 63.2 mol%.

The type strain is YIM 91118^T (=CCTCC AB 206094^T =KCTC 12848^T), isolated from a saline soil sample in Xinjiang province, north-west China.

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