Note

Microbulbifer salipaludis sp. nov., a moderate halophile isolated from a Korean salt marsh

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A Gram-negative, non-motile, non-spore-forming, moderately halophilic rod (strain SM-1^T) was isolated from salt marsh around the junction of the Youngsan River and the Yellow Sea in Korea and subjected to a polyphasic taxonomic study. This organism grew optimally at 37 °C and was able to grow at 10 and 45 °C. It grew optimally in the presence of 2–3 % (w/v) NaCl. The major fatty acids in strain SM-1^T were iso-C_{15:0} and C_{16:0}. Strain SM-1^T and *Microbulbifer hydrolyticus* DSM 11525^T were characterized by having ubiquinone-8 as the predominant respiratory lipoquinone. The DNA G+C content of strain SM-1^T was 59 mol%. Phylogenetic analysis based on 16S rDNA sequences showed that strain SM-1^T formed a coherent cluster with *M. hydrolyticus*; this relationship was supported by a bootstrap resampling value of 100 %. The level of 16S rDNA identity between strain SM-1^T and the type strain of *M. hydrolyticus* was 98.6 %. The mean level of DNA–DNA relatedness between strain SM-1^T and the type strain of *M. hydrolyticus* was 20.6 %. Therefore, on the basis of phenotypic properties, phylogeny and genomic data, strain SM-1^T should be placed in the genus *Microbulbifer* as a member of a novel species, for which the name *Microbulbifer salipaludis* sp. nov. is proposed. The type strain of the novel species is strain SM-1^T (=KCCM 41586^T =JCM 11542^T).

The genus *Microbulbifer* was proposed by González *et al.* (1997) to accommodate a novel rod-shaped and strictly aerobic marine bacterium belonging to the *Proteobacteria*. There is currently only one species, *Microbulbifer hydrolyticus*, included in this genus. Phylogenetic analyses based on 16S rDNA sequences showed that the genus *Microbulbifer* falls within the γ -*Proteobacteria* and is most closely related to the genera *Marinobacter*, *Marinomonas* and *Oceanospirillum* (Anzai *et al.*, 2000; González *et al.*, 1997; Solano & Sanchez-Amat, 1999). However, the genus *Microbulbifer* has less than 91 % 16S rDNA sequence identity to all previously described members of the γ -*Proteobacteria* (González *et al.*, 1997). There are also numerous phenotypic differences between the genus *Microbulbifer* and other members of this subclass (González *et al.*, 1997). The genus *Microbulbifer* is characterized chemotaxonomically by having iso-C_{15:0} and iso-C_{17:1}ω9c as the major fatty acids and by a DNA G+C content of 57.7 mol%

(González *et al.*, 1997). Isolates belonging to this genus have been isolated from a marine enrichment community growing on pulp-mill effluent, and the original inoculum was from a salt marsh on the coast of Georgia, USA (González *et al.*, 1997). Recently, a moderately halophilic bacterial strain, SM-1^T, was isolated from salt marsh around the junction of the Youngsan River and the Yellow Sea in Korea. This isolate was considered to be a *Microbulbifer*-like strain from 16S rDNA sequence comparison results. Accordingly, strain SM-1^T was subjected to polyphasic characterization to investigate the possibility that it represents a second species of the genus *Microbulbifer*. In this work, the morphological, phenotypic, phylogenetic and genomic characteristics of strain SM-1^T are described. On the basis of the data presented below, a novel species of the genus *Microbulbifer*, *Microbulbifer salipaludis* sp. nov., is proposed for strain SM-1^T.

Strain SM-1^T was isolated by the dilution-plating technique on marine agar (MA) (Difco). Cell biomass of strain SM-1^T and *M. hydrolyticus* DSM 11525^T for respiratory

The GenBank accession number for the 16S rDNA sequence of strain SM-1^T is AF479688.

lipoquinone analysis and for DNA extraction was obtained from marine broth (MB) (Difco) cultures at 37 °C. The two strains were cultivated on a horizontal shaker at 150 r.p.m. and the broth cultures were checked microscopically for purity before being harvested by centrifugation. For fatty acid methyl ester analysis, cell mass of strain SM-1^T and *M. hydrolyticus* DSM 11525^T was obtained from agar plates after growth for 3 days at 37 °C on MA. Cell morphology was examined by light microscopy and TEM. The presence or absence of flagella was examined by TEM using cells from exponentially growing cultures. The cells were negatively stained with 1% (w/v) phosphotungstic acid and after air drying, the grids were examined with a model CM-20 TEM (Philips). Catalase activity was determined by bubble production in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolysis of casein and starch were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin was determined according to the method of Lanyi (1987) with addition of 3% NaCl. Hydrolysis of hypoxanthine, tyrosine, xanthine and Tween 80 was performed on MA using substrate concentrations described previously (Cowan & Steel, 1965). Hydrolysis of chitin (Sigma) and birch-wood xylan (Sigma) was determined on solid and in liquid marine salts basal medium (Baumann & Baumann, 1981) respectively containing 0.5% chitin or xylan as the sole carbon source. *M. hydrolyticus* DSM 11525^T was used as a positive control for hydrolysis tests of chitin and xylan. Additional enzyme activities were determined using the API ZYM system (bioMérieux). Acid production from carbohydrates was determined as described by Leifson (1963). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with MA that had been prepared anaerobically. Growth at various NaCl concentrations was investigated on MA or in MB. Growth at various temperatures (4–55 °C) was measured on MA.

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

16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rDNA was performed 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 using 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 using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Jukes & Cantor (1969) with the program DNADIST. The stability of relationships was assessed by bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset using the programs SEQBOOT 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 microdilution wells.

Cells of strain SM-1^T were rods, approximately 0.3–0.5 µm wide and 1.2–3.0 µm long after 3 days cultivation at 37 °C on MA. In microscopic examinations, numerous vesicles were observed. The Gram-staining reaction was negative. Cells of strain SM-1^T had no flagella. No spores were observed. Colonies on MA were greyish-yellow in colour, smooth, circular to slightly irregular, flat with erose margins and 2.0–4.0 mm in diameter after 3 days growth at 37 °C. Strain SM-1^T grew optimally at 37 °C; it grew at 10 and 45 °C, but not at 4 °C or above 46 °C. Strain SM-1^T grew optimally at pH 7.0–8.0; it grew weakly at pH 5.0 and no growth was observed at pH 4.5. Strain SM-1^T grew optimally in the presence of 2–3% (w/v) NaCl. It grew in the presence of 10% NaCl, but growth did not occur without NaCl or in the presence of more than 11% NaCl. Strain SM-1^T did not grow under anaerobic conditions on MA. Strain SM-1^T showed catalase and oxidase activities. Aesculin, starch, Tween 80 and xylan were hydrolysed. Agar, casein and tyrosine were hydrolysed weakly. No hydrolysis of chitin, hypoxanthine or xanthine was observed. Acid was produced from D-cellobiose, D-glucose, maltose and L-rhamnose. The following enzymes were present in strain SM-1^T and *M. hydrolyticus* DSM 11525^T when assayed with the API ZYM system: alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. *N*-Acetyl-β-glucosaminidase was present only in strain SM-1^T. The phenotypic properties of strain SM-1^T are summarized in Table 1, together with those of *M. hydrolyticus*.

The predominant respiratory lipoquinone found in strain SM-1^T and *M. hydrolyticus* DSM 11525^T was an unsaturated ubiquinone with eight isoprene units (Q-8). Strain SM-1^T and *M. hydrolyticus* DSM 11525^T respectively contained approximately 96 and 95% Q-8. Strain SM-1^T had a cellular fatty acid profile that contained major

Table 1. Differential phenotypic characteristics of strain SM-1^T and *M. hydrolyticus*

Both species were rods and positive for hydrolysis of starch, Tween 80 and xylan, catalase, oxidase, alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and acid production from D-cellobiose, D-glucose and maltose. Both species were negative for spore formation, Gram-staining, motility, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, α -fucosidase, acid production from adonitol, D-fructose, D-galactose, lactose, D-mannitol, D-mannose, D-melezitose, melibiose, myo-inositol, D-raffinose, ribose, D-sorbitol, stachyose, sucrose and D-trehalose, decomposition of hypoxanthine and xanthine and growth in the absence of NaCl. +, Positive; –, negative; w, weakly positive. Data for *M. hydrolyticus* were taken from González *et al.* (1997) unless indicated otherwise.

Characteristic	Strain SM-1 ^T	<i>M. hydrolyticus</i>
Colour of colonies	Greyish yellow	Cream
Decomposition of:		
Aesculin	–	+*
Agar	w	–
Casein	w	+*
Chitin	–	+
Tyrosine	w	–*
<i>N</i> -Acetyl- β -glucosaminidase	+	–†
Acid production from:		
L-Arabinose	–	+*
L-Rhamnose	+	–*
D-Xylose	–	+*
Growth at pH 6.0	+	–
Growth in 10% NaCl	+	–
Maximum growth temperature (°C)	45	41
DNA G+C content (mol%)	59	58

*Data for strain DSM 11525^T from this study.

†API ZYM data for strain DSM 11525^T from this study.

amounts of saturated, unsaturated and branched fatty acids, similar to that of the type strain of *M. hydrolyticus* (Table 2). Strain SM-1^T contained iso-C_{15:0} and C_{16:0} as the major fatty acids. The genomic DNA G+C content of strain SM-1^T was 59 mol%.

An almost complete 16S rDNA sequence of 1492 nt (approx. 96% of the *Escherichia coli* 16S rRNA sequence) was determined for strain SM-1^T. Strain SM-1^T had highest 16S rDNA identity to members of the γ -*Proteobacteria*. In the phylogenetic tree based on the neighbour-joining algorithm, strain SM-1^T formed a coherent cluster with *M. hydrolyticus* (Fig. 1). The clustering fidelity between strain SM-1^T and the type strain of *M. hydrolyticus* was supported by bootstrap resampling analysis at a confidence level of 100%. This bootstrap resampling value was also found in the tree generated with maximum-parsimony algorithms. The level

Table 2. Cellular fatty acid composition of strain SM-1^T and *M. hydrolyticus* DSM 11525^T

Values are percentages of total fatty acids. –, Not detected; i, iso; ai, anteiso. Fatty acids representing less than 0.5% were omitted.

Fatty acid	Strain SM-1 ^T	<i>M. hydrolyticus</i> DSM 11525 ^T
C _{10:0}	2.4	1.7
C _{10:0} 3-OH	1.2	1.0
i-C _{11:0}	4.8	5.7
i-C _{11:0} 3-OH	5.7	6.2
C _{14:0}	2.6	1.2
i-C _{15:0}	19.4	24.4
i-C _{15:1} F*	0.7	1.0
C _{15:0}	1.7	1.5
C _{16:0}	16.3	11.4
C _{16:0} 2-OH	0.9	–
i-C _{17:1} ω 9c	9.5	10.1
i-C _{17:0}	5.5	10.4
C _{17:1} ω 8c	1.0	0.5
cyclo-C _{17:0}	–	5.7
C _{17:0}	2.2	2.9
i-C _{17:0} 3-OH	0.9	–
C _{18:1} ω 5c	0.7	–
C _{18:1} ω 7c	11.8	8.9
C _{18:0}	1.4	1.6
cyclo-C _{19:0} ω 8c	–	1.0
Summed feature 3†	7.1	2.7
Summed feature 4†	1.7	–

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

†Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains one or more of following fatty acids: iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c. Summed feature 4 contains one or more of following fatty acids: anteiso-C_{17:1} B and/or iso-C_{17:1} I.

of 16S rDNA sequence identity between strain SM-1^T and *M. hydrolyticus* ATCC 700072^T was 98.6%. Strain SM-1^T exhibited levels of 16S rDNA identity of less than 91.3% to other species used in the phylogenetic analysis (Fig. 1). DNA–DNA hybridization was performed to determine the genomic relatedness between strain SM-1^T and *M. hydrolyticus* DSM 11525^T. The two strains exhibited a mean DNA relatedness of 20.6% when each of their DNAs was used separately as the labelled DNA probe.

Phylogenetic inference based on 16S rDNA sequences revealed that strain SM-1^T is the nearest phylogenetic neighbour of *M. hydrolyticus* and that both species are closely related to the genera *Pseudomonas*, *Marinobacter*, *Oceanospirillum*, *Halomonas* and *Alcanivorax* (Fig. 1). This phylogenetic relationship was also observed in previous studies (Anzai *et al.*, 2000; González *et al.*, 1997; Solano & Sanchez-Amat, 1999). 16S rDNA sequence comparisons revealed that strain SM-1^T has nucleotide sequence identity of 98.6% to the type strain of *M. hydrolyticus*, but

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

relatively low levels of nucleotide identity (less than 91.3%) to other previously described members of the γ -Proteobacteria. These data suggest that strain SM-1^T may be a member of the genus *Microbulbifer*. Phylogenetic analysis based on 16S rDNA sequences is now recognized as the most important method for the exact classification or identification of prokaryotic organisms (Woese, 1987; Stackebrandt *et al.*, 1997). Nevertheless, chemotaxonomic characterization may be necessary to support the results of monothetic classification by 16S rDNA sequence analysis. Comparative chemotaxonomic analyses confirmed the conclusion from the phylogenetic classification that strain SM-1^T belongs to the genus *Microbulbifer*. The chemotaxonomic data observed for strain SM-1^T, i.e. major respiratory lipoquinone, cellular fatty acid profile and G+C content, are almost identical to those of *M. hydrolyticus*. However, strain SM-1^T can be clearly differentiated from members of the genera *Pseudomonas*, *Marinobacter*, *Oceanospirillum*, *Halomonas* and *Alcanivorax* by cellular fatty acid profile and G+C content (Franzmann & Tindall, 1990; González *et al.*, 1997; Huu *et al.*, 1999; Oyaizu & Komagata, 1983; Sakane & Yokota, 1994; Yakimov *et al.*, 1998). Strain SM-1^T can also be distinguished from the genera *Marinobacter* and *Halomonas* and authentic *Pseudomonas* species by its predominant respiratory lipoquinone. The genus *Halomonas* and authentic *Pseudomonas* species have ubiquinone-9 as the predominant respiratory lipoquinone (Franzmann & Tindall, 1990; Oyaizu & Komagata, 1983). The genus *Marinobacter* has been found to contain ubiquinone-9 as the predominant respiratory lipoquinone in a recent study. Accordingly, phylogenetic inference and available chemotaxonomic data indicate that strain SM-1^T is a member of the genus *Microbulbifer*.

Strain SM-1^T is similar to *M. hydrolyticus* in its morphological characteristics. However, there are some differences between strain SM-1^T and *M. hydrolyticus* in physiological characteristics, such as growth at pH 6.0, NaCl tolerance, maximum growth temperature and acid production from carbohydrates (Table 1). Whereas strain SM-1^T grows in the presence of 10% NaCl, *M. hydrolyticus* shows no growth (Table 1). Strain SM-1^T has a maximum growth temperature of 45 °C (optimum of 37 °C), whereas *M. hydrolyticus* has a maximum growth temperature of 41 °C

(optimum of 37 °C) (Table 1). In addition, strain SM-1^T and *M. hydrolyticus* DSM 11525^T have differences in the presence of *N*-acetyl- β -glucosaminidase and in acid production from L-arabinose, L-rhamnose and D-xylose (Table 1). The 16S rDNA sequences of strain SM-1^T and the type strain of *M. hydrolyticus* have 20 bp sequence differences in the region compared. The level of DNA-DNA relatedness between strain SM-1^T and the type strain of *M. hydrolyticus* is much lower than 70%, a value which has been recognized for delineating a species (Wayne *et al.*, 1987). These data clearly indicate that strain SM-1^T and *M. hydrolyticus* are members of different genomic species. Therefore, on the basis of phenotypic characteristics, phylogenetic data and genomic distinctiveness, strain SM-1^T should be placed in the genus *Microbulbifer* as a novel species, for which the name *Microbulbifer salipaludis* sp. nov. is proposed.

Description of *Microbulbifer salipaludis* sp. nov.

Microbulbifer salipaludis (sa.li.pa.lu'dis. L. n. *sal* salt; L. gen. n. *paludis* of a swamp; N.L. gen. n. *salipaludis* of a salt marsh).

Gram-negative, non-motile, non-spore-forming rods, 0.3–0.5 $\times$ 1.2–3.0 μ m. In microscopic examinations, numerous vesicles are observed. Gram-staining reaction is negative. Colonies are smooth, circular, slightly irregular, flat with erose margins, greyish-yellow in colour and 2.0–4.0 mm in diameter after 3 days cultivation at 37 °C on MA. Optimal growth temperature is 37 °C. Growth occurs at 10 and 45 °C, but not at 4 °C or above 46 °C. Optimal growth pH is 7.0–8.0. Weak growth occurs at pH 5.0, but not at pH 4.5. Optimal growth occurs in the presence of 2–3% NaCl. Growth occurs in the presence of 10% NaCl, but not in the absence of NaCl or in the presence of more than 11% NaCl. No growth occurs under anaerobic conditions on MA. Catalase- and oxidase-positive. Aesculin, starch, Tween 80 and xylan are hydrolysed. Agar, casein and tyrosine are weakly hydrolysed. Chitin, hypoxanthine and xanthine are not hydrolysed. Acid is produced from D-cellobiose, D-glucose, maltose and L-rhamnose. Acid is not produced from adonitol, L-arabinose, D-fructose, D-galactose, *myo*-inositol, lactose, D-mannitol, D-mannose,

D-melezitose, melibiose, D-raffinose, D-ribose, D-sorbitol, stachyose, sucrose, D-trehalose or D-xylose. The following enzymes are present when assayed with the API ZYM system: alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase. 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, α -mannosidase and α -fucosidase. The predominant respiratory lipoquinone is ubiquinone-8. The major fatty acids are iso-C_{15:0} and C_{16:0}.

The type strain is strain SM-1^T (=KCCM 41586^T =JCM 11542^T), which has a DNA G+C content of 59 mol% (determined by HPLC) and was isolated from a salt marsh around the junction of the Youngsan River and the Yellow Sea in Korea.

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