Microbulbifer mangrovi sp. nov., a polysaccharide degrading bacterium isolated from the mangroves of India

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The GenBank accession number for the 16S rRNA gene sequence of strain DD13^T is HQ24446. FAME analysis of strain DD13^T and other *Microbulbifer* species (supplementary table S1) and detection of multiple polysaccharide degrading activity by plate screening method (supplementary figure S2) is provided as a supplementary file.

Abstract

A rod shaped, Gram-negative, non-motile, aerobic, and non-endospore forming bacterium, designated as strain DD13^T was isolated from the mangrove ecosystem of Goa, India. Strain DD13^T degraded polysaccharides such as agar, alginate, chitin, cellulose, laminarin, pectin, pullulan, starch, carrageenan, xylan and β-glucan. The optimum pH and temperature for growth was 7 and 36°C respectively. The bacterium grew optimally in the presence of 3% NaCl (w/v). The DNA G+C content was 61.4 mol%. The predominant fatty acid in strain DD13^T was iso-$C_{15:0}$ whereas ubiquinone-8 was detected as the major respiratory lipoquinone. Phylogenetic studies based on 16S rRNA gene sequence analysis demonstrated that the strain DD13^T formed a coherent cluster with *Microbulbifer* genus. Strain DD13^T exhibited 16S rRNA sequence similarity level of 98.9 to 97.1% with *M. hydrolyticus* IRE-31^T, *M. salipalidus* JCM11542^T, *M. agarilyticus* JAMB A3^T, *M. celer* KCTC 12973^T and *M. elongatus* DSM 6810^T. However, level of DNA-DNA relatedness between strain DD13^T and the five type strains of *Microbulbifer* species were in the range of 26-33%. Additionally, strain DD13^T demonstrates several phenotypic differences with these type strains of *Microbulbifer* species. Thus strain DD13^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer mangrovi* sp. nov. is proposed with the type strain DD13^T (= KCTC 23483^T = JCM 17729^T).

The genus *Microbulbifer* was originally described by Gonzàlez *et al.,* (1997) to describe strictly aerobic, rod-shaped members of Proteobacteria having numerous vesicles on its membrane surface. Subsequently, several novel species belonging to the genus *Microbulbifer* with capability to degrade several polysaccharides have been isolated from various habitats such as salt marsh, intertidal sediment, solar saltern, sediments (marine and deep sea), marine algae and

mangrove forests (Yoon *et al.*, 2003, 2004, 2007; Miyazaki *et al.*, 2008; Wang *et al*., 2009; Zhang *et al*., 2012; Nishijima *et al.*, 2009; Baba *et al*., 2011). Additionally, the presence of iso $C_{15:0}$ as the major fatty acid and Q-8 as the predominant isoprenoid quinone are characteristic features of the genus *Microbulbifer*. Further, *Microbulbifer* sp. are also known to demonstrate a characteristic rod-coccus cycle associated with different stages of their growth stages (Nishijima *et al*., 2009).

Mangrove ecosystem, the connecting link between terrestrial and marine ecosystem is rich in organic matter. Diverse floral and faunal communities thrive in the different niches existing in this ecosystem. Further, the litter which is composed of mangrove foliage and vegetative remains contribute to the induction of complex polysaccharides (CPs) such as cellulose, xylan, pectin, pullulan etc in this ecosystem. The colonization of CPs decomposing microorganisms with litter to form the detritus is an important ecological process and plays an important role in carbon recycling. In the present study, we report the taxonomic characteristics of a novel species belonging to genus *Microbulbifer* that was isolated from the mangrove ecosystem of Goa, India and is capable of degrading several polysaccharides.

Bacterial strain DD13^T was isolated from a water sample collected from mangroves of Goa, India (15°30’35”N and 73°52’63’’E). The mangrove forest is situated along the Mandovi estuary of Goa and detailed physico-chemical characteristics of water have been reported (Singh *et al*., 2009). The water sample was collected in sterile bottles and later serially diluted in an artificial sea water medium (ASW) (Ghadi *et al.*, 1997). An aliquot was spread plated on ASW containing 2% agar supplemented with 1% sodium alginate. The plates were incubated at 30°C

for 48-72 h. Single colonies depicting depression or clearance zones were picked and purity was confirmed by repeated streaking. The alginolytic activity of the isolates was detected by flooding culture plates with 10% cetyl pyridinium chloride (Gacesa & Wusteman 1990). One of the alginolytic isolate that demonstrated multiple polysaccharide degrading ability was designated as strain DD13^T and was subjected to polyphasic characterization to determine the exact taxonomic position.

The morphological and growth characteristics of strain DD13^T was investigated by growing on marine agar 2216 (MA) at 36°C. Growth was determined at different temperatures (4, 10, 15, 20, 25, 30, 32, 34-39 and 45°C). The pH range (3-12) for bacterial growth was assessed in customized marine broth (MB) whose pH was adjusted by adding respective buffers [50 mM of respective buffers: citrate (pH 3-6); Tris base (pH 7-8); borate (pH 9); glycine-NaOH (pH 10); sodium bicarbonate (pH 11); potassium chloride (pH 12)]. Modified MB (devoid of NaCl) was supplemented with NaCl in the range of 0-10% (w/v) for investigating growth at different concentrations of NaCl.

Unless otherwise stated, biochemical studies of strain DD13^T were analyzed in ASW medium at 36°C. Further, whenever reference type strains of *Microbulbifer* were used for comparison, they were also grown in ASW at their respective optimum temperatures. Catalase and oxidase activity, hydrolysis of casein, starch and Tweens 20, 40, 60, 80 were determined as described by Cowan & Steel (1965). Hydrolysis of esculin and urea, reduction of nitrate and carbohydrate utilization profiles were analyzed using HiAssorted^{TM} biochemical test kit KB002 and HiCarbohydrate^{TM} kit KB009 respectively (HiMedia Pvt Ltd, India). The multiple

polysaccharase activity was detected by plate screening method after 48 h on ASW agar plates or ASW agar plates supplemented with 0.2% of any one polysaccharide [alginic acid (mixed polymer of guluronate and mannuronate), chitin (Poly {N-acety-1,4,β-D-glucopyranosoamine}), carboxymethyl-cellulose (sodium salt), laminarin (from *Laminaria digitata*), pectin (poly D-galacturonic acid methyl ester), pullulan (standard), starch (from potato), carrageenan (from Irish moss), β-glucan (from barley) or xylan (from oat spelts)]. (Ruijssenaars & Hartmans, 2001; Morgan *et al*.,1979; Teather & Wood, 1982; Gacesa & Wusteman 1990; Gonzàlez-Candelas., *et al*., 1995; Stanier, 1942). Laminarin, pullulan and β-glucan were from Sigma whereas other polysaccharides were obtained from HiMedia Pvt Ltd, India

Bacterial strain DD13^T and type strains of closely related *Microbulbifer* species were grown in MB at 35ºC up to late-exponential phase. Fatty acid compositions were determined following the instructions of Sherlock Microbial Identification System (Sasser, 1990) using a Finnigan TRACE DSQ GC-MS system (Thermo Fisher Scientific) equipped with a DB-5 column (J&W Scientific) under a helium flow of 1.5 ml min^{-1} and an oven temperature program increasing from 140°C (5 min) to 280°C (5 min) at 4°C min^{-1}. Further, strain DD13^T cultivated in MB at 35°C for 24 h was used for isolation of genomic DNA (Maloy, 1990) and isoprenoid quinone analysis using reverse phase HPLC (Komagata & Suzuki, 1987). The DNA G+C content was determined using the method of Tamaoka & Komagata (1984). DNA–DNA hybridization (in triplicate) was measured fluorometrically at 48˚C for 4 h as described by Ezaki *et al.* (1989).

PCR amplification and sequencing of the 16S rRNA gene were carried out as described (Hauben *et al*., 1997). The 16S rRNA sequence was determined using Big Dye terminator v3.1 Cycle

Sequencing kit and the run was carried out in an automated DNA sequencer, model 3730XL (Applied Biosystems). The 16S rRNA gene sequence of strain DD13^T (1536 bp) was compared and analyzed with respective reference gene sequences by NCBI-BLAST program for identification of closely related type strains with validly published bacterial names. The 16S rRNA gene sequences of closest homologs were downloaded from GenBank and EMBL databases and multiple alignments were performed with CLUSTAL_X program (version 1.83; Thompson et al., 1997). The alignment gaps and missing data were edited. The evolutionary distance matrices were generated according to Jukes & Cantor (1969). Phylogenetic trees were inferred using the maximum-parsimony, maximum-likelihood and neighbor-joining methods with the MEGA program (version 5.0; Tamura et al., 2011). The robustness of the topology of phylogenetic trees was evaluated by a bootstrap analysis with 10,000 replications.

The 16S rRNA gene sequence of strain DD13^T (HQ 424446) was a continuous stretch of 1536 bp. A BLAST analysis of the 16S rRNA sequence of strain DD13^T indicated that it belonged to *Gammaproteobacteria* and was closely related to the genus *Microbulbifer*. The sequence similarity level of 16S rRNA of strain DD13^T in comparison to *M. hydrolyticus* IRE-31^T, *M. salipalidus* JCM 11542^T, *M.agarilyticus* JAMB A3^T, *M. celer* KCTC 12973^T and *M. elongatus* DSM 6810^T were 98.9, 98.5, 97.5, 97.2 and 97.1 % respectively. The 16S rRNA gene sequence identity with other members of the genus *Microbulbifer* was in the range 96.7–93.6 %. The 16S rRNA phylogenetic tree based on the neighbor-joining algorithm, depicted strain DD13^T to be in the same clade comprising the *Microbulbifer* species (Fig. 1). Phylogenetic analysis based on neighbor-joining method showed that strain DD13^T formed an evolutionarily distinct lineage within the cluster comprising *M. hydrolyticus* IRE-31^T, *M. salipaludis* JCM 11542^T, *M.*

agarilyticus JAMB A3^T, *M. celer* KCTC 12973^T and *M. elongatus* DSM 6810^T, supported by a bootstrap resampling value of 100 % (Fig.1). The same relationship was also inferred from phylogenetic trees obtained using maximum-parsimony and maximum-likelihood methods.

Strain DD13^T was isolated as greyish yellow colored colony on MA and formed depressions when grown on ASW agar plates containing 1% (w/v) sodium alginate. Strain DD13^T is catalase and oxidase positive and can degrade eleven different polysaccharides. Besides the presence of iso-$C_{15:0}$ as major fatty acid in strain DD13^T that confirmed its affiliation to *Microbulbifer*, iso-$C_{17:1\omega9c}$ $C_{18:1\omega7c}$ and $C_{16:0}$ were also detected as other prominent fatty acids. No significant differences were observed when fatty acid profile of strain DD13^T were compared to fatty acid profile of related type strains of *Microbulbifer* species (Supplementary Table S1). Detailed results of phenotypic and biochemical studies of strain DD13^T are shown in Table 1 or mentioned in the species description.

On the basis of physiological characteristics, strain DD13^T is easily differentiated from the closely related *Microbulbifer* species (Table 1). Unlike other *Microbulbifer* species, strain DD 13^T is able to grown in MB without NaCl. Further strain DD13^T does not hydrolyzes casein and produces H_2S whereas the other closely related *Microbulbifer* species used for comparison in present study hydrolyzed casein and did not produce H_2S (Table 1). Additionally, strain DD13^T demonstrated several differences with closely related *Microbulbifer* species with regards to percentage of G+C of DNA, ability to grow in MB amended with 10% NaCl as well as its ability to grow at pH 5 and 10 (Table 1). When compared to other *Microbulbifer* species, strain DD13^T, degrades eleven tested complex polysaccharides such as agar, alginate, chitin, cellulose,

laminarin, pectin, pullulan, starch, carrageenan, xylan and β-glucan (Supplementary Figure S2). *Saccharophagus degradans*, isolated from salt marsh is the only other polysaccharide degrading strain reported to degrade ten polysaccharides (Ekborg *et al*., 2005). Amongst the *Microbulbifer* species tested, only strain DD13^T and *M. elongatus* DSM 6810^T were observed to degrade pectin (Table 1).

Strain DD13^T exhibited 16S rRNA gene sequence similarity level of 97.1 to 98.9% to the type species of *Microbulbifer* chosen in the present study. However, DNA–DNA hybridization tests of the genomic DNA of DD13^T with the type strains of *M. hydrolyticus* IRE-31^T, *M. salipaludis* JCM 11542^T, *M. celer* KCTC 12973^T, *M. agarilyticus* JAMB-A3^T and *M. elongatus* DSM 6810^T depicted similarity of 28□%, 33□%, 27□%, 26□%, and 32□%, respectively. These similarities are below the generally accepted species differentiation limit of 70□% (Wayne et al., 1987). Thus strain DD13^T is not related to these species and is a novel species of the genus *Microbulbifer*.

Thus based on chemotaxonomic characteristics, phylogenetic data and genomic distinctiveness, strain DD13^T should be placed in the genus *Microbulbifer* as a novel species, for which the name *Microbulbifer mangrovi* sp. nov. is proposed with strain DD13^T as the type strain.

Description of *Microbulbifer mangrovi* sp. nov.

Microbulbifer mangrovi (man.gro'vi. N.L. gen. neut. n. mangrovi of mangrove; Latinized to *mangrovum* refers to the isolation of this bacterium from the mangrove ecosystem).

Strain DD13^T is aerobic, Gram-negative, non-motile and rod-shaped with an average length of 1.2 μm and a width of 0.3μm. Both rods and predominant coccoid cells were observed in old cultures. Colonies on MA after 48 h were greyish yellow colored that were 2–3 mm in diameter, circular, slightly convex, smooth and glossy. Growth was observed in the range of 20-38ºC with optimal growth at 36 ºC. No growth was observed at 4, 15 or above 39ºC. Growth was observed in the pH range of 5 to 9.0 and optimal at pH 7. Growth occurs in MB medium without NaCl. Growth was observed in MB medium containing NaCl up to 10% with optimal growth at 3% NaCl. Anaerobic growth does not occur on MA. Tweens 20, 40, 60 and 80 are hydrolyzed, but urea, L-tyrosine and xanthine are not. Nitrate is not reduced to nitrite. The following substrates were utilized as carbon substrates and acid production was observed: D-cellobiose, maltose, D-arabinose and aesculin, D-glucose, D-xylose. The following compounds are not utilized as carbon source: D-fructose, D-galactose, D-mannose, trehalose, L-arabinose, sucrose, melibiose, D-mannitol, D-melezitose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, inositol, citrate, succinate, benzoate, L-malate, salicin, formate, L-arginine and L-glutamate. Methyl red, Voges-Proskauer, lysine decarboxylase and ornithine decarboxylase tests are negative. Gelatin and casein are not hydrolyzed. ONPG (*o*-nitrophenyl *β*-D-galactopyranoside) test is negative. Catalase and oxidase tests are positive whereas indole production is negative. H_2S production was weakly positive. The strain hydrolyzes several polysaccharides including agar, alginate, chitin, cellulose, laminarin, pectin, pullulan, starch, carrageenan, β-glucan and xylan. The major ubiquinone is Q-8. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:1\omega9c}$ $C_{18:1\omega7c}$ and $C_{16:0}$. The DNA G+C content is 61.4 mol%.

The type strain, DD13^T (KCTC 23483^T = JCM 17729^T) was isolated from mangroves of Goa, India.

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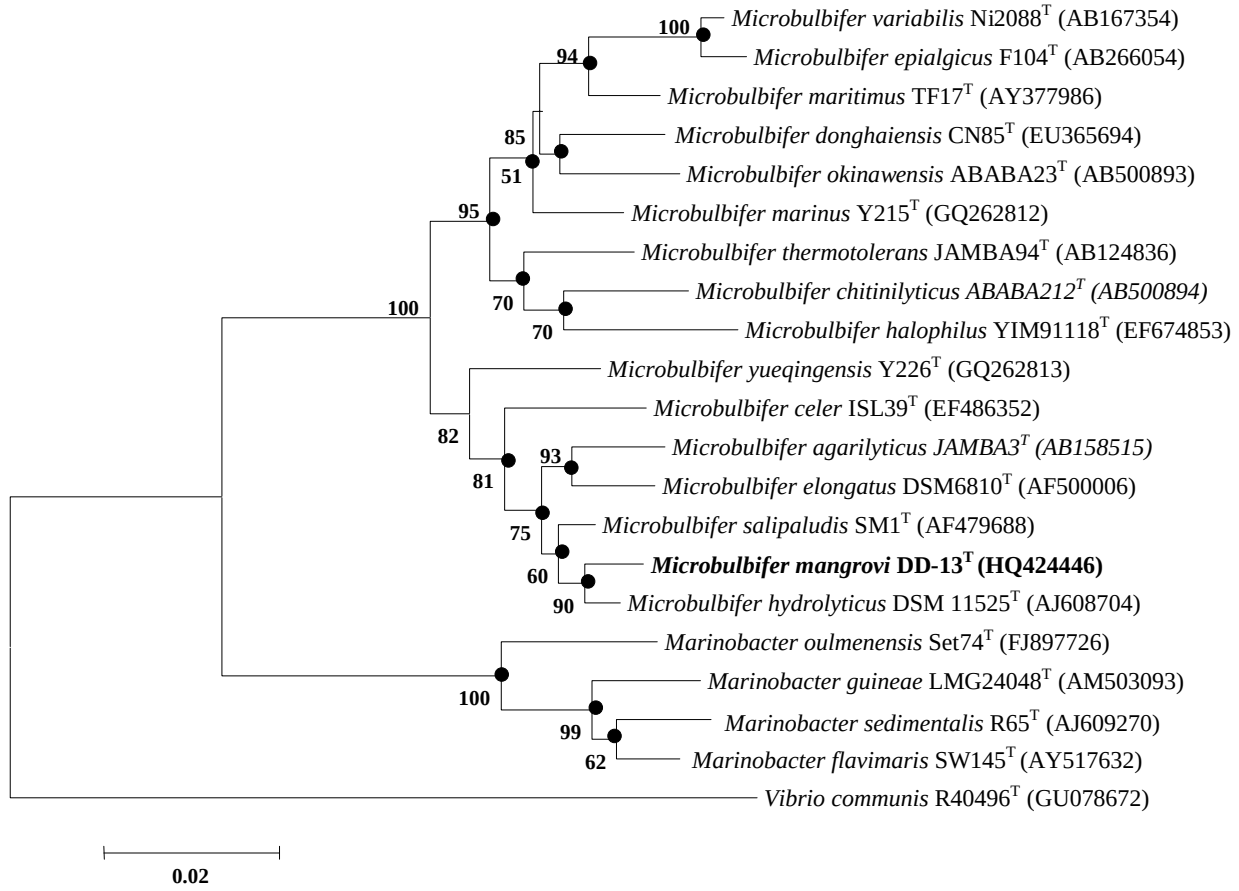

Fig.1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, representing the position of strain DD-13^T, *Microbulbifer* species and other related taxa. Bootstrap percentages are based on 10,000 replications. >50% are shown at branch points. • denote that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Vibrio communis* R40496^T was used as an out-group. Bar 0.02 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics of strain DD-13^T and other related *Microbulbifer* species

Strain Species: 1, DD-13^T, data from this study; 2, *M. hydrolyticus* IRE 31^T; 3, *M. salipaludis* JCM 11542^T; 4, *M.celer* KCTC 12973^T; 5, *M. agarilyticus* JAMB A3^T; 6, *M. elongatus* DSM 6810$^{T.}$ All of the species are positive for the following: Rod shaped, catalase and oxidase activities, ornithine utilization*, hydrolysis of starch, xylan, agar and cellobiose; All species are negative for spore formation, Gram-staining, Urease test*, Sodium gluconate*, Dulcitol*, Inositol , α- Methyl-D- glucoside*, α- Methyl-D- mannoside* and Sorbose utilization*. #Data for species 2, 3, 4 is from Gonzàlez *et al* (1997), Yoon *et al* (2003) and Yoon *et al* (2007) respectively whereas for species 5 and 6 is from Miyazaki *et al* (2008) and Yoon *et al* 2007 respectively. (+, Positive reaction; -, negative reaction; W, weakly positive reaction; *Data obtained from this study; ND- not determined.)

Tests	**1**	**2**	**3**	**4**	**5**	**6**
Colony colour	Greyish yellow	Cream	Greyish yellow	Greyish yellow	Cream	Yellowish brown
Motility	-	-	+	-	+	-
#G+C (%)	61.4	57.7	59	57.7	55.2	58.2
Growth at 0% NaCl	+	-	-	-	-	-

Growth at 10% NaCl	+	-	+	+	-	-
Growth at pH 5	+	-	-*	-*	-	+*
Growth at pH 10	+	-	-*	+*	-	+*
H_2S production	+	-	-	-	-	-
Nitrate reduction	-	-	+	-	+	-
Casein hydrolysis	-	+	+	+	+	+
Amino acid utilization						
L-Alanine	+	+	+*	+*	-*	+
L-Leucine	-	+	-*	+*	-*	+
L-Proline	+	+	+*	+*	+*	-
L-Serine	-	+	-	-*	-*	+
Polysaccharide degradation						
Chitin	+	+	-	-*	+	+
Pullulan	+	-*	-*	-*	ND	ND
Pectin	+	-*	-*	-*	-*	+*
Alginate	+	+	-*	-*	-*	+*
β Glucan	W	-*	+*	+*	ND	ND
Laminarin	+	-*	-*	+*	ND	ND
Carrageenan	+	+*	W	-*	+*	+*
Carbohydrate utilization						
D-Galactose	-	+*	-	-	-*	+
D-Glucose	+	+	+	-	+	+
D-Xylose	+	-*	W*	-	-*	-*
Maltose	+	+*	+	-	+	+

D- Arabinose	+	-*	+*	+*	-	+
Trehalose	-	+*	+*	-	-	W*
Melibiose	-	+*	-*	-	-*	W*
Inulin	-	-*	-*	-*	-*	+*
Sorbitol	-	+*	+*	-	-	+*
Salicin	-	+*	-*	-	-*	-*
Raffinose	-	+*	-	-	-	-
Malonate	-	+*	W*	W*	W*	-*

325

326

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