Oceanisphaera litoralis gen. nov., sp. nov., a novel halophilic bacterium from marine bottom sediments

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A polyphasic taxonomic study was performed to characterize a new bacterial isolate, designated KMM 3654^T, from a marine bottom sand sample. The strain was Gram-negative, encapsulated, aerobic, moderately halophilic and grew between 0.5 and 10 % NaCl and at 4–42 °C. Its DNA G + C content was 56.4 mol%. Isolate KMM 3654^T was phylogenetically closely related to members of the genus *Oceanimonas*, showing 96.7 and 95.6 % 16S rRNA gene sequence similarity to *Oceanimonas doudoroffii* DSM 7028^T and *Oceanimonas baumannii* ATCC 700832^T, respectively. Strain KMM 3654^T shared some physiological and chemotaxonomic properties with these two *Oceanimonas* species, but differed from them in morphology, growth at 4 °C, urease activity, weak phenol degradation and utilization of phenylacetate. On the basis of phenotypic and phylogenetic evidence, *Oceanisphaera litoralis* gen. nov., sp. nov. is proposed, with the type strain KMM 3654^T (= DSM 15406^T).

Strain KMM 3654^T was isolated from a bottom sand sample that was collected from the coastal sea-water area of Peter the Great Bay, Sea of Japan, Russia, in June 2002. A bottom sand sample was retrieved at a depth of 0.2 m, placed in a sterile tube and diluted serially with sterile sea water. An aliquot of each dilution was spread on marine 2216 agar (MA; Difco) and inoculated at 28 °C for 7 days. Cultures were stored at –80 °C in liquid nutrient medium supplemented with 30 % (v/v) glycerol. Strain KMM 3654^T was grown routinely on MA or tryptone soy agar (TSA) or in marine broth 2216 (MB) (all from Difco).

Cell morphology was examined by transmission and scanning electron microscopy of cells grown in MB after 30 h incubation. Cells were fixed with 1 % (v/v) glutaraldehyde and negatively stained with 4 % (w/v) aqueous uranyl acetate and carbon film. Samples were examined by using a Zeiss transmission electron microscope (model TEM910) at an acceleration voltage of 80 kV at calibrated magnifications. Gram-reaction, oxidase, catalase and hydrolysis of starch, casein, DNA, chitin and gelatin were tested as described by Baumann *et al.* (1972) and Smibert & Krieg

(1994). Growth at 4–45 °C was determined on MA and TSA. Tolerance of NaCl was tested by using glucose–peptone nutrient medium, prepared with artificial sea water and supplemented with NaCl concentrations of 0, 0.5, 1, 3, 5, 8, 10, 12 and 15 % (w/v). Ability to use phenol as the sole carbon source was determined on minimal media that contained 2 or 5 % NaCl (w/v) and 4 mM phenol for up to 7 days incubation. Leifson's oxidation–fermentation medium for marine bacteria (Leifson, 1963) was used to test acid production from carbohydrates with 1 % (w/v) of each compound. Other biochemical tests were carried out by using API 20NE test kits (bioMérieux) according to the manufacturer's instructions, except that the culture was suspended in 2 % (w/v) NaCl solution, and by the Biolog GN MicroPlate panel. For the latter tests, strain KMM 3654^T was grown on MA at 28 °C for 24 h and Biolog microtitre plates were inoculated with cells suspended in 2 % (w/v) NaCl solution. Results were read automatically with a spectrophotometer after 24 and 48 h incubation at 28 °C. For lipid analysis, the strain was cultivated on MA at 28 °C for 2 days. Fatty acid methyl esters were prepared from cells by acid-catalysed transmethylation and analysed by GLC. Whole-cell fatty acids and phospholipids were examined according to procedures described previously (Svetashev *et al.*, 1995; Ivanova *et al.*, 2000). DNA G + C content was

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of KMM 3654^T is AJ550470.

Fig. 1. Dendrogram of 16S rRNA gene sequence relatedness, showing the phylogenetic position of strain KMM 3654^T next to members of the genus *Oceanimonas* and less closely related species of the γ -*Proteobacteria*. Numbers at branching points refer to bootstrap values (500 resamplings). Bar, 2 inferred nucleotide substitutions per 100 nucleotides.

determined by HPLC according to the method of Mesbah *et al.* (1989).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced directly by using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. An Applied Biosystems 310 Genetic Analyzer was used for electrophoresis of sequence reaction products. The 16S rDNA sequence of strain KMM 3654^T was aligned manually with nucleotide sequences obtained from GenBank/EMBL. The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Accession numbers of reference strains used in the phylogenetic analysis are shown in Fig. 1.

Phylogenetic analysis based on the almost-complete 16S rRNA gene sequence (1505 nt) of strain KMM 3654^T indicated that *O. doudoroffii* DSM 7028^T and *O. baumannii* ATCC 700832^T (Brown *et al.*, 2001) were the closest phylogenetic neighbours, respectively displaying 96.7 and 95.6% sequence similarity to strain KMM 3654^T. *Ferrimonas balearica* DSM 9799^T (Rosselló-Mora *et al.*, 1995), *Tolumonas auensis* DSM 9187^T (Fischer-Romero *et al.*, 1996) and members of the families *Vibrionaceae*, *Enterobacteriaceae* and *Aeromonadaceae* were more distantly related (<92% sequence similarity). This relationship is displayed in the 16S rRNA gene sequence dendrogram based on the additive treeing algorithm of DeSoete (1983) (Fig. 1). Based on moderate sequence similarity of <97%, the novel isolate can be considered to be a separate taxonomic entity from the two described species of the genus *Oceanimonas* (Stackebrandt & Goebel, 1994), which share only 38.6% DNA similarity (Brown *et al.*, 2001).

Chemotaxonomically, isolate KMM 3654^T shared properties that were also reported for the two species of the genus *Oceanimonas* (Brown *et al.*, 2001), i.e. whole-cell fatty acids, phospholipids and DNA G+C content of 56.4 mol%, which is similar to those reported for *O. doudoroffii* DSM 7028^T and *O. baumannii* ATCC 700832^T (each 54 mol%). High amounts (up to 90% of the total) of fatty acids C_{16:0}, C_{16:1cis} and C_{18:1cis} found in strain KMM 3654^T were also reported for *Oceanimonas* species (Brown *et al.*, 2001). Quantitative analysis is given in the species description. KMM 3654^T contained phosphatidylethanolamine and

phosphatidylglycerol (43.7 and 46.9%, respectively) as the main phospholipids. These compounds were also found in the two *Oceanimonas* species; KMM 3654^T differed from these species in the lack of fatty acid C_{12:0} and in a higher proportion (9.4%) of diphosphatidylglycerol.

Strain KMM 3654^T was aerobic, Gram-negative, moderately halophilic and oxidase- and catalase-positive. Cells were coccoid, 1.0–1.2 µm in diameter, encapsulated and motile by a single polar flagellum (Fig. 2a). Cells grown on hard media occurred as single motile or non-motile cells, due to their ability to lose flagella easily, and formed aggregates and some fibrillar structures when cultivated in liquid media (Fig. 2b). The isolate required sodium ions for growth and grew in 0.5–10% NaCl at 4–42 °C, with optima between 1 and 3% NaCl and 28 and 35 °C, respectively. After 24 h on MA, strain KMM 3654^T produced smooth,

Fig. 2. Transmission (a) and scanning (b) electron micrographs of strain KMM 3654^T grown in MB, showing spherical cell shape, polar flagella and cell aggregates. Bars, (a) 1 and (b) 2 µm.

shining, slightly yellow colonies with regular edges that were 3–5 mm in diameter. Diffusion of yellowish pigment into the medium was observed. Growth was weak or delayed for 5–7 days when cultivated in minimal salts medium that contained 2 % NaCl and phenol as the sole carbon and energy source, which was used to cultivate strains of *Oceanimonas* (Brown *et al.*, 2001), but growth did not occur in this medium when the salinity was raised to 5 %. The main phenotypic characteristics of strain KMM 3654^T and related *Oceanimonas* species are shown in Table 1.

Unlike members of the genus *Oceanimonas*, the new isolate is yellowish-pigmented, encapsulated, coccoid, able to form aggregates and external fibrillar-like structures, able to grow at 4 °C and up to 10 % NaCl, produces urease and utilizes phenylacetate and does not utilize ethanol, succinate, L-alanine or L-proline. Heissenberger *et al.* (1996) observed production of exopolymer and, in addition, fibrillar material that connected marine bacterial cells. The capsular envelope has been hypothesized to play a basic role in bacterial attachment to surfaces in marine environments. Taking into consideration the fact that the habitat of KMM 3654^T is marine sand sediment, the

formation of cell aggregates and capsular and fibrillar-like materials may be a useful cellular adaptation for attachment to surfaces. Strain KMM 3654^T lacked the ability to degrade phenol, which is positive for *O. doudoroffii* and *O. baumannii* (Brown *et al.*, 2001). The new isolate exhibited weak reactions for utilization of Tween 40, Tween 80, α -ketobutyric acid, α -ketovaleric acid and L-glutamic acid and did not utilize most of the carbohydrates, organic acids or amino acids provided by the API 20NE and Biolog substrate panels (Table 1).

Strain KMM 3654^T can be distinguished from members of the genus *Oceanimonas* by using a combination of biochemical characteristics (Table 1) and phylogenetic distance (Fig. 1). Based on these results, we propose to classify isolate KMM 3654^T in a novel genus and species, *Oceanisphaera litoralis* gen. nov., sp. nov., with the type strain KMM 3654^T (= DSM 15406^T).

Description of *Oceanisphaera* gen. nov.

Oceanisphaera (O.ce.a.ni.sphae'ra. L. masc. n. *oceanus* ocean; L. fem. n. *sphaera* ball, globe, sphere; N.L. fem. n. *Oceanisphaera* oceanic sphere).

Spherical cells, 1.0–1.2 µm in diameter and motile by means of flagella. Gram-negative chemoorganotroph with absolute requirement for sodium ions. Aerobic. Moderately halophilic and oxidase- and catalase-positive. Major phospholipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Major fatty acids are C_{16:0}, C_{16:1}ω7c and C_{18:1}ω7c. DNA G+C content is 56.4 mol% (HPLC). Isolated from a marine environment. Phylogenetically related to the genus *Oceanimonas* in the γ -Proteobacteria. The type species is *Oceanisphaera litoralis*.

Description of *Oceanisphaera litoralis* sp. nov.

Oceanisphaera litoralis (li.to.ra'lis. L. fem. adj. *litoralis* of or belonging to the seashore).

Gram-negative, aerobic, oxidase- and catalase-positive, motile by a single polar flagellum, spherical, encapsulated single cells that are 1.0–1.2 µm in diameter. May be non-motile. Able to form aggregates and fibrillar structures when cultivated in liquid media. Moderately halophilic, does not grow without sodium ions; grows in 0.5–10 % NaCl at 4–42 °C, but not at 44 °C. On MA, strain KMM 3654^T produces smooth, shining, yellowish colonies with regular edges that are 3–5 mm in diameter after 24 h incubation at 28 °C. Gelatin, casein, aesculin, starch, DNA and chitin are not hydrolysed. No acid is produced from glucose, sucrose, maltose, lactose, N-acetylglucosamine, arabinose, rhamnose, galactose, glycerol or mannitol. Positive for nitrate reduction, urease activity and utilization of malate, citrate and phenylacetate. Metabolic properties are indicated in Table 1. According to Biolog system identification tests, KMM 3654^T weakly utilizes Tween 40, Tween 80, α -ketobutyric acid, α -ketovaleric acid and L-glutamic acid. The rest of the organic substrates included in the Biolog

Table 1. Phenotypic characteristics of strain KMM 3654^T and related *Oceanimonas* species

Taxa: 1, KMM 3654^T; 2, *Oceanimonas doudoroffii* DSM 7028^T; 3, *Oceanimonas baumannii* ATCC 700832^T. Data for *Oceanimonas* species were taken from Brown *et al.* (2001). All strains are positive for oxidase, catalase, Na⁺ requirement for growth, nitrate reduction*, malate* and citrate* utilization and negative for indole production*, arginine dihydrolase*, gelatin and aesculin hydrolysis* and utilization of glucose*, arabinose*, mannose*, mannitol*, N-acetylglucosamine*, maltose*, gluconate*, adipate*, sucrose, L-leucine, L-valine and L-tyrosine.

Characteristic	1	2	3
Cell morphology	Coccoid	Rod-shaped	Rod-shaped
Cell size (µm)	1.0–1.2	0.7–1.2 × 2.0–2.5	2 × 1
Growth temperature (°C)	4–42	10–41	10–41
Urease*	+	–	–
Utilization of:			
Caprate*	–	+	+
Phenylacetate*	+	–	–
Galactose	–	–	+
Glycerol	–	–	+
Ethanol	–	+	+
Phenol	w	+	+
Succinate	–	+	+
L-Glutamate	w	+	+
L-Proline	–	+	+
L-Alanine	–	+	+
DNA G+C content (mol%)	56.4	54	54

*Determined by the API 20NE test (this study and Brown *et al.*, 2001).

panel are not utilized. Main fatty acids are C_{16:0} (21.6%), C_{16:1 ω 7c} (41.0%) and C_{18:1 ω 7c} (27.5%); minor amounts of C_{15:0} (1.8%), iso-C_{16:0} (1.6%), C_{17:0} (1.9%) and C_{17:1 ω 8c} (1.9%) are present. Phospholipids consist of phosphatidylethanolamine (43.7%), phosphatidylglycerol (46.9%) and diphosphatidylglycerol (9.4%). DNA G+C content is 56.4 mol% (HPLC).

The type strain is KMM 3654^T (=DSM 15406^T). Isolated from a bottom sand sample, Peter the Great Bay, Sea of Japan, Pacific Ocean, Russia.

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