Oceanisphaera donghaensis sp. nov., a halophilic bacterium from the East Sea, Korea

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A taxonomic study was carried out on two isolates, strains BL1^T and BL11, from marine sediment collected from the East Sea, Korea. Comparative 16S rRNA gene sequence studies showed that these isolates clearly affiliated with the *Gammaproteobacteria*. BL1^T and BL11 were most closely related to *Oceanisphaera litoralis* KMM 3654^T (97·6 and 97·7 % 16S rRNA gene sequence similarity, respectively). The level of 16S rRNA gene sequence similarity between strains BL1^T and BL11 was 99·7 %. The two isolates were Gram-negative, aerobic, moderately halophilic, and grew in 0·5–8·0 % NaCl and at 4–42 °C. Strains BL1^T and BL11 shared some physiological and biochemical properties with *O. litoralis* KMM 3654^T, although they differed in that BL1^T and BL11 were able to utilize ethanol, proline and alanine. The G+C contents of the genomic DNA of strains BL1^T and BL11 were 56·6 and 57·1 mol%, respectively. Both strains possessed $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{18:1}\omega7c$ as the major fatty acids. DNA–DNA relatedness data indicated that strains BL1^T and BL11 represent a genomic species that is separate from *O. litoralis* KMM 3654^T. On the basis of polyphasic evidence, it is proposed that strain BL1^T (=KCTC 12522^T=DSM 17589^T) represents the type strain of a novel species, *Oceanisphaera donghaensis* sp. nov.

The genus *Oceanisphaera* was created by Romanenko *et al.* (2003) to accommodate Gram-negative, aerobic, moderately halophilic, and oxidase- and catalase-positive species. At present, *Oceanisphaera litoralis* is the only species in this genus. During screening of manganese-oxidizing strains, two novel manganese-oxidizing bacterial strains, BL1^T and BL11, were isolated from marine sediment of the East Sea, Korea, and selected for further characterization by a polyphasic approach. BL1^T and BL11 were isolated using an artificial marine agar medium (Stein *et al.*, 2001) containing 1 mM $MnCl_2$. A sediment sample was placed in a sterile conical tube and diluted serially with filtered sea water. An aliquot of each dilution was spread on the solid medium and incubated at 17 °C for 2 weeks. Colonies showing a brown colour on the medium were selected as manganese oxidizers (Stein *et al.*, 2001). Single colonies were purified by transferring them onto new plates and subjecting them to an additional incubation for 3 days at 30 °C. Cultured strains were stocked as a glycerol suspension (20 %, w/v) at −70 °C.

Bacterial genomic DNA was extracted using a commercial genomic DNA extraction kit (Bioneer). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set (9F and 1512R) and purified PCR products were sequenced by Solgent (Daejeon, Korea) (Yoon *et al.*, 1998). The full 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed with the program CLUSTAL_X (Thompson *et al.*, 1997). Gaps were edited using

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BL1^{T}and BL11 are DQ190441 and DQ190440, respectively.

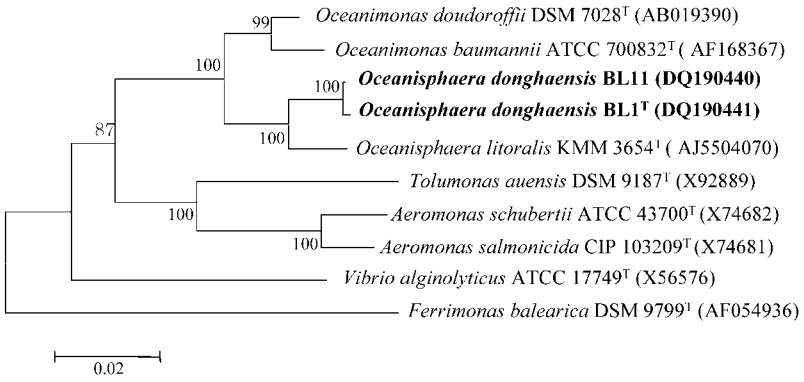

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *Oceanisphaera donghaensis* BL1^T and BL11 and their nearest neighbours based on 16S rRNA gene sequences. Bar, 2 substitutions per 100 nucleotide positions. Bootstrap values are expressed as percentages of 500 replications.

the program BIOEDIT (Hall, 1999). Evolutionary distances were calculated using the Kimura (1983) two-parameter model. Phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) with the program MEGA3 (Kumar *et al.*, 2004); bootstrap values were based on 500 replications (Felsenstein, 1985). Phylogenetic analysis of strains BL1^T and BL11 revealed that they belong to the *Gammaproteobacteria*; the highest degrees of 16S rRNA gene sequence similarity were found with *Oceanisphaera litoralis* KMM 3654^T (97·6 and 97·7 %, respectively), *Oceanimonas doudoroffii* DSM 7028^T (96·2 and 96·2 %, respectively) and *Oceanimonas baumannii* ATCC 700832^T (95·1 and 95·2 %, respectively). In the phylogenetic tree (Fig. 1), strains BL1^T and BL11 clearly belonged to the lineage *Oceanisphaera*, as shown by the high bootstrap value (100 %).

The Gram reaction was performed by the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (Nikon) and transmission electron microscopy (Carl Zeiss) after negative staining with 1 % (w/v) phosphotungstic acid. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Strains BL1^T and BL11 were Gram-negative, spherical and aerobic (Fig. 2). Cells contained flagella and formed aggregates when cultivated in liquid media. Colonies grown on Marine 2216 agar plates (Difco) for 3 days were circular, convex, yellowish in colour and 1·3–2·0 mm in diameter. This result supports affiliation of strain BL1^T to the genus *Oceanisphaera* and differentiates it from members of the genus *Oceanimonas*.

Cellular fatty acids of strains BL1^T and BL11 were analysed after growth on trypticase soy agar (TSA; Difco) for 2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). The fatty acids analysed by GC (Hewlett Packard 6890) were identified by the Microbial Identification software package. Chromosomal DNA extracted for 16S rRNA gene amplification was used for determination of the G+C content. RNA in the DNA solution was removed by incubation with a mixture of ribonuclease A and T1 (each at 20 units ml^{-1}) at 30 °C for 1 h. The G+C content of the chromosomal DNA was analysed as described by Mesbah *et al.* (1989) using reverse-phase HPLC. Phospholipid analysis was done as described previously (Komagata & Suzuki, 1987; Vaskovsky *et al.*, 1975). The major cellular fatty acid profiles of strain BL1^T and BL11 were composed of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{18:1}\omega7c$ (Table 1). These fatty acid profiles were similar to that of *Oceanisphaera litoralis* KMM 3654^T (Table 1). However, there were differences in the proportions of fatty acids between this study and the study of Romanenko *et al.* (2003); these differences may be caused by different cultivation, extraction or analytical conditions. The G+C contents of genomic DNA of strains BL1^T and BL11 were 56·6 and 57·1 mol%, respectively. BL1^T and BL11 contained phosphatidylethanolamine (58·6 and 54·1 %, respectively) and phosphatidylglycerol (38·2 and 42·9 %, respectively) as the main phospholipids. The polar lipid profile of BL1^T and BL11 was similar to that reported for *Oceanisphaera litoralis* KMM 3654^T.

Utilization of various substrates as sole carbon source and some physiological characteristics were determined with API 32GN and API 20NE galleries according to the manufacturer's instructions (bioMérieux). Hydrolysis of

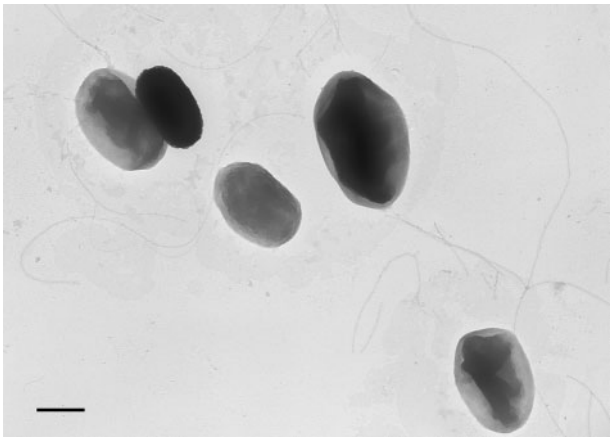

Fig. 2. Negatively stained transmission electron micrograph of *Oceanisphaera donghaensis* BL1^T cells. Bar, 0·5 μm.

Table 1. Cellular fatty acid content (%) of strains BL1^T and BL11 and *Oceanisphaera litoralis* KMM 3654^T

Fatty acids representing less than 0·3 % in all strains were omitted. ND, Not detected; ECL, equivalent chain-length.

Fatty acid	BL1^T	BL11	KMM 3654^T
Straight-chain			
$C_{12:0}$	6·09	5·45	10·31
$C_{14:0}$	0·30	0·31	1·00
$C_{15:0}$	1·01	0·35	0·46
$C_{16:0}$	15·92	19·47	16·45
$C_{17:0}$	0·53	0·67	0·30
$C_{18:0}$	0·31	0·51	ND
Branched			
iso-$C_{16:0}$	1·85	1·57	0·65
iso-$C_{17:0}$	0·36	0·79	ND
Unsaturated			
$C_{17:1}\omega8c$	0·68	0·42	0·39
$C_{18:1}\omega7c$	18·66	19·07	14·01
Summed features*			
2	5·13	5·02	8·23
3	46·20	42·39	45·08
7	0·68	0·71	ND
Unknown			
ECL 14·502	0·62	0·57	0·64

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contains one or more of iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH. Summed feature 3 contains one or more of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 7 contains one or more of an unknown fatty acid of ECL 18·846, $C_{19:0}$ cyclo $\omega10c$ and/or $C_{19:1}\omega6c$.

Table 2. Differential phenotypic characteristics of strains BL1^T and BL11 and *Oceanisphaera litoralis* KMM 3654^T

Taxa: 1, *Oceanisphaera donghaensis* BL1^T; 2, *Oceanisphaera donghaensis* BL11; 3, *Oceanisphaera litoralis* KMM 3654^T. Data for *Oceanisphaera litoralis* KMM 3654^T were from Romanenko *et al.* (2003). All strains grow at 4–42 °C and are coccoid bacteria. All strains are positive for oxidase, catalase, Na^+ growth requirement, nitrate reduction, and malate and citrate utilization; all are negative for arginine dihydrolase, gelatin and aesculin hydrolysis, and utilization of caprate, galactose, glycerol, succinate, glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, adipate, sucrose, L-leucine, L-valine and L-tyrosine. All utilize Tween 80 weakly. Characteristics are scored as: W, weak; +, positive; −, negative.

Characteristic	1	2	3
Cell diameter (μm)	0·5–1·5	0·5–1·5	1·0–1·2
Production of acid from:			
Citrate*	+	−	+
Malate*	W	W	+
Hydrolysis of:			
Urease*	−	−	+
Utilization of:			
Phenylacetate*	−	−	+
Ethanol	+	+	−
Phenol†	−	−	W
L-Glutamate	+	+	W
L-Proline	+	+	−
L-Alanine	+	+	−
DNA G+C content (mol%)	56·6	57·1	56·4

*Determined by API 32GN and 20NE tests (this study and Romanenko *et al.*, 2003).
†Phenol concentration 4 mM.

casein, starch and Tween 80 was determined as described by Cowan & Steel (1965) with modified artificial sea water. The artificial sea water contained (per litre distilled water) 23·6 g NaCl, 0·64 g KCl and 4·53 g $MgCl_2.6H_2O$ (Levring, 1946); rèactions were read after 5 days. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested after 5 days incubation on artificial sea water supplemented with 0–15 % (w/v) NaCl. On marine 2216 agar (Difco), strain BL1^T was able to grow at 4–42 °C, but not at 2 or 45 °C. Comparative results of selective physiological characteristics of strains BL1^T and BL11 and *Oceanisphaera litoralis* KMM 3654^T are shown in Table 2.

DNA–DNA hybridization experiments were carried out with BL1^T, BL11 and *Oceanisphaera litoralis* KMM 3654^T using the method described by Ezaki *et al.* (1989). Strains BL1^T and BL11 showed less than 10 % DNA–DNA relatedness with *Oceanisphaera litoralis* KMM 3654^T. The mean level of DNA–DNA relatedness between BL1^T and BL11 was 86 %. DNA–DNA relatedness data indicate that strains BL1^T and BL11 are representatives of a genomic species that is separate from *Oceanisphaera litoralis* KMM 3654^T.

On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons described above, strains BL1^T and BL11 represent a novel species, for which the name *Oceanisphaera donghaensis* sp. nov. is proposed.

Description of *Oceanisphaera donghaensis* sp. nov.

Oceanisphaera donghaensis (dong.ha.en′sis. N.L. fem. adj. *donghaensis* of Donghae, the Korean name for the East Sea in Korea from which the strains were isolated).

Cells are Gram-negative, oxidase-positive, catalase-positive, manganese-oxidizing and spherical. They occur singly and are 1·0–1·2 μm in diameter. Contains a single polar flagellum. Moderately halophilic and grows in 0·5–8·0 % NaCl at 4–42 °C. Favourable growth occurs aerobically producing circular colonies with regular edges within 2 days, with diameters of approximately 1·3–2·0 mm. The type strain, BL1^T, requires Na^+ for growth, reduces nitrate, utilizes malate and citrate, and is negative for arginine dihydrolase,

gelatin and aesculin hydrolysis, and utilization of caprate, glycerol, succinate, L-leucine, L-valine and L-tyrosine. No acid is produced from galactose, glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, adipate or sucrose. Carbon and nitrogen source utilization and enzymic activities are shown in Table 2. The major cellular fatty acids of BL1^T are $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{18:1}\omega7c$. Polar lipids of the type strain include phosphatidylethanolamine (58·6 %), phosphatidylglycerol (38·2 %) and diphosphatidylglycerol (3·2 %). DNA G+C content of strain BL1^T is 56·6 mol% (as determined by HPLC).

The type strain is BL1^T (=KCTC 12522^T=DSM 17589^T), isolated from marine sediment of the East Sea, Korea.

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