Oceanisphaera sediminis sp. nov., isolated from marine sediment

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Two strains, designated TW92^T and TW93, were isolated from marine sediment collected from the south coast of Korea. Cells of both strains were Gram-staining-negative, coccus-shaped, aerobic, motile and catalase- and oxidase-positive. Strain TW92^T grew optimally in the presence of 2 % (w/v) NaCl (range 1–5 %) while strain TW93 grew optimally in the presence of 1 % (w/v) NaCl (range 0–12 %), and both strains had an optimal growth temperature of 30 °C (range 4–37 °C). Strains TW92^T and TW93 had the same optimum pH (pH 7), but differed in their ability to grow at pH 10. Phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strains TW92^T and TW93 were most closely related to *Oceanisphaera donghaensis* BL1^T, with 98.8 % and 98.7 % similarity, respectively. Pairwise similarity between the 16S rRNA gene sequences of strains TW92^T and TW93 was 99.9 %. The major fatty acids of both strains were summed features 3 (comprising $C_{16:1}\omega7c$/iso-C_{15} 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. Both strains possessed the ubiquinone Q-8 as the predominant respiratory quinone and phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol as the polar lipids. The genomic DNA G+C contents of strains TW92^T and TW93 were 58.5 and 59.6 mol%, respectively. Genomic relatedness values based on DNA–DNA hybridization of strains TW92^T and TW93 with related species were below 47 % and 31 %, respectively. DNA–DNA hybridization values between strains TW92^T and TW93 were above 85 %. On the basis of a taxonomic study using polyphasic analysis, it is proposed that the two isolates represent a novel species, *Oceanisphaera sediminis* sp. nov., with strain TW92^T (=KACC 15117^T=JCM 17329^T) as the type strain and strain TW93 (=KACC 15118=JCM 17330) as an additional strain.

The annual mass mortality of cage-cultured invertebrates severely damages the aquaculture industry along the south coast of Korea. To investigate whether perturbation of the bacterial community contributes to this mass mortality, the bacterial composition of sediment collected from an ark clam farm during a mass mortality event was determined using a culture-dependent strategy. During the investigation, two novel *Oceanisphaera*-like bacterial strains, designated TW92^T and TW93, were isolated. The genus *Oceanisphaera* in the class *Gammaproteobacteria* was first introduced by Romanenko *et al.* (2003) with the type species *Oceanisphaera litoralis*. At present, four species belonging to the genus *Oceanisphaera* have been described: *O. litoralis* (Romanenko *et al.*, 2003), *Oceanisphaera donghaensis* (Park *et al.*, 2006), '*Oceanisphaera arctica*' (Srinivas *et al.*, 2012) and *Oceanisphaera ostreae* (Choi *et al.*, 2011), but only three of these currently have validly published names. The majority of species of the genus *Oceanisphaera* have been isolated from aquatic environments. The species of this genus are distinguished from other genera by their morphology, fatty acid composition ($C_{16:0}$ and $C_{18:1}\omega7c$), ubiquinone type (Q-8), and phylogenetic distance. Based on phenotypic, genotypic and taxonomic analyses, a novel species of the genus *Oceanisphaera* is proposed.

Strains TW92^T and TW93 were isolated from marine sediment collected from a cage-cultured ark clam farm in the Gang-jin bay of Korea. Isolation was carried out using the standard dilution-plating method at 25 °C on marine

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Abbreviations: DDH, DNA–DNA hybridization; MA, marine agar; MB, marine broth.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TW92^T and TW93 are HQ171441 and HQ171442, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

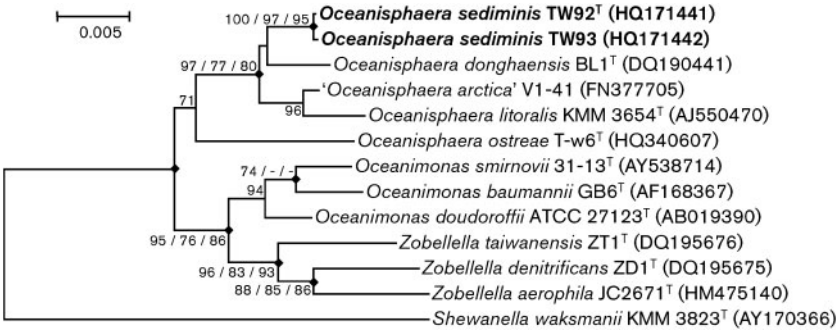

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences indicating positions of strains TW92^T, TW93 and close relatives. Filled diamonds represent identical branches that are present in phylogenetic consensus trees constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms. Numbers at nodes indicate bootstrap values as percentages of 1000 replicates. Values <70 % are not shown at the branch points. Bar, 0.005 substitutions per nucleotide.

agar (MA; BBL). Pure cultures of both strains were obtained by repeated streaking. All tests were performed in triplicate. Gram staining was determined using a kit (bioMérieux). Cell morphology was examined using a light microscope (ECLIPSE 50*i*; Nikon) and an energy-filtering transmission electron microscope (LIBRA 120; Zeiss). Cellular motility was examined using semi-solid agar media (Tittsler & Sandholzer, 1936). Growth under anaerobic conditions was monitored by incubation for 7 days in an anaerobic chamber (N_2 : H_2 : CO_2, 90 : 5 : 5) on MA plates. Catalase and oxidase activities were confirmed by observing bubble production in 3 % (v/v) hydrogen peroxide solution and by 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. To determine optimal growth conditions for strains TW92^T and TW93, the growth of both isolates was tested under various conditions. Growth temperature tests were performed at 4, 10, 15, 25, 30, 37, 45 and 55 °C in marine broth (MB; BBL). The pH range and optimum pH for growth were examined in MB supplemented with MES to achieve pH values of pH 4, 5 and 6; TAPS to achieve pH values of 7, 8 and 9; or Na_2HPO_4 to achieve pH values of 10 and 11. Salt requirement and tolerance were assessed using media that contained all of the constituents of MB except NaCl, supplemented with increasing concentrations of NaCl (0, 1, 2, 3, 4, 5, 8, 10, 12, 15 and 20 %, w/v). The turbidity of each culture was measured by optical density at 600 nm using a spectrophotometer (SYNERGY MX; BioTek) after incubation for 24 h, 48 h and 7 days. Cells of strains TW92^T and TW93 were Gram-stain-negative, coccoid (1.0–1.5 μm in diameter), motile by a single polar flagellum (Fig. S1, available in IJSEM online), and catalase- and oxidase-positive. Anaerobic growth was observed. The strains shared an optimum temperature for growth of 30 °C (range 4–42 °C) and an optimum pH for growth of pH 7 (range pH 6–9 for strain TW92^T and pH 6–10 for strain TW93). Strain TW92^T had an optimum salinity at 2 % (w/v) NaCl (range 1–5 %), while strain TW93 had an optimum salinity at 1 % (w/v) NaCl (range 0–12 %).

The 16S rRNA gene sequences of each strain were amplified by colony PCR using PCR pre-mix (WIZBIO) and two universal bacteria-specific primers: 8F and 1492R (Baker *et al.*, 2003). Amplified products were purified (QIAquick PCR Purification kit; Qiagen) and then sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The reaction mixture was analysed using an automated system (PRISM 3730XL DNA analyzer; Applied Biosystems). The almost complete 16S rRNA gene sequences of the isolates were assembled using SeqMan (DNASTAR) and then compared with other sequences in the EzTaxon server (Chun *et al.*, 2007). As a result, the strains were identified as members of the genus *Oceanisphaera*. Strains TW92^T and TW93 exhibited high 16S rRNA gene sequence similarity with *O. donghaensis* BL1^T (98.8 % and 98.7 %, respectively), '*O. arctica*' V1-41 (98.2 % and 98.1 %, respectively), *O. litoralis* KMM 3654^T (98.1 % and 98.0 %, respectively) and *O. ostreae* T-w6^T (96.9 %). The 16S rRNA gene sequence similarity between strains TW92^T and TW93 was 99.9 % with a single nucleotide difference. The three strains, *O. donghaensis* BL1^T (=DSM 17589^T), *O. litoralis* KMM 3654^T (=DSM 15406^T) and '*O. arctica*' V1-41 (=KCTC 23013), that exhibited greater than 97.0 % similarity in 16S rRNA gene sequence to both strains TW92^T and TW93 were obtained from the Korean Collection for Type Cultures (KCTC) or the German Culture Collection (DSMZ) for comparison as reference species.

For phylogenetic analysis, the 16S rRNA gene sequences of strains TW92^T and TW93 and their nearest relatives and associated species were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994). The aligned sequences were confirmed manually using BioEdit software (Hall, 1999). Phylogenetic consensus trees based on the aligned sequences and evolutionary analyses were constructed using MEGA version 5 (Tamura *et al.*, 2011) with three algorithms: neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) based on 1000 randomly chosen bootstrap replications. The phylogenetic trees demonstrated that strains TW92^T and TW93 constituted a monophyletic clade with other species of the genus *Oceanisphaera* (Fig. 1).

Sole carbon source assimilation and acid production from carbohydrates were tested on GN2 MicroPlates (Biolog) using inoculating fluid (Biolog) supplemented with 1 % (w/v) NaCl, and API 50CH test strips (bioMérieux) using 50 CHB/E medium (bioMérieux), respectively, according to

Table 1. Comparison of the morphological and physiological properties of strains TW92^T and TW93 with those of the closest related species

Strains: 1, TW92^T; 2, TW93; 3, *O. donghaensis* BL1^T; 4, '*O. arctica*' VI-41; 5, *O. litoralis* KMM 3654^T. All data were from the current study except where indicated. Data for carbon source assimilation, acid production from carbohydrate and enzyme activity were determined by GN2 MicroPlate (Biolog), API 50CHB, and API ZYM or API 20NE test strips (bioMérieux), respectively. All strains were positive for catalase and oxidase. All strains assimilated dextrin, pyruvic acid methyl ester and succinamic acid (GN2 MicroPlate). All strains produced acid from D-tagatose and 5-ketogluconate (API 50CHB). All strains were positive for alkaline phosphatase, esterase (C4) and leucine arylamidase (API ZYM). +, Positive; −, negative; W, weakly positive; ND, no data reported.

Characteristic	1	2	3	4	5
Temperature (°C)					
Range*	4–42	4–42	4–42	4–37	4–42
Optimum*	30	30	ND	18–30	28–35
Salinity (%, w/v)					
Range*	1–5	0–12	0.5–8.0	0–3	0.5–10
Optimum*	2	1	ND	2	1–3
Motility*	+	+	+	−	+
Assimilation of:					
D-Arabitol	−	−	−	−	W
Maltose	−	−	−	−	W
3-Methyl glucose	+	+	+	W	+
Methyl α-D-glucoside	W	W	+	W	+
Methyl β-D-glucoside	W	W	+	W	+
Methyl α-D-mannoside	−	−	−	+	−
Palatinose	−	−	−	W	−
Sedoheptulosan	−	−	−	+	W
Xylitol	W	−	+	+	−
Acetic acid	W	+	+	+	W
α-Ketovaleric acid	W	+	+	+	+
Lactamide	W	W	+	W	W
D-Lactic acid methyl ester	−	−	W	−	W
L-Lactic acid	−	−	−	+	W
D-Malic acid	−	−	−	−	W
L-Malic acid	+	+	+	+	W
Succinic acid monomethyl ester	−	−	W	W	W
Propionic acid	+	W	+	+	+
Pyruvic acid	−	−	W	+	−
Succinic acid	−	−	−	+	−
L-Alaninamide	+	+	W	+	W
D-Alanine	−	−	+	+	−
L-Alanyl glycine	+	+	+	−	−
L-Glutamic acid	+	+	+	+	W
Glycyl L-glutamic acid	−	−	W	−	−
L-Serine	−	−	+	+	+
Putrescine	W	+	+	+	W
2,3-Butanediol	W	W	−	W	−
Glycerol	+	+	+	W	−
Adenosine	+	+	−	+	−
Adenosine-5′-monophosphate	+	−	−	−	−
Acid production from:					
D-Ribose	+	+	W	+	+
D-Fructose	−	−	−	W	+
Enzyme activity					
Esterase lipase (C8)	W	W	W	−	−
Valine arylamidase	W	W	−	W	W
Acid phosphatase	W	W	W	+	W
Naphthol-AS-BI-phosphohydrolase	W	W	W	+	W
β-Glucuronidase	−	−	−	−	+

Table 1. cont.

Characteristic	1	2	3	4	5
Reduction of nitrates to nitrites	+	w	+	+	w
Urease	−	−	−	+	+

*Data for reference species are from Park *et al.* (2006), Srinivas *et al.* (2012) and Romanenko *et al.* (2003).

the manufacturers' instructions. Reactions were recorded after 72 h of incubation at 30 °C. Enzyme activities were characterized using API 20NE and API ZYM test strips (bioMérieux), according to the manufacturer's instructions. The results of the biochemical tests and distinguishable characteristics are presented in Table 1. Strains TW92^T and TW93 differed from each other in their assimilation of xylitol and adenosine-5′-monophosphate, and their ability to reduce nitrates to nitrites.

For chemotaxonomic investigations, cultures of strains TW92^T, TW93 and the reference species were grown on MA plates at 30 °C for 48 h and then harvested. For analysis of the cellular fatty acid profile, fatty acids of strains TW92^T, TW93 and the reference species were extracted as described by Sherlock Microbial Identification System (MIDI, 1999), analysed by GC (6890; Hewlett Packard), and then identified with the Microbial Identification software package (Sasser, 1990) (Sherlock version 4.0) based on the TSBA40 database. Isoprenoid quinone was extracted from freeze-dried cells as described by Collins & Jones (1981a) and identified by reverse-phase HPLC (Collins & Jones, 1981b) using a Thermo ODS HYPERSIL (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Xin *et al.* (2000), separated by two-dimensional TLC on a silica plate (Merck), and then identified by spraying the plate with appropriate detection reagents (Tindall, 1990): molybdatophosphoric acid for total lipids, ninhydrin reagent for amino-containing lipids, Zinzadze reagent for phospholipids and α-naphthol reagent for glycolipids. The major cellular fatty acids of strains TW92^T and TW93 were summed feature 3 (comprising $C_{16:1}\omega7c$/iso-C_{15} 2-OH, 45.1 and 44.8 %, respectively), $C_{16:0}$ (18.6 and 18.9 %, respectively) and $C_{18:1}\omega7c$ (14.6 and 16.3 %, respectively). The major components of both strains (summed feature 3, $C_{16:0}$ and $C_{18:1}\omega7c$) were similar to those of the reference species, whereas the minor components differed. The complete cellular fatty acid profiles of strains TW92^T, TW93 and the reference species are presented in Table 2. The predominant quinone of the strains was ubiquinone Q-8, in agreement with their affiliation to the genus *Oceanisphaera* (Choi *et al.*, 2011). According to previous research of Tindall (2005), ubiquinones are generally found in members of the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. The polar lipids of both strains comprised phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol (Fig. S2). Chemotaxonomic parameters, including fatty acid composition, quinone type and polar lipids, revealed that strains TW92^T and TW93 belong to the genus *Oceanisphaera* and represent a novel species.

Table 2. Cellular fatty acid content (%) of strains TW92^T and TW93 and their closest phylogenetic relatives

Strains: 1, TW92^T; 2, TW93; 3, *O. donghaensis* BL1^T; 4, '*O. arctica*' VI-41; 5, *O. litoralis* KMM 3654^T. All data were obtained from the current study. Fatty acids that represented <0.5 % in all species were omitted. tr, Trace amount (<0.5 %); −, not detected.

Fatty acid	1	2	3	4	5
Saturated acids					
$C_{10:0}$	tr	−	tr	tr	tr
$C_{12:0}$	9.7	9.6	9.8	12.0	10.5
$C_{14:0}$	0.8	0.6	0.7	0.8	tr
$C_{16:0}$	18.6	18.9	18.8	14.8	15.1
$C_{17:0}$	tr	tr	−	−	tr
Unsaturated acids					
$C_{16:1}\omega5c$	−	−	−	−	tr
$C_{17:1}\omega8c$	tr	tr	tr	−	0.5
$C_{18:1}\omega9c$	tr	−	−	−	−
$C_{18:1}\omega7c$	14.6	16.3	13.8	14.4	20.4
$C_{20:1}\omega7c$	tr	−	−	tr	−
Branched acids					
$C_{12:0}$ 3-OH	tr	tr	tr	0.5	tr
iso-$C_{14:0}$ 3-OH	−	−	−	−	tr
iso-$C_{15:0}$	tr	−	−	−	−
iso-$C_{16:0}$	tr	tr	tr	−	0.6
iso-$C_{17:0}$	tr	−	−	−	−
anteiso-$C_{17:0}$	tr	−	−	−	−
$C_{18:1}$ 11 methyl $\omega7c$	−	−	tr	−	tr
Summed features*					
2	7.6	7.3	8.0	9.3	7.6
3	45.1	44.8	45.1	46.1	42.0
4	−	−	−	−	tr
7	0.5	0.6	0.6	0.5	−
Unknown					
ECL 11.799	−	−	−	tr	tr
ECL 13.957	−	−	0.7	−	−
ECL 14.502	0.6	0.5	0.5	0.5	0.6

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed features 2 comprised $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I. Summed features 3 comprised $C_{16:1}\omega7c$/iso-C_{15} 2-OH. Summed features 4 comprised anteiso-$C_{17:1}$ B/i I. Summed features 7 comprised unknown ECL 18.846/$C_{19:1}\omega6c$.

Genomic DNA was extracted according to the method of Rochelle *et al.* (1992) and purified using an UltraClean kit (MoBio), according to the manufacturer's instructions. The DNA G+C content of strains TW92^T and TW93 was determined by fluorimetric method using SYBR Gold I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). Samples of genomic DNA of *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Ruminococcus obeum* ATCC 29174^T were used as references for calibration (Sambrook *et al.*, 1989). The DNA G+C contents of strains TW92^T and TW93 were 58.5 mol% and 59.6 mol%, respectively. The distinct species status of strains TW92^T, TW93 and the reference species was demonstrated by DNA–DNA hybridization (DDH), performed by reciprocal analysis using genome-probing microarrays (Bae *et al.*, 2005; Chang *et al.*, 2008). The DDH values of strain TW92^T were 47±7 %, 18±4 % and 12±2 % with *O. donghaensis* BL1^T, '*O. arctica*' V1-41, and *O. litoralis* KMM 3654^T, respectively. The DDH values of strain TW93 were 19±3 %, 31±4 % and 28±4 % for *O. donghaensis* BL1^T '*O. arctica*' V1-41 and *O. litoralis* KMM 3654^T, respectively. The DDH value of strain TW92^T with strain TW93 was 88±6 % (reciprocal 85±3 %). Complete results from reciprocal DDH analysis are provided in Table S1. Taken together, the results indicate that strains TW92^T and TW93 are representative of a genospecies and that both isolates are novel strains at the species level (Wayne *et al.*, 1987).

Based on differential phenotypic, phylogenetic and genotypic characteristics, strains TW92^T and TW93 represent a novel species of the genus *Oceanisphaera*, for which the name *Oceanisphaera sediminis* sp. nov. is proposed.

Description of *Oceanisphaera sediminis* sp. nov.

Oceanisphaera sediminis (se.di′mi.nis. L. gen. n. *sediminis* of a sediment).

Cells are facultatively aerobic, Gram-staining-negative, coccoid (1.0–1.5 μm in diameter), motile by means of a polar flagellum and slightly halophilic. On MA, aerobically grown colonies are circular, moist, translucent, raised with entire margin, beige and 0.7–1.0 mm in diameter after 48 h incubation at 30 °C. Growth occurs at 4–42 °C (optimum 30 °C), in the presence of 1–5 % (w/v) NaCl (optimum 2 %) and at pH 6–9 (optimum pH 7). Catalase- and oxidase-positive. Assimilates dextrin, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, xylitol, acetic acid, α-ketovaleric acid, lactamide, L-malic acid, pyruvic acid methyl ester, propionic acid, succinamic acid, L-alaninamide, L-alanyl glycine, L-glutamic acid, putrescine, 2,3-butanediol, glycerol, adenosine and adenosine-5′-monophosphate on Biolog GN2. Acid is produced from D-ribose, D-tagatose and 5-ketogluconate on API 50CH test strips. The following API ZYM test enzymes scored as positive: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. The following API ZYM test enzymes scored as negative: lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Positive result in tests for the reduction of nitrates to nitrites, but negative for indole production from L-tryptophan, D-glucose fermentation, arginine dihydrolase, urease, aesculin ferric citrate hydrolysis, gelatin hydrolysis and β-glucosidase activity on API 20NE test strips. The predominant cellular fatty acids are summed feature 3 (comprising $C_{16:1}\omega7c$/iso-C_{15} 2-OH), $C_{16:0}$ and $C_{18:1}\omega7c$. The major respiratory quinone is Q-8. The polar lipids contain phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol.

The type strain TW92^T (=KACC 15117^T=JCM 17329^T) and an additional strain TW93 (=KACC 15118=JCM 17330) were isolated from marine sediment on the south coast of Korea. The DNA G+C content of the type strain TW92^T is 58.5 mol%. Strain TW93 differs from the type strain by growth in the presence of 0–12 % (optimum 2 %) (w/v) NaCl, the inability to assimilate xylitol and adenosine-5′-monophosphate, the extent of activity for nitrate reduction and DNA G+C content (59.6 mol%).

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