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| 보 문 |

Isolation and Identification of *Oceanisphaera* sp. JJM57 from Marine Red Algae *Laurencia* sp. (Ceramiales: Rhodomelaceae)

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해양 홍조류 *Laurencia* sp. (Ceramiales: Rhodomelaceae)에서 분리한 *Oceanisphaera* sp. JJM57의 분리 및 동정

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A taxonomic study was carried out to assess the phylogenetic characteristics of isolate JJM57 from marine red algae *Laurencia* sp. collected from intertidal zone in Jeju Island, South Korea. Comparative analysis of 16S rRNA gene sequence shows that this isolate belongs to the genus *Oceanisphaera*. It shows 98.02% and 97.7% sequence similarity with *Oceanisphera litoralis* DSM 15406^T and *Oceanisphera donghaensis* KCTC 12522^T, respectively. Strain JJM57 is a Gram-negative, aerobic, moderately halophilic bacterium able to grow in different NaCl concentration ranges from 0.5 to 8.0% and at varying temperatures from 4 to 37℃. Sharing some of the physiological and biochemical properties with *O. litoralis* and *O. donghaensis*, JJM57 strain differs in the utilization of ethanol, proline, and alanine. The G+C contents of the strain JJM57 is 61.94 mol% and it is rich in $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$, and $C_{18:1}$ *ω7c* fatty acids. The DNA-DNA relatedness data separates the strain JJM57 from other species such as *O. litoralis* and *O. donghaensis*. On the basis of these polyphasic evidences, present study proposed that strain JJM57 (=KCTC 22371 =AM983543 =CCUG 60764) represents a novel bacterial species of *Oceanisphaera*.

***Keywords*:** 16S rRNA, *Oceanisphera litoralis*, *Oceanisphera donghaensis*, *Oceanisphaera* sp., Red algae

The marine red algae, *Laurencia* (Rhodomelaceae, Ceramiales) is a most diverse group with about 140 algal species, spreads in the Pacific basin and warm tropical seas including South Korea except in the Arctic and Antarctic regions (Nam and Saito, 1995; Masuda *et al*., 1996). Previous reports reveal the existence of clear distinction between *Laurencia* and *Osmundea* (Saito, 1967; Nam *et al*., 1994) and with two subgenera namely *Laurencia* and *Chondrophycus* (Nam and Sohn, 1994; Nam and Saito, 1995). The genus *Laurencia* is a prolific synthesizer of halogenated metabolites and has been reported to produce more than 250 diverse compounds, while the subgenus *Chondrophycus* also been reported to produce such metabolites (Erickson, 1983). These compounds are classified into four groups: sesquiterpenoid, diterpenoid, triterpenoid, and C_{15} acetogenin (Fenical and Norris, 1975; Howard *et al*., 1980; Erickson, 1983; Masuda *et al*., 1996). The genus *Oceanisphaera* was proposed by Romanenko *et al.* (2003) to accommodate as Gram-negative, aerobic, moderately halophilic, and oxidase- and catalase-positive species. Recently, two species of the genus, namely *Oceanisphaera donghaensis* and *O. litoralis* have been described, isolated from marine sediment (Romanenko *et al*., 2003; Park *et al*., 2006). In course

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of our study on marine microbial diversity, an *Oceanisphaera*-like strain JJM57 was isolated from marine red alga and selected for further polyphasic taxonomic investigation. Hence, the present study aims to isolate, identify and characterizes a novel bacterial species, *Oceanisphaera halophilus* sp. nov (strain JJM57), an important bacterial addition to the genera for the first time from marine red algae *Laurencia* (Rhodomelaceae, Ceramiales) collected from intertidal zone in Jeju Island, South Korea.

Materials and Methods

Marine red algae *Laurencia* species (Rhodomelaceae, Ceramiales) were collected from intertidal zone in Jeju Island and washed in sterile distilled water, grinded in an agate mortar and sieved with 150 Tyler mesh. Then the sample was diluted with sterilized seawater and spreads in a marine agar 2216 plate (MA; Difco). The plates were incubated at 25℃ for 2 days and the cultures were stored at -80℃ in marine broth (MB) supplemented with 20% (v/v) glycerol until used for identification.

A portion of the suspension from the glycerol stock was transferred into 10-ml fresh medium and incubated at 25℃. After three successive transfers, the suspension was plated in a solid medium to isolate pure cultures. The cell morphological and physiological characteristics of the pure colonies were done aerobically at 15℃ on MA. The cell morphology was determined using light microscope and Gram's stain done with bioMérieux Gram stain kit on 2 day old cultures, according to the manufacturer's instructions. Strains growth was determined at different temperatures (4–40℃) and pH (4.0–12.0) on both MA and MB; sodium ion requirement and tolerance for various NaCl concentrations (0–14%) were assessed using Nutrient agar (NA, Difco).

For cell morphology, cells were fixed with 1% (v/v) glutaraldehyde and negatively stained with 4% (w/v) aqueous uranyl acetate and carbon film. Cell motility was observed under an Olympus light microscope equipped with phase-contrast optics (magnification × 400), and the presence of flagella was examined using transmission electron microscopy (TEM). Gliding motility was assessed as described by Bowman (2000). Oxidase and catalase activities, degradation of agar, DNA and starch contents were examined according to the methods described by Smibert and Krieg (1994). Casein hydrolysis was determined according to the method reported by Norris *et al.* (1985); Flexirubin pigment production and cellulose hydrolysis were determined as described by Bowman (2000); hydrolysis of chitin and Tweens 20, 40, and 80 was determined according to Baumann and Baumann (1981). Other physiological and biochemical properties were tested using API 20E, API 20NE, API 50CH, and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Isoprenoid quinones were extracted from lyophilized cells and analysed as described by Collins (1985). Cellular fatty acids were analysed according to the instructions of Sherlock Microbial Identification System (MIDI, version 6). 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 by Svetashev *et al.* (1995) and Ivanova *et al.* (2000). For lipid analysis, the strain was cultured on MA at 25℃ for 2 days.

Bacterial genomic DNA was extracted using a commercial genomic DNA extraction kit (Bioneer, Korea). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set 27F and 1492R and the purified PCR product was sequenced by Solgent Co. Ltd (Korea). The full 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR) (Kim *et al.*, 2012). Alignment of sequences was carried out using CLUSTAL X software (Thompson *et al.*, 1997). Phylogenetic analyses were performed using the neighbour-joining (Saitou and Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Evolutionary distances for the neighbour-joining method were calculated using the method of Jukes and Cantor (Jukes and Cantor, 1969). A bootstrap analysis (Felsenstein, 1981) was performed for estimating the tree topology, with 1,000 resamplings of the dataset.

Results and Discussion

The isolated strain JJM57 are Gram-negative, aerobic, halophilic bacterium shows positive oxidase and catalase activity. Colonies morphology of the isolated strain appears as yellow-colored circular, convex with entire margin with approximately 6.0 μm diameter after 5 days incubation at 25℃ on MA. TEM analysis

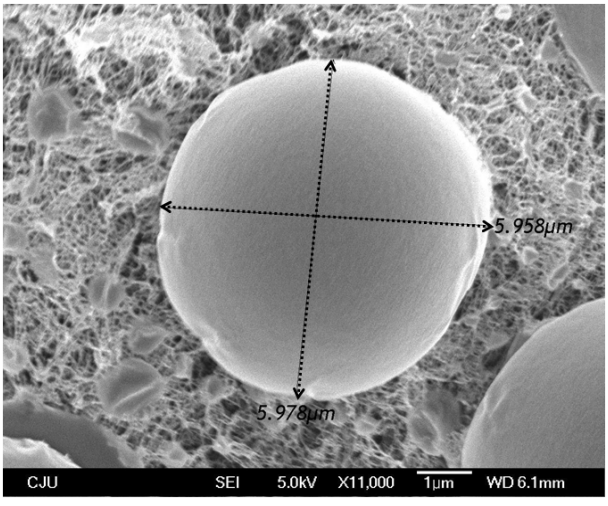

Fig. 1. Scanning electron micrograph of the strain JJM57. Bar, 1 μm.

Table 1. Characteristics used to differentiate of the present isolate (1) strain JJM57 from the two recognized members of the genus *Oceanisphaera* of (2) *O. litoralis* (Romanenko *et al*., 2003), and (3) *O. donghaensis* (Park *et al*., 2006)

Characteristic	1	2	3
Cell morphology	Coccoid	Coccoid	Coccoid
Cell size (μm)	6.0	1.0-1.2	0.5-1.5
Motility	+	+	+
Colony color	Yellowish	Yellow	Yellow
Temperature range for growth (℃)	4–37	4–42	4–42
NaCl range for growth (%)	1.0–11	0.5–10	0.5–8.0
Hydrolysis of:			
Urease[a]	-	+	-
Aesculin	+	-	-
Tween 80	+	W	W
Production of acid from			
Citrate[a]	+	+	+
Malate[a]	+	+	W
Utilization of:			
Phenylacetate[a]	-	+	-
L-Glutamate	+	W	+
L-Proline	+	-	+
L-Alanine	+	-	+
Ethanol	+	-	+
Phenol[b]	W	W	-
DNA G+C content (mol%)	61.94	56.4	56.6

Symbols: +, positive; -, negative; W, weak or delayed, All strains grow at 4–35℃ and are coccoid bacteria. All strains are positive for oxidase, catalase, Na^+ growth requirement, nitrate reduction, and malate and citrate utilization. All strains are negative for arginine dihydrolase, gelatin and utilization of caprate, glucose, mannose, *N*-acetylglucosamine, maltose, gluconate, adipate, L-leucine.
[a] Determined by API 20NE tests (this study and Romanenko *et al*., 2003; Park *et al*., 2006).
[b] Phenol concentration 4 mM.

revealed that the cells are of coccoid type with size ranges from 5.958 μm and 6.978 μm (Fig. 1). The results of biochemical and physiological tests are given in the species description in Table 1. Isolates showed no spore formation and its slightly halophilic nature. Sodium ions required for growth of the strain JJM57 ranged between 1–11% NaCl and the optimum growth was observed between 2 and 5% with no growth in 11% NaCl. The strain JJM57 growth occurs between 4 and 37℃ while the optimum growth temperature found between 25 and 28℃ and no growth at 40℃. This strain grows in the pH ranged from 5 to 10 and optimum growth occurred at pH 7.0 to 7.3. This stain utilizes the carbon and nitrogen sources by enzymatic activities of phenylacetate. The stain JJM57 showed positive for the reaction to produced alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, trypsin, and *N*-acetyl-β-glucosaminidase but not lipase (C14), valine arylamidase, α-chymotrypsin, β-glucuronidase, α -glucosidase, β-glucosidase, α-mannosidase or α-fucosidase. The tests for the fermentation of inositol, sorbitol, rhamnose, melibiose, and amygdalin were also positive.

The DNA G+C content of the strain JJM57 was similar to those described for *O. litoralis* DSM 15406^T (98.02%) and *O. donghaensis* KCTC 12522^T (97.7%), respectively. Also these values were in consistent with the G+C content of the genus *Oceanisphaera*, with slightly lower values of 56.5% and 56.6% (Romanenko *et al*., 2003; Park *et al*., 2006). The DNA G+C content of the present strain JJM57 was 61.94 mol%. The major fatty acids are summed feature 2 of Iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH (6.40%), summed feature 3 of $C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2-OH (41.03%), and summed feature 7 of $C_{19:0}$ cyclo ω10*c* and/or $C_{19:1}$ ω6*c* (0.5%) and this profile was similar to that of the four recognized members of genus *Oceanisphaera* (Tables 2 and 3).

The present strain JJM57 complete (1,425 bp) 16S rRNA gene sequence was determined a preliminary BLAST search against GenBank showed relationship with the members of the family *Alteromonadaceae*. *Vibrio alginolyticus* ATCC 17749^T (GenBank accession no. X56575) was used as outgroup in constructing the phylogenetic tree. Sequence similarity calculations obtained after a neighbour-joining analysis revealed that strain JJM57 was clustered with *O. litoralis* DSM 15406^T (98.02%) and *O. donghaensis* KCTC 12522^T (97.7%) (Fig. 2).

Description of *Oceanisphaera halophilus* sp. nov.

Oceanisphaera halophilus (hal·o·phil. N.L. n. *halophil* an

Table 2. Fatty acid compositions of the present (1) strain JJM57 compared to the genus *Oceanisphaera* of (2) *O. litoralis* (Romanenko *et al*., 2003), and (3) *O. donghaensis* (Park *et al*., 2006)

Fatty acid	1	2	3
Saturated fatty acids:			
C12:0	6.75	10.31	6.09
C14:0	0.35	1.00	0.30
C15:0		0.46	1.01
C16:0	22.47	16.45	15.92
C17:0	0.67	0.30	0.53
C18:0	0.71	ND	0.31
Branched fatty acids:			
Iso-$C_{16:0}$	0.34	0.65	1.85
Iso-$C_{17:0}$		ND	0.36
Unsaturated fatty acids:			
$C_{17:1}$ $\omega 8c$		0.39	0.68
$C_{18:1}$ $\omega 7c$	17.79	14.01	18.68
Summed feature 2[a]	6.40	8.23	5.13
Summed feature 3[a]	41.03	45.08	46.20
Summed feature 7[a]	0.52	ND	0.68
Unknown fatty acid 13.957(ECL)	0.63	ND	ND
Unknown fatty acid 14.502(ECL)	0.70	0.64	0.62

Fatty acids representing less than 0.3% in all strains were omitted. ND, Not detected; ECL, equivalent chain-length.
[a] Summed feature 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 $C_{16:1}$ $\omega 7c$ 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 $\omega 10c$ and/or $C_{19:1}$ $\omega 6c$.

organism living in a salty environment that requires a high concentration of salt for optimal growth and survival; Gr. Adj. *philus* loving; N.L. fem. Adj. *halophilus* an organism loving in a salty environment for optimal growth and survival).

Cells are Gram-negative with single polar flagellum, oxidase-positive, catalase-positive, and they occur singly with diameter of 5.95–5.97 μm. Strain JJM57 is moderately halophilic and grows in 1–11% NaCl at 4–37℃. Favorable growth occurs aerobically producing circular colonies with regular edges within 2 days, with diameters of approximately 6.0 μm. The strain, JJM57, requires Na^+ for growth; reduces nitrate, utilizes malate and citrate. But this strain is negative for arginine dihydrolase, gelatin and utilization of glycerol, succinate, L-valine and L-tyrosine. No acid is produced from galactose, glucose, arabinose, mannose, *N*-acetylglucosamine, maltose, gluconate, adipate or sucrose test. Strain, JJM57 produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, trypsin, and *N*-acetyl-β-glucosaminidase but not lipase (C14), valine arylamidase, α-chymotrypsin, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase or α-fucosidase. It utilize carbon and nitrogen source for enzymic activities (Table 1). The major cellular fatty acids of JJM57 are $C_{16:1}$ $\omega 7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{18:1}$ $\omega 7c$. Polar lipids of the strain JJM57 include phosphatidylethanolamine (52.3%), phosphatidylglycerol (42.6%), and diphosphatidylglycerol (5.1%). DNA G+C content of strain JJM57 is 61.94 mol% as determined by HPLC.

The strain JJM57 (=KCTC 22371 =AM983543 =CCUG 60764) was isolated from marine red algae genera *Laurencia* (Rhodomelaceae, Ceramiales) in Jeju Island, South Korea.

적 요

본 연구는 한국 제주도 조간대에 서식하는 홍조류로부터 분리된 JIM57 균주의 계통학적 특성을 조사하기 위하여 수행되었

Table 3. Major polar lipid and minor polar lipid of the present (1) strain JJM57 compared to the genus *Oceanisphaera* of (2) *O. litoralis* (Romanenko *et al*., 2003), and (3) *O. donghaensis* (Park *et al*., 2006)

Characteristic	1	2	3
Quinone	ND	ND	ND
Polar lipid			
Major polar lipid	Phosphatidylethanolamine (52.3%) Phosphatidylglycerol (42.6%)	Phosphatidylethanolamine (43.7%) Phosphatidylglycerol (46.9%)	Phosphatidylethanolamine (58.6%) Phosphatidylglycerol (38.2%)
Minor polar lipid	diphosphatidylglycerol (5.1%)	diphosphatidylglycerol (9.4%)	diphosphatidylglycerol (3.2%)

다. 16S rRNA gene 염기서열을 분석한 결과, 본 균주는 *Oceanisphaera* 속과 대단히 유사하였으며, *Oceanisphaera litoralis* DSM 15406^{T}와 98.02%, *O. donghaessis* KCTC 12522^{T}와 97.7%의 염기서열 상동성을 나타내었다. 본 균주는 그람양성의 호기성 구균으로써, 0.5-8.0%의 NaCl 및 4-47℃에서 생육할 수 있었다. 본 균주는 *Oceanisphaera litoralis* DSM 15406^{T}와 일부 생리학적 및 생화학적 특성을 공유하였으나 ethanol, proline 및 alanine 이용성에서는 차이가 있었다. 본 균주 genomic DNA의 GC 함량은 61.94 mol%였으며, 주요 균체 지방산 지방산으로서 $C_{16:1}$ $\omega7c$, iso-$C_{15:0}$ 2-OH, $C_{16:0}$, and $C_{18:1}$ $\omega7c$를 함유하고 있었다. 또한 DNA-DNA 상동성을 조사한 결과, JIM57 균주는 *O. litoralis* DSM 15406^T 및 *O. donghaessis* KCTC 12522^{T}와 별개의 종임을 알 수 있었다. 이러한 결과들을 종합한 결과, JIM57 균주(=KCTC 22371 =AM 983543 =CCUG 60764)는 *O. litoralis* DSM 15406^T 및 *O. donghaessis* KCTC 12522^{T}와 다른 특성을 나타내는 것으로 확인되어 *Oceanisphaera*의 새로운 종임을 제안하였다.

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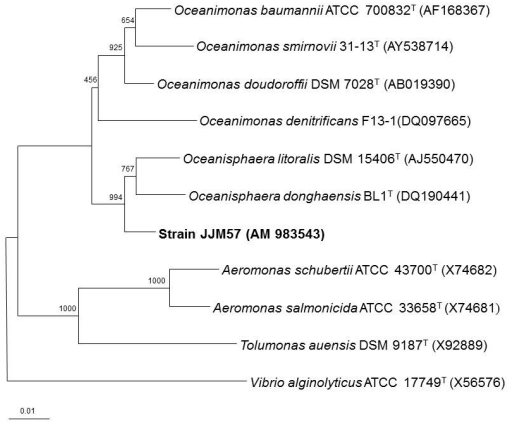

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain JJM57 within the radiation of the genus *Oceanisphaera*. The tree was constructed from evolutionary distance matrix by using neighbour-joining method (Thompson *et al*., 1997). The sequence of *Vibrio alginolyticus* ATCC 17749^T (X56575) was used as an outgroup. Asterisks represent the branches also found in both maximum-likelihood and maximum-parsimony trees (Felsenstein, 1981). Bootstrap percentages (from 1,000 replications) >50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

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