Kordia algicida gen. nov., sp. nov., an algicidal bacterium isolated from red tide

Jae Hak Sohn,[1] Jung-Hyun Lee,[1] Hana Yi,[2] Jongsik Chun,[2]
Kyung Sook Bae,[3] Tae-Young Ahn[4] and Sang-Jin Kim[1]

[1]Microbiology Laboratory, Korea Ocean Research and Development Institute, Ansan PO Box 29, 425-600, Republic of Korea

[2]School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

[3]Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, PO Box 115, Yusong, Taejon 305-600, Republic of Korea

[4]Department of Microbiology, Dankook University, Cheon-An 330-150, Republic of Korea

Correspondence
Sang-Jin Kim
s-jkim@kordi.re.kr

A bacterium (named OT-1$^{\mathrm{T}}$) that showed algicidal activity was isolated from sea water of Masan Bay, Korea, during an outbreak of red tide. Phylogenetic analysis based on 16S rDNA sequences showed that the isolate formed a distinct phyletic lineage within the family *Flavobacteriaceae* of the *Cytophaga–Flavobacterium–Bacteroides* group. No species with a validly published name showed $\geqslant$93 % 16S rRNA gene sequence similarity to strain OT-1$^{\mathrm{T}}$. The isolate had major amounts of iso-branched and 3-hydroxy iso-branched fatty acids and menaquinone 6 and a DNA G+C content of 34 mol%; these chemotaxonomic characters also supported the placement of the organism in the family *Flavobacteriaceae*. The strain was Gram-negative, yellow-pigmented, non-motile, non-gliding, flexirubin-negative, strictly aerobic, catalase-negative, oxidase-positive and halophilic. Na^{+}, Ca^{2+} and Mg^{2+} ions were obligately required for growth. The strain utilized various sugars as sole carbon sources and degraded gelatin, skimmed milk and starch. Several phenotypic characters can be used to differentiate the test strain from phylogenetically related marine bacterial genera. On the basis of polyphasic evidence, it is proposed that strain OT-1$^{\mathrm{T}}$ should be assigned to the family *Flavobacteriaceae* as *Kordia algicida* gen. nov., sp. nov. The type strain is OT-1$^{\mathrm{T}}$ (=KCTC 8814P$^{\mathrm{T}}$=NBRC 1000336$^{\mathrm{T}}$).

In coastal sea water where red tide occurs frequently, marine bacteria play an important role for the interactions of phytoplankton, in the sense of decreasing and developing algal blooming (Yoshinaga *et al.*, 1995a). Algicidal bacteria that can kill and lyse causative phytoplanktons of red tides could potentially be of great use in reducing and preventing harmful algal blooms. During the past decades, algicidal bacteria isolated from marine and coastal environments have been assigned to the genera *Alteromonas*, *Cytophaga*, *Flavobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Saprospira* and *Vibrio* (Baker & Herson, 1978; Ishio *et al.*, 1989; Sawayama *et al.*, 1991, 1993; Imai *et al.*, 1993, 1995, 2001; Yoshinaga *et al.*, 1995b; Lovejoy *et al.*, 1998; Adachi *et al.*, 2002).

In the screening of algicidal bacteria, a moderately halophilic, yellow-pigmented bacterium, designed OT-1$^{\mathrm{T}}$, was isolated from the surface water of Masan Bay, Korea, during an outbreak of red tide. This bacterium has the ability to kill and lyse several marine microalgal species. In this study, we report the taxonomic properties of strain OT-1$^{\mathrm{T}}$, for which the name *Kordia algicida* gen. nov., sp. nov. is proposed.

Isolation

A sea water sample was collected from a depth of 1 m in Masan Bay, Republic of Korea, in which algal bloom caused by a marine microalga, *Skeletonema costatum*, occurred. The sample was filtered through a 1·2 μm membrane filter, co-cultured with *S. costatum* and incubated at 20 °C under cycling light (approx. 5000 lx for 14 h) and dark (10 h) periods. *S. costatum* cells in a co-culture tube were killed completely after 3 days. The bacterium that was responsible for killing *S. costatum*, designed OT-1$^{\mathrm{T}}$, was isolated from the co-culture tube by using ZoBell 2216e agar medium (ZoBell, 1946). The organism also showed algicidal activity against other algal species, namely *Thalassiosira* sp., *Heterosigma akashiwo* and *Cochlodinium polykrikoides*. The test

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Abbreviations: CFB, *Cytophaga–Flavobacterium–Bacteroides*; O/F, oxidation–fermentation.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain OT-1$^{\mathrm{T}}$ is AY195836.

strain was maintained as a glycerol suspension (20 %, w/v) at −80 °C.

16S rDNA analysis

Genomic DNA was prepared by using a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. 16S rDNA was enzymically amplified, purified and sequenced according to Chun & Goodfellow (1995). The sequence of strain OT-1^T was aligned manually with representative sequences of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group that were obtained from GenBank. Phylogenetic trees were inferred by using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out by using the PHYDIT (available at http://plaza.snu.ac.kr/~jchun/phydit/) and PAUP 4.0 (Swofford, 1998) programs, as described previously (Chun *et al.*, 2000).

On the basis of 16S rDNA analysis, strain OT-1^T showed low levels of sequence similarity to species with validly published names; highest similarities were found to the type strains of *Cytophaga latercula* (92·5 %), *Flexibacter tractuosus* (92·1 %), *Salegentibacter salegens* (91·0 %), *Cellulophaga lytica* (90·9 %), *Cellulophaga fucicola* (90·7 %), *Tenacibaculum maritimum* (90·5 %) and *Polaribacter franzmannii* (90·1 %). The position of strain OT-1^T was not stable within the CFB complex, as the branching positions in the trees varied depending on the phylogenetic method employed (Fig. 1). However, it is evident from 16S rDNA analysis that our isolate is distant from all species with validly published names and represents a new phyletic lineage that merits novel genus status in the family *Flavobacteriaceae*.

Morphology and physiological properties

Tests for Gram stain, spore stain and motility were performed as described by Lewin & Lounsbery (1969). Gliding motility was observed by direct microscopic examination of the edge of colonies in the exponential phase on a plate that contained CY agar [3 g casitone, 1 g yeast extract, 1 g $CaCl_2.2H_2O$, 40 g artificial sea salts (Sigma) and 15 g agar in 1000 ml distilled water], according to Bernardet *et al.* (2002). The isolate was a Gram-negative, non-spore-forming, non-motile and non-gliding rod. For scanning electron microscopy, strain OT-1^T was fixed for 3 h with 4 % glutaraldehyde. After washing with 50 mM HEPES buffer (pH 7·5) and post-fixation with 2 % OsO_4 solution for 6–8 h, the material was dehydrated in a series of graded ethanol solutions. The specimen was freeze-dried, coated with gold in an ion sputter and examined with a scanning electron microscope. After 24 h growth in ZoBell 2216e broth at 25 °C, single cells were 2–5 μm in length and 0·3–0·5 μm in diameter (Fig. 2). The colony colour of strain OT-1^T was yellowish on ZoBell 2216e medium, but white on peptone/sea water agar medium. The bathychromatic shift test with 20 % KOH revealed no flexirubin pigments. After 3 days cultivation, colonies of strain OT-1^T were about 1·2 mm in diameter, slightly convex (elevation), entire (margin) and round (configuration).

Growth characteristics were examined by using ZoBell 2216e as the basal medium. Strain OT-1^T grew at temperatures between 5 and 40 °C; optimal growth occurred at 30 °C. The pH range for growth was determined by measuring OD_{660} after 24 and 48 h incubation at 25 °C in ZoBell 2216e broth. The organism grew at pH values from 6 to 10; optimal growth occurred at around pH 7. Specific ion requirements and NaCl tolerance were tested by using synthetic ZoBell medium (5 g Bacto peptone, 1 g yeast

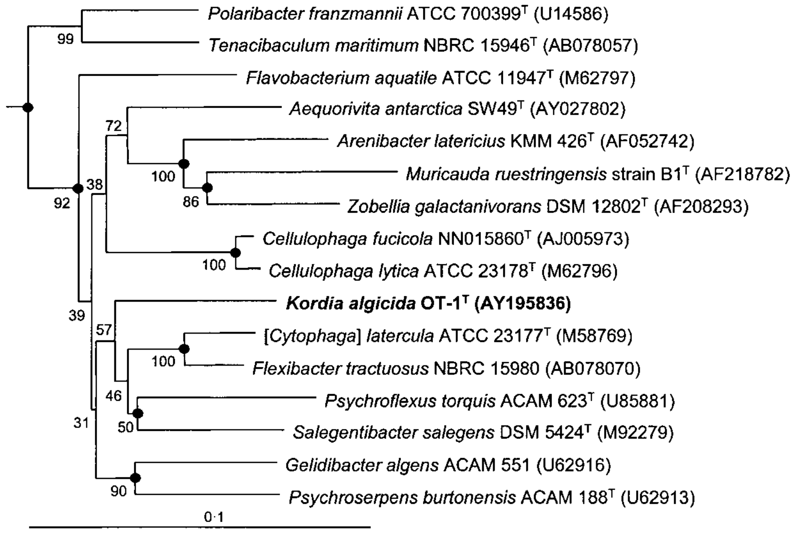

Fig. 1. Neighbour-joining tree based on nearly complete 16S rDNA sequences, showing relationships between strain OT-1^T and members of the CFB group. Numbers at nodes are levels of bootstrap support (%), based on neighbour-joining analyses of 1000 resampled datasets; solid circles indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. Bar, 0·1 nucleotide substitution per position.

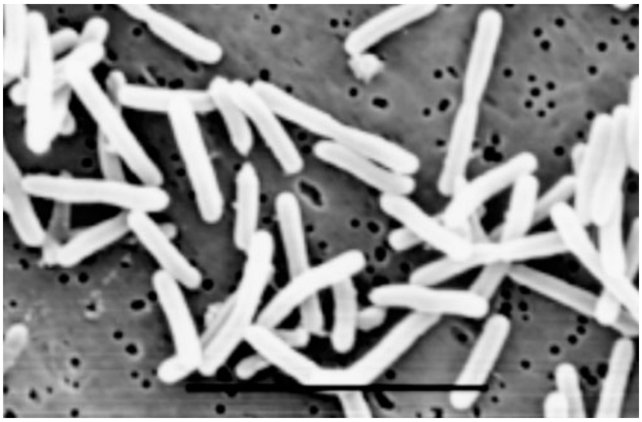

Fig. 2. Scanning electron micrograph of strain OT-1^T. Bar, 5 μm.

extract, 10 mg $FePO_4$ in 900 ml distilled water) that was supplemented with combinatorial artificial sea salts or NaCl. Growth in the synthetic medium was determined by measuring OD_{660} after 24 and 48 h incubation at 25 °C. The strain showed absolute requirement for cations, namely Na^+, Mg^{2+} and Ca^{2+}, as no growth was observed in medium that had not been supplemented with any of these ions. This strain grew at NaCl concentrations between 1 and 5 %; optimal growth occurred in 3 % NaCl.

Physiological and biochemical characters were examined as described by Lewin & Lounsbery (1969). Liquefaction of gelatin (10 %) and degradation of agar (1 %), skimmed milk (20 %), starch (0·5 %), CM-cellulose (3 %), cellulose (Avicell, 1 %), chitin (2 %), inulin (1 %), alginate (3 %) and pectin (2 %) were examined. Additional biochemical tests were performed by using API 20NE strips (bioMérieux) following the manufacturer's instructions, except that the solution used for bacterial suspension was the modified one (5 g $MgCl_2.6H_2O$, 1 g $CaCl_2.2H_2O$, 25 g NaCl in 1 l distilled water) instead of saline solution. As strain OT-1^T was unable to grow in the Aux medium of the API 20NE test kit, no data were obtained from 12 carbohydrate assimilation and glucose acidification test cupules. Thus, acid production from glucose and oxidation–fermentation (O/F; glucose) test media (Lewin & Lounsbery, 1969), supplemented with 50 % aged sea water, and a Biolog GN2 MicroPlate were used instead.

Strain OT-1^T was negative for catalase, urease, β-glucosidase (aesculinase), arginine dihydrolase, β-galactosidase, H_2S production, indole production, acid production from glucose and the O/F test (glucose). However, the isolate reduced NO_3 to N_2 and had oxidase activity. The test strain showed positive reactions for gelatin, skimmed milk and starch degradation, but was negative for degradation of cellulose, CM-cellulose, agar, chitin, alginate, pectin and inulin.

Utilization of nitrogen source was determined as described by Lewin & Lounsbery (1969). The strain did not grow on urea, NH_4 or NO_3 (supplied as inorganic nitrogen sources), but grew when Casaminoic acid, sodium glutamate, peptone, tryptone or yeast extract were supplied instead of an inorganic nitrogen source.

The organism's ability to oxidize various carbon compounds was examined by using a Biolog GN2 MicroPlate. The strain was grown for 2 days at 25 °C on trypicase soy agar (TSA; Difco) by using aged sea water. An inoculum was prepared in modified suspension solution (5 g $MgCl_2.6H_2O$, 1 g $CaCl_2.2H_2O$, 25 g NaCl in 1 l distilled water) instead of saline water. On the basis of the Biolog system, the test strain oxidized various carbon compounds, as given in the species description.

Chemotaxonomy

Fatty acid methyl esters were prepared from biomass that was scraped from TSA supplemented with 75 % aged sea water, incubated at 25 °C for 3 days and analysed by using GC, according to the instructions of the Microbial Identification system (MIDI). The organism had the following fatty acids: iso-$C_{15:0}$ (41·2 %), iso-$C_{17:0}$ 3-OH (22·2 %), iso-$C_{15:0}$ 3-OH (13·8 %), iso-$C_{16:0}$ 3-OH (4·0 %), a mixture of iso-$C_{15:0}$ 2-OH and $C_{16:1}\omega7c$ (3·3 %; the MIDI system could not differentiate between these two fatty acids), anteiso-$C_{15:0}$ (2·8 %), iso-$C_{17:1}\omega9c$ (2·3 %), $C_{15:0}$ (1·5 %) and unknown fatty acids (8·9 %). These levels of branched hydroxy fatty acids are often found in the CFB group (Gosink *et al.*, 1998). Isoprenoid quinones were analysed by using HPLC, according to Minnikin *et al.* (1984) and Collins (1985). The predominant isoprenoid quinone of the test strain was menaquinone 6. The genera *Flavobacterium* (Bernardet *et al.*, 1996), *Salegentibacter* (McCammon & Bowman, 2000) and *Tenacibaculum* (Suzuki *et al.*, 2001) were also reported to have MK-6; this is different from the genera *Cytophaga* (Nakagawa & Yamasato, 1993; Nakagawa *et al.*, 1997) and *Flexibacter* (Kleinig *et al.*, 1974; Fujita *et al.*, 1996), which contain MK-7 as their major isoprenoid quinone. DNA was prepared according to Wolff & Gemmill (1997) and DNA G + C contents were determined by HPLC of deoxyribonucleosides as described by Mesbah *et al.* (1989), using a reverse-phase column (Supelcosil LC-18-S; Supelco). The DNA G + C content of strain OT-1^T was 34 mol%.

Pigment absorption spectra were determined by using a model UV-2401PC spectrophotometer (Shimadzu) after ethanol extraction (Lewin & Lounsbery, 1969). The resultant absorption spectrum, which included peaks at 448 and 475 nm and a shoulder at 425 nm, was similar to those of carotenoids.

Taxonomic conclusions

Very low 16S rDNA sequence similarity values (<93 %) to species with validly published names and the formation of an independent phyletic lineage indicate clearly that our isolate can be assigned to a novel genus in the CFB group and, in particular, to a clade whose members contain menaquinone 6 (Suzuki *et al.*, 2001). In addition, several phenotypic characters can be used to differentiate our isolate from its phylogenetic neighbours from marine sources (Table 1). On the basis of the polyphasic evidence presented in this study, we propose that the algicidal strain OT-1^T should be classified in a novel genus in the family *Flavobacteriaceae* as *Kordia algicida* gen. nov., sp. nov.

Description of *Kordia* gen. nov.

Kordia (Kor′di.a. N.L. fem. n. *Kordia* arbitrary name derived from the abbreviation KORDI, which stands for Korea Ocean Research and Development Institute).

Cells are strictly aerobic, Gram-negative, non-motile and non-gliding rods. Carotenoid-type pigments are present. Flexirubin-type pigments are absent. Oxidase is produced, but catalase is not. Several carbohydrates are used as sole carbon sources. Unable to grow in the absence of any of the ions Na^+, Ca^{2+} and Mg^{2+}. Requires 1–5 % (w/v) NaCl for growth, with optimal growth at 3 % NaCl. Gelatin, skimmed milk and starch are degraded. Major cellular fatty acids are saturated iso-branched and 3-hydroxy iso-branched fatty acids. Respiratory quinone is menaquinone 6. DNA G+C content is 34 mol%. The type and only species to date is *Kordia algicida*.

Description of *Kordia algicida* sp. nov.

Kordia algicida (al.gi′ci.da. L. fem. n. *alga -ae* alga; L. suff. *-cida* from L. v. *caedere* to cut or to kill; N.L. n. *algicida* alga-killer).

Cell widths range from 0·3 to 0·5 μm; cell lengths range from 2 to 5 μm. Resting stages are absent. Colonies on solid media (ZoBell 2216e and TSA plus sea water) are yellowish, slightly convex (elevation), entire (margin) and round (configuration). Urease and *β*-glucosidase (aesculinase) are absent. H_2S and indole are not produced. Nitrate is reduced to nitrite. *α*-Cyclodextrin, dextrin, glycogen, *N*-acetyl-D-glucosamine, adonitol, *i*-erythritol, gentiobiose, *α*-D-glucose,

Table 1. Differential characteristics for strain OT-1^T and phylogenetically related genera in the family *Flavobacteriaceae*

Strain or genus: 1, strain OT-1^T; 2, *Aequorivita*; 3, *Arenibacter*; 4, *Cellulophaga*; 5, *Cytophaga*; 6, *Flavobacterium*; 7, *Flexibacter*; 8, *Gelidibacter*; 9, *Muricauda*; 10, *Polaribacter*; 11, *Psychroflexus*; 12, *Psychroserpens*; 13, *Salegentibacter*; 14, *Tenacibaculum*; 15, *Zobellia*. Data are from this study and others (Reichenbach, 1989; Dobson *et al.*, 1993; Fujita *et al.*, 1996; Bowman *et al.*, 1997; Gosink *et al.*, 1998; Johansen *et al.*, 1999; McCammon & Bowman, 2000; Barbeyron *et al.*, 2001; Bruns *et al.*, 2001; Ivanova *et al.*, 2001; Suzuki *et al.*, 2001; Bowman & Nichols, 2002). Abbreviations and symbols: O, orange; S, salmon; Y, yellow; F, filament; H, helices; R, rod; AP, with appendage; G, with gas vesicle; A, aerobe; FA, facultative anaerobe; SA, strict aerobe; ND, no data available; NG, no growth; V, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pigment	Y	O/Y	O	O/Y	O/Y	ND	O	Y	Y	O/S	O	Y	Y	Y	O/Y
Flexirubin reaction	−	−	−	−	V	V	V	−	−	−	−	−	−	−	+
Cell shape	R	R/F	R	R	R	ND	R/F	R/F	R-AP	R/F-G	R/F	R/F/H	R	R/F	R
Gliding motility	−	−	−	+	V	+	+	+	+	−	V	−	−	+	+
Requirement for:															
Sea salts	+	V	ND	V	−	−	ND	+	+	+	V	+	−	+	+
Organic nitrogen	+	+	−	−	−	ND	ND	+	ND	ND	V	+	−	+	ND
Relation to oxygen	SA	SA	SA	SA	SA	SA	SA	SA	FA	A	ND	SA	SA	SA	SA
Growth at:															
4 °C	+	+	−	V	V	ND	ND	+	−	+	+	+	+	−	−
25 °C	+	+	+	+	+	+	ND	V	+	−	V	−	+	+	+
Nitrate reduction	+	−	+	V	V	V	V	−	−	V	−	−	+	V	+
Acid production from carbohydrate	−	−	ND	V	ND	ND	ND	+	ND	+	ND	−	ND	ND	+
Catalase	−	+	+	+	V	+	V	+	−	+	V	+	+	+	+
Oxidase	+	ND	+	V	+	+	+	−	+	V	+	−	+	+	+
Urease	−	V	+	V	ND	ND	ND	−	ND	−	ND	−	ND	ND	−
Agarase	−	−	−	+	V	−	V	−	−	−	−	−	+	−	+
Amylase	+	V	−	+	V	V	V	+	−	+	+	−	+	V	V
β-Galactosidase	−	−	ND	V	ND	V	V	−	ND	V	−	V	+	ND	+
Caseinase	+	V	−	V	V	ND	V	V	ND	NG	−	+	−	+	ND
Aesculinase	−	V	ND	ND	−	V	ND	+	ND	V	V	−	+	−	+
Gelatinase	+	+	−	V	−	+	V	V	−	V	V	V	+	V	+
DNA G+C content (mol%)	34	33–39	37–38	32–42	34–42	32–37	37–47	36–38	41	31–33	31–39	27–29	37–38	31–33	42–44
Isoprenoid quinone	MK-6	ND	ND	ND	MK-7	MK-6	MK-7	ND	ND	ND	ND	ND	MK-6	MK-6	ND

maltose, D-mannitol, D-mannose, D-raffinose, sucrose, citric acid, D-glucuronic acid, α-ketoglutaric acid, DL-lactic acid, quinic acid, succinic acid, alaninamide, L-asparatic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, L-proline, L-threonine, DL-carnitine, uridine, glucose 1-phosphate and glucose 6-phosphate are oxidized, but not Tween 40, Tween 80, *N*-acetyl-D-galactosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, *m*-inositol, α-D-lactose, lactulose, D-melibiose, methyl β-D-glucoside, D-psicose, L-rhamnose, D-sorbitol, D-trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-histidine, hydroxy L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, γ-aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol or DL-α-glycerol phosphate. Acid is not produced from glucose. Negative for O/F test (glucose). Does not utilize urea, NH_4 or NO_3 as sole nitrogen sources. Degrades gelatin, skimmed milk and starch, but not cellulose, CM-cellulose, agar, chitin, alginate, pectin or inulin. Grows optimally at 30 °C, pH 7–8 and 3 % NaCl. Does not grow in ZoBell 2216e broth with 0 or 6 % NaCl, nor at pH 5. Requires sea salts (Na^+, Ca^{2+} and Mg^{2+}) for growth. Distinctive features of the fatty acid profile are high levels of iso-$C_{15:0}$ (41·2 %) and the presence of iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH and iso-$C_{17:0}$ 3-OH.

The type strain is OT-1^T (=KCTC $8814P^T$=NBRC 1000336^T). Isolated from a sea water sample associated with red tide in Masan Bay, Republic of Korea.

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