Seohaeicola zhoushanensis sp. nov., isolated from seawater

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A novel, Gram-stain-negative, motile, facultatively anaerobic bacterium, designated strain NF48^T, was isolated from surface seawater around Zhoushan Islands. Cells were rod-shaped (1.1–3.9×0.5–0.9 μm). Strain NF48^T was able to grow at 10–40 °C (optimum 30 °C), at pH 5.5–9.0 (optimum 6.5–8.0) and with 0.5–7.0 % (w/v) NaCl (optimum 2.0 %). The genomic DNA G+C content was 65.5 mol%. Chemotaxonomic analysis showed that the main isoprenoid quinone was Q-10 and the major fatty acids were $C_{18:1}\omega7c$ and $C_{16:0}$. The major polar lipids of strain NF48^T were phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain NF48^T belonged to the genus *Seohaeicola* with similarity values of 95.5–97.2 % to members of this genus, and was most closely related to *Seohaeicola nanhaiensis* SS011A0-7#2-2^T (97.2 %). On the basis of the phenotypic, chemotaxonomic and phylogenetic characteristics, strain NF48^T is suggested to represent a novel species of the genus *Seohaeicola*, for which the name *Seohaeicola zhoushanensis* sp. nov. is proposed. The type strain is NF48^T (=MCCC 1K01157^T=KCTC 42650^T).

The genus *Seohaeicola*, belonging to the family *Rhodobacteraceae*, was proposed by Yoon *et al.* (2009). At the time of writing, the genus consists of three recognized species, *Seohaeicola saemankumensis* (Yoon *et al.*, 2009), *Seohaeicola westpacificensis* (Xian *et al.*, 2014) and *Seohaeicola nanhaiensis* (Xie *et al.*, 2014), which were isolated from a tidal flat of the Yellow Sea in Korea, deep West Pacific seawater and sediment of the South China Sea at a depth of 1500 m, respectively. The type species of the genus is *S. saemankumensis*, which has various cell shapes, such as rod, oval and coccoid (Yoon *et al.*, 2009). Cells of *S. westpacificensis* and *S. nanhaiensis* resemble a spindle with two narrow poles (Xian *et al.*, 2014) and are rod-shaped, respectively. The predominant ubiquinone of the genus is Q-10. All members of the genus *Seohaeicola* are positive for activity of esterase (C4), esterase lipase (C8) and leucine arylamidase. In this study, a novel species, isolated from surface seawater around Zhoushan Islands, Zhejiang province, China, is described based on a polyphasic taxonomic analysis.

The surface seawater sample was collected in April 2014 around Zhoushan Islands (30° 07′ 59.56″ N 122° 47′ 41.55″ E) of the East China Sea. The sample was diluted using ten-fold dilution series methods and spread on modified 2216E agar medium (Oppenheimer, 1952). The plates were then incubated at 28 °C. The modified 2216E agar medium contained (per litre distilled water): yeast extract 0.5 g, tryptic case peptone 0.1 g, NaCl 19.45 g, $MgCl_2{\cdot}6H_2O$ 12.6 g, $MgSO_4{\cdot}7H_2O$ 6.64 g, $CaCl_2{\cdot}2H_2O$ 1.8 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, KBr 0.08 g, H_3BO_3 22 mg, $NaSiO_3{\cdot}9H_2O$ 93 mg, NaF 2.4 mg, NH_4NO_3 2.4 mg, Na_2HPO_4 8 mg, $SrCl_2$ 57 mg, ferric citrate 0.1 g, agar 20 g, pH 7.2, adjusted with NaOH. After 3 days of incubation, one cream-coloured colony was picked and named strain NF48^T; the strain was subsequently purified and preserved at −80 °C in the same medium supplemented with 30 % (v/v) glycerol.

Cell morphology and motility were observed by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) after cells were incubated on marine agar 2216 (MA) at 30 °C for 2 days. The temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NF48^T is KP063901.

Four supplementary figures are available with the online Supplementary Material.

37, 40 and 45 °C. The pH range for growth was determined at different pH values (pH 4.5–10, at increments of 0.5 pH units) and supplemented with buffering agents 40 mM MES (pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5) and CAPSO (pH 9.0–10.0), respectively. A modified marine broth (MB) was used for NaCl tolerance test, in which NaCl was omitted (0 %) or added at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 % (w/v) final concentration. Growth under anaerobic conditions was determined at 30 °C in modified MB medium supplemented with 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite, 20 mM sodium nitrate, 5 g L-arginine l^{-1}, 0.5 g cysteine l^{-1}, 1 g cysteine l^{-1} and 1 mg resazurin l^{-1} (Pan *et al.*, 2014). The strain was incubated in Hungate tubes with a gas phase of 100 % N_2. Pigment was tested according to Hu *et al.* (2015).

Antibiotic sensitivity was tested on MA with antibiotic discs, each disc containing neomycin (30 μg), penicillin G (10 μg), gentamicin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), carbenicillin (100 μg), rifampicin (5 μg), streptomycin (10 μg), kanamycin (30 μg), tetracycline (30 μg), ampicillin (10 μg), lincomycin (15 μg) or polymyxin B (100 U), respectively. The strain was incubated at 30 °C for 6 h before the antibiotic discs were attached to the MA. Sensitivity to antibiotics was observed after 2 days.

Single carbon source assimilation tests were performed in modified MB which contained 0.01 % yeast extract but lacked trypticase peptone. In addition, the modified MB was supplemented with the corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %) or organic acid (0.2 %). Acid production was tested using modified MOF medium (Leifson, 1963) which contained (per litre distilled water): Casitone (BD) 1 g, yeast extract 0.1 g, $(NH_4)_2SO_4$ 0.5 g, Tris buffer 0.5 g, phenol red 0.01 g, NaCl 13.75 g, $MgCl_2{\cdot}6H_2O$ 7.75 g, $MgSO_4{\cdot}7H_2O$ 2.0 g, $CaCl_2$ 0.5 g, KCl 1.0 g, $FeSO_4$ 0.001 g, adjusted to pH 7.5 with HCl. Oxidase activity was determined using oxidase reagent (bioMérieux). Catalase activity was determined by observing bubble production in 5 % (v/v) H_2O_2. Hydrolysis of casein and gelatin were tested on MA supplemented with 1 % skimmed milk (Difco) and 1 % gelatin, respectively. Degradation of starch was tested on MA supplemented with 0.2 % soluble starch (Smibert, 1994). Hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Zhu *et al.* (2011). MA containing 0.5 % L-tyrosine was used to test the degradation of L-tyrosine. Hydrolysis of hypoxanthine and xanthine were tested on MA supplemented with 0.4 % hypoxanthine and xanthine, respectively. H_2S production, methyl red and Voges–Proskauer reactions and indole production were determined as described by Wu *et al.* (2010). Other physiological characteristics and enzyme activities were also tested by using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions.

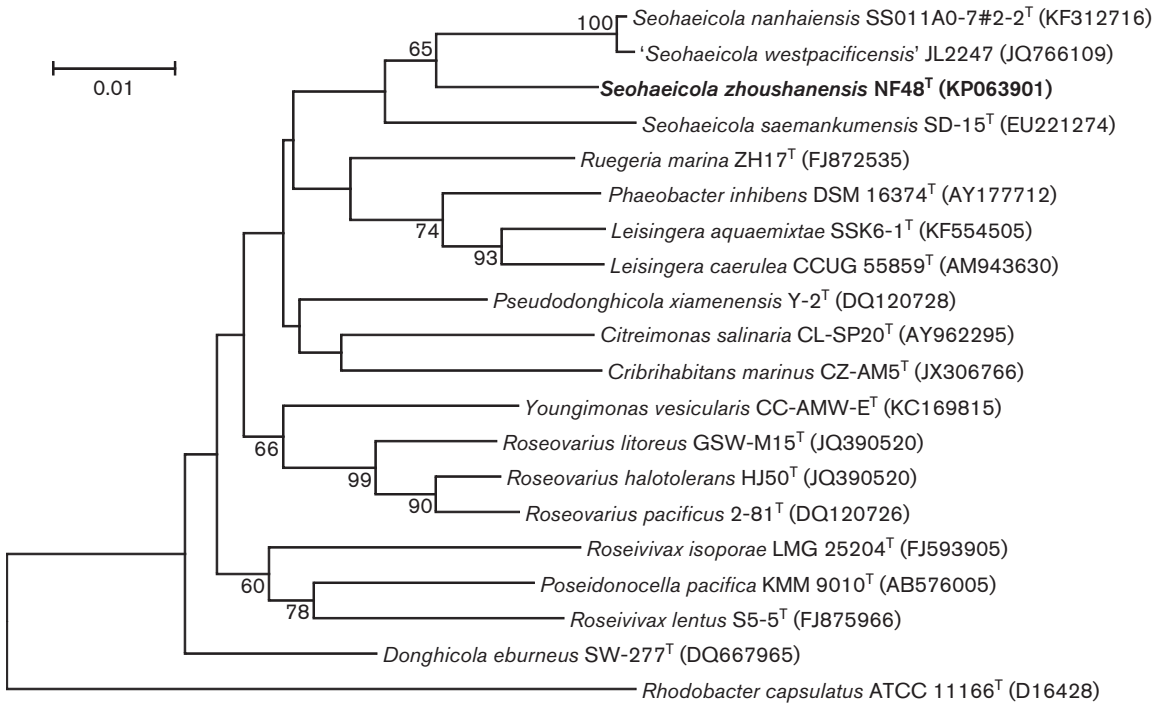

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships among strain NF48^T, other members of the genus *Seohaeicola* and other related genera. Bootstrap values are based on 1000 replicates; only values ≥50 % are shown. Bar, 0.01 substitutions per nucleotide position. *Rhodobacter capsulatus* ATCC 11166^T was used as an outgroup.

For cellular fatty acid analysis, cells of strain NF48^T, *S. nanhaiensis* SS011A0-7#2-2^T and *S. westpacificensis* JL2247^T were obtained and freeze-dried after incubation in MB at 30 °C for 24 h (Kuykendall *et al.*, 1988) while *S. saemankumensis* SD-15^T was incubated for 3 days because of slow growth. Fatty acids were then analysed according to the standard protocol of the Microbial Identification System (MIDI; Microbial ID). Isoprenoid quinones were analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). The polar lipids were extracted and separated by two-dimensional TLC on silica gel plates (10×10 cm; Merck 5554) (Minnikin *et al.*, 1977). The solvent systems of the two dimensions were prepared as described by Jia *et al.* (2014). The plates were then heated at 120 °C for 10–15 min after spraying with 50 % (v/v) sulfuric acid ethanol solution. Other reagents such as ninhydrin and molybdenum blue (Sigma) were used to detect aminolipids and phospholipids, respectively. In addition, the silica gel plates were sprayed with 5 % phosphomolybdic acid and heated at 140 °C for 10–15 min to identify the total polar lipids.

The Quick Bacteria Genomic DNA Extraction Kit (Dong-Sheng Biotech) was used to extract genomic DNA. The 16S rRNA gene was amplified by PCR using two universal primers, 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AGAAAGGAGGTGATCCAGCC-3′) (Embley, 1991), and the PCR products were then purified and cloned into pMD 19-T vector (TaKaRa) for sequencing. The 16S rRNA gene sequence was identified on the ExTaxon-e service (Kim *et al.*, 2012). Multiple sequence alignment was accomplished via the CLUSTAL W program of the MEGA 5 package (Tamura *et al.*, 2011). The neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods were used to reconstruct phylogenetic trees using the MEGA 5 software (Tamura *et al.*, 2011). Bootstrap values of three phylogenetic trees were based on 1000 replicates. The algorithm of Kimura's two-parameter model (Kimura, 1980) was chosen for the neighbour-joining method. For G+C content analysis, the genomic DNA was extracted as previously described and treated with P1 nuclease and calf intestine alkaline phosphatase before the G+C content was determined by reversed-phase HPLC (Mesbah & Whitman, 1989).

On the basis of 16S rRNA gene sequence similarity, strain NF48^T was a close relative of the three species of the genus *Seohaeicola*, sharing 97.2, 97.0 and 95.5 % similarity with *S. nanhaiensis* SS011A0-7#2-2^T, *S. westpacificensis* JL2247^T and *S. saemankumensis* SD-15^T, respectively. The four strains formed a cluster in the neighbour-joining (Fig. 1), maximum-likelihood (Fig. S1, available in the online Supplementary Matrerial) and maximum-parsimony (Fig. S2) trees, indicating that strain NF48^{T}may represent a novel species of the genus *Seohaeicola*.

Strain NF48^T did not produce bacteriochlorophyll *a*, which was consistent with data for other species of the genus *Seohaeicola*. The physiological and biochemical characteristics of strain NF48^T in comparison to the reference strains are shown in Table 1. All strains were negative for indole production, and hydrolysis of starch, hypoxanthine, xanthine and Tween 40. Strain NF48^T and members of the genus *Seohaeicola* shared numerous similarities, such as being negative for activities of lipase (C14), trypsin, chymotrypsin, *N*-acetyl-β-glucosaminidase, α-galactosidase, β-galactosidase,

Table 1. Differential characteristics between strain NF48^T and closely related species

Strains: 1, NF48^T; 2, *S. nanhaiensis* SS011A0-7#2-2^T; 3, *S. westpacificensis* JL2247^T; 4, *S. saemankumensis* SD-15^T. +, Positive; –, negative; W, weakly positive; ND, not detected/not reported.

Characteristic	1	2	3	4
Motility	+	–	+	–
Colony colour	Cream	Light yellow	Cream	Pale yellow
Growth at:				
4 °C	–	+	+*	–†
pH 9.5	–	–	+*	+†
API 20NE results:				
Nitrate reduction	+	–	–	+
API ZYM results:				
Valine arylamidase	+	+	+	W
Cystine arylamidase	W	W	W	–
α-Glucosidase	+	–	–	–
Voges–Proskauer test	–	–	–	+
Hydrolysis of:				
Gelatin	+	+	+	–
Tyrosine	+	–	–	–
Tween 20	+	+	+	–
Tween 60	–	+	+	–
Tween 80	+	–	+	+
Production of:				
H_2S	+	+	+	–
Utilization of:				
D-Glucose	+	+	+	–
Xylitol	+	–	–	–
Mannitol	–	W	+	–
Erythritol	W	W	W	–
Acid production from:				
Galactose	–	+	+	–
Sorbitol	–	–	W	W
D-Fructose	W	+	+	–
Mannose	–	–	+	–
Methyl red test	–	–	–	+
Susceptibility to:				
Polymyxin B	+	+	+*	–†
Tetracycline	–	–‡	+*	+†
DNA G+C content (mol%)	65.5	67.9‡	72.6*	63.4†

*Data from Xian *et al.* (2014).
†Data from Yoon *et al.* (2009).
‡Data from Xie *et al.* (2014).

β-glucosidase, α-mannosidase and α-fucosidase. All strains were sensitive to neomycin, penicillin G, streptomycin, ampicillin, gentamicin, kanamycin, carbenicillin and chloramphenicol. However, some characteristics were found to discriminate strain NF48^T from the reference strains. Unlike *S. westpacificensis* JL2247^T and *S. saemankumensis* SD-15^T, strain NF48^T could not grow at pH 9.5. *S. nanhaiensis* SS011A0-7#2-2^T and *S. westpacificensis* JL2247^T could grow at 4 °C, but strain NF48^T could not. In the API ZYM system, α-galactosidase was positive for strain NF48^T but negative for the reference strains. Strain NF48^T could hydrolyse tyrosine, in contrast to the recognized members of the genus *Seohaeicola.* In contrast to the closest phylogenetic neighbour *S. nanhaiensis* SS011A0-7#2-2^T, strain NF48^T could reduce nitrate to nitrite and hydrolyse Tween 80, but could not hydrolyse Tween 60. Strain NF48^T could utilize xylitol but could not utilize mannitol, which were in contrast to *S. nanhaiensis* SS011A0-7#2-2^T. Moreover, compared with *S. nanhaiensis* SS011A0-7#2-2^T, strain NF48^T could not produce acid from galactose.

The fatty acid profiles of strain NF48^T and the reference strains are listed in Table 2. The predominant fatty acid in all four strains was $C_{18:1}\omega7c$ and the strains had similarities in major components (≥5 %), such as cyclo $C_{19:0}\omega8c$. However, there were some characteristics that distinguished strain NF48^T from the reference strains. $C_{16:0}$ was a major fatty acid in strain NF48^T but not in *S. nanhaiensis* SS011A0-7#2-2^T or *S. westpacificensis* JL2247^T. $C_{16:0}$ 2-OH was a major fatty acid in strain NF48^T but was absent in *S. saemankumensis* SD-15^T. The fatty acid 11-methyl $C_{18:1}\omega7c$ was a major component in strain NF48^T but not in the closest phylogenetic neighbour *S. nanhaiensis* SS011A0-7#2-2^T. Moreover, an unknown fatty acid (ECL 11.799) was detected only in *S. saemankumensis* SD-15^T. The main respiratory quinone detected in strain NF48^T was ubiquinone-10 (Q-10), as in *S. saemankumensis* SD-15^T, *S. westpacificensis* JL2247^T and *S. nanhaiensis* SS011A0-7#2-2^T. The major polar lipids of strain NF48^T were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and four unidentified lipids (Fig. S3), a profile that was similar to those of other members of the genus *Seohaeicola*. However, in contrast to strain NF48^T, one phosphatidylmonomethylethanolamine was detected only in *S. nanhaiensis* SS011A0-7#2-2^T and *S. westpacificensis* JL2247^T. One phospholipid was not detected in strain NF48^T but found in other reference strains. Lipid L3 was detected only in strain NF48^T while lipids L5 and L6 were found only in *S. nanhaiensis* SS011A0-7#2-2^T and *S. westpacificensis* JL2247^T, respectively. Besides, two phospholipids (PL2 and PL3) were detected only in *S. saemankumensis* SD-15^T.

The G+C content of strain NF48^T was 65.5 mol%, which was similar to *S. saemankumensis* SD-15^T (63.4 mol%) (Yoon *et al.*, 2009) and *S. nanhaiensis* SS011A0-7#2-2^T (67.9 %) (Xie *et al.*, 2014), but lower than *S. westpacificensis* JL2247^T (72.6 mol%) (Xian *et al.*, 2014). Based on 16S rRNA gene sequences, the phylogenetic analysis revealed that strain NF48^T belonged to the genus *Seohaeicola*, and the most closely related strain was *S. nanhaiensis* SS011A0-7#2-2^T (97.2 % similarity). This similarity value was lower than the 16S rRNA gene threshold value for species delineation, 98.65 % (Kim *et al.*, 2014).

Based on the phenotypic, phylogenetic and chemotaxonomic properties presented in this study, strain NF48^T represents a novel species in the genus *Seohaeicola,* for which the name *Seohaeicola zhoushanensis* sp. nov. is proposed.

Description of *Seohaeicola zhoushanensis* sp. nov.

Seohaeicola zhoushanensis (zhou.shan.en'sis. N.L. fem. adj. *zhoushanensis* pertaining to Zhoushan Islands in China, where the type strain was isolated).

Cells are rod-shaped (1.1–3.9 μm×0.5–0.9 μm) with flagella (Fig. S4). Colonies grown on MA are milky, circular and translucent. Growth occurs at 10–40 °C (optimum 30 °C), at pH 5.5–9.0 (optimum 6.5–8.0) and with 0.5–7.0 % (w/v) NaCl (optimum 2.0 %). Weak growth is observed after incubation at 30 °C for 15 days under anaerobic conditions. Does not produce bacteriochlorophyll *a*. Sensitive to neomycin, penicillin G, streptomycin, erythromycin, rifampicin, ampicillin, gentamicin, kanamycin, carbenicillin,

Table 2. Cellular fatty acid contents of strain NF48^T and the reference strains

Strains: 1, NF48^T; 2, *S. nanhaiensis* SS011A0-7#2-2^T; 3, *S. westpacificensis* JL2247^T; 4, *S. saemankumensis* SD-15^T. Fatty acids comprising less than 1 % in all of four strains are not shown. –, Not detected.

Fatty acid	1	2	3	4
Straight-chain				
$C_{16:0}$	14.9	3.6	4.1	7.0
$C_{17:0}$	1.0	1.8	1.7	0.2
$C_{18:0}$	0.6	0.7	1.1	0.4
Unsaturated				
11-methyl $C_{18:1}\omega7c$	5.9	4.2	6.9	14.6
$C_{17:1}\omega8c$	0.5	1.0	1.0	–
$C_{18:1}\omega7c$	48.3	56.9	55.2	52.2
cyclo $C_{19:0}\omega8c$	12.9	9.4	14.7	9.7
Hydroxy				
$C_{10:0}$ 3-OH	4.2	4.7	3.6	0.5
$C_{12:1}$ 3-OH	3.6	4.4	3.2	–
$C_{16:0}$ 2-OH	5.7	8.0	5.0	–
Summed features*				
3	0.7	0.5	0.5	2.8
7	–	–	–	3.4
Unknown				
ECL 11.799	–	–	–	7.0

*Summed features represent two or more fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contains $C_{16:1}\omega7c$ and/or iso-C_{15} 2-OH, while summed feature 7 contains unknown ECL 18.846 and/or $C_{19:1}\omega6c$.

chloramphenicol and polymyxin B, but resistant to tetracycline and lincomycin. Oxidase- and catalase-positive. Gelatin, Tween 20, Tween 80 and L-tyrosine can be hydrolysed, but starch, hypoxanthine, xanthine, Tween 40 and Tween 60 cannot. Negative for indole production, methyl red and Voges–Proskauer test, but positive for H_2S production. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, α-glucosidase and naphthol-AS-BI-phosphohydrolase are positive; cystine arylamidase is weakly positive; lipase (C14), trypsin, α-chymotrypsin, *N*-acetyl-β-glucosaminidase, α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. In API 20NE tests, reduction of nitrate to nitrite, and assimilation of malate and adipate are positive, but reduction of nitrate to N_2, indole production, fermentation of D-glucose, arginine dihydrolase activity, hydrolysis of urea, aesculin, gelatin and β-galactosidase, and assimilation of L-arabinose, D-mannose, D-mannitol, maltose, *N*-acetyl-glucosamine, gluconate, capric acid and citric acid are negative. In the substrate utilization tests, D-glucose, xylitol, raffinose, sodium citrate and sodium acetate can be used for growth, while starch, D-ribose, D-fructose, D-xylose, trehalose, α-lactose, D-galatose, sucrose, inositol, maltose, mannitol, rhamnose, α-ketoglutaric acid and D-(−)-salicin cannot. Produces acid from D-glucose, D-ribose, D-xylose and raffinose, but not from α-lactose, D-galactose, D-mannose, maltose, sucrose, mannitol, rhamnose, trehalose, sorbitol, dulcitol or erythritol. The main respiratory quinone is ubiquinone 10 (Q-10). The major fatty acids ($\geq$5 % of the total fatty acids) are $C_{18:1}\omega7c$, $C_{16:0}$, cyclo $C_{19:0}\omega8c$, 11-methyl $C_{18:1}\omega7c$ and $C_{16:0}$ 2-OH. Major polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and two unidentified lipids.

The type strain, NF48^T (=KCTC 42650^T=MCCC 1K01157^T), was isolated from surface seawater around Zhoushan Islands in the East China Sea. The G+C content of the genomic DNA of the type strain is 65.5 mol%.

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