Maritimibacter alkaliphilus gen. nov., sp. nov., a genome-sequenced marine bacterium of the *Roseobacter* clade in the order *Rhodobacterales*

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A Gram-negative, chemoheterotrophic, strictly aerobic, alkaliphilic, rod-shaped marine bacterium, designated HTCC2654^T, was isolated from the western Sargasso Sea by using a dilution-to-extinction culturing method. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain HTCC2654^T belonged to the *Roseobacter* clade of the order *Rhodobacterales*. The 16S rRNA gene sequence similarity of the strain with respect to other members of the *Roseobacter* clade ranged from 90.4 to 95.1 %. In the phylogenetic analyses, the strain formed an independent phyletic line and could not be assigned to any other known genera of the *Rhodobacterales*. The DNA G+C content of strain HTCC2654^T was 61.7 mol% by HPLC and 64.1 mol% from genome sequences. The predominant constituents of the cellular fatty acids were $C_{16:0}$ 2-OH (27.3 %), 11-methyl $C_{18:1}\omega7c$ (19.6 %) and $C_{18:1}\omega7c$ (17.3 %), and the major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and phosphatidylcholine, which served to differentiate the strain from other members of the *Roseobacter* clade. On the basis of the taxonomic data obtained in this study, strain HTCC2654^T represents a novel genus and species, for which the name *Maritimibacter alkaliphilus* gen. nov., sp. nov. is proposed. The type strain is HTCC2654^T (=KCCM 42376^T=NBRC 102057^T).

The *Roseobacter* clade (Giovannoni & Rappé, 2000) in the order *Rhodobacterales* encompasses diverse members of marine alphaproteobacteria. Both culture-independent and culture-dependent surveys have shown that this clade is one of the most abundant 16S rRNA gene lineages in marine ecosystems. Since 2002, cultivation approaches involving high-throughput culturing using a dilution-to-extinction method (Button *et al.*, 1993; Connon & Giovannoni, 2002) have allowed many hitherto uncultured members of the *Roseobacter* clade to be cultured. Among the isolates in the *Roseobacter* clade that were obtained by high-throughput culturing, six strains, designated HTCC2516^T, HTCC2597^T, HTCC2601^T, HTCC2150, HTCC2255 and HTCC2654^T, have been subjected to full genome shotgun sequencing (http://www.moore.org/microgenome). The drafts for their whole genome sequences have been deposited in the GenBank database. The binomial names of strains HTCC2516^T, HTCC2597^T and HTCC2601^T have been validly published, on the basis of polyphasic taxonomy, as *Oceanicola granulosus* (Cho & Giovannoni, 2004), *Oceanicola batsensis* (Cho & Giovannoni, 2004) and *Pelagibaca bermudensis* (Cho & Giovannoni, 2006), respectively. Strains HTCC2150 and HTCC2255, isolated from the Oregon coast of the USA, do not form colonies on standard solid agar plates (such as marine agar 2216), which made polyphasic characterization difficult. Strain HTCC2654^T, isolated from the western Sargasso Sea (Atlantic Ocean) grew well on marine agar 2216 (MA; Difco), and thus here we can report on the characterization of this strain. On the basis of our taxonomic evaluations, strain HTCC2654^T represents a novel genus and species in the *Roseobacter* clade.

The extinction culture of strain HTCC2654^T was obtained using previously described high-throughput culturing approaches (Cho & Giovannoni, 2003; Connon & Giovannoni, 2002). The strain was subsequently purified as single colonies on MA after incubation at 25 °C for 4 days, and was stored as 10 % (v/v) glycerol suspensions in liquid nitrogen and in a deep-freezer at −86 °C. Unless stated otherwise, the strain was grown routinely on MA at 30 °C for characterization studies.

DNA extraction, 16S rRNA gene amplification, and sequencing of the PCR products were performed as described previously (Cho & Giovannoni, 2003). Almost-complete

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HTCC2654^T is DQ915443.

A thin-layer chromatogram indicating the polar lipids of strain HTCC2654^T and a transmission electron micrograph of a cell of strain HTCC2654^T are available with the online version of this paper.

16S rRNA gene sequences (1407 bp) of strain HTCC2654^T were obtained and used for phylogenetic analyses. Comparisons of the 16S rRNA gene sequence of strain HTCC2654^T with those held in GenBank and Ribosomal Database Project II (Cole *et al.*, 2005) showed that the strain belonged to the order *Rhodobacterales* of the class *Alphaproteobacteria*. To determine the phylogenetic relationships between strain HTCC2654^T and other members of the *Roseobacter* clade, phylogenetic inferences were performed with the ARB software package (Ludwig *et al.*, 2004) and PAUP* 4.0 beta 10 (Swofford, 2002). Reference sequences comprising more than 1300 bp were included in the phylogenetic analysis, and 1185 unambiguously aligned nucleotide positions were used. Comparisons with the ARB database showed that strain HTCC2654^T was distantly related to the members of the *Roseobacter* clade. The 16S rRNA gene sequence similarity of the strain with respect to species of the *Rhodobacterales* ranged from 90.4 to 95.1 %. The most closely related recognized species were *Roseovarius crassostreae* (95.1 % sequence similarity), followed by *O. batsensis* (94.5 %), *Roseovarius tolerans* (94.5 %), *Thalassobius mediterraneus* (94.4 %), *Jannaschia rubra* (94.1 %) and *Jannaschia cystaugens* (94.1 %). To clarify the phylogenetic position of the strain, phylogenetic trees were generated by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) approaches. The robustness of the neighbour-joining and maximum-parsimony trees was evaluated by performing bootstrap analyses based on 1000 resamplings. In all of the phylogenetic trees, strain HTCC2654^T failed to form any robust phylogenetic clades with members of the *Roseobacter* clade, but did form an independent phyletic line (Fig. 1). Although the genera *Roseovarius*, *Oceanicola*, *Thalassobius* and *Jannaschia* were related to strain HTCC2654^T with 94.1–95.1 % 16S rRNA gene sequence similarity, strain HTCC2654^T was not phylogenetically associated with any other genera of the *Roseobacter* clade. The most closely related species (on the basis of sequence similarity to strain HTCC2654^T), i.e. *R. crassostreae*, was phylogenetically separated from the three other *Roseovarius* species (Fig. 1), suggesting a need for the reclassification of *R. crassostreae* to another taxon. The distant relationship between strain HTCC2654^T and members of the order *Rhodobacterales* from the above phylogenetic analyses suggested that this strain represented a novel genus within the order *Rhodobacterales* in the class *Alphaproteobacteria*.

The genome size and DNA G+C content of strain HTCC2654^T were computed from the draft genome sequence (GenBank accession number AAMT00000000), consisting of six contigs, which was prepared by the J. Craig Venter Research Institute (Rockville, MD, USA). In addition, the DNA G+C content was analysed by using HPLC with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco) (Mesbah *et al.*, 1989). Respiratory quinones were investigated, using reversed-phase HPLC, by the

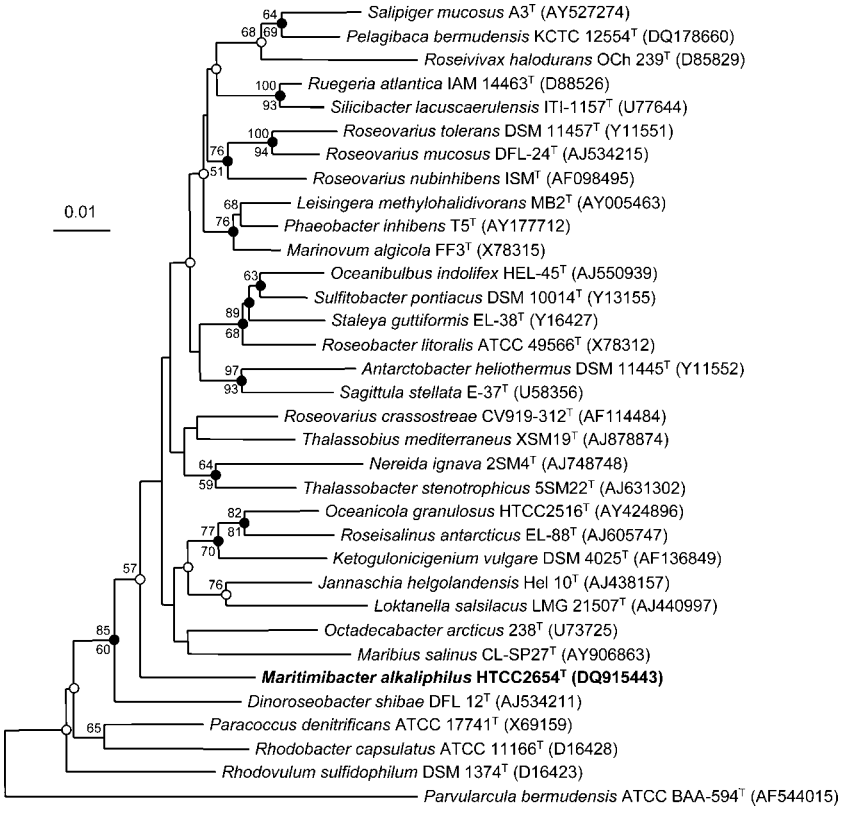

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain HTCC2654^T and representatives of the order *Rhodobacterales*. Bootstrap percentages (above 50 %) from both the neighbour-joining approach (above the nodes) and the maximum-parsimony approach (below the nodes) are shown. Filled and open circles indicate nodes recovered reproducibly by all treeing methods or by two treeing methods, respectively. Bar, 0.01 substitutions per nucleotide position.

Korean Culture Center of Microorganisms (Seoul, Korea). Cellular fatty acid methyl esters were prepared from a culture grown on MA at 30 °C for 3 days, and analysed, according to the instructions of the Microbial Identification System (MIDI), by the Center. The estimated genome size, based on genome sequencing data, was approximately 4.5 mbp, coding for 4757 open reading frames. The DNA G+C content of strain HTCC2654^T was 64.1 mol% from genome sequences and 61.7 mol% with the HPLC method. The only respiratory quinone detected was Q-10, which is a typical quinone of members of the *Roseobacter* clade. Polar lipids were extracted with chloroform/methanol/0.3 % NaCl (90 : 100 : 30, by vol.), purified and then identified as described by Minnikin *et al.* (1984). The major polar lipids found in strain HTCC2654^T were phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol (see Supplementary Fig. S1 available in IJSEM Online), the profile being similar to those of the genera *Ruegeria* and *Jannaschia*, except that they lack diphosphatidylglycerol. The major fatty acids found in strain HTCC2654^T, i.e. $C_{16:0}$ 2-OH (27.3 %), 11-methyl $C_{18:1}\omega7c$ (19.6 %), $C_{18:1}\omega7c$ (17.3 %) and $C_{16:0}$ (15.3 %), were different with respect to other members of the *Roseobacter* clade (Table 1). Characteristics that serve to differentiate strain HTCC2654^T from other species of the *Roseobacter* clade are listed in Table 1.

Phenotypic characterization was carried out as described in previous studies (Cho & Giovannoni, 2004; Smibert & Krieg, 1994), at 30 °C with MA as the basal medium. Cell morphology was examined by using energy-filtering transmission electron microscopy (LIBLA120; Carl Zeiss) and phase-contrast and epifluorescence microscopy (Nikon 80i; Nikon). The presence of poly-β-hydroxyalkanoate granules was checked for by using epifluorescence microscopy after staining of the cells with Nile blue A (Ostle & Holt, 1982). Anaerobic growth was tested on MA at 30 °C using both the MGC anaerobic system and AnaeroPACK Anaero (Mitsubishi Gas Chemical Company). Colony morphology, size and colour were examined using cultures grown aerobically on MA at 30 °C for 3 days. For detecting bacteriochlorophyll *a* and carotenoids, pigments of strain HTCC2654^T were extracted with acetone/methanol (1 : 1, v/v) and absorption spectra were determined using a scanning UV/visible spectrophotometer (Optizen 2120UV; Mechasis). The pH range and optimum were examined at pH values in the range 4.0–12.0. The pH was adjusted with 0.1 M HCl and 0.1 M NaOH. The NaCl concentration range and optimum for growth were determined in a medium that contained the following (l^{-1}): 1.0 g $MgCl_2.6H_2O$, 5.0 g $MgSO_4.7H_2O$, 0.7 g KCl, 0.5 g NH_4Cl, 0.27 g KH_2PO_4, 0.15 g $CaCl_2.2H_2O$, 0.1 g KBr, 0.04 g $SrCl_2.6H_2O$, 0.025 g H_3BO_3, 5.0 g peptone and 1.0 g yeast extract (pH 8.0) with 0–20 % NaCl (w/v). The catalase test was performed by adding 3.0 % hydrogen peroxide to fresh colonies, and oxidase activity was determined using Kovács' solution (Kovács, 1956). Biochemical tests and carbon-source utilization tests were carried out on API 20NE, API ZYM (both from bioMérieux) and Biolog GN2 microplates, with artificial seawater (l^{-1}: 25.0 g NaCl, 1.0 g $MgCl_2.6H_2O$, 5.0 g $MgSO_4.7H_2O$, 0.7 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.5 g NH_4Cl, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2.6H_2O$ and 0.025 g H_3BO_3), according to the manufacturers' instructions. The following antimicrobial agents were tested: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg).

Morphological, physiological, and biochemical characteristics of strain HTCC2654^T are listed in the genus and species descriptions and in Table 1. The strain was found to be Gram-negative, chemoheterotrophic, strictly aerobic, slightly alkaliphilic, to require NaCl for growth and to consist of non-motile, rod-shaped cells (see Supplementary Fig. S2 available in IJSEM Online). The strain did not produce bacteriochlorophyll *a* or poly-β-hydroxyalkanoate granules. These phenotypic characteristics, together with chemotaxonomic properties, serve to differentiate the strain from related genera of the *Roseobacter* clade (Table 1). The most important chemotaxonomic property that differentiates strain HTCC2654^T from other members of the clade is the cellular fatty acid composition. Generally, the most dominant cellular fatty acid detected in members of the *Roseobacter* clade is *cis*-7-octadecenoic acid, with a content of approximately 60–80 % (Martens *et al.*, 2006). The percentage of *cis*-7-octadecenoic acid in strain HTCC2654^T was only 17.3 %, whereas hydroxyl ($C_{16:0}$ 2-OH, 27.3 %) and methyl (11-methyl $C_{18:1}\omega7c$, 19.6 %) fatty acids were abundant. On the basis of phenotypic and chemotaxonomic traits, strain HTCC2654^T cannot be characterized as a member of any of the known genera within the *Roseobacter* clade.

The combined phenotypic, chemotaxonomic, and phylogenetic evidence conclusively demonstrates that strain HTCC2654^T represents a novel genus and species in the *Roseobacter* clade (order *Rhodobacterales*), for which the name *Maritimibacter alkaliphilus* gen. nov., sp. nov. is proposed.

Description of *Maritimibacter* gen. nov.

Maritimibacter (Ma.ri.ti′mi.bac′ter. L. adj. *maritimus* of the sea; N.L. masc. n. *bacter* a rod, bacterium; N.L. masc. n. *Maritimibacter* a rod-shaped bacterium of the sea).

Cells are Gram-negative, non-motile, strictly aerobic rods that are 1.4–2.5 μm long and 0.7–0.9 μm wide. Carotenoid pigments and bacteriochlorophyll *a* are not found. Do not produce exopolysaccharides or poly-β-hydroxyalkanoate granules. Chemoheterotrophic and require NaCl for growth. The predominant fatty acids are $C_{16:0}$ 2-OH, 11-methyl $C_{18:1}\omega7c$ and $C_{18:1}\omega7c$. The only respiratory quinone detected is Q-10. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol are the major polar lipids. The genus is phylogenetically affiliated to the

Table 1. Differential characteristics of strain HTCC2654^T and other related taxa within the *Roseobacter* clade

Taxa: 1, strain HTCC2654^T; 2, *R. crassostreae* (data from Boettcher *et al.*, 2005); 3, *Roseovarius* (Biebl *et al.*, 2005; González *et al.*, 2003; Labrenz *et al.*, 1999); 4, *Oceanicola* (Cho & Giovannoni, 2004; Gu *et al.*, 2007); 5, *Thalassobius* (Arahal *et al.*, 2005; Rüger & Höfle, 1992); 6, *Jannaschia* (Adachi *et al.*, 2004; Choi *et al.*, 2006; Macián *et al.*, 2005; Wagner-Döbler *et al.*, 2003). +, Positive reaction; −, negative reaction; V, variable data; ND, not determined.

Characteristic	1	2	3	4	5	6
Cell shape*	SR	R	R or SR	SR	CR	R
Pigmentation	Beige	Pink to beige	Red to beige	Yellow	Colourless to brown	Whitish to dark red
Flagella	−	+	−	−	V†	V‡
Growth at:						
40 °C	−	+	+	V§	−	−
4 °C	−	−	V‖	V§	−	V
pH 10–12	+	−	−	−	−	−
Bacteriochlorophyll *a*	−	−	V	−	−	−¶
Nitrate reduction	−	+	−	V#	V†	V**
Acid from glucose	+	−	−	−	−	−
Hydrolysis of:						
Gelatin	−	−	V††	−	V†	−
Aesculin	−	−	ND	V‡‡	ND	V
Major fatty acid composition (%):§§						
$C_{16:0}$	15.3	3.8	6.2–13.1	7.0–15.0	2.9–3.0	−
$C_{18:1}\omega7c$	17.3	85.3	69.4–76.4	31.0–81.2	68.8–84.6	45.0–79.4
$C_{16:0}$ 2-OH	27.3	−	−	−	−	−
$C_{18:1}$ 2-OH	7.7	−	−	−	−	−
11-Methyl $C_{18:1}\omega7c$	19.6	0.7	0–2.1	0–8.1	−	0–7.6
Polar lipids‖‖	PC, PG, PE	ND	PC, PG, PE, DPG¶¶	ND	ND	PC, PG, PE, DPG##
DNA G+C content (mol%)	61.7	59.0	62.9–66	64.7–71.5	57–59	59.1–64.6

*CR, Coccoid to rod-shape; R, rod; SR, short rod.

†*T. mediterraneus* is negative and *Thalassobius gelatinovorus* is positive for this characteristic.

‡All species are positive except *Jannaschia helgolandensis*.

§All species are positive except *Oceanicola nanhaiensis*.

‖All species are negative except *R. tolerans*.

¶Data from *J. rubra* and *J. seosinensis*.

#All species are negative except *O. nanhaiensis* (weakly positive).

**All species are negative except *Jannaschia seosinensis*.

††All species are negative except *Roseovarius mucosus*.

‡‡*O. granulosus* is positive and *O. batsensis* is negative for this characteristic.

§§For cellular fatty acid analyses, all species except three (*R. crassostreae*, *Roseovarius nubinhibens* and *J. helgolandensis*) were grown on MA. *R. crassostreae*, *R. nubinhibens* and *J. helgolandensis* were grown on SWT (Boettcher *et al.*, 2005), YSST (González *et al.*, 2003) and LBSS (Wagner-Döbler *et al.*, 2003) media, respectively.

‖‖DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

¶¶Data from *R. tolerans* and *R. mucosus*.

##Data from *J. helgolandensis*.

Roseobacter clade in the order *Rhodobacterales*. The type, and only, species is *Maritimibacter alkaliphilus*.

Description of *Maritimibacter alkaliphilus* sp. nov.

Maritimibacter alkaliphilus [al.ka.li′phi.lus. N.L. n. *alkali* (from the Arabic word *al-qaliy*) the ashes of saltwort; Gr. adj. *philos* loving; N.L. masc. adj. *alkaliphilus* loving alkaline conditions].

In addition to having the traits reported for the genus, colonies on MA are circular, smooth, convex, opaque, beige-coloured and 0.8–1.3 mm in diameter. Growth occurs at 16–37 °C, optimally at 30 °C, but not below 10 °C or above 42 °C. Growth occurs at pH 4–12 and 0.5–7.5 % NaCl,

optimally at pH 10 and 2.5–3.0 % NaCl. Oxidase- and catalase-positive. Does not produce indole. Produces acid from glucose utilization. Urea is hydrolysed. Negative for nitrate reduction, arginine dihydrolase, aesculin hydrolysis, gelatin liquefaction and β-galactosidase. Positive (using API ZYM) for alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase. In tests with Biolog GN2 microplates, the following carbon substrates are utilized: dextrin, glycogen, Tweens 40 and 80, maltose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, DL-lactic acid, succinic acid, succinamic acid, L-glutamic acid, inosine and thymidine. The following carbon substrates are not utilized: α-cyclodextrin, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, adonitol, L-arabinose, D-arabitol, D-mannitol, α-D-lactose, lactulose, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, *cis*-aconitic acid, citric acid, D-galacturonic acid, D-glucosaminic acid, D-galactonic acid, D-gluconic acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, D-glucuronic acid, formic acid, α-ketoglutaric acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-proline, L-threonine, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, DL-carnitine, urocanic acid, γ-aminobutyric acid, uridine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate. Sensitive to ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg) but resistant to penicillin G (10 μg). Cellular fatty acids comprise $C_{16:0}$ 2-OH (27.3 %), 11-methyl $C_{18:1}\omega7c$ (19.6 %), $C_{18:1}\omega7c$ (17.3 %), $C_{16:0}$ (15.3 %), $C_{18:1}$ 2-OH (7.7 %), cyclo $C_{19:0}\omega8c$ (4.8 %), $C_{16:1}\omega7c$ plus i-$C_{15:0}$ 2-OH (2.6 %), $C_{15:0}$ 2-OH (1.0 %), $C_{14:0}$ (0.7 %), $C_{10:0}$ 3-OH (0.6 %), $C_{17:0}$ (0.5 %), $C_{18:0}$ (0.5 %), $C_{17:0}$ 2-OH (0.5 %), $C_{15:0}$ (0.4 %), $C_{14:0}$ 2-OH (0.3 %), 10-methyl $C_{19:0}$ (0.2 %), $C_{10:0}$ (0.2 %), $C_{18:0}$ 2-OH (0.2 %), $C_{12:0}$ (0.2 %) and $C_{16:1}$ 2-OH (0.1 %). The DNA G+C content of the type strain is 61.7 mol% by HPLC and 64.1 mol% by genome shotgun sequencing.

The type strain, HTCC2654^T (=KCCM 42376^T=NBRC 102057^T), was isolated from Bermuda Atlantic Time Series Station in the western Sargasso Sea, Atlantic Ocean.

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