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# *Microbaculum marinum* gen. nov., sp. nov., isolated from deep seawater

Yue Su,[1] Shuaibo Han,[2] Ruijun Wang,[1] Xiaoyun Yu,[2] Geyi Fu,[1] Can Chen,[1] Chongya Zhang,[1] Yu Li,[2] Cong Sun[3,*] and Min Wu[1,2,*]

**Abstract**

A Gram-stain-negative, aerobic, rod-shaped and non-motile bacterium, designated HSF11$^T$, was isolated from deep seawater of the South China Sea. Growth was observed at 20–40 °C (optimum 35 °C), pH 5.0–8.5 (optimum pH 7.0–7.5) and with 0–7 % NaCl (optimum 2 %). Bacteriochlorophyll *a* and poly-$\beta$-hydroxybutyrate (PHB) granules were not detected. Nitrate could be reduced to nitrite. The major fatty acids (≥5 %) of strain HSF11$^T$ were $C_{19:0}$cyclo $\omega$8*c*, $C_{18:0}$ and $C_{16:0}$. The isoprenoid quinone was Q-10. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, two unknown phospholipids (PL1, PL2), one unknown aminolipid (AL1) and three unknown lipids (L1, L3, L4). The genomic DNA G+C content was 70.7 mol%. 16S rRNA gene sequence analysis indicated that strain HSF11$^T$ was most closely related to *Tepidamorphus gemmatus* DSM 19345$^T$ (95.7 % 16S rRNA gene sequence similarity) and *Lutibaculum baratangense* KCTC 22669$^T$ (95.2 %). On the basis of the genotypic, phenotypic, phylogenetic and chemotaxonomic characteristics, strain HSF11$^T$ represents a novel species of a new genus, for which the name *Microbaculum marinum* gen. nov., sp. nov. is proposed. The type strain of the type species is HSF11$^T$ (=KCTC 52 363$^T$=MCCC 1K03192$^T$).

The family *Rhodobiaceae* belongs to the order *Rhizobiales* of the class *Alphaproteobacteria*. At the time of writing, the family *Rhodobiaceae* comprised eight genera according to LPSN ([1]; www.bacterio.net/index.html): *Afifella* [2], *Anderseniella* [3], *Lutibaculum* [4], *Parvibaculum* [5], *Rhodobium* [6], *Rhodoligotrophos* [7], *Roseospirillum* [8] and *Tepidamorphus* [9]. Members of the family were isolated from various habitats, such as a saltern, sediment, a mud volcano, freshwater and hot spring. Here we present phenotypic, genotypic and chemotaxonomic characterization of strain HSF11$^T$, which was isolated from deep seawater in the South China Sea.

Strain HSF11$^T$ was isolated from a deep seawater sample which was collected in October 2011 from the South China Sea (19° 22′ N 115° 38′ E) at a depth of 2.5 km. A standard dilution-plating method [10] on modified marine agar 2216 [11] at 28 °C was used for isolation. After incubation for 15 days, diverse colonies were formed on the modified MA, including strain HSF11$^T$, based on colony morphology [4]. After purification, the strain was preserved as suspensions with 30 % (v/v) glycerol at −80 °C and freeze-dried for long-term preservation. Reference strains in this study were *Tepidamorphus gemmatus* DSM 19345$^T$ and *Lutibaculum baratangense* KCTC 22669$^T$.

The Gram-staining reaction was tested with the method as described by Claus [12] and observed by optical microscopy (BX40; Olympus). Gliding motility was performed by the hanging-drop method [13]. Transmission electron microscopy (JEM-1230; JEOL) was used to detect the presence of flagellum when cells were in the exponential phase of growth on a marine agar 2216 (MA; Difco) plate at 35 °C. Poly-$\beta$-hydroxybutyrate (PHB) granules were assessed by staining with Sudan Black [14]. Bacteriochlorophyll *a* was extracted and tested according to Kumar *et al.* [4]. The temperature range for growth was determined in marine broth 2216 (MB; Difco) at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45, 50 and 55 °C. The pH range for growth in MB was measured from pH 4.5 to pH 10.0 with an interval of 0.5 pH, supplementing with buffering agents 40 mM MES (for pH 4.5–6.0), MOPS (for pH 6.5–7.0), Tricine (for pH 7.5–8.5) and CAPSO (for pH 9.0–10.0), respectively. Tolerance of NaCl was tested with NaCl concentrations at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 % (w/v).

**Author affiliations:** [1]Ocean College, Zhejiang University, Zhoushan 316000, PR China; [2]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China; [3]College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, PR China.
***Correspondence:** Cong Sun, michael_sc@sina.com; Min Wu, wumin@zju.edu.cn
**Keywords:** deep seawater; *Proteobacteria*.
**Abbreviations:** FAMEs, fatty acid methyl esters; PHB, poly-$\beta$-hydroxybutyrate.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HSF11$^T$ is KU195387.
Four supplementary figures and one supplementary table are available with the online Supplementary Material.

Oxidase activity was determined by using the oxidase reagent (bioMérieux). Catalase activity was tested by observing bubble production after addition of 3 % (v/v) $H_2O_2$to the cells. $H_2S$ production, indole production, methyl red (MR) and Voges–Proskauer (VP) tests were assayed according to Zhang *et al.* [15]. Hydrolysis of skimmed milk, gelatin and CM-cellulose were separately determined on MA with 1 % (w/v) skimmed milk, 1 % (w/v) gelatin and 1 % (w/v) CM-cellulose, respectively. Degradation of filter paper was tested in MB with the addition of five filter paper discs. Degradation of starch and L-tyrosine and hydrolysis of Tweens 20, 40, 60 and 80 were examined as described by Sun *et al.* [16]. Other biochemical properties and enzyme activities were tested using API ZYM and API 20NE kits (bioMérieux) following the manufacturer's instructions.

Single carbon source assimilation tests were performed in modified MB which contained 0.03 % yeast extract and removal of trypticase peptone. Yeast extract was found to be essential for growth. The modified MB was supplemented with the corresponding filter-sterilized 0.4 % (w/v) carbon source, such as sugar, alcohol and organic acid. Assimilation of carbon source was scored by Su *et al.* [17]. Acid production was examined with the API 50CH kit (bioMérieux) and modified MOF medium [18] which contained (per litre distilled water): 1 g Casitone (BD), 0.1 g Yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.5 g Tris buffer, 0.01 g Phenol red, 13.75 g NaCl, 7.75 g $MgCl_2 . 6H_2O$, 2.0 g $MgSO_4 . 7H_2O$, 0.5 g $CaCl_2$, 1 g KCl and 0.001 g $FeSO_4$, adjusting pH with HCl to 7.5.

Anaerobic growth was observed in the modified MB with sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM) and L-arginine (5 g $l^{-1}$) as electron acceptors, cysteine (1 g $^{-1}$) as reductant and resazurin (1 $mg^{-1}$) as oxygen indicator. Hungate tubes filled with $N_2$ were used for the test. MA plates with exponential phase cells spread and incubated at 35 °C for 6 h were used for antimicrobial susceptibility tests. The pretreated plate attached with antibiotics discs was incubated at 35 °C for one week. The following antibiotics were tested (μg per disc unless otherwise stated): carbenicillin (100), bacitracin (0.04 U), gentamicin (100), erythromycin (15), chloramphenicol (30), kanamycin (30), streptomycin (10), rifampicin (5), ampicillin (10), tetracycline (30), cotrimoxazole (1.25), nalidixic acid (30) and minocycline (30).

The 16S rRNA gene of strain HSF11$^T$ was amplified by PCR using universal primer pair 27F (5′-GAGAGTTTGATC MTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTG TTACGAC-3′), and then cloned into the pMD 19-T vector (TaKaRa) for sequencing. The complete 16S rRNA gene sequence was identified using the EzTaxon-e service [19]. Multiple sequences alignment was performed with the CLUSTAL X program of the MEGA 5 software package [20]. Phylogenetic trees were reconstructed using the neighbour-joining [21], maximum-likelihood [22] and maximum-parsimony [23] methods with the MEGA 5 package. Bootstrap analysis was based on 1000 replications. The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* [24].

For fatty acid methyl esters (FAMEs), polar lipid and isoprenoid quinone analyses, cells of strain HSF11$^T$ and *L. baratangense* KCTC 22669$^T$ in the exponential phase after incubation in MB at 35 °C for 2 days were freeze-dried, while *T. gemmatus* DSM 19345$^T$ was incubated at 35 °C for 5 days because of slow growth in MB. FAMEs were extracted as described by Kuykendall *et al.* [25] and analysed by using the Sherlock Microbial Identification System (MIDI). Polar lipids were extracted with 80 ml chloroform/methanol/water (1 : 2 : 1, by vol), separated on silica gel plates (10×10 cm, Merck 5554) and then analysed as described by Fang *et al.* [26] and Xu *et al.* [27]. Isoprenoid quinones were analysed as described by Minnikin *et al.* [28].

Cells of strain HSF11$^T$ were Gram-stain-negative rods measuring 0.4–0.5 μm wide and 1.0–1.3 μm long (Fig. S1, available in the online Supplementary Material). They were strictly aerobic and non-motile without flagella. Strain HSF11$^T$ grew at 20–40 °C (optimum 35 °C), at pH 5.0–8.5 (optimum pH 7.0–7.5) and with 0–7 % NaCl (optimum 2 %). Strain HSF11$^T$ was susceptible to carbenicillin (100 μg), erythromycin (15 μg), rifampicin (5 μg) and ampicillin (10 μg), partly susceptible to gentamicin (100 μg), chloramphenicol (30 μg) and streptomycin (10 μg) and resistant to bacitracin (0.04 U), kanamycin (30 μg), tetracycline (30 μg), cotrimoxazole (1.25 μg), nalidixic acid (30 μg) and minocycline (30 μg). Detailed physiological and biochemical properties are displayed in Table 1 and the species description.

On the basis of 16S rRNA gene sequence similarity, strain HSF11$^T$ was affiliated with the family *Rhodobiaceae* and shared high sequence similarities with the genera *Tepidamorphus* and *Lutibaculum*, of which type species were *T. gemmatus* DSM 19345$^T$ (95.7 % 16S rRNA gene sequence similarity) and *L. baratangense* KCTC 22669$^T$ (95.2 %), respectively, sharing low sequence similarities (<95 %) with other species of the family *Rhodobiaceae*. After reconstruction of neighbour-joining (Fig. 1), maximum-likelihood (Fig. S3) and maximum-parsimony (Fig. S4) trees, the three above strains formed an independent cluster, which clearly showed that strain HSF11$^T$ belonged to the family *Rhodobiaceae* and represented a novel genus.

The genomic DNA G+C content of strain HSF11$^T$ was 70.7 mol%, which was higher than *T. gemmatus* DSM 19345$^T$ (66.9±0.2 mol%) [9] and *L. baratangense* KCTC 22669$^T$ (70.5 mol%) [4]. Q-10 was the only isoprenoid quinone in strain HSF11$^T$, which was the same as *T. gemmatus* DSM 19345$^T$, but different from *L. baratangense* KCTC 22669$^T$ (Q-10, Q-9) [4]. The fatty acid profiles of strain HSF11$^T$ and the reference strains are shown in Table S1. The major fatty acids (≥5 %) of strain HSF11$^T$ were $C_{19:0}$ cyclo *ω8c* (79.8 %), $C_{18:0}$ (8.9 %) and $C_{16:0}$ (5.9 %). Compared with *L. baratangense* KCTC 22669$^T$, whose predominant component was $C_{18:1}\omega7c$, strain HSF11$^T$ possessed $C_{19:0}$ cyclo *ω8c* as its predominant component. For

**Table 1.** Comparison of the phenotypic characteristics of strain HSF11$^T$ and related reference strains

Strains: 1, HSF11$^T$; 2, *T. gemmatus* DSM 19345$^T$; 3, *L. baratangense* KCTC 22669$^T$. All strains are positive for oxidase and catalase and negative for hydrolysis of starch, L-tyrosine, CM-cellulose, filter paper and Tweens 20 and 80, indole production, methyl red and Voges–Proskaur tests. +, Positive; w, weakly positive; −, negative.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Isolation source | Deep seawater | Hot spring*[a] | Mud volcano[b] |
| Cell size (width×length; μm) | 0.4–0.5×1.0–1.3 | 0.5–2.0×1.0–1.5[a] | 0.9–1.2×1.5–2[b] |
| Flagella | − | +[a] | +[b] |
| Motility | − | +[a] | +[b] |
| Temperature range (optimum) (°C) | 20–40 (35) | 30–50 (45–50)[a] | 25–37 (30–37)[b] |
| pH range (optimum) | 5.0–8.5 (7.0–7.5) | 6.5–9.5 (7.5–8.5)[a] | 7–9 (8)[b] |
| Salt tolerance (optimum) (%, w/v) | 7 (2) | 3 (0–1)[a] | 4 (2)[b] |
| Hydrolysis of: | | | |
| Gelatin | w | + | + |
| Skimmed milk | − | + | − |
| Tween 40 | − | + | − |
| Tween 60 | − | + | − |
| API ZYM tests | | | |
| Lipase (C14) | − | − | w |
| Valine arylamidase | + | w | w |
| Cystine arylamidase | w | − | + |
| α-Chymotrypsin | − | − | + |
| α-Glucosidase | − | − | + |
| API 20NE tests | | | |
| Urease | + | + | − |
| Acid production from: | | | |
| D-Galactose | − | + | + |
| D-Glucose | − | + | + |
| D-Fucose | − | + | − |
| Melibiose | − | + | + |
| Potassium 2-ketogluconate | + | − | − |
| Potassium 5-ketogluconate | + | − | − |
| Utilization of: | | | |
| D-Xylose | w | − | + |
| D-Ribose | − | + | + |
| D-Fructose | w | + | w |
| Succinate | + | − | + |
| Malonate | − | w | + |
| Acetate | + | w | + |
| DNA G+C content (mol%) | 70.7 | 66.9±0.2[a] | 70.5[b] |
| Isoprenoid quinone | Q-10 | Q-10[a] | Q-10, Q-9[b] |
| Major fatty acids | $C_{19:0}$ cyclo ω8*c*, $C_{18:0}$, $C_{16:0}$ | $C_{19:0}$ cyclo ω8*c*, $C_{18:1}\omega7c$, $C_{18:0}$, 11-methyl $C_{18:1}\omega7c$, $C_{20:1}\omega7c$ | $C_{18:1}\omega7c$, $C_{19:0}$ cyclo ω8*c* |
| Polar lipids† | DPG, PG, PL1, PL2, AL1, L1, L3, L4 | DPG, PG, PC, PE, PL2, AL1, AL2, AL3 | DPG, PG, PE, PL2, L1, L2, L3 |

*Data from: *a*, Albuquerque *et al.* [9]; *b*, Kumar *et al.* [4].

†DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PL, unknown phospholipid; AL, unknown aminolipid; L, unknown lipid.

*T. gemmatus* DSM 19345$^T$, although $C_{19:0}$ cyclo ω8*c* was also detected to account for the maximum proportion of the total fatty acids, strain HSF11$^T$ contained a much higher amount of $C_{19:0}$ cyclo ω8*c* (79.8 %), more than 1.5 times that of *T. gemmatus* DSM 19345$^T$ (45.8 %). Moreover, $C_{20:1}\omega7c$ was absent from strain HSF11$^T$, while there was

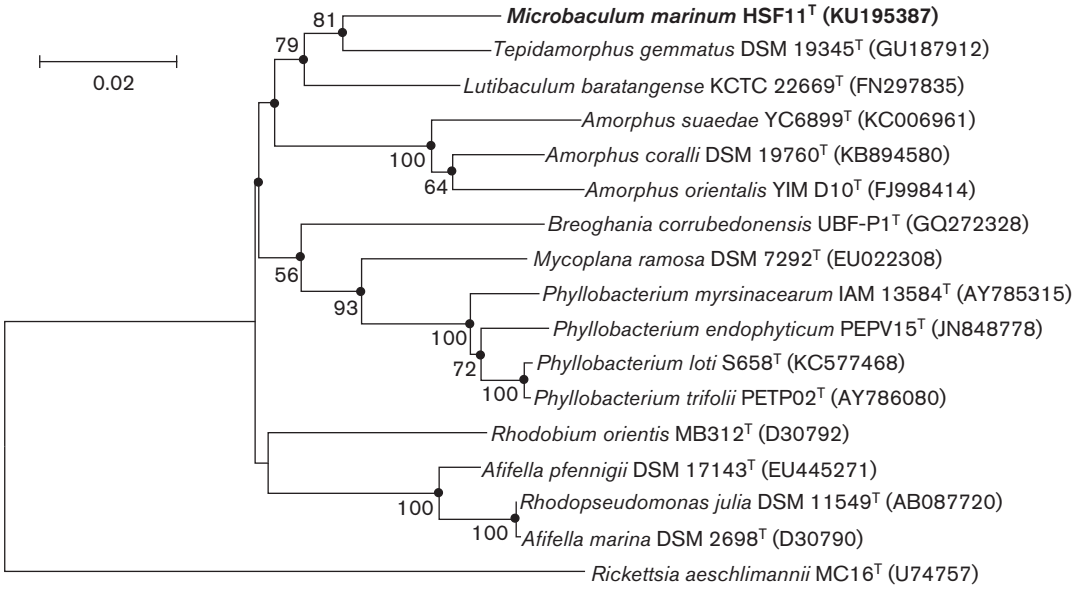


**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship of strain HSF11$^T$ with related taxa. *Rickettsia aeschlimannii* MC16$^T$ was used as an outgroup. Numbers at nodes are bootstrap values based on 1000 replications; only values ≥50 % are shown. Filled circles indicate nodes also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 0.02 substitutions per nucleotide position.

more than 5 % $C_{20:1}\omega7c$ detected in *T. gemmatus* DSM 19345$^T$. In contrast with a trace amount (0.9 %) in strain HSF11$^T$, 11-methyl $C_{18:1}\omega7c$ was found at 9.3 % in *T. gemmatus* DSM 19345$^T$. In addition, $C_{18:1}\omega7c$ represented less than 1 % of the total fatty acids in strain HSF11$^T$, but was detected at 17.1 and 57.6 % in *T. gemmatus* DSM 19345$^T$ and *L. baratangense* KCTC 22669$^T$, respectively.

There were also differences in the polar lipid profiles between strain HSF11$^T$ and the two reference strains. Strain HSF11$^T$ contained diphosphatidylglycerol, phosphatidylglycerol, two unknown phospholipids (PL1, PL2), one unknown aminolipid (AL1) and three unknown lipids (L1, L3, L4). Compared with the presence of phosphatidylethanolamine in both *T. gemmatus* DSM 19345$^T$ and *L. baratangense* KCTC 22669$^T$, the absence of phosphatidylethanolamine in strain HSF11$^T$ distinguished strain HSF11$^T$ from the reference strains. Furthermore, phosphatidylcholine and AL3 were found in *T. gemmatus* DSM 19345$^T$, but were not detected in strain HSF11$^T$. The detailed differences in polar lipid profiles are shown in Fig. S2.

Apart from the above, there were also some differences in phenotypic characteristics between strain HSF11$^T$ and the reference strains. Cells of strain HSF11$^T$ had a narrower width (0.4–0.5 µm) than *T. gemmatus* DSM 19345$^T$ (0.5–2.0 µm) and *L. baratangense* KCTC 22669$^T$ (0.9–1.2 µm). In contrast to the two reference strains with flagella, strain HSF11$^T$ had no flagella. Strain HSF11$^T$ had a higher NaCl tolerance (7 %) than *T. gemmatus* DSM 19345$^T$ (3 %) and *L. baratangense* KCTC 22669$^T$ (4 %). Compared with *T. gemmatus* DSM 19345$^T$, strain HSF11$^T$ could not hydrolyse skimmed milk, Tween 40 or Tween 60. In API ZYM tests, lipase (C14), $\alpha$-chymotrypsin and $\alpha$-glucosidase were negative in strain HSF11$^T$, but positive in *L. baratangense* KCTC 22669$^T$. Strain HSF11$^T$ could not produce acid from D-galactose and D-glucose, while the two reference strains could. More detailed differences are displayed in Table 1.

On the basis of the phylogenetic, genomic, chemotaxonomic and phenotypic characteristics, we propose that strain HSF11$^T$ represents as a novel species of a new genus of the family *Rhodobiaceae*, with the name *Microbaculum marinum* gen. nov, sp. nov.

## DESCRIPTION OF *MICROBACULUM* GEN. NOV.

*Microbaculum* (Mi.cro.ba′cu.lum. Gr. adj. *mikros* small; L. neut. n. *baculum* rod; N.L. neut. n. *Microbaculum* a small rod).

Gram-stain-negative, rod-shaped, aerobic and non-motile. Oxidase and catalase activities are positive. The isoprenoid quinone is Q-10. The major fatty acids (≥5 % of the total fatty acids) are $C_{19:0}$ cyclo $\omega8c$, $C_{18:0}$ and $C_{16:0}$. The total polar lipids are DPG, PG, two unknown phospholipids (PL1, PL2), one unknown aminolipid (AL1) and three unknown lipids (L1, L3, L4). Phylogenetically, the genus is affiliated to the family *Rhodobiaceae* of the order *Rhizobiales*.

The type species is *Microbaculum marinum*.

## DESCRIPTION OF *MICROBACULUM MARINUM* SP. NOV.

*Microbaculum marinum* (ma.ri′num. L. neut. adj. *marinum* belonging to the sea, marine).

Displays the following properties in addition to those given in the genus description. Colonies on MA after incubation for 4 days at 35 °C are circular, opaque, regular edged, beige, shiny, convex and 1.0–1.5 mm in diameter. Cells are approximately 0.4–0.5 µm wide and 1.0–1.3 µm long. Growth occurs at 20–40 °C (optimum 35 °C), at pH 5.0–8.5 (optimum pH 7.0–7.5) and with 0–7 % NaCl (optimum 2 %). Bacteriochlorophyll *a* and PHB granules are not detected. Gelatinase activity is weakly positive. Hydrolysis of skimmed milk, starch, L-tyrosine, CM-cellulose, filter paper and Tweens 20, 40, 60 and 80 are negative, as well as indole production, $H_2S$ production, methyl red and Voges–Proskaur tests. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, weakly positive for cystine arylamidase and negative for lipase (C14), $\alpha$-chymotrypsin, $\alpha$-galactosidase, $\beta$-galactosidase, $\beta$-glucuronidase, $\alpha$-glucosidase, $\beta$-glucosidase, *N*-acetyl-$\beta$-glucosaminidase, $\alpha$-mannosidase and $\alpha$-fucosidase. In API 20NE tests, nitrate is reduced to nitrite but not to nitrogen. Positive for urease, weakly positive for hydrolysis of gelatin and negative for production of indole, arginine dihydrolase, fermentation of glucose, hydrolysis of aesculin, $\beta$-galactosidase, assimilation of glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, caprate, adipate, malate, citrate and phenylacetate. In API 50CH tests, acid is produced from glycerol, L-arabitol, D-xylose, D-fucose, potassium 2-ketogluconate and potassium 5-ketogluconate. In utilization of carbon sources test, positive for pyruvate, succinate and acetate, weakly positive for D-glucose, D-xylose, D-galactose, D-fructose and D-arabinose and negative for L-sorbin, D-ribose, maltose, trehalose, sucrose, lactose, melibiose, cellobiose, D-mannose, erythritol, xylitol, sorbitol, insitol, mannitol, ribitol, salicin, citrate and malonate.

The type strain, HSF11$^T$ (=KCTC 52363$^T$=MCCC 1K03192$^T$) was isolated from deep seawater of the South China Sea at a depth of 2.5 km. The genomic DNA G+C content of the type strain is 70.7 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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