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Alcanivorax profundi sp. nov., isolated from deep seawater of the Mariana Trench

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Abstract

A Gram-stain-negative, rod-shaped, non-motile, strictly aerobic strain, designated as MTEO17^T, was isolated from a 1000 m deep seawater sample of the Mariana Trench. Growth was observed at 10–45 °C (optimum, 37 °C), in the presence of 0.0–12.0 % NaCl (w/v; optimum, 3.0 %) and at pH 6.0–10.0 (optimum, pH 7.0–8.0). Phylogenetic analysis, based on the 16S rRNA gene sequence, revealed that strain MTEO17^T belonged to the genus *Alcanivorax* and showed the highest sequence similarity of 97.9 % to *Alcanivorax nanhaiticus* MCCC 1A05629^T. The estimated average nucleotide identity and DNA–DNA hybridization values between strain MTEO17^T and *A. nanhaiticus* MCCC 1A05629^T were 78.98 and 23.80 %, respectively. The significant dominant fatty acids were $C_{16:0}$, summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$) and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$). The polar lipids comprised two phosphatidylethanolamines, one phosphatidylglycerol, one unidentified phospholipid and four unidentified polar lipids. The DNA G+C content of strain MTEO17^T was 57.5 %. On the basis of the polyphasic evidence, strain MTEO17^T is proposed to represent a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax profundi* sp. nov. is proposed. The type strain is MTEO17^T (=KCTC 52694^T=MCCC 1K03252^T).

The genus *Alcanivorax*, to date the best-known hydrocarbonoclastic bacteria affiliated to *Gammaproteobacteria*, was first proposed by Yakimov *et al.* [1] with the description of *Alcanivorax borkumensis* isolated from enriched cultures of seawater and sediment collected from the North Sea. At the time of writing, the genus *Alcanivorax* contained 11 recognized species isolated exclusively from the marine environment: *Alcanivorax borkumensis* [1], *Alcanivorax jadensis* [2, 3], *Alcanivorax venustensis* [3], *Alcanivorax dieselolei* [4], *Alcanivorax balearicus* [5], *Alcanivorax hongdengensis* [6], *Alcanivorax pacificus* [7], *Alcanivorax marinus* [8], *Alcanivorax xenomutans* [9], *Alcanivorax gelatiniphagus* [10] and *Alcanivorax nanhaiticus* [11]. All the *Alcanivorax* species are Gram-negative, aerobic, chemoheterotrophic, rod-shaped and show high salt tolerance and the capability of aliphatic hydrocarbon utilization. In the present study, a novel *Alcanivorax* strain, designated as MTEO17^T, was isolated from a deep seawater sample collected at a depth of 1000 m in the Mariana Trench (11° 22.569′ N, 142° 18.105′ E) during a cruise of R/V *Dong Fang Hong* 2 on January, 2016. The accurate phenotypic and taxonomic characteristics of strain MTEO17^T, a potential novel species of the genus *Alcanivorax*, is reported.

The seawater sample was collected using a Sealogger CTD (SBE 25, Sea-Bird Co.) rosette water sampler, and was serially diluted with 0.85 % (w/v) sterile sodium chloride solution. The dilutions were spread on marine agar 2216E (MA; Becton Dickinson), and incubated at room temperature onboard and at 37 °C in the laboratory. Strain MTEO17^T, which formed a white, round and transparent colony, was picked and streaked three times on MA for purification. The strain was preserved in sterile 0.85 % (w/v) sodium chloride solution supplemented with 15 % (v/v) glycerol at −80 °C. *A. nanhaiticus* MCCC 1A05629^T, *A. hongdengensis* MCCC 1A01496^T and *A. jadensis* MCCC 1A01030^T (obtained from the Marine Culture Collection of China), and *A. borkumensis* CGMCC 1.2815^T(obtained from the

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Abbreviations: ANI, average nucleotide identity; COG, cluster of orthologous group; GGDC, Genome-to-Genome Distance Calculator; GL, glycolipid; L, unidentified lipid; MA, marine agar; MB, marine broth; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid.
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The GenBank accession number for the 16S rRNA gene sequence of *Alcanivorax profundi* MTEO17^T *is* KY352038. The Whole Genome Shotgun project of strain MTEO17^T is deposited in GenBank under the accession number QYYA00000000.
Three supplementary figures and one supplementary table are available with the online version of this article.

Table 1. Differential characteristics of *Alcanivorax profundi* sp. nov. and other members of the genus *Alcanivorax*

Strains: 1, *Alcanivorax profundi* sp. nov. MTEO17^T; 2, *Alcanivorax nanhaiticus* MCCC 1A05629^T; 3, *Alcanivorax borkumensis* CGMCC 1.2815^T; 4, *Alcanivorax jadensis* MCCC 1A01030^T; 5, *Alcanivorax hongdengensis* MCCC 1A01496^T. All strains were non-motile, negative for alginase, cellulase, casease, DNase and chitinase; positive for hydrolysis of Tweens 20, 40 and 80. In the API 20NE system, the strains were negative for all substrates except for β-galactosidase, gelatin and capric acid. In the API ZYM system, all strains are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase; negative for cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase. Data for catalase, oxidase and G+C content for the reference strains are from the study of Lai *et al.* [11].

Characteristic	1	2	3	4	5
Range (optimum) for growth:					
Temperature (°C)	10–45 (37)	4–42 (28)	4–42 (28)	10–45 (28)	10–42 (28)
NaCl (%)	0–12 (3)	0.5–15 (3)	1–12 (3)	0.5–15 (3)	0.5–15 (3)
Hydrolysis of:					
Catalase	−	+	+	−	−
Oxidase	+	+	W	+	−
Utilization of:					
β-Galactosidase	−	−	−	+*	−
Gelatin	+	−	+	−	+
Capric acid	−	+	−	−	−
Enzyme activities:					
Lipase (C14)	−	+	+	+	-*
Valine arylamidase	+	W	W	W	W
Trypsin	−	+*	−	+*	−
N-acetyl-β-glucosaminidase	−	−	+*	−	−
DNA G+C content (%)	57.50	56.44	54.70	58.37	60.68

*Results differ from original studies.

China General Microbiological Culture Collection Centre) were used as reference strains. They were cultured under the same conditions as strain MTEO17^T [MA/marine broth 2216 (MB); 37 °C], unless otherwise specified.

Cell morphology and flagellation were determined by transmission electron microscopy (JEM-1200EX, JEOL) after negative staining with 1 % (w/v) phosphotungstic acid (Fig. S1, available in the online version of this article). Gram-staining was performed using standard methods [12]. Gliding motility was tested using the hanging drop method [13] after cultures were grown on MA at 37 °C for 1 day. Anaerobic growth was tested on MA in a jar filled with nitrogen and a packet of AnaeroPack-Anaero (Mitsubishi Gas Chemical) at 37 °C for up to 1 month. The temperature range for growth was determined by incubation of cells on MA at 10–47 °C (10, 16, 20, 24, 28, 32, 37, 42, 45 and 47 °C) for 7 days, and at 0 °C and 4 °C for at least 30 days. Tolerance of pH was evaluated by incubation of cells in MB (5 g peptone, 1 g yeast extract and 0.01 g $FePO_4$ in 1 l seawater) and measurement of the optical densities (wavelength 590 nm) in MB tubes. pH was assessed over the range 5.0–11.0 at intervals of 1 unit using the following buffer systems: MES (pH 5.0–6.0), MOPS (pH 7.0), tricine (pH 8.0), TAPS (pH 9.0), CAPS (pH 10.0) and Na_2CO_3/$NaHCO_3$ (pH 11.0). Tolerance of salinity was also evaluated using MB medium. The NaCl concentration was adjusted to 0 to 15.0 % (w/v) at intervals of 1.0 % (including 0.5 %) by mixing different proportions of distilled water and seawater. Catalase and oxidase activities, and hydrolysis of alginate, starch, casein, gelatin, cellulose, chitin and Tweens 20, 40 and 80 were determined according to the methods described by Tindall *et al.* [14] with the exception that distilled water was replaced by sterile seawater. DNase activity was examined using DNase agar (Qingdao 96 Hope Bio-technology) according to the manufacturer's instructions. API ZYM, API 20NE and API 50CHB/E strips (bioMérieux) and Gram-negative MicroPlate (Biolog) testing were carried out according to the manufacturer's instructions after a 2 day growth on MA at 37 °C, except that the strips were inoculated with sterile seawater [15]. The morphological, physiological and biochemical characteristics of strain MTEO17^T and the other reference strains are shown in Table 1.

For cellular fatty acid analysis, cells were incubated on M2 medium (5.0 g CH_3COONa, 0.5 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.5 g sucrose, 0.05 g sodium citrate, 0.05 g DL-malic acid, 1.0 g NH_4NO_3, 0.2 g NH_4Cl, 0.5 g KH_2PO_4, 16 g agar and 1 l seawater; pH 7.6) at 37 °C for 2 days until the bacterial cells reached the late-exponential stage of growth according to the four quadrant streak method [16]. Cellular fatty acids were extracted and analysed according to the instructions of the MIDI system (Sherlock Microbial Identification System, version 6.10) and the TSBA6.0 database of the Microbial Identification System [16]. For polar lipids and respiratory quinone analyses, cell biomass was collected

Table 2. Cellular fatty acid contents (%) of strain MTEO17^T and reference species of the genus *Alcanivorax*

Strains: 1, *A. profundi* sp. nov. MTEO17^T; 2, *A. nanhaiticus* MCCC 1A05629^T; 3, *A. borkumensis* CGMCC 1.2815^T. All data are from this study. Values are percentages of the total fatty acids. Fatty acids accounting for less than 1 % in all strains are not shown. TR, Traces (<1 %); —, not detected. Major components (>10 %) are highlighted in bold.

Fatty acid	1	2	3
$C_{10:0}$	3.5	3.6	3.7
$C_{10:0}$ 3-OH	TR	TR	4.4
$C_{12:0}$	4.9	3.8	7.6
$C_{12:0}$ 2-OH	2.3	2.5	2.5
$C_{12:1}$ 3-OH	TR	0.18	–
$C_{12:0}$ 3-OH	7.9	7.0	**12.8**
$C_{14:0}$	1.2	1.5	2.4
$C_{16:0}$	**30.7**	**27.1**	**22.7**
$C_{18:0}$	TR	1.1	2.71
Summed feature 3*	**16.8**	**18.1**	**26.6**
Summed feature 8*	**30.6**	**33.4**	**14.6**

*Summed feature 3 contains $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; summed feature 8 contains $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

by centrifugation and cryodesiccation after 48 h cultivation with shaking in MB at 37 °C. Polar lipids were extracted according to the procedures described by Minnikin *et al.* [17] and separated by two-dimensional TLC on silica gel 60 F254 plates (Merck) using chloroform/methanol/water (65 : 25 : 4, v/v) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v) for the second dimension [18]. Individual lipids were identified by spraying with appropriate detection reagents [19]. In brief, 5 % ethanolic molybdophosphoric acid was used to detect all lipids. Ninhydrin spray was applied to determine most of the lipids with free amino groups, while spraying the plate with the lipid phosphate reagent of Dittmer and Lester after the ninhydrin spray could reveal the presence of phospholipids. Respiratory quinones were extracted with chloroform/methanol (2 : 1, v/v), fractionated by silica-gel TLC and identified by HPLC [20]. DNA was extracted according to the procedure of Moore *et al.* [21].

The major cellular fatty acids of strain MTEO17^T were $C_{16:0}$, summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$) and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), occupying proportions of 30.7, 30.6, and 16.8 %, respectively. These were also the top three predominant components in reference strains *A. nanhaiticus* MCCC 1A05629^T and *A. borkumensis* CGMCC 1.2815^T, although their relative proportions varied (Table 2). Specifically, strain MTEO17^T contained the highest proportion of $C_{16:0}$ and the lowest proportion of summed feature 3 among the three strains. Furthermore, all three strains had $C_{12:0}$ 3-OH and $C_{12:0}$ as the fourth and fifth most abundant types of fatty acids, respectively. The polar lipids of strain MTEO17^T were two phosphatidylethanolamines (PEs), one phosphatidylglycerol (PG), one unidentified phospholipid (PL) and four unidentified polar lipids (Ls; Fig. S2a). PE and PG were also predominant in *A. nanhaiticus* MCCC 1A05629^T (Fig. S2b) and *A. borkumensis* CGMCC 1.2815^T (Fig. S2c), whereas other components such as PL and glycolipid varied between these species. The polar lipid profile of *A. nanhaiticus* MCCC 1A05629^T measured between Lai *et al.* [11] and this study was similar, with a predominance of PE, PG and PL. No respiratory quinone was detected, similar to that of other *Alcanivorax* species.

The genomic DNA of strain MTEO17^T was extracted with the classic phenol-chloroform method [22]. Amplification of the 16S rRNA gene was performed with the universal bacterial primer B8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and B1510R (5′-GGTTACCTTGTTACGACTT-3′). Amplicons were cloned and sequenced at BGI (Qingdao, China), and the resulting sequence was manually edited and submitted to the GenBank database. Taxonomic assignment of MTEO17^T and pairwise 16S rRNA gene similarities were determined by using the EzBioCloud server (www.ezbiocloud.net/) [23]. The related 16S rRNA gene sequences were retrieved and aligned with the CLUSTAL_X program [24]. Phylogenetic trees were reconstructed in MEGA version 7.0 [25] with three algorithms: neighbour-joining, maximum-likelihood and maximum-parsimony. The Kimura two-parameter model was used in the reconstruction of the neighbour-joining and maximum-likelihood trees [26]. Bootstrap values of 1000 replicates were performed. The genome sequence of MTEO17^T was sequenced on the Illumina HiSeq platform and assembled using the assembly pipeline A5 [27]. Gene prediction and annotation was performed using the RAST server [28]. The draft genome features of strain MTEO17^T are summarized in Table S1. The DNA G+C content was calculated based on the genome sequence. The average nucleotide identity (ANI) was calculated with the USEARCH-based OrthoANIu algorithm embedded in EzBioCloud (www.ezbiocloud.net/) [29]. The genome-to-genome distance analysis was performed using the Genome-to-Genome Distance Calculator (GGDC 2.1; http://ggdc.dsmz.de/ggdc.php) [30].

The 16S rRNA gene sequence of strain MTEO17^T was 1499 nt long and showed the highest sequence similarity of 97.9 % to *A. nanhaiticus* MCCC 1A05629^T and 96.4 % to *A. borkumensis* CGMCC 1.2815^T. Strain MTEO17^T was positioned within members of *Alcanivorax* as evidenced by the phylogenetic trees based on three algorithms and they formed a monophyletic cluster (Fig. 1). The relatively low sequence similarity of strain MTEO17^T to the type strains of recognized species in the genus *Alcanivorax* suggested that it may represent a novel species [31]. Additionally, the genomic DNA G+C content of strain MTEO17^T (57.5 %, Table S1) fell into the range (54.7–60.68 %) of other *Alcanivorax* strains. The ANI value between strain MTEO17^T and *A. nanhaiticus* MCCC 1A05629^T was 78.98 %, lower than the ANI cut-off value (95–96 %) proposed for delineating

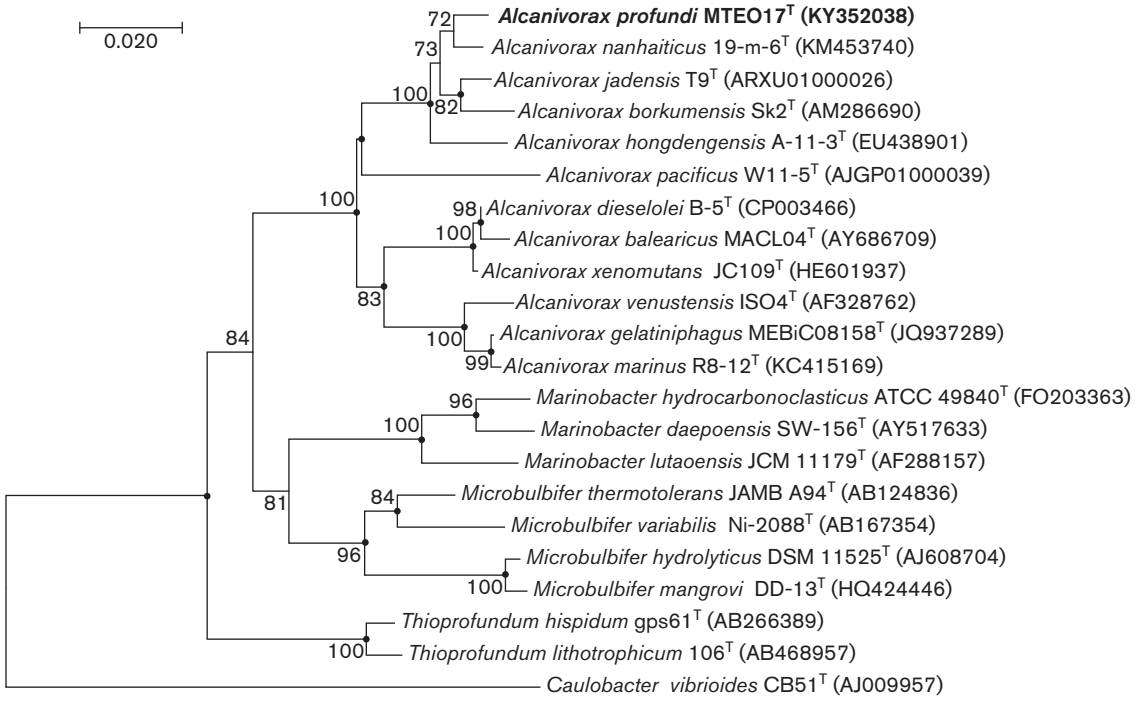

Fig. 1. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1499 nt) showing the phylogenetic position of strain MTEO17^T and other closely related species. Percentage bootstrap values above 70 % (1000 replicates) are shown at branch nodes. Closed circles indicate that the corresponding nodes were also recovered in tree generated with the maximum-likelihood and maximum-parsimony algorithms. *Caulobacter vibrioides* CB51^T (AJ009957) was used as an outgroup. Bar, 0.02 nucleotide substitutions per nucleotide position.

bacterial species [31]. Consistently, the DNA–DNA hybridization (DDH) value estimated by the GGDC was 23.80 % between strain MTEO17^T and *A. nanhaiticus* MCCC 1A05629^T. The 16S rRNA gene sequence similarity, ANI and DDH values suggest strain MTEO17^T as a distinctive species in the genus *Alcanivorax*. In addition, strain MTEO17^T harboured two genes encoding alkane-1 monooxygenase (alkane hydroxylase, *alkB*), whereas four were obtained from *A. nanhaiticus* MCCC 1A05629^T. Functional comparison based on the clusters of orthologous groups of proteins (COGs) database [32] showed that strain MTEO17^T, although with a relatively small genome size, harboured a higher abundance of COG H (coenzyme transport and metabolism), I (Lipid transport and metabolism), J (translation, ribosomal structure and biogenesis) and P (inorganic ion transport and metabolism) than *A. nanhaiticus* MCCC 1A05629^T (Fig. S3).

Strain MTEO17^T shared the same features with the reference strains of the genus *Alcanivorax* in terms of positive activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, hydrolysis of Tweens 20, 40 and 80, and negative activities of cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, alginase, cellulase, casease, DNase and chitinase. Similar to other *Alcanivorax* species, no respiratory quinone was detected in MTEO17^T. All strains shared the same dominant components of cellular fatty acids that were $C_{16:0}$, summed feature 8 and summed feature 3. PE and PG were present in these species, but other components such as PL and GL varied in different species (Fig. S2). Moreover, phylogenetic trees clustered strain MTEO17^T within the genus *Alcanivorax*. However, strain MTEO17^T and reference strains showed differences in some features, including the temperature and NaCl ranges that support growth, catalase and oxidase activities (Table 1) and the relative proportion of some fatty acids (Table 2). Therefore, the phenotypic and physiological characteristics and phylogenetic inferences mentioned above jointly suggest that strain MTEO17^T belongs to the genus *Alcanivorax* and represents a novel species in this genus, for which the name *Alcanivorax profundi* sp. nov. is proposed

DESCRIPTION OF *ALCANIVORAX PROFUNDI* SP. NOV.

Alcanivorax profundi (pro.fun′di. L. gen. n. *profundi* of/from the depths of the sea).

Cells are Gram-stain-negative, strictly aerobic, rod-shaped and non-motile. The cell size is approximately 0.4–0.6 µm long and 0.2–0.3 µm wide after culturing on MA for 2 days at 37 °C. The colonies are white transparent in colour and

0.5–1 mm in diameter on MA after cultured at 37 °C for 2 days. Growth occurs at 10–45 °C (optimum 37 °C), in the presence of 0.0–12.0 % NaCl (w/v, optimum 3.0 %) and at pH 6.0–10.0 (optimum pH 7.0–8.0). Oxidase is positive, whereas catalase is negative. Positive for hydrolysis of gelatin and Tweens (20, 40 and 80), but negative for hydrolysis of alginate, starch, DNA, casein, chitin and cellulose. In the 20NE strips, positive for aesculin ferric citrate and gelatin hydrolysis. The positive result for gelatin hydrolysis is consistent between 20NE strip analysis and the plate-based test for extracellular enzymatic activity. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present; however, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. In API 50CHB/E strips, acid is produced from erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, methyl β-D-xylopyranoside, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. There are positive reactions in the Biolog GN2 MicroPlate system for α-cyclodextrin, Tween 40 and 80, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, β-hydroxybutyric acid, propionic acid, and sebacic acid; weak for α-ketobutyric acid, α-ketoglutaric acid and α-ketovaleric acid. Positive results for Tweens 40 and 80 is consistent between the Biolog GN2 MicroPlate and the plate-based test on extracellular enzymatic activity. The dominant fatty acids are $C_{16:0}$, summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$) and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$). The polar lipids comprise two PEs, one PG, one unidentified PL and four unidentified polar Ls.

The type strain, MTEO17^T (=KCTC 52694^T=MCCC 1K03252^T), was isolated from deep seawater sample collected from the Mariana Trench. The DNA G+C content of the type strain is 57.5 %.

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

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