

Methyloprofundus sedimenti gen. nov., sp. nov., an obligate methanotroph from ocean sediment belonging to the ‘deep sea-1’ clade of marine methanotrophs

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We report the isolation and growth characteristics of a gammaproteobacterial methane-oxidizing bacterium (*Methylococcaceae* strain WF1^T, ‘whale fall 1’) that shares 98% 16S rRNA gene sequence identity with uncultivated free-living methanotrophs and the methanotrophic endosymbionts of deep-sea mussels, ≤94.6% 16S rRNA gene sequence identity with species of the genus *Methylobacter* and ≤93.6% 16S rRNA gene sequence identity with species of the genera *Methylomonas* and *Methylosarcina*. Strain WF1^T represents the first cultivar from the ‘deep sea-1’ clade of marine methanotrophs, which includes members that participate in methane oxidation in sediments and the water column in addition to mussel endosymbionts. Cells of strain WF1^T were elongated cocci, approximately 1.5 μm in diameter, and occurred singly, in pairs and in clumps. The cell wall was Gram-negative, and stacked intracytoplasmic membranes and storage granules were evident. The genomic DNA G + C content of WF1^T was 40.5 mol%, significantly lower than that of currently described cultivars, and the major fatty acids were 16:0, 16:1ω9c, 16:1ω9t, 16:1ω8c and 16:2ω9,14. Growth occurred in liquid media at an optimal temperature of 23 °C, and was dependent on the presence of methane or methanol. Atmospheric nitrogen could serve as the sole nitrogen source for WF1^T, a capacity that had not been functionally demonstrated previously in members of *Methylobacter*. On the basis of its unique morphological, physiological and phylogenetic properties, this strain represents the type species within a new genus, and we propose the name *Methyloprofundus sedimenti* gen. nov., sp. nov. The type strain of *Methyloprofundus sedimenti* is WF1^T (=LMG 28393^T=ATCC BAA-2619^T).

The marine methane cycle represents both a source and a sink to the global greenhouse gas inventory, yet relatively few of the micro-organisms that participate in this cycle have been cultivated, making controlled analysis of their role in the mitigation of methane release from the ocean challenging. Cultivated marine methanotrophs include *Methylobacter marinus*, *Methylomarinum vadi*, *Methylo-microbium pelagicum* and *Methylomicrobium japanense* (Bowman *et al.*, 1993; Hirayama *et al.*, 2013; Kalyuzhnaya *et al.*, 2008; Sieburth *et al.*, 1987). In ocean sediments, assemblages of anaerobic archaea and diverse communities

of aerobic bacteria oxidize methane to cellular carbon (reviewed by Reeburgh, 2007). In ocean waters, significantly less methanotrophic diversity has been documented, with only a handful of proteobacterial methane-oxidizing bacteria (MOB) identified as planktonic aerobic methanotrophs (Li *et al.*, 2014; Swan *et al.*, 2011; Tavormina *et al.*, 2008). The gammaproteobacterial MOB clade ‘deep sea-1’ (Lüke & Frenzel, 2011) comprises uncultivated members that participate in methane oxidation in the water column (Hayashi *et al.*, 2007; Li *et al.*, 2014) and sediments (Elsaied *et al.*, 2004; Redmond *et al.*, 2010). Some members of ‘deep sea-1’ also form endosymbiotic relationships with *Bathymodiolus* mussels (Duperron *et al.*, 2007; Raggi *et al.*, 2013; Spiridonova *et al.*, 2006), thereby facilitating these animals’ life cycles near methane seeps on the cold, dark ocean floor. More recently, *Idas* mussels living near organic falls (e.g. whale falls and wood falls) have also been shown to host ‘deep sea-1’-associated intracellular methanotrophs

Abbreviations: FAME, fatty acid methyl ester; FISH, fluorescence *in situ* hybridization; MOB, methane-oxidizing bacteria; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pmoA* and *mxoF* gene sequences of strain WF1^T are KF484906–KF484908.

(reviewed by Duperron *et al.*, 2013; Petersen & Dubilier, 2009). Thus, the 'deep sea-1' clade is interesting not only for its role in the marine methane cycle as it pertains to both sediments and the water column, but also as a potential window into events that lead to endosymbiont acquisition in animals. Here, we report the cultivation and initial characterization of the first representative of this clade.

Strain WF1^T ('whale fall 1') was isolated from marine sediment in Monterey Canyon off the coast of California, USA (36.708° N 122.105° W; 1828 m below sea-level). Surface (0–1 cm) sediment in close proximity to a whale fall (whale 1820, 'Patrick'; Braby *et al.*, 2007) was collected on 6 June 2011 using the R/V *Western Flyer* and ROV *Doc Ricketts*, owned and operated by the Monterey Bay Aquarium Research Institute. This whale fall lies 500 m from a methane cold seep and 23 km from shore. Physico-chemical values recorded during sampling included temperature (2.2 °C), salinity (34.6 practical salinity units) and oxygen (166.7 µM; 3.712 ml l⁻¹). Approximately 10 g sediment was placed in a foil-wrapped 120 ml serum bottle sealed with a rubber stopper and crimp cap and incubated at 4 °C without shaking, under laboratory air with added methane and propane each equalling 30% (v/v) of the headspace volume. Propane was initially included to allow for concurrent enrichment of short-chain-hydrocarbon oxidizers (e.g. Redmond *et al.*, 2010), and was excluded from subsequent incubations specifically aimed at methanotroph isolation. After 12 months, approximately 10 µl sediment was inoculated onto solid nitrate mineral salts medium (NMS; ATCC 1306) modified as follows: trace element solution from medium ATCC 1306 was replaced with trace element solution from medium DSM141 (Robb *et al.*, 1995), vitamin solution from medium DSM141 (*ibid.*; used as a 1000-fold stock) was added and NaCl was added to a final concentration of 0.6 M (3.5%, w/v). This modified NMS was solidified with 2% Bacto agar (Becton Dickinson). Both agar plates and slants (in Balch-style serum tubes) were inoculated for single colonies and placed under laboratory air with added methane (30% v/v in the headspace). Plates and slants were incubated at 10 and 23 °C and examined periodically for growth, and the original enrichment bottle was maintained at 4 °C.

Four weeks after inoculation of plates and slants, 60 individual colonies were probed molecularly for the gene central to methane metabolism in MOB, *pmoA*, encoding the particulate methane monooxygenase (pMMO) A-subunit, and four colonies tested positive for identical *pmoA* sequences. These four colonies were passaged several times on agar under methane, but growth diminished with each passage, suggestive of potential contamination. One isolate was transferred into liquid modified NMS medium, where it was successfully passaged. In the weeks following transition to liquid medium, a contaminating strain was identified as a member of *Ahrensia* (Uchino *et al.*, 1998) on the basis of microscopy and 16S rRNA gene sequence (Lane, 1991). Dilution to extinction in liquid medium,

coupled with a reduction of oxygen concentration in the headspace (50% N₂, 30% CH₄, 20% laboratory air), promoted methanotroph growth relative to *Ahrensia* and, after several months of successive passaging, no contaminating strains were detected. The culture was considered pure when general 16S rRNA primers exclusively amplified the methanotroph 16S rRNA gene, when methane-free incubations supplemented with 0.1% (w/v) yeast extract did not promote any growth and when all cells in the culture hybridized to the gammaproteobacterial methanotroph-specific fluorescence *in situ* hybridization (FISH) probe MetI-444 (Lösekann *et al.*, 2007). Strain WF1^T was stored at -80 °C following recent guidelines that promote better revival of methanotroph strains (Hoefman *et al.*, 2012).

Unless otherwise noted, strain WF1^T was grown in modified liquid NMS medium under 50% N₂, 30% CH₄ and 20% laboratory air, and tubes were incubated at 10 °C with rocking. Under these growth conditions, the strain formed a uniformly distributed culture and attained an OD₄₅₀ of 0.3; direct cell counts indicated that this represents approximately 5 × 10⁷ cells ml⁻¹. In the absence of rocking or shaking, cells settled to the bottom of the tube and were easily redistributed into solution via swirling. There was no pellicle formation.

Physiological properties

All physiological testing was performed by adjusting the standard medium and diluting exponential-phase cells 100-fold into these media. Growth was monitored as a change in OD₄₅₀. To test the effect of temperature on growth, triplicate cultures of strain WF1^T were inoculated in 30 ml Balch-style serum tubes and incubated with rocking at 4, 10, 18, 23, 26, 29 and 32 °C. Strain WF1^T exhibited doubling times of 52 h at 4 °C, 14 h at 10 °C, 9.5 h at 18 °C, 9 h at 23 °C and 11 h at 26 °C, and did not grow at or above 29 °C. To test the effect of pH on growth, the concentrations of KH₂PO₄ and Na₂HPO₄ were adjusted to vary the pH by 0.5 unit increments (pH 4.5–8.0), while maintaining a constant phosphate concentration. The strain grew at pH 6–8; the optimum pH for growth was 6.5–7.5. To test the effect of salt concentration on growth, the NaCl concentration was adjusted to achieve final concentrations of 0, 0.17, 0.34, 0.51, 0.68, 0.85, 1.02 and 1.2 M (0, 1, 2, 3, 4, 5, 6 and 7%, w/v). The strain required NaCl for growth, and grew at concentrations from 0.17 to 0.68 M, with optimal growth at 0.34 M NaCl (2%, w/v).

Effects of heat and desiccation

To test the ability of strain WF1^T to withstand heat, exponentially growing cells were heated to 40, 50, 60, 70 or 80 °C for 10 min, diluted 1:10 into fresh medium, incubated under standard conditions and inspected for growth at 3, 7 and 14 days. The ability of strain WF1^T to withstand desiccation was tested as described previously (Whittenbury *et al.*, 1970). Spore formation was tested by

heating a 6-week-old stationary-phase culture to 80 °C for 20 min, diluting 1 : 100 into fresh medium and incubating under standard conditions, and observed for growth at 3, 7 and 14 days. The strain did not survive any of these treatments, and spore formation was not observed.

Morphological characteristics, FISH and transmission electron microscopy

Cells were observed using light microscopy, phase-contrast microscopy, FISH and transmission electron microscopy. For light, phase-contrast and fluorescence microscopy, an Olympus BX51 microscope was used at $\times 1000$ magnification under oil immersion. Phase-contrast was employed to assess motility of wet-mount cells in exponential and stationary-phase cultures. Under light microscopy, cells appeared coccoid or slightly elongated and frequently occurred in pairs and clumps. Under wet-mount phase-contrast microscopy, cells were non-motile and appeared to have capsules. Capsules were confirmed with negative staining (Fig. 1a), and are common among gammaproteobacterial methanotrophs (Bowman *et al.*, 1993). FISH was routinely employed to verify culture purity. For FISH, 0.5 ml late-exponential-phase liquid culture was fixed with 2 % formaldehyde for 2 h at room temperature. 16S rRNA-targeted fluorescently labelled oligonucleotide probes (Integrated DNA Technologies) specific for all bacteria (Fluos-labelled EUB 338 I-III; Amann *et al.*, 1990; Daims *et al.*, 1999) and gammaproteobacterial methanotrophs (Cy3-labelled MetI-444; Lösekann *et al.*, 2007) and 4',6-diamidino-2-phenylindole (DAPI) were used to assess culture purity. Controls ('no probe' and Cy3-labelled NonEUB338; Wallner *et al.*, 1993) were included to examine the possibility of non-specific probe binding. Under fluorescence microscopy (FISH), a single morphological type was observed: elongated coccoid cells that contained intracellular areas resistant to staining, consistent with the presence of storage granules (Fig. 1b).

To prepare cells for transmission electron microscopy, 5 ml late-exponential-phase culture was fixed for 2 h on ice with 2 % glutaraldehyde and then rinsed with 1 \times PBS. Cells were washed and resuspended three times in 1 ml buffer A (50 mM sodium cacodylate, pH 7.4) plus 0.6 M NaCl, followed by post-fixation in buffer A plus 1 % OsO₄ for 30 min on ice. Cells were then rinsed five times in 1 ml double-distilled water, and stained with 2 % uranyl acetate in water overnight. Cells were dehydrated on ice in a graded ethanol series (20, 50, 70, 90 and 100 %) and infiltrated in 1 : 1 Durcupan ACM resin/ethanol for 30 min followed by polymerization at 60 °C for 48 h. Sections (200 nm) were obtained on a Leica ultramicrotome. Cells were imaged on a Zeiss EM 109 transmission electron microscope, operated at 50 kV. Electron microscopy revealed a typical Gram-negative cell wall, intracytoplasmic membrane stacks (~ 10 –25 layers) and storage granules, probably polyhydroxyalkanoate or polyhydroxybutyrate (Fig. 1c).

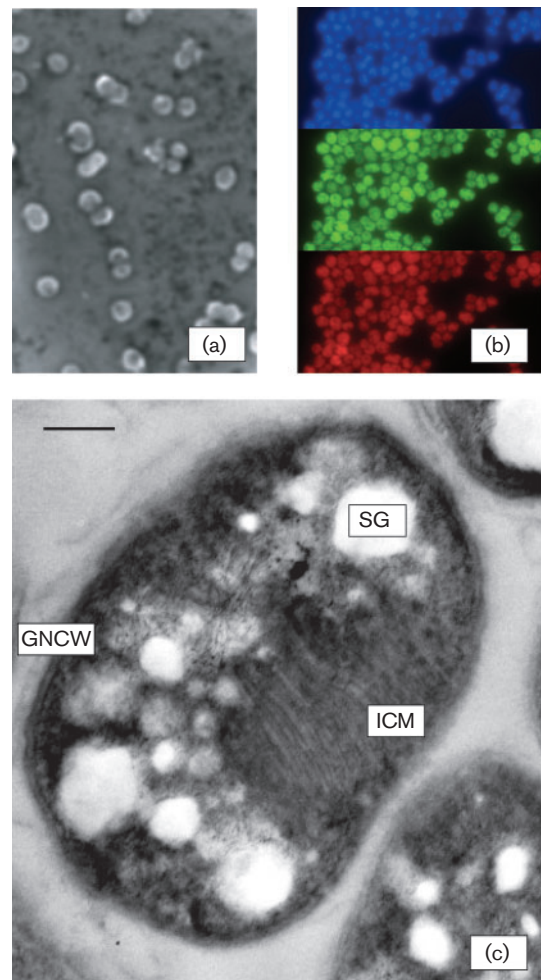


Fig. 1. Morphology of strain WF1^T. (a) Negative stain ($\times 1000$), showing the presence of capsules in stationary-phase cells. (b) Fluorescence microscopy ($\times 1000$). DAPI (blue), EUB338 I-III (green) and MetI-444 (red) FISH illustrating the purity of the culture as well as stain-recalcitrant intracellular areas. (c) Thin section of cells of strain WF1^T, illustrating typical type I stacked intracytoplasmic membranes (ICM), storage granules (SG) and a typical Gram-negative cell wall (GNCW). Bar, 0.2 μ m.

Carbon and nitrogen source utilization

Methane or methanol was required for growth; methane supported growth at all concentrations tested [5–50 % (v/v) headspace], and methanol supported growth in the absence of methane at all concentrations tested (0.1, 0.2, 0.3, 0.4 and 0.5 %, v/v). Growth of members of the genus *Methylobacter* may be inhibited by direct addition of methanol (>0.1 %, v/v) to the medium (pp. 258–259 in Bowman, 2005), making the growth of WF1^T under all methanol concentrations tested a distinguishing feature of this strain. Alternative potential carbon sources including glucose, sucrose, acetate, citrate, pyruvate, succinate, formate, formaldehyde, formamide, yeast extract, glycerol, dimethylformamide and ethanol were tested for their ability to

support growth in the absence of methane by supplementing modified NMS with each respective carbon source to 0.1 % (w/v). For these carbon source utilization tests, exponential-phase cells were diluted 1:100, incubated under standard conditions and examined for growth after 7, 14 and 25 days. Each of these alternative carbon sources failed to support growth. Alternative potential nitrogen sources including ammonium, Tris, urea, nitrite, yeast extract, glucosamine, trimethylamine, formamide, taurine, thiamine, cysteine, glycine, isoleucine, leucine, lysine, methionine, proline, tryptophan and valine were tested for their ability to support growth in the absence of nitrate by replacing nitrate with each respective nitrogen source in modified NMS at a final concentration of 0.05 % (w/v). Atmospheric nitrogen was tested for its ability to serve as a sole nitrogen source by preparing medium without any added nitrogen sources, adding exponential-phase cells at a 1:100 dilution and passaging three times in this manner to exclude the possibility that trace nitrate in the original inoculum was serving as the nitrogen source. For these nitrogen source utilization tests, exponential-phase cells were diluted 1:100, incubated under standard conditions and examined for growth after 7, 14 and 25 days. Atmospheric nitrogen, nitrate, ammonium, urea, yeast extract, glucosamine, leucine, lysine and, to a lesser degree, cysteine were able to serve as nitrogen sources. Although genomes of members of the genus *Methylobacter* contain genes for nitrogen fixation, a direct demonstration of nitrogen fixation from a member of *Methylobacter* has not been reported (Bowman, 2005), revealing a unique potential of the 'deep sea-1' clade relative to its closest cultured relatives. The utilization by strain WF1^T of lysine and leucine as nitrogen sources further distinguishes this strain from previously described species of the genera *Methylobacter* and *Methylomonas* (Bowman *et al.*, 1993). A requirement for vitamin supplementation was tested by preparing NMS medium without added vitamins. Exponential-phase cells were added to this medium at a 1:100 dilution and passaged three times in this manner to exclude the possibility that trace vitamins in the original inoculum were promoting growth. Strain WF1^T initially grew in vitamin-free medium, but growth diminished in subsequent passages, suggesting that growth in culture is promoted by the presence of vitamins.

Fatty acid extraction and analysis

Frozen cell pellets were lyophilized and pre-weighed prior to extraction. Lipid extraction and trans-esterification to fatty acid methyl esters (FAMES) were done simultaneously at room temperature with 1 ml hexane and 2 ml 0.5 M NaOH in anhydrous methanol. The reaction was neutralized after 10 min with 70 µl glacial acetic acid and the FAMES were subsequently extracted with 2 ml water and 2 ml hexane (Griffiths *et al.*, 2010). A solution of tricosane (40 ng µl⁻¹) was used as an internal standard for extraction efficiency and quantification and was added prior to trans-esterification.

Double bond positions were identified from 3-pyridylcarbinol esters of fatty acids (Christie & Han, 2010). The reaction catalyst was prepared in a N₂-flushed vial by combining 200 µl 3-(hydroxymethyl)pyridine and 100 µl 1 M t-butoxide in tetrahydrofuran. FAMES were dissolved in dry dichloromethane and then added by syringe to the reaction catalyst. The mixture was heated at 40 °C for 20 min. After cooling to room temperature, the 3-pyridylcarbinol esters were extracted with water and hexane, dried over Na₂SO₄ and concentrated prior to analysis.

FAMES and 3-pyridylcarbinol esters of fatty acids were analysed by gas chromatography-mass spectrometry (GC-MS) on a Thermo-Scientific Trace-DSQ with a ZB-5ms column (30 m × 0.25 mm, 0.25 µm i.d.) using a programmed temperature vaporization injector operated in splitless mode. The oven temperature program was 100 to 320 °C (held for 20 min) at 6 °C min⁻¹. Quantification was done using a flame-ionization detector. Peak areas were determined using Thermo Xcalibur version 2.1. Compound identification was based on comparison of retention time and mass spectra to known standards as well as to the NIST mass spectra library version 2.0.

The cellular fatty acid profile of strain WF1^T, containing primarily C₁₆ fatty acids, is consistent with those of other type I methanotrophic bacteria (Bowman *et al.*, 1991, 1993); however, it is differentiated by the presence of 16:2 fatty acids (Bodelier *et al.*, 2009). Three C_{16:1} fatty acids were present in similar proportions in strain WF1^T: 16:1ω9c (28.8%), 16:1ω9t (26.9%) and 16:1ω8c (22.3%). Additionally, 16:0 (15%) and 16:2ω9,14 (7.1%) were detected in fatty acid extracts. A summary of the distinguishing features of strain WF1^T is provided in Table 1.

Sequence analysis

DNA was extracted from 200 ml of a late-exponential-phase culture following established methods (Schmidt *et al.*, 1991) and further purified to a level suitable for PCR and sequencing (Saunders & Burke, 1990). PCR amplifications were performed using established primers and methods for the 16S rRNA gene, *pmoA* and the methanol dehydrogenase gene *mxoA* (Lane, 1991; McDonald & Murrell, 1997; Tavormina *et al.*, 2008, 2010). PCR amplicons were sequenced using a commercial service (Laragen, Culver City, CA, USA) and BigDye chemistry. For genome sequencing, purified DNA (10 µg) was sent to SeqMatic (Union City, CA, USA) for Illumina sequencing on the MiSeq platform. Data were uploaded to <http://rast.nmpdr.org/> for automatic annotation.

A total of 4 065 599 bp on 684 contiguous DNA fragments were recovered in the partial genome sequence of strain WF1^T. The genomic G+C content was 40.5 mol%, significantly lower than those of previously described methanotrophic cultivars, including *Methylobacter luteus* (49 mol%), the type species of the genus *Methylobacter*, and *Methylobacter psychrophilus* (46 mol%), as well as the Antarctic methanotroph *Methylosphaera hansonii* (43.5 mol%)

Table 1. Characteristics of strain WF1^T in comparison with related methanotrophic genera

Taxa: 1, strain WF1^T; 2, *Methylobacter* (14 strains) (data from Bowman *et al.*, 1993, 1995; Warttiainen *et al.*, 2006); 3, *Methylomonas* (54 strains) (Bowman *et al.*, 1993, 1995; Danilova *et al.*, 2013; Fang *et al.*, 2000; Kalyuzhnaya *et al.*, 1999, 2008); 4, *Methylomicrobium* (15 strains) (Bowman *et al.*, 1993, 1995; Kalyuzhnaya *et al.*, 2008); 5, *Methylomarinum* (Hirayama *et al.*, 2013); 6, *Methylosarcina* (Wise *et al.*, 2001); 7, *Methylosoma* (Rahalkar *et al.*, 2007); 8, *Methylovulum* (Iguchi *et al.*, 2011); 9, *Methylophaera* (Bowman *et al.*, 1997). ND, No data available; v, variable between taxa; PLFA, phospholipid fatty acid; RuMP, ribulose monophosphate.

Characteristic	1	2	3	4	5	6	7	8	9
Temperature for growth (°C)									
Range	4–26	3.5–37	5–37	15–37	20–44	22–37	16–30	5–34	0–21
Optimum	18–23	ND	ND	25–30	37–43	ND	25	24–32	10–13
pH for growth									
Range	6–8	5–9	3.8–9	5.5–10.5	4.5–8.1	5–9	5–9	6–7.5	ND
Optimum	6.5–7.5	ND	ND	ND	6.2–7.0	ND	6–8	ND	ND
Salinity range (% w/v)									
Range	1–4	0–3.0	0–2.5	0–8.8	1.0–8.0	0–1	ND	0–0.2	ND
Optimum	2	ND	ND	ND	2.0–3.0	ND	<0.5	ND	ND
Cell morphology	Cocci, ellipsoids, rods	Cocci, ellipsoids, rods	Ellipsoids, rods	Cocci, ellipsoids, rods	Short rods	Cocci, rods	Cocci, ellipsoids, rods	Cocci, rods	Cocci
Cyst formation	–	+/-	+	–	–	+	+	–	–
Motility	–	+/-	+	+	+	+	–	–	–
Major PLFAs (%)*	16:0 (15), 16:1ω8c (22), 16:1ω9c (29), 16:1ω9t (27), 16:2ω9,14 (7)	14:0 (7–11), 16:0 (7–9), 16:1ω8 (0–35), 16:1ω7 (23–58), 16:1ω6c (4–6), 16:1ω5c (6–8), 16:1ω5t (10–26)	14:0 (19–25), 16:0 (5–9), 16:1ω8c (19–41), 16:1ω7c (7–15), 16:1ω6c (5–13), 16:1ω5c (2–6), 16:1ω5t (8–16)	16:0 (12–18), 16:1ω8c (13–19), 16:1ω7c (14–20), 16:1ω6c (6–14), 16:1ω5c (5–7), 16:1ω5t (10–30), 18:1ω7c (0–27)	16:0 (22–32), 16:1ω7c (36–51), 16:1ω5t (7–24)	16:0 (18–20), 16:1ω8c (14–16), 16:1ω7c (25–32), 16:1ω6c (7–9), 16:1ω5t (19–30)	14:0 (9), 16:0 (9), 16:1ω7c (60), 16:1ω6c (15)	14:0 (34), 16:0 (47), 16:0 3-OH (8), 17:1ω6c (6)	16:0 (14–15), 16:1ω8c (37–41), 16:1ω7c (16–19), 16:1ω6c (17–18)
sMMO	–	–	+/-	+/-	–	–	–	+	–
C ₁ assimilation pathway	RuMP	RuMP	RuMP	RuMP	RuMP	RuMP	ND	RuMP	ND
Presence of <i>nifH</i> †	+	v	v	–	–	–	+	+	ND
Nitrogen fixation (function)†	+	–	v	–	–	–	+	–	+
DNA G+C content (mol%)	40.5	46–55	48.5–59.0	46–51	51.0–51.6	54.1–54.3	49.9	49.3	43.5–45.9
Maximum 16S rRNA gene sequence identity to strain WF1 ^T (BLAST) (%)	(100)	95	94	93	92	94	92	90	93
Genomic ANI to strain WF1 ^T (%)‡	(100)	79.3–80.5	81.5	81.7–82.7	NG	NA	NG	80.3	NG

*PLFAs representing more than 5% of the total cellular pool are shown. Data for PLFA abundances for reference taxa were taken from Bowman *et al.* (1993, 1995, 1997), Hirayama *et al.* (2013), Iguchi *et al.* (2011), Rahalkar *et al.* (2007) and Warttiainen *et al.* (2006).

†Presence of *nifH* gene, or assessment of function either by acetylene reduction assay or by growth on atmospheric nitrogen. In addition to articles describing specific methanotrophic taxa, see Auman *et al.* (2001).

‡Two-way average nucleotide identity (ANI) (<http://enve-omics.ce.gatech.edu/ani/>). NA, Insufficient hits for two-way ANI calculation; NG, no genome data available.

(Bowman *et al.*, 1997). Interestingly, low G+C content is one of the hallmarks of obligate and facultative intracellular symbionts and pathogens, possibly resulting from the genetic isolation of small starting populations (Moran *et al.*, 2008). While it is currently not known whether strain WF1^T is capable of establishing an endosymbiotic relationship with mussels, this feature is consistent with the phylogenetic placement of this strain within a clade that harbours environmentally acquired endosymbionts of methanotrophic mussels.

Preliminary annotation of the partial genome of strain WF1^T revealed genetic capacity common to many gamma-proteobacterial cultivars, including single copies of genes for 16S rRNA, 23S rRNA and *pmoCAB*. *pxm* genes and genes encoding soluble methane monooxygenase (sMMO) were not present in the partial genome of strain WF1^T. Several genes encoding enzymes relevant to the serine pathway of formaldehyde assimilation were identified; however, a complete serine pathway could not be constructed from the partial genome. Likewise, the Calvin–Benson–Bassham cycle could not be constructed. Genes encoding a complete ribulose monophosphate (RuMP) pathway of carbon assimilation were identified, as were genes for nitrogen fixation, capsule formation and glycogen, polyphosphate and polyhydroxyalkanoate (polyhydroxybutyrate) storage. Genes predicted to encode urease and a leucine-specific transporter were also identified in the genome, which may pertain to the ability of strain WF1^T to utilize urea and leucine as nitrogen sources. Genes predicted to encode cytochrome *c* oxidase and catalase were also present, and a complete ubiquinone biosynthetic pathway was identified.

Phylogenetic analysis

To reconstruct a phylogenetic tree for the 16S rRNA gene, sequences from type species of the family *Methylococcaceae* were used to infer a tree by maximum-likelihood using the PhyML package (Guindon *et al.*, 2010) and the HKY evolutionary model in the software program ARB version 5.5 (<http://www.arb-home.de/>; Ludwig *et al.*, 2004). Nearly full-length 16S rRNA gene sequences were used, where the shortest sequence was 1396 bases (GenBank accession no. GU584415). The sequences were aligned in the SSURef-111-SILVA-NR database (<http://www.arb-silva.de/>; Quast *et al.*, 2013) and masked using the bacterial filter provided. Clone 2E-055 (GenBank accession no. FJ981085; 683 bases) was inserted into the tree using the parsimony insertion algorithm in ARB. The reliability of the tree was estimated by bootstrapping in software program Geneious version 7.1.7 (<http://www.geneious.com/>) using PhyML maximum-likelihood, the HKY model and 1000 replicates. Sequences that served as an outgroup (not shown) included those of *Methylocapsa acidiphila* B2^T (GenBank accession no. AJ278726), *Methylocystis parvus* OBBP^T (Y18945) and *Methylosinus trichosporium* OB3b^T (ADVE01000118). To reconstruct a phylogenetic tree for the pMMO A-subunit,

the *pmoA* gene sequence of strain WF1^T was translated *in silico* to protein sequence, aligned with pMMO A sequences from cultured methanotrophs within the family *Methylococcaceae* with validly published names and relevant environmental sequences using MUSCLE (<http://www.phylogeny.fr>; Dereeper *et al.*, 2008) and trimmed to residues 63–189 (positions according to the sequence of *Methylococcus capsulatus* Bath). The resulting alignment was analysed via the neighbour-joining and maximum-likelihood treeing methods, with the AMO-A sequence of ‘*Nitrosomonas cryotolerans*’ ATCC 49181 (GenBank accession no. AF314753) as the outgroup.

The 16S rRNA gene sequence from strain WF1^T was 97–99% identical to multiple 16S rRNA gene sequences assigned to uncultured methanotrophic endosymbionts of bathymodiolin mussels (Duperron *et al.*, 2007; Raggi *et al.*, 2013; Spiridonova *et al.*, 2006) and sequences recovered from the marine water column and marine sediments (Elsaied *et al.*, 2004; Li *et al.*, 2014; Redmond *et al.*, 2010; Schauer *et al.*, 2011; Schmidtova *et al.*, 2009). These sequences formed a clade separate from cultured methanotrophic genera. When compared to existing cultivars, the 16S rRNA gene sequence of strain WF1^T was most closely related to those of members of *Methylobacter* (≤94.6% sequence identity), *Methylosarcina* (≤93.6% sequence identity) and *Methylomonas* (93.6% sequence identity) (Fig. 2a, Table 1). Phylogenetic analysis of the pMMO A protein revealed similar patterns of relatedness between strain WF1^T, environmental sequences and methanotrophic cultivars (Fig. 2b). This relationship was strongly supported for both the 16S rRNA gene sequences (100% bootstrap support, 1000 replicates) and the deduced pMMO A protein sequences (90% bootstrap support, 1000 replicates).

Because of the unique morphological and physiological features of strain WF1^T and its phylogenetic divergence from established cultivars, and the monophyletic relationship with the ‘deep sea-1’ clade of uncultured microorganisms, which includes sediment-, water column- and mussel-hosted members, we propose the name *Methyloprofundus sedimenti* gen. nov., sp. nov.

Description of *Methyloprofundus* gen. nov.

Methyloprofundus [Me.thy'lo.pro.fun'dus. N.L. pref. *methyl-* pertaining to the methyl radical, from N.L. n. *methylum* (from Fr. adj. *méthyle*) the methyl group; L. masc. adj. *profundus* of the deep; N.L. masc. n. *Methyloprofundus* a methyl-using bacterium from the deep sea].

Deep-sea marine bacteria with Gram-negative cell wall. Cells are coccoid to slightly elongated, frequently occurring in pairs, and stacked intracytoplasmic membranes are evident. Utilize methane and methanol as sole carbon sources. Possess pMMO and assimilate carbon via the ribulose monophosphate pathway. Do not possess sMMO. The genus is a member of the family *Methylococcaceae*. The type species is *Methyloprofundus sedimenti*.

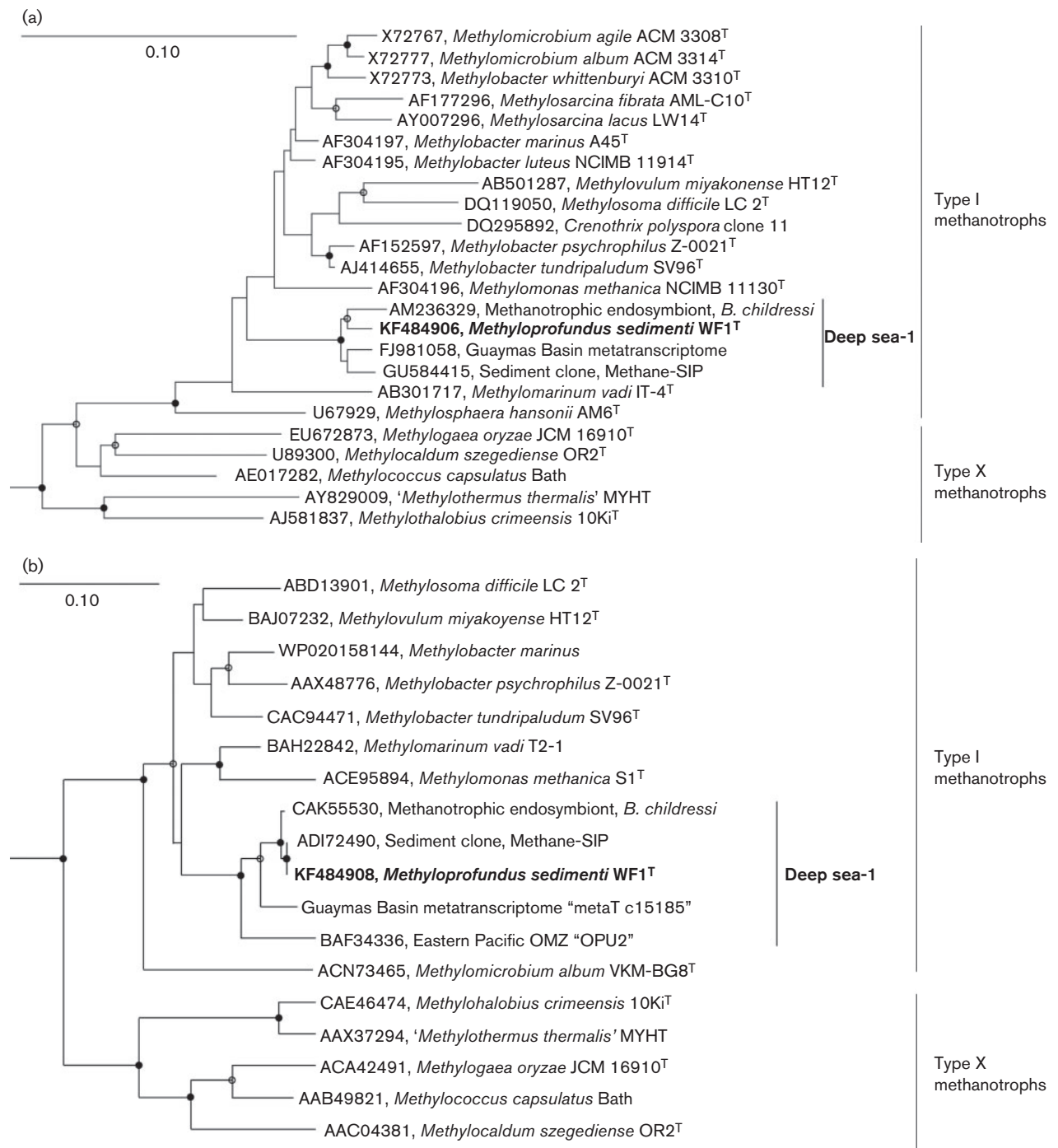


Fig. 2. (a) 16S rRNA gene phylogeny showing the placement of strain WF1^T within the order *Methylococcales* of the class *Gammaproteobacteria*. The tree was inferred with maximum-likelihood, the HKY evolutionary model and 1000 bootstraps. Bootstrap support of 60% or greater is indicated by open circles and 80% or greater by filled circles. Bar, 0.10 substitutions per site. (b) pMMO-A phylogeny. Neighbour-joining tree based on predicted protein sequences (residues 63–189 from the sequence of *Methylococcus capsulatus* Bath) from strain WF1^T and cultured type I methanotrophs. The branch labelled 'deep sea-1' includes strain WF1^T and representative uncultured sequences from the water column [OPU2 (Hayashi *et al.*, 2007); Guaymas Basin metaT c15185 (Li *et al.*, 2014)], sediment (Methane-SIP; Redmond *et al.*, 2010) and endosymbionts of mussels (endosymbiont of *B. childressi*; Duperron *et al.*, 2007). Bootstrap support of 60% or greater is indicated by open circles and 80% or greater by solid circles (1000 replicates). Bar, 0.10 substitutions per site.

Description of *Methyloprofundus sedimenti* sp. nov.

Methyloprofundus sedimenti (se.di.men'ti. N.L. masc. adj. *sedimenti* of sediment).

In addition to the characteristics of the genus, the following traits are observed. Cells are 1–1.5 µm in diameter and non-motile. Requires NaCl for growth, with optimal NaCl concentration of 2%. Optimal growth temperature is 18–23 °C; growth does not occur at or above 29 °C. Utilizes nitrate, ammonium, yeast extract, glucosamine, urea, cysteine, leucine, lysine and atmospheric nitrogen as sole nitrogen sources. Predominant fatty acids are 16:0, 16:1 ω 9c, 16:1 ω 9t, 16:1 ω 8c and 16:2 ω 9,14. Does not form colonies on NMS solid medium.

Strain WF1^T (=LMG 28393^T=ATCC BAA-2619^T) is the type strain, isolated from marine sediment near a whale fall in Monterey Canyon, California. The G+C content of genomic DNA of the type strain is 40.5 mol%.

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