Genus

Proteobacteria/Gammaproteobacteria/Methylococcales/Methylococcaceae/

Methyloprofundus

Tavormina, Hatzenpichler, McGlynn, Chadwick, Dawson, Connon, and Orphan 2015, 256[VP]

Patricia L. Tavormina, *Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA, USA*

Me.thy.lo.pro.fun'dus. N.L. neut. n. *methylum* the methyl radical, L. adj. *profundus* deep, N.L. masc. n. *Methyloprofundus* a methyl-using bacterium from the deep sea.

The genus *Methyloprofundus* is represented by aerobic methanotrophic bacteria in the deep ocean and is notable for the ability of some members to form endosymbiotic relationships with methane-seep-associated mussels. In chemosynthetic marine environments including deep-sea methane seeps, such symbiotic events help support the establishment of extensive faunal communities. The genus *Methyloprofundus* accommodates aerobic, slightly halophilic obligate methanotrophs that use the ribulose monophosphate pathway. Members of the genus form elongated cocci, frequently occurring in singles and pairs. Extensive stacked membranes are present throughout the interior of the cell. Cells are nonmotile, do not form cysts or spores, and do not form colonies on solid media. *Methyloprofundus* grows at temperatures between 4 and 26 °C and is not tolerant of heat or desiccation. Members of genus *Methyloprofundus* have been detected exclusively in the deep ocean, most typically in methane-rich seeps and sediments, and within bacteriocytes of seep-associated mussels in *Bathymodiolus*. Members of *Methyloprofundus* are also occasionally detected in the deep water column.

The mol% G+C content: **40.5%,** assessed by genomic sequence analysis.

Type species: **Methyloprofundus sedimenti** (Tavormina, Hatzenpichler, McGlynn, Chadwick, Dawson, Connon, and Orphan 2015, 256[VP].

Cocci and short rods, 1.0–1.5 µm in diameter, occurring in singles, pairs, and clumps. **Gram negative**, and nonmotile. **Strictly aerobic** respiratory metabolism with oxygen as the terminal electron acceptor. **Pure cultures do not form colonies**; liquid cultures display even distribution; there is a slight pinkish tinge to pelleted cells. Mesophilic to psychrotolerant and slightly halophilic. **Methane or methanol is used as carbon source**; multicarbon compounds do not support growth. Methane is oxidized by the particulate methane monooxygenase, pMMO. The ribulose monophosphate pathway is used to assimilate formaldehyde into cellular carbon. Nitrate can serve as nitrogen source; may fix atmospheric nitrogen; additional nitrogen sources can be utilized for growth. Major fatty acids are **$C_{16:0}$, $C_{16:1}$ (ω6, ω7*c*, and ω8), and $C_{16:2}$ ω2,6.** Ubiquinone-8 is the dominant lipoquinone. Natural distribution is restricted to the deep ocean. Some members form **endosymbiotic relationships with methane seep-associated *Bathymodiolus* mussels**.

The mol% G+C content: **40.5%,** assessed by genomic sequence analysis.

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Type species: **Methyloprofundus sedimenti** (Tavormina, Hatzenpichler, McGlynn, Chadwick, Dawson, Connon, and Orphan 2015, 251[VP].

Number of validated species: 1.

Family classification: The genus *Methyloprofundus* is classified within the family *Methylococcaceae*.

Further descriptive information

Cell morphology

Cells are up to 1.5 µm in diameter and somewhat irregularly shaped, most often appearing as elongated cocci and short rods. Cells occur as singles and pairs in actively growing cultures, and as clumps in stationary phase (Figure 1a).

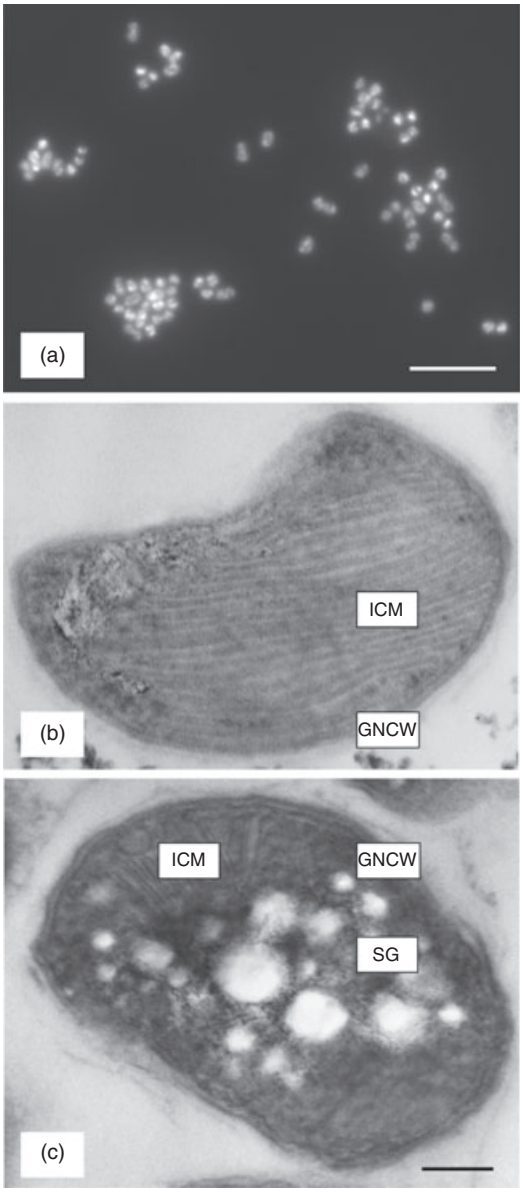
Cell wall composition; fine structure

Cell walls are Gram negative, and capsules are visible with negative staining. Some imaging techniques, including phase contrast and fluorescence microscopy, reveal a granular appearance to cells (Figure 1a). Stacked intracytoplasmic membranes (ICM) and storage granules occupy the vast majority of intracellular space (Figure 1b, c). Cells are nonmotile and flagella are not observed. The dominant fatty acids in actively growing *Methyloprofundus* are, in roughly equal proportions, $C_{16:1}$ ω8 (29%), $C_{16:1}$ ω7*c* (26%), and $C_{16:1}$ ω6 (21%). $C_{16:0}$ fatty acids accounted for an additional 17%, and $C_{16:2}$ ω9,14 fatty acids account for an additional 6%. Ubiquinone-8 is the major quinone in membranes, and this is not unusual for gammaproteobacterial methanotrophs. $C_{16:2}$ fatty acids are unusual among gammaproteobacterial methanotrophs, and these may be unique to genus *Methyloprofundus*.

Cultural characteristics

As a pure culture, *Methyloprofundus* does not grow on solid media. This methanotroph can, however, be readily grown and maintained in liquid culture, where it typically distributes evenly as a pale cream-colored suspension. No pellicle is observed, and cells settle in the absence of swirling. A slight pink tinge is evident in pelleted cells. Actively growing cultures develop a distinctive, moderately pungent odor that most individuals describe as unpleasant. This odor dissipates rapidly upon exposure to air. *Methyloprofundus* is psychrotolerant and halophilic with a growth optimum of 18–23 °C and 2% NaCl. The pH optimum for growth is between 6.5 and 7.5.

FIGURE 1. Representative views of *Methyloprofundus sedimenti* strain WF1. (a) Cells stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells in actively growing cultures appear as singles, pairs, and clumps. Bar, 10 µm. (b) Transmission electron microscopic view. Intracytoplasmic membranes (ICM) fill large portions of the cytoplasmic space; and the cell wall is Gram negative (GNCW). (c) In portions of the cell interior that do not contain extensive ICM, storage granules (SG) are frequently evident, particularly when cells are grown under standard conditions. Bar in (b) and (c), 0.2 µm.



Nutrition and growth conditions

Methyloprofundus is an obligate methanotroph, requiring methane or methanol as a carbon source for growth. Methane is oxidized by the membrane-bound particulate methane monooxygenase, which is encoded in the genome of the type species. pMMO (*pmo*)-mediated methane oxidation is the only expected pathway for methane utilization in *Methyloprofundus*, as soluble methane monooxygenase is not encoded in the genome of the type species. The complete ribulose monophosphate pathway (RuMP) is also predicted from the genome, and cells are expected to assimilate carbon via the RuMP pathway. This C_1 assimilation pathway is typical for gammaproteobacterial methanotrophs.

Multiple nitrogen sources at low concentration (0.05%) can be utilized by *Methyloprofundus*. These sources include nitrate, ammonium, urea, yeast extract, glucosamine, leucine, lysine, and cysteine. The genome encodes a complete nitrogen fixation pathway and atmospheric nitrogen may also serve as a nitrogen source *in situ*. Vitamins may stimulate growth, although the physiological basis for this observation is not yet known. Oxygen is required for growth.

Pathogenicity

Methyloprofundus is not pathogenic to humans; however, the genus is notable in that as-yet uncultured members of the genus form endosymbiotic relationships with *Bathymodiolus* mussels occurring near deep-sea methane seeps (Duperron et al., 2007; Raggi et al., 2013; Spiridonova et al., 2006). This endosymbiotic relationship is constrained to bacteriocyte cells in mussel gills, and mussels appear to derive carbon from methane through this interaction. A single report also indicates symbiosis between members of *Methyloprofundus* and *Idas* mussels (Duperron et al., 2013; Petersen and Dubilier, 2009).

Ecology and habitat

The bulk of information regarding the ecology of *Methyloprofundus* is based on molecular environmental studies, and *in situ* hybridization works on *Bathymodiolus* mussel specimens. Sequences (*pmoA* and 16S rRNA genes) affiliated with genus *Methyloprofundus* have been detected in the oxygen minimum zone of the eastern Pacific (Hayashi et al., 2007), in the deep water column following the extensive 2010 Gulf of Mexico oil spill (Rivers et al., 2013), and in multiple deep-sea methane seep environments (Li et al., 2014; Redmond et al., 2010; Ruff et al., 2013). In seep environments, *Methyloprofundus* typically occupies surficial sediments (0–3 cm depth). Gene signatures have not been reported from deeper sediments (>3 cm), shallow marine waters, or freshwater or terrestrial environments. Thus, the genus is largely limited to marine methane seep sediment environments, with additional occasional detection in deep-water column samples. This distribution pattern provides the basis for the genus name. *Methyloprofundus* can occasionally be detected in nonseep deep-sea sediments at very low abundance. Members of this genus are present in high abundance in the bacteriocyte cells of *Bathymodiolus* gills (Duperron et al., 2007; Halary et al., 2008).

Enrichment/isolation procedures

Attempts to isolate members of *Methyloprofundus* from bacteriocyte gill tissue have not produced viable pure cultures to date. On the other hand, free-living members of *Methyloprofundus* can be enriched from deep-sea environmental samples, and this approach coupled with dilution-to-extinction allowed isolation of the type species of the genus. Enrichment involves an initial incubation of several months or longer under methane. Following enrichment, colonies are generated on solid media and screened for the *pmoA* gene, using polymerase chain reaction (PCR), which identifies candidate methanotroph colonies. Sequencing the PCR amplicons identifies the affiliation of each candidate methanotroph colony. Repeated dilution to extinction of candidate colonies in liquid culture can successfully purify *Methyloprofundus* from contaminating bacteria. A variety of sediment samples have shown enrichment of members of *Methyloprofundus* using this strategy. These unpublished enrichments have not yet resulted in the isolation of additional pure species.

Maintenance procedures

Methyloprofundus is best propagated in liquid culture using a slight modification to standard media for methanotroph cultivation. Nitrate mineral salts (NMS, ATCC medium 1306) provide the base media for this genus. Trace elements from DSM medium 141 replace the trace elements from ATCC medium 1306. Vitamin solution from DSM medium 141 (1000X) is added, and salt is adjusted to 2%. This resulting medium is sterilized by filtration. Freshly prepared medium promotes optimal growth. Cultures are grown in stoppered tubes, bottles, or flasks; culture volume should not exceed 30% of the vessel volume. Limiting culture volume to 10% of vessel results in higher final cell density. Refreshing the headspace during late logarithmic phase can also increase

final cell density. After stoppering and crimping vessel, methane is added at a final volume of 5–20% headspace and a final pressure of 1–2 bars. Ammonium mineral salts (AMS) can replace NMS in the above-mentioned media with satisfactory results.

Cultures are best incubated at temperatures between 4 and 23 °C. Maximal growth rates for the type species occur between 18 and 23 °C. Cells require rocking or gentle swirling during incubation for best growth.

A several day lag period is expected upon recovering active cultures from frozen stocks. Upon entering active growth, cells will divide at a minimum of every 9 h under optimal growth conditions. Growth is monitored as a change in optical density at 450 nm. An OD_{450} of 0.3–0.4 is typical at saturation. Stationary phase cultures kept at or below room temperature remain viable for at least several months.

Methyloprofundus does not survive heat or desiccation, and therefore, lyophilization is not expected to be an effective long-term storage method. Frozen stocks can be successfully prepared; however, standard preservation media (e.g., glycerol) are inadequate to preserve *Methyloprofundus* stocks. Instead, storage of *Methyloprofundus* requires trehalose-based preservation medium (Hoefman et al., 2012). Preparation of freezer stocks is best accomplished by growing several hundred milliliters of *Methyloprofundus* to mid log phase ($OD_{450} \sim 0.1$). This large volume is sterilely pelleted at 2000 rpm for 20 min, and the supernatant decanted. The pellet is resuspended in several milliliters of trehalose-based preservation medium (Hoefman et al., 2012) under methane. This dense culture is incubated with gentle rocking or swirling for approximately 12 h, to allow cells to incorporate trehalose. After this incubation, dimethylsulfoxide (DMSO) is added to a 5% final concentration, and small aliquots of this dense preparation are frozen at −80 °C.

From frozen stocks, cells are most successfully revived in freshly prepared media. Best results for reviving cells from frozen stocks are observed when a single frozen aliquot is allowed to thaw at room temperature, a small volume of fresh and sterile media is added to the aliquot, and the entire diluted aliquot is transferred gently to a prepared culture vessel. Avoid vortexing or excessive pipetting as the cells may be fragile at this stage. A dilution to extinction series can be generated if any concern exists regarding culture purity.

Procedures for testing special characters

For microscopic identification of *Methyloprofundus* in complex environmental samples, fluorescent *in situ* hybridization (FISH) is recommended. The oligonucleotide probe MetI-444 (Losekann et al., 2007) works well in this application

FIGURE 2. Phylogenetic trees indicating placement of *Methyloprofundus sedimenti* within family *Methylococcaceae*. Both trees were inferred with maximum likelihood using 100 bootstraps. Bootstrap support (>50%, open circles and >80%, closed circles) is indicated at nodes. The bars indicate substitutions per site. Ammonia-oxidizing betaproteobacteria *Nitrosomonas cryotolerans* and *Nitrosopira briensis* served as outgroups (not shown) for both trees. (a) 16S rRNA gene-based phylogeny. (b) PmoA-based phylogeny.

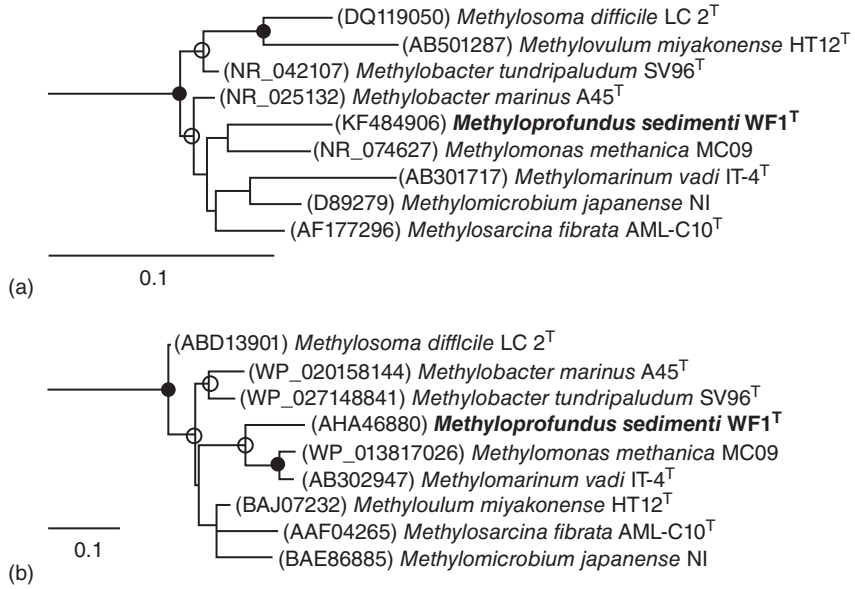


TABLE 1. Features that distinguish genus *Methyloprofundus* from closely related genera

Characteristic		1	2	3	4	5	6
Temperature for growth (°C)	Range	4–26	3.5–37	5–37	15–37	20–44	30–55
	Optimum	18–23	ND	ND	25–30	37–43	45–50
pH for growth	Range	6–8	5–9	3.8–9	5.5–10.5	4.5–7.0	5.3–6.9
	Optimum	6.5–7.5	ND	ND	ND	6.2–6.6	6.0–6.4
Salinity range (%, w/v)	Range	1–4	0–3	0–2.5	0–8.8	1.0–8.0	1.0–5.0
	Optimum	2	ND	ND	ND	2–3	3.0
Motility		−	±	+	+	+	+
Major PLFAs (%)[a]		$C_{16:0}$ (17), $C_{16:1}$ ω8 (29), $C_{16:1}$ ω7*c* (26), $C_{16:1}$ ω6 (21), $C_{16:2}$ ω2,6 (6)	$C_{14:0}$ (7–11), $C_{16:0}$ (7–9), $C_{16:1}$ ω8 (0–35), $C_{16:1}$ ω7 (23–58), $C_{16:1}$ ω6*c* (4–6), $C_{16:1}$ ω5*c* (6–8), $C_{16:1}$ ω5*t* (10–26)	$C_{4:0}$ (19–25), $C_{16:0}$ (5–9), $C_{16:1}$ ω8*c* (19–41), $C_{16:1}$ ω7*c* (7–15), $C_{16:1}$ ω6*c* (5–13), $C_{16:1}$ ω5*c* (2–6), $C_{16:1}$ ω5*t* (8–16)	$C_{16:0}$ (12–18), $C_{16:1}$ ω8*c* (13–19), $C_{16:1}$ ω7*c* (14–20), $C_{16:1}$ ω6*c* (6–14), $C_{16:1}$ ω5*c* (5–7), $C_{16:1}$ ω5*t* (10–30), $C_{18:1}$ ω7*c* (0–27)	$C_{16:0}$ (21.8–32), $C_{16:1}$ ω7*c* (35.5–50.8), $C_{16:1}$ ω5*t* (7.4–24.3),	$C_{16:0}$ (43), $C_{18:1}$ (39.1)
DNA G + C content (mol%)		40.5	48.5–59	46–51	51–51.6	50.9–51.7	66

Taxa: 1, *Methyloprofundus sedimenti*; 2, *Methylobacter* (14 strains) (Bowman et al., 1995; Bowman et al., 1993; Wartiainen et al., 2006); 3, *Methylomonas* (54 strains) (Bowman et al., 1995; Bowman et al., 1993; Danilova et al., 2013; Fang et al., 2000; Kalyuzhnaya et al., 2008; Kalyuzhnaya et al., 1999); 4, *Methylomicrobium* (15 strains) (Bowman et al., 1995; Bowman et al., 1993; Kalyuzhnaya et al., 2008); 5, *Methylomarinum*, (Hirayama et al., 2013); and 6, *Methylomarinovum* (Hirayama et al., 2014).

[a]Fatty acid assignments for *Methyloprofundus* are corrected from the defining publication and were kindly provided by Dr. Matthias Kellerman. Trace (<1%) $C_{16:1}$ ω7*t* and $C_{16:1}$ ω5 were also detected in this analysis.

and can be used at a formaldehyde concentration of 30% during hybridization. For molecular detection of *Methyloprofundus* from environmental samples, DNAs, or candidate colonies, PCR can be used. Primers that detect the *pmoA* gene (pmoA189f, mb661r; (Costello and Lidstrom, 1999)) are satisfactory for such investigations; resulting amplicons are either cloned (environmental samples) or sequenced directly (colonies) to verify phylogenetic affiliation. It can be beneficial to dilute template samples 10-fold and 100-fold for successful PCR amplification; concentrated samples can inhibit the PCR.

Differentiation from other closely related genera

Among cultivated methanotrophs, *Methyloprofundus* is most closely related to members of *Methylobacter, Methylomonas*, and *Methylomicrobium* (Figure 2). However, *Methyloprofundus* differs from these genera by virtue of a substantially lower G + C content, utilization of leucine and lysine as nitrogen sources, and the presence of $C_{16:2}$ unsaturated fatty acids. Additional features distinguishing *Methyloprofundus* from related methanotrophic are provided in Table 1.

Taxonomic comments

Methyloprofundus (type species and uncultured members) forms a monophyletic clade with family *Methylococcaceae* and is most closely related to genera *Methylobacter* (95% 16S rRNA gene similarity; 81.7–82.7% average nucleotide identity), *Methylomonas* (94% 16S rRNA gene similarity; 81.5% average nucleotide identity), and *Methylomicrobium* (93% 16S rRNA gene similarity; 79.3–80.5% average nucleotide identity). The mol% DNA G + C content of *Methyloprofundus* is the lowest documented to date among cultivated methanotrophs. Of some interest, low DNA G + C content is one defining hallmark among intracellular symbionts and pathogens. Isolation of additional members of *Methyloprofundus* is necessary to firmly establish the range of DNA G + C content in the genus.

List of species of the genus *Methyloprofundus sedimenti*

Methyloprofundus sedimenti Tavormina, Hatzenpichler, McGlynn, Chadwick, Dawson, Connon, and Orphan 2015, 258^{VP}

se.di.men'ti. L. gen. n. *sedimenti* of sediment.

Cells are approximately 1.3 µm in length. Cells do not grow at or above 29 °C and do not form colonies on solid NMS medium. NaCl in the range 1–4% is required for growth in NMS. The type strain of this species, WF1 (whale fall 1), was isolated from surficial (0–1 cm) sediment from Monterey Canyon, California, USA, 23 km from shore. Sediment was sampled near a whale fall, at a depth of 1828 m below sea level.

DNA G+C content (mol%): 40.5 (genome analysis).
Type strain: WF1, LMG 28393, ATCC BAA-2619.
EMBL/GenBank accession (16S rRNA gene): KF484906.

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