

## A Novel Cold-adapted *Methylovulum* species, with a High C16:1 $\omega$ 5c Content, Isolated from an Arctic Thermal Spring in Spitsbergen

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A novel cold-adapted methane-oxidizing bacterium, termed TFB, was isolated from the thermoglacial Arctic karst spring, Trollosen, located in the South Spitsbergen National Park (Norway). The source water is cold and extremely low in phosphate and nitrate. The isolate belongs to the *Methylovulum* genus of gammaproteobacterial methanotrophs, with the closest phylogenetic affiliation with *Methylovulum miyakonense* and *Methylovulum psychrotolerans* (96.2 and 96.1% 16S rRNA gene sequence similarities, respectively). TFB is a strict aerobe that only grows in the presence of methane or methanol. It fixes atmospheric nitrogen and contains Type I intracellular membranes. The growth temperature range was 2–22°C, with an optimum at 13–18°C. The functional genes *pmoA*, *mxoF*, and *nifH* were identified by PCR, whereas *mmoX* and *cbbl* were not. C16:1 $\omega$ 5c was identified as the major fatty acid constituent, at an amount (>49%) not previously found in any methanotrophs, and is likely to play a major role in cold adaptation. Strain TFB may be regarded as a new psychrotolerant or psychrophilic species within the genus *Methylovulum*. The recovery of this cold-adapted bacterium from a neutral Arctic thermal spring increases our knowledge of the diversity and adaptation of extremophilic gammaproteobacterial methanotrophs in the candidate family “*Methylomonadaceae*”.

**Key words:** arctic spring, cold adapted, fatty acids, methanotrophs, pMMO

Aerobic methane oxidation is performed by a unique group of methylotrophic bacteria found in various methane-containing environments, e.g. soil, lake sediments, rice paddies, landfills, tundra, and thermal and cold ecosystems (Hanson and Hanson, 1996; Dunfield *et al.*, 2007), in which they play an important role in controlling the release of methane into the atmosphere (Conrad, 1996; Reeburgh, 2007). Most methane in nature is formed through anaerobic microbial processes by methanogenic archaea. However, thermogenic methane, a non-microbial source, is generated through the abiotic decomposition of buried organic materials and geochemical processes. This geological methane is freed to the atmosphere via seeps, mud volcanoes, gas venting, and geothermal areas (Etiope and Klusman, 2002; Fiebig *et al.*, 2007; Nazaries *et al.*, 2013).

Methane-oxidizing bacteria (MOB), or methanotrophs, possess highly specialized metabolism and have the ability to grow using only methane and a few other one-carbon compounds as their source of carbon and energy. These microorganisms currently belong to the phyla *Proteobacteria*, *Verrucomicrobia*, and *Methylomirabilaeota* (the candidate phylum NC10) (Houghton *et al.*, 2019). A reclassification of the phylum *Proteobacteria* has more recently been recommended, which includes twenty-seven genera of proteobacterial methanotrophs (five genera of the

class *Alphaproteobacteria* and twenty-two genera of the class *Gammaproteobacteria*) (Rahalkar *et al.*, 2007; Trotsenko and Murrell, 2008; Deutzmann *et al.*, 2014; Hirayama *et al.*, 2014; Khalifa *et al.*, 2015; Tavormina *et al.*, 2015), and two genera of the phylum *Verrucomicrobia* were also suggested (Op Den Camp *et al.*, 2009; Sharp *et al.*, 2014; Van Teeseling *et al.*, 2014). The taxonomic structure at the family level, within the order *Methylococcales* of gammaproteobacterial methanotrophs, was reconstructed using genome-based phylogeny, and three family lineages were proposed as *Methylomonadaceae* (Type Ia) (Parks *et al.*, 2018), *Methylococcaceae* (Type Ib) (Orata *et al.*, 2018), and *Methylothermaceae* (Type Ic) (Hirayama *et al.*, 2014).

Several key functional genes, *pmoA* (encoding a subunit of the particulate methane monooxygenase: pMMO), *mmoX* (encoding a subunit of the soluble methane monooxygenase: sMMO), and *mxoF* (encoding the large subunit of methanol dehydrogenase: MDH), have been used to identify aerobic gammaproteobacterial methanotrophs from various environments. The *pmoA* gene is often used as a phylogenetic marker for the detection of aerobic methanotrophs in distinct habitats, including cold methane seeps in the floodplains of west Siberian rivers (Knief, 2015; Oshkin *et al.*, 2016). The alphaproteobacterial genera (Type IIb) *Methylocella* (Dedysh *et al.*, 2004) and *Methyloferula* (Vorobev *et al.*, 2011) were described as psychrotolerant and acidophilic methanotrophs. They were isolated from cold ecosystems (wetlands) and only contain sMMO enzyme systems.

Methanotrophs generally occur in relatively cold environments, e.g. tundra soil, ground waters, arctic wetland soil, saline meromictic lakes, polar lakes, permafrost, and marine sediments (Bowman *et al.*, 1997; Trotsenko and

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Khmelenina, 2005; Wartiainen *et al.*, 2006). Until now, only two true psychrophilic species (optimal growth temperatures between 3.5 and 13°C), *Methylosphaera hansonii* (Bowman *et al.*, 1997) and *Methylobacter psychrophilus* (Omelchenko *et al.*, 1996), have been described and isolated from the surface sediments of an Antarctic meromictic lake and Russian Arctic tundra soil, respectively. *Msp. hansonii* requires salt-water for growth, whereas *Mbc. psychrophilus* does not need NaCl for growth. Several psychrotolerant Type Ia methanotrophs from various permanently cold habitats have been reported as new genera and species, including *Methylovulum psychrotolerans* (Oshkin *et al.*, 2016), *Methyloglobulus morosus* (Deutzmann *et al.*, 2014), *Methylovulum miyakonense* (Iguchi *et al.*, 2010), *Methyloprofundus sedimenti* WF1<sup>T</sup> (Tavormina *et al.*, 2015), *Methylosoma difficile* (Rahalkar *et al.*, 2007), *Methylobacter tundripaludum* (Wartiainen *et al.*, 2006), and *Methylomonas scandinavica* (Kalyuzhnaya *et al.*, 1999). Three methanotrophic Type Ia bacteria, strain M200, *Methylomonas* strain M5 (Kip *et al.*, 2011), and *Methylomonas paludis* strain MG30 (Danilova *et al.*, 2013), were recently isolated from peat ecosystems. They were reported as being acid-tolerant (growth below a pH of 4.5). Furthermore, two sheathed and filamentous freshwater microorganisms, *Crenothrix polyspora* Cohn 1870 (Stoecker *et al.*, 2006) and *Clonothrix fusca* Roze 1896 (Vigliotta *et al.*, 2007), were identified and associated with gammaproteobacterial methane oxidizers. They appear to be psychrotolerant, but have not yet been grown in pure cultures.

Spitsbergen is the largest island of the high Arctic Svalbard archipelago, located north of Norway. It has a geologically active past and hot groundwater and glacial meltwater is mixed in several places, leading to karst springs at which the water stays above freezing temperature throughout the year (Lauritzen and Bottrell, 1994). One of the largest thermal springs on South Spitsbergen is Trollosen, located in the southernmost part (Fig. 1). Trollosen discharges water at a maximum rate of 18 m<sup>3</sup> s<sup>-1</sup>, which varies with the seasons. Water temperature varies between 4 and 15°C. The smell of hydrogen sulphide (H<sub>2</sub>S) and the presence of organic slime

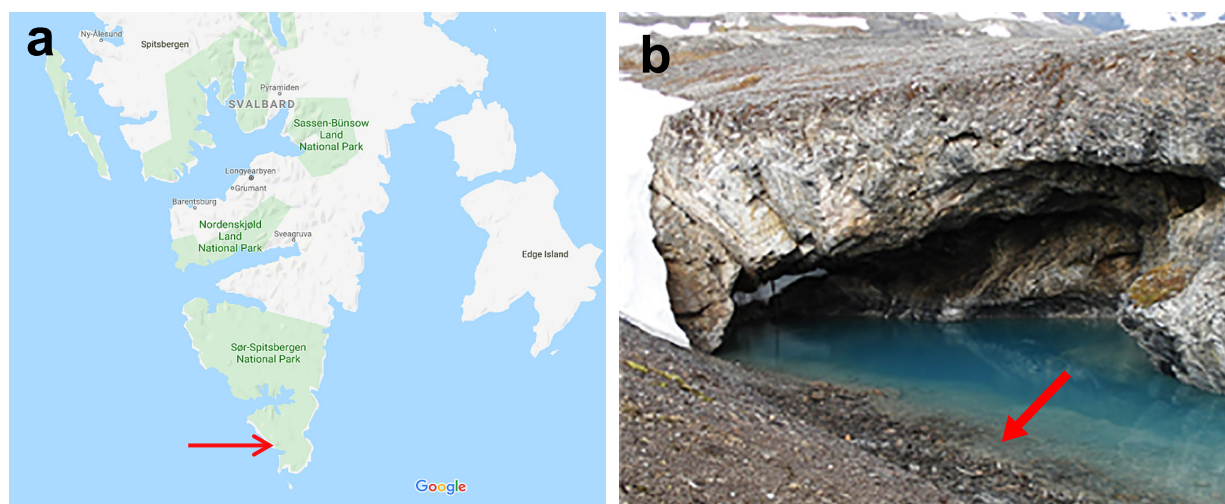
in discharged water provides evidence of an active microbial community in the Trollosen aquifer (Lauritzen and Bottrell, 1994). In a previous study based on cultivation-independent analyses, sulphur-oxidizing chemolithotrophic *Proteobacteria* were found to dominate the bacterial community in this spring, with minor contributions by heterotrophs, including methane oxidizers (Reigstad *et al.*, 2011). In the present study, water and slime samples were collected to investigate methane-utilizing bacteria in the Trollosen spring. A novel cold-adapted methanotrophic bacterium belonging to the candidate family “*Methylomonadaceae*” of the class *Gammaproteobacteria* was recovered.

## Materials and Methods

### Sampling and growth conditions

In June 2006, approximately 40 mL of water, containing white slimy filaments, was collected from the thermal spring, Trollosen (76°72'N, 16°28'E), in the South Spitsbergen National Park during a joint expedition with another group from the University of Bergen (Reigstad *et al.*, 2011). Samples were collected in Falcon tubes and stored at 8°C before being used as an inoculum for the enrichment and cultivation of methanotrophs. The *in situ* temperature and pH of the sample were 15°C and 6.8, respectively. The 3×1 m cave opening of the spring is dominated by glacial meltwater from the nearby Vitkovskij glacier and discharged approximately 8 m<sup>3</sup> s<sup>-1</sup> at the time of sampling. On the day of sampling, the water contained (in decreasing order of concentrations in mM): Cl<sup>-</sup>, 56; Na, 2.76; Ca, 2.46; Mg, 0.81; ammonia, 0.19; SO<sub>4</sub><sup>2-</sup>, 0.18; Li, 0.1; and lesser amounts of Al, Fe, B, Mn, As, Mo, and Ba. The amounts of nitrate and phosphate were below the detection limit (Reigstad *et al.*, 2011).

A previously described low-salt mineral medium (LMM) with pH 6.8 and vitamins was used for enrichment conditions (Islam *et al.*, 2015), except that KNO<sub>3</sub> was replaced with NH<sub>4</sub>Cl (0.1 g L<sup>-1</sup>). Thereafter, 2 mL of the water sample was added directly to 20 mL of LMM in a 120-mL serum bottle. The bottle was closed with butyl rubber caps and aluminium crimped seals. A mixture of methane (80%) (purity of methane, 99.5%; Yara Praxair), air (10%), and CO<sub>2</sub> (10%) was added aseptically via a syringe into the headspace. The bottle was incubated at 18°C in the dark and without shaking for three weeks. The gas mixture was replaced every seven days. After the incubation, the enrichment culture became



**Fig. 1.** Sampling location. (a) Map of South Spitsbergen National Park in Svalbard designating the sampling site (<https://www.google.com/maps>). (b) A close-up photograph of the Arctic thermal spring (Trollosen). The arrow shows the sampling location.

visibly turbid and was examined using a phase-contrast microscope (Eclipse E400 microscope; Nikon). Thereafter, 2 mL of the culture was transferred to fresh LMM and re-incubated under the same conditions as those described above.

#### *Enrichment, isolation, and morphology*

To recover a true methane oxidizer, the environmental enrichment sample was transferred five times into fresh LMM and incubated with methane, air, and CO<sub>2</sub>. Serial dilutions (10<sup>-6</sup> to 10<sup>-8</sup>) were prepared and 0.1-mL aliquots were spread onto either gelrite (20 g L<sup>-1</sup>, Gelzan™ CM; Sigma-Aldrich) or agar (Difco) plates containing LMM. The plates were then incubated for four weeks at 18°C in jars filled with a methane/air (2:1) mixture. Individual colonies were collected, streaked onto fresh plates, and re-incubated for three weeks. A single colony was then selected and examined by phase-contrast microscopy. The selected colony was transferred to fresh liquid LMM and incubated for three weeks under the same growth conditions. After a pure culture was extracted, LMM was used for its routine cultivation at 15 and 18°C for two weeks. The purity of the culture was assessed by phase-contrast and electron microscopies, observations of single colony growth on plates, and a heterotrophic contamination test using Luria-Bertani broth (1 to 5% [v/v]) and glucose (10 mM). In addition, growth was examined in the diluted and undiluted nitrate or ammonium mineral salt media used to cultivate the methanotrophs (Whittenbury *et al.*, 1970). Morphology was assessed using a phase-contrast microscope and Jeol-1230 electron microscope. Exponentially-grown cells were harvested by centrifugation and used to prepare ultrathin sections for transmission electron microscopy (TEM), as described previously (Islam *et al.*, 2015).

#### *Utilizable carbon and nitrogen sources, pH, temperature, and salt concentration*

Various organic compounds were tested as possible carbon and energy sources at a concentration of 10 mM in fresh LMM. The following substrates were tested: glucose, acetate, pyruvate, lactate, malate, succinate, ethanol, sucrose, fructose, maltose, mannitol, sorbitol, Luria-Bertani broth, and yeast extract. Growth on methanol, methylamine, formate, and formaldehyde was tested at concentrations between 0.03% and 0.2% (v/v) in LMM. To prevent vaporization, bottles were capped with butyl-rubber stoppers. Growth was also tested in nitrogen-free LMM (without KNO<sub>3</sub>) and adjusted to pH 6.8 in triplicate, and the only nitrogen source was N<sub>2</sub> from the air. After three weeks of incubation, resultant growth was evaluated. The growth temperature range was tested at 0, 2, 5, 10, 13, 15, 18, 20, 22, 25, and 30°C (at pH 6.8). The effects of pH on growth were investigated and the test for antibiotic sensitivity was performed as previously described (Islam *et al.*, 2020). The effects of salt concentrations on growth were also assessed by adding NaCl (0.1, 0.5, 1, 2.0, and 3% [w/v]) to LMM. Growth was assessed after two weeks of incubation using optimal culture conditions.

#### *Acetylene inhibition test, naphthalene assay, and fatty acid analysis*

To assess the effects of acetylene, 4% of acetylene (v/v) was added into the headspace after five days of incubation with methane or methanol. Triplicate flasks were used for the methane oxidation inhibition test. One flask without acetylene was used as a control. The naphthalene-oxidation assay was performed as described previously (Islam *et al.*, 2020). A phospholipid fatty acid (PLFA) analysis of cells grown at 15°C was performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

#### *DNA isolation and 16S rRNA and functional gene analyses*

Genomic DNA was isolated using the CTAB procedure (Murray and Thompson, 1980). The 16S rRNA gene was amplified as described previously (Islam *et al.*, 2008) using a Peltier thermal

cycler (PTC-200; MJ Research). PCR products were purified and sequenced using the BigDye kit for automated DNA sequencers (ABI 3700 PE; Applied Biosystems). Amplification of the functional genes *pmoA*, *mmoX*, *mxoF*, *nifH* (nitrogenase iron protein), and *cbbL* (the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase, RubisCO) was performed using genomic DNA (Table S1). PCR was accomplished using Dynazyme™ High-Fidelity DNA Polymerase (Finnzymes) and the PCR program was previously described (Islam *et al.*, 2015). *Methylococcus capsulatus* (strain Bath) DNA was used as a positive control. MilliQ water was used as a negative control. The PCR products of the gene *pmoA* were cloned using pCR4-TOPO (Invitrogen) according to the manufacturer's protocol. The four independent clones of the *pmoA* gene were sequenced and confirmed to be identical. The Southern blotting technique was utilized for the verification of pMMO and sMMO. DNA from *Mcc. capsulatus* (Bath) and *Methylophilum kamchatkense* (Kam1) were used as positive and negative controls, respectively. Genomic DNA from the pure isolate was digested with EcoRI and HindIII. Fragments of DNA were separated using agarose gel electrophoresis and transferred onto Hybond-N nylon membranes (Amersham Biosciences). The probes utilized for hybridization were made by PCR using the *pmoA* and *mmoX* primer sets (Table S1) and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the DNA labeling kit (Amersham Biosciences) as previously described (Baxter *et al.*, 2002; Islam *et al.*, 2008).

#### *Phylogenetic analysis and accession numbers*

The 16S rRNA sequences and deduced protein sequences of the functional genes (using the ExPASy Translate tool; <https://web.expasy.org/translate>) were evaluated with available sequences in the GenBank database using BLASTn and BLASTp (the NCBI tools), respectively (Table S2). 16S rRNA gene and deduced amino acid (PmoA) sequences were aligned using the CLUSTAL W algorithm. Phylogenetic trees were constructed using different methods, such as the Neighbor-Joining, Maximum Likelihood, and Minimum-Evolution methods, based on the following models: Kimura 2-parameter, Maximum composite likelihood, Tamura 3-parameter, Jukes-Cantor, Jones Taylor-Thornton (JTT), Dayhoff, and Poisson. These models were implemented in the MEGA7 software package (Kumar *et al.*, 2016). The sequences of the 16S rRNA, *pmoA*, *mxoF*, and *nifH* genes of strain TFB have been deposited in GenBank under accession numbers GQ130272, KX282487, KX282488, and KX282489, respectively.

## **Results and Discussion**

#### *Isolation of strain TFB and morphological characteristics*

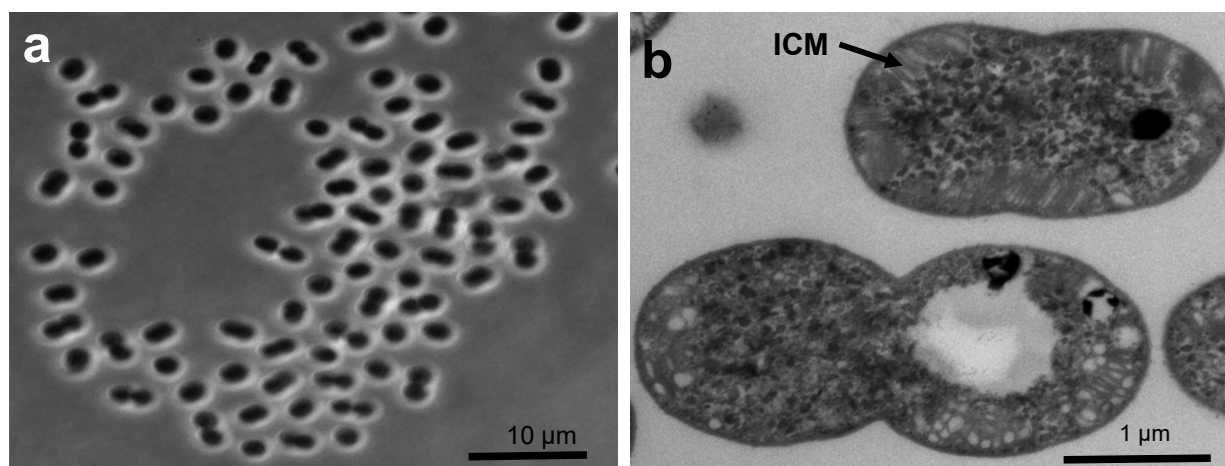
After three weeks of incubation at 18°C with methane, the enrichment was turbid, and phase-contrast microscopy revealed the presence of non-motile, straight, or curved rod-shaped cells, small cocci, and large coccoid cells with a thick mucous capsule. The large coccoid cells demonstrated poor growth with NH<sub>4</sub>Cl in enriched LMM. After five successive transfers into fresh LMM (with KNO<sub>3</sub>), the fraction of large coccoid cells was larger. After 3–4 weeks of incubation, the following colonies were observed on gelrite plates: transparent (approximately 1.4 to 1.8 mm in diameter), light yellow (approximately 0.5 to 1.0 mm), small white (0.8 to 1 mm), and large white (2.0 to 2.5 mm). Transparent colonies were not present on the agar plates (even after five weeks of incubation). However, small and large white colonies were observed. To detect a true methane oxidizer, three different colonies were transferred to fresh LMM separately and incubated with methane, air, and CO<sub>2</sub>. Only a single transparent colony from the gelrite plate showed growth in

methane after two weeks of incubation, whereas no growth was observed for the white or light-yellow colonies. A true cold-adapted methanotrophic isolate was obtained from the transparent colonies on the gelrite plates. The isolate, called TFB, was a large coccoid/elliptical rod with a length of 1.5–2.0  $\mu\text{m}$  and diameter of 1.2–1.5  $\mu\text{m}$ . The strain grew in LMM with methane or methanol (0.2%). It did not grow in the presence of methane or methanol under anaerobic conditions or in the absence of methane or methanol. These cells were non-motile and occurred individually or in pairs. An investigation by electron microscopy revealed the presence of intracytoplasmic membranes (ICM) stacked (as vesicular disks) throughout TFB cells, which is a common feature of the methanotroph family *Methylomonadaceae* (Fig. 2). Spores or cysts were not observed in TFB using TEM. The isolate had a polysaccharide-like matrix around it, which was also observed in the acid-tolerant methanotrophic strain M200 (Kip *et al.*, 2011). Similar bacteria, also described as

large morphotype 2 psychrophilic methanotrophs, have been observed in enrichments from Russian Arctic tundra soil (Berestovskaya *et al.*, 2002). Moreover, these large cocci, with thick mucous capsules, grew at a pH and temperature between 5–7 and 5–10°C, respectively.

#### Physiological characteristics

A comparison of the major characteristics of strain TFB with related cold-adapted (*e.g.* psychrophilic and psychrotolerant) and mesophilic Type Ia methanotrophs is shown in Table 1. Strain TFB utilized only methane and methanol as the sole carbon and energy sources, respectively. It did not grow on substrates containing multi-carbon compounds or in complex media, evincing obligate methanotrophy. Growth in methanol was observed at concentrations between 0.05 to 0.2% (v/v). Cells utilized nitrate and ammonium salts as nitrogen sources. In the absence of vitamins, TFB grew very poorly. The isolate grew on nitrogen-free



**Fig. 2.** Cell morphology of strain TFB. (a) Phase-contrast micrograph of cells grown in LMM, containing methane, for 7 days. (b) Transmission electron micrograph showing intracytoplasmic membrane (ICM) systems. Bars, 10  $\mu\text{m}$  (a) and 1  $\mu\text{m}$  (b).

**Table 1.** Comparisons of major characteristics of the cold-adapted strain TFB and Type Ia gammaproteobacterial methanotrophs. **Strains:** **1)** Strain TFB (this study); **2)** *Methylovulum psychrotolerans* (strains Sph1<sup>T</sup>, Sph2, and OZ2) (Oshkin *et al.*, 2016); **3)** Methanotrophic strain M200 (Kip *et al.*, 2011); **4)** *Methylovulum miyakonense* HT12<sup>T</sup> (Iguchi *et al.*, 2010); **5)** *Methyloprofundus sedimenti* WF1<sup>T</sup> (Tavormina *et al.*, 2015); **6)** *Methylosoma difficile* LC 2<sup>T</sup> (Rahalkar *et al.*, 2007); **7)** *Methyloglobulus morosus* KoM1<sup>T</sup> (Deutzmann *et al.*, 2014); **8)** *Methylobacter tundripaludum* SV96<sup>T</sup> (Wartiainen *et al.*, 2006); and **9)** *Methylosphaera hansonii* ACAM 549<sup>T</sup> (Bowman *et al.*, 1997). +, positive results; –, negative results; nr, not reported.

Characteristics	1	2	3	4	5	6	7	8	9
Cell morphology	Cocci, elliptical rods	Cocci	Cocci	Cocci or rods	Cocci, elliptical rods	Cocci, elliptical rods	Short rods	Rods	Cocci
pMMO	+	+	+	+	+	+	+	+	+
sMMO	–	–	–	+	–	–	–	–	–
<i>mxoF</i> gene	+	+	nr	+	+	+	+	+	nr
<i>nifH</i> gene/N <sub>2</sub> fixation	+	+	+	+	+	+	+	+	+
Pigmentation	Transparent	Light pink	Pink	Pale brown	nr	Pale pink	Red pink	Pale pink	– <sup>d</sup>
Growth temp. (opt.)	2–22 (13–18)	2–36 (20–25)	4–30 (nr)	5–34 (24–32)	4–26 (18–23)	10–30 (25)	4–30 (20)	10–30 (23)	0–21 (10–13)
pH range	5.2–8.5 (6.5–7.2)	4.0–8.9 (6.0–7.0)	4.1–7.0 (5.5)	6.0–7.5	6.0–8.0 (6.5–7.5)	5–9 (6–8)	5–8.5 (6–8)	5.5–7.9	7.0
Growth on methanol	+	+	+	+	+	+	+	– <sup>e</sup>	+
Vitamin required	+	–	–	–	+	+	–	–	– <sup>e</sup>
G+C content (mol%) <sup>a</sup>	nd	51.4–51.9	nr	49.3	40.5	49.9	47.7	47	43–46
G+C content (mol%) <sup>b</sup>	52.2	–	52.2	53.4	–	52.7	54	50.7	–
Source	Arctic thermal Spring	Cold methane seeps and freshwater lake	Acidic <i>Sphagnum</i> peat	Forest soil	Marine sediment	Lake sediment	Lake sediment	Wetland soil	Antarctic meromictic lake

<sup>a</sup>The G+C content was assessed by HPLC (DSMZ; Mesbah *et al.*, 1989; Kamagata and Mikami, 1991). <sup>b</sup>16S rRNA, *pmoA*, *mxoF*, and *nifH* sequences were used to measure the DNA G+C content (mol%). <sup>c</sup>Shows poor to no growth on methanol. <sup>d</sup>Highly purified agar, agarose, and gelrite were likewise unsuccessful for pigmentation. <sup>e</sup>Seawater required.

LMM, demonstrating its ability to fix atmospheric N<sub>2</sub>, whereas growth was more optimal in LMM containing KNO<sub>3</sub>. Strain TFB grew between 2 and 22°C, with optimal growth occurring between 13 to 18°C (Fig. S1). This result implies that strain TFB is more psychrotolerant/psychrophilic than the acid-tolerant methanotrophic strain M200 and mesophilic *Mvm. miyakonense* HT12<sup>T</sup>. Although *Mvm. psychrotolerans* strains grew efficiently at temperatures lower than 10°C, their optimal growth temperature was 20–25°C (Oshkin *et al.*, 2016). No growth was observed at 25°C, indicating that strain TFB is not a member of the mesophilic Type Ia methanotrophs. The specific growth rate ( $\mu$ ) and doubling time at the optimum temperature were 0.017 h<sup>-1</sup> and 18 h, respectively. At 15°C, strain TFB grew at a pH interval of 5.2–8.5; however, optimal growth was observed at a pH 6.8–7.2. It did not show any growth at pH 5.0 or 9.0, demonstrating that strain TFB is a neutrophilic bacterium rather than a member of the acid-tolerant or alkaline methanotrophs. The strain did not require excess NaCl for growth and grew favorably in LMM with 0.1% NaCl (w/v). When NaCl concentrations exceeded 0.5% (w/v), growth was inhibited. Acetylene acts as a suicide substrate for methanotrophs containing pMMO (Prior and Dalton, 1985). The effects of acetylene (4%) on TFB showed that growth and methane oxidation were completely suppressed, which confirmed the presence of pMMO as the functional methane oxidization enzyme. This result is consistent with previous findings obtained on other Type Ia, Type Ib, and verrucomicrobial methanotrophs (Bedard and Knowles, 1989; Islam *et al.*, 2008).

### Fatty acid profile

Strain TFB exhibited a unique fatty acid profile from the known psychrophilic and psychrotolerant methanotrophic genera of MOB (Table 2). The predominant fatty acids were C16:1 $\omega$ 5c, C14:0, and C16:1 $\omega$ 7c. The fatty acid C16:1 $\omega$ 5c is generally found in related psychrotolerant species, such as *Mvm. psychrotolerans* and the methanotrophic strain M200. The genera *Methyloterricola oryzae* 73a<sup>T</sup> (a member of Type Ib in the family *Methylococcaceae*) contained 28.3% of C16:1 $\omega$ 5c, whereas strain TFB contained 49.65%. This was relatively high, and such a high percentage of C16:1 $\omega$ 5c has not previously been reported in any gammaproteobacterial methanotrophs described to date. A recent study reported that *Mvm. psychrotolerans* adapts to decreasing growth temperatures by increasing unsaturation in bulk fatty acids, including C14 and C16, as well as in hopanoids (Bale *et al.*, 2019). At 4°C, *Mvm. psychrotolerans* possesses 88–91% unsaturated fatty acids (mostly isomers of C16:1 and C14:1 $\omega$ 7), and 79–80% when grown at 20°C (Bale *et al.*, 2019). The high amount of C16:1 $\omega$ 5c in strain TFB indicates that it plays an important role in cold adaptation and may be used as a diagnostic feature in identifying cold-adapted methanotrophs and differentiating them from other mesophilic or thermotolerant methanotrophs. The amounts of C14:0, C16:1 $\omega$ 7c, and C16:0 were similar to those in other psychrophilic, psychrotolerant, and mesophilic methanotrophic genera. Additionally, C16:1 $\omega$ 7c in strain TFB showed a significant difference from *Mvm. miyakonense* strain HT12<sup>T</sup>, while acid-

**Table 2.** Comparison of cellular fatty acid compositions of cold-adapted strain TFB with other psychrophilic, psychrotolerant, and mesophilic Type Ia and Type Ib methanotrophs. 1) Strain TFB (this study); 2) *Methylovulum psychrotolerans* (strains Sph1<sup>T</sup>, Sph2, and OZ2) (Oshkin *et al.*, 2016); 3) Methanotrophic strain M200 (Kip *et al.*, 2011); 4) *Methylovulum miyakonense* HT12<sup>T</sup>; 5) *Methyloprofundus sedimenti* WF1<sup>T</sup> (Tavormina *et al.*, 2015); 6) *Methylsoma difficile* LC 2<sup>T</sup> (Rahalkar *et al.*, 2007); 7) *Methyloglobulus morosus* Kom1<sup>T</sup> (Deutzmann *et al.*, 2014); 8) *Methylobacter tundripaludum* SV96<sup>T</sup> (Wartiainen *et al.*, 2006); 9) *Methylsphaera hansonii* ACAM 549<sup>T</sup> (Bowman *et al.*, 1997); 10) *Methyloterricola oryzae* 73a<sup>T</sup> (Frindt *et al.*, 2017); and 11) *Methylococcaceae* strains BRS-k6, GFS-K6, and AK-K6 (Islam *et al.*, 2015). Values are given as a percentage of total fatty acids.

Fatty acids	1	2	3	4	5	6	7	8	9	10	11
C12:0	0.96	–	–	–	–	2.74	0.1	–	–	–	–
C13:1	1.53	–	–	–	–	–	–	–	–	–	–
<b>C14:0</b>	<b>18.28</b>	<b>7.1–9.3</b>	<b>7.5</b>	<b>34.2</b>	–	<b>8.55</b>	<b>0.7</b>	34.9	<b>2–3</b>	–	3.73–8.43
C15:0	1.22	–	0.7	2.97	–	0.79	–	<b>23.4</b>	1–2	–	–
C16:1 $\omega$ 8c	–	22.7–30.1	37.4	–	22.3	–	<b>55.3</b>	8.2	37–41	–	–
<b>C16:1<math>\omega</math>7c<sup>a</sup></b>	<b>16.52</b>	<b>22.5–33.0</b>	<b>14.9</b>	–	3–	<b>60.0</b>	<b>5.8</b>	–	<b>16–19</b>	<b>26.9</b>	57.93–69.41
C16:1 $\omega$ 6c	–	5.7–6.2	9.4	6.40	–	15.0	28.7	26.3	17–18	8.7	–
<b>C16:1<math>\omega</math>5c</b>	<b>49.65</b>	<b>17.3–19.2</b>	<b>24.3</b>	–	–	–	–	–	–	<b>28.3</b>	<b>11.38–30.02</b>
C16:1 $\omega$ 7t	–	–	–	–	–	–	6.8	–	2–3	–	–
C16:1 $\omega$ 9t	–	–	–	–	26.9	–	–	–	–	–	–
C16:0	9.18	6.2–11.4	4.1	46.9	15	8.5	–	–	14–15	30.9	4.72–11.37
C16:1 $\omega$ 9c	–	–	–	–	28.8	–	–	–	–	–	–
C16:1 $\omega$ 10c	–	–	–	–	–	–	–	–	–	2.4	–
C16:1 $\omega$ 11c	–	–	–	–	–	2.44	–	–	–	–	–
C16:0 3OH	2.66	–	–	8.00	–	1.31	1.0	–	–	–	1.50–1.54
C16:2 $\omega$ 9,14	–	–	–	–	7.1	–	–	–	–	–	–
C18:1 $\omega$ 7c	–	0.7–1.2	–	–	–	–	0.9	–	1–2	1.7	–
C18:1 $\omega$ 9c	–	0–1.1	–	–	–	–	–	–	–	–	–
$\beta$ OH-nC16:0	–	2.8–4.6	–	–	–	–	–	–	–	–	–
Growth temp. (°C)	15	20	nr*	24	23	25	20	20	10	30	25

\* not reported

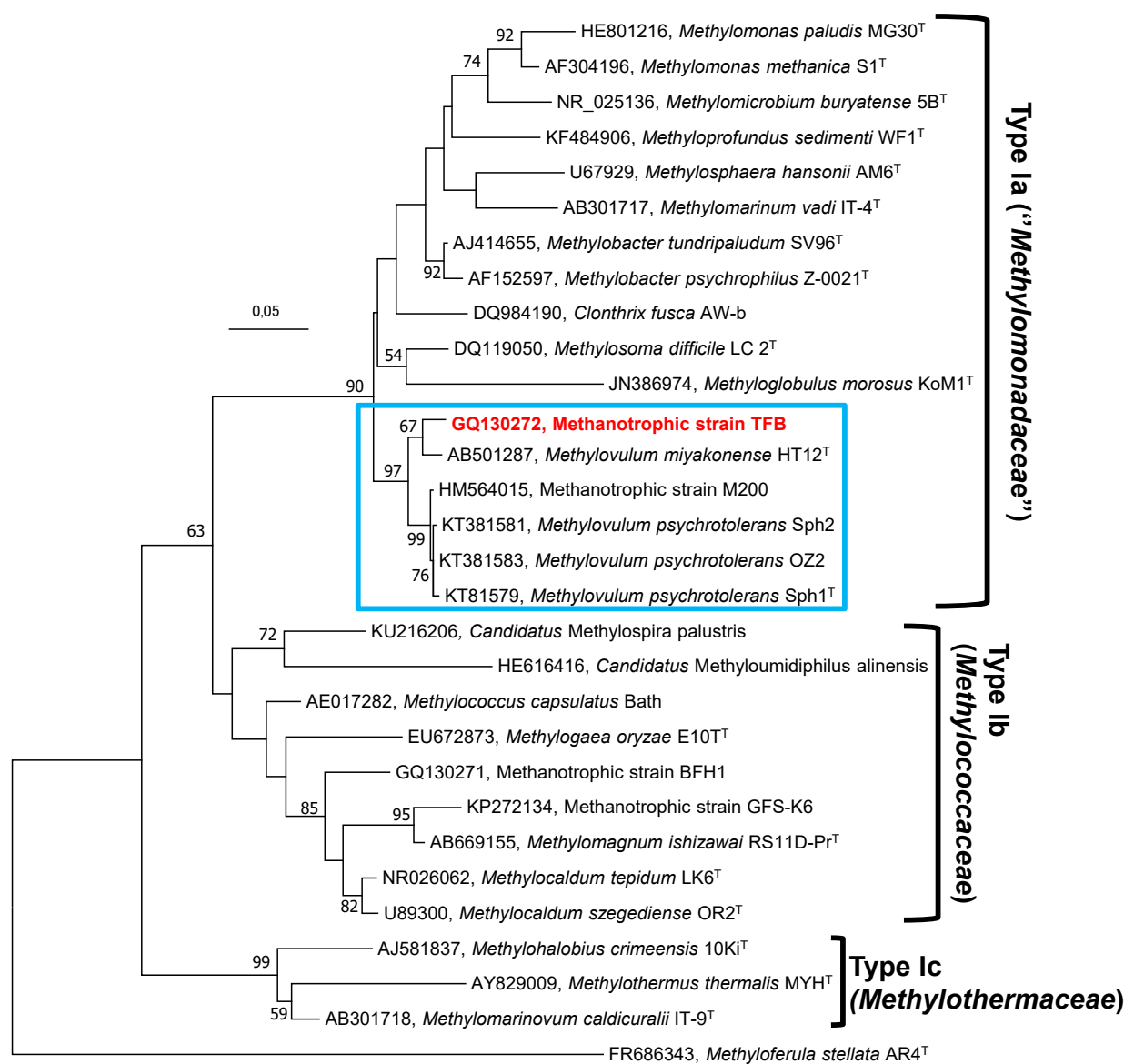
tolerant methanotrophic strain M200 contained the fatty acid C16:1 $\omega$ 8c (37.4%), which was completely absent in strain TFB.

### Functional genes and phylogenetic analyses

Partial fragments of the functional genes *pmoA*, *mxrA*, and *nifH* were amplified and sequenced. The results obtained indicated a close relationship to corresponding genes in other Type Ia methanotrophs. PCR amplification trials on *mmoX* and subsequent Southern blotting (Table S3) yielded negative results that substantiated the negative naphthalene assay, thereby confirming the lack of genes encoding for sMMO in strain TFB. Furthermore, no feasible PCR band for the *cbbL* gene was detected, revealing that the strain may not utilize genes encoding RuBisCO for autotrophic CO<sub>2</sub> fixation. The presence of RuBisCO in certain Type

Ib methanotrophs (such as *Methylococcus*, *Methylocaldum*, *Methylomagnus*, *Methyloterricola*, *Methylospira*, and methanotrophic strains BFH1, BFH2, and LS7-MC) has been reported (Baxter *et al.*, 2002; Danilova *et al.*, 2013; Islam *et al.*, 2015; Islam *et al.*, 2016; Frindte *et al.*, 2017), whereas Type Ia methanotrophs, as well as some Type Ib methanotrophs (such as *Methylomonas*, *Methylomicrobium*, *Methylomarinum*, *Methylotetracoccus*, *Methyloparacoccus*, and *Methylogaea*), do not contain RuBisCO (Baxter *et al.*, 2002; Hirayama *et al.*, 2013; Ghashghavi *et al.*, 2019). The nearly complete 16S rRNA gene (1411 bases) and PmoA (169 amino acids) sequences of the methanotrophic strain TFB were used to construct phylogenetic trees.

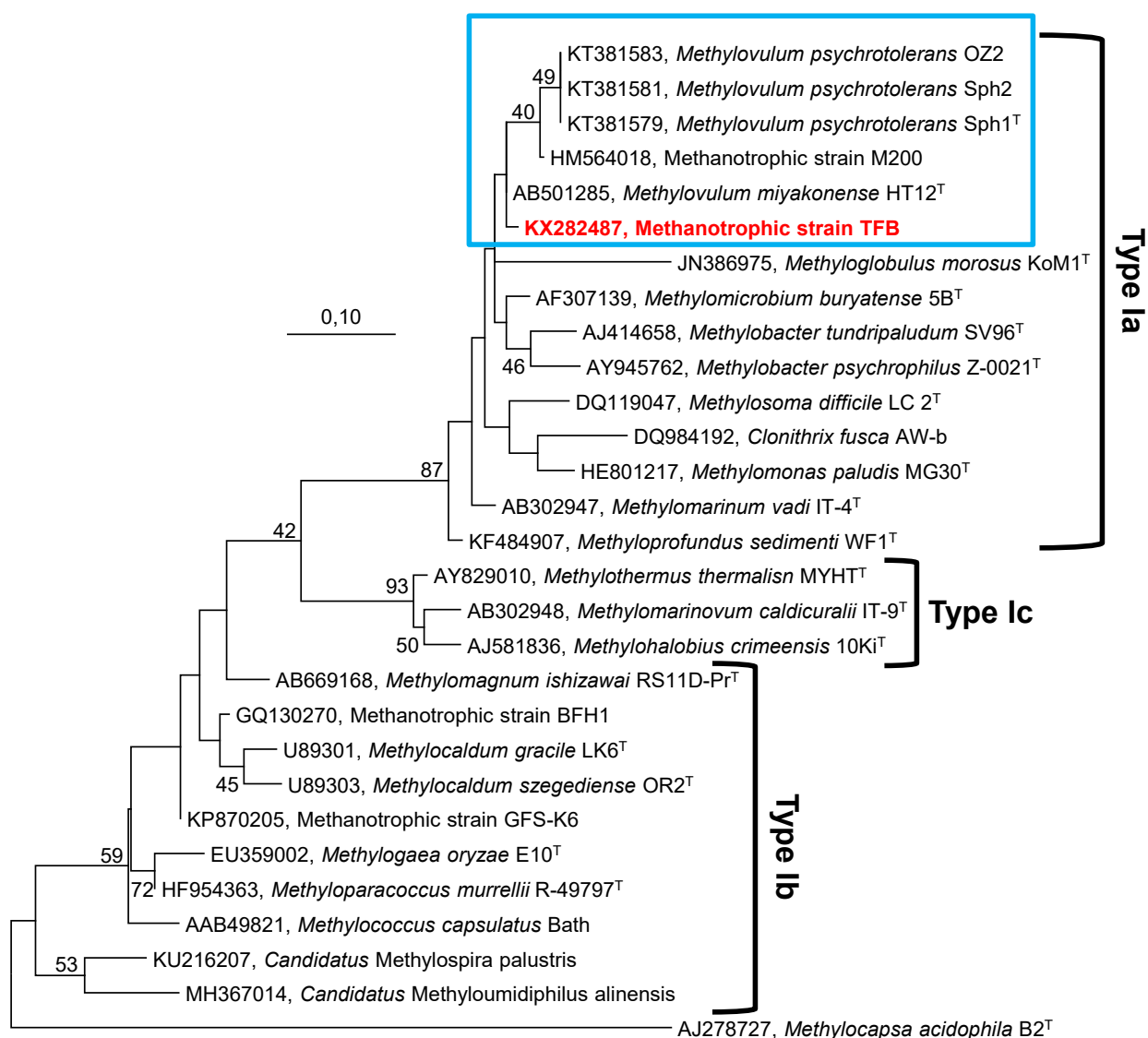
Members of psychrotolerant Type Ia methanotrophic strains, such as *Msm. difficile* LC 2<sup>T</sup> (Rahalkar *et al.*, 2007), *Mbc. tundripaludum* SV96<sup>T</sup> (Wartiainen *et al.*, 2006), and



**Fig. 3.** Molecular phylogenetic analysis (16S rRNA gene sequences) using the Maximum Likelihood method based on the Kimura 2-parameter model of strain TFB (indicated in bold red) and other related Type Ia, Type Ib, and Type Ic gammaproteobacterial methanotrophs. Evolutionary analyses were conducted in MEGA7. Nodes supported by bootstrap values (percentages of 1,000 data resamplings)  $\geq 50\%$  are shown at each node. The Type IIB methanotroph, *Methyloferula stellata* AR4 (FR686343) of the class *Alphaproteobacteria* (in the family *Beijerinckiaceae*) was used as an outgroup. Bar, 0.05 substitutions per nucleotide.

*Mbc. psychrophilus* Z-0021<sup>T</sup> (Omelchenko *et al.*, 1996), have 16S rRNA gene sequence identities of 93.0–93.6% to strain TFB. According to further 16S rRNA gene analyses, the methanotrophic bacterium M200 (Kip *et al.*, 2011), *Mvm. psychrotolerans* (strains Sph1<sup>T</sup>, Sph2, and OZ2), and *Mvm. miyakonense* HT12<sup>T</sup> (Iguchi *et al.*, 2010) were identified as being closely related to strain TFB, with 96.6, 96.4–96.2, and 96.0% sequence similarities, respectively (Table S2). Furthermore, a phylogenetic analysis of the deduced amino acid sequence of *pmoA* showed high sequence similarities with strain M200 (98.8%), *Mvm. psychrotolerans* (98.8%), *Mvm. miyakonense* (100%), *Mbc. tundripaludum* (99.3%), *Mbc. psychrophilus* (97.5%), *Msm. difficile* (97.6%), and *C. fusca* (96.3%). The BLASTp search of MxaF protein sequences from the strain TFB showed the strongest associations with *Mvm. miyakonense* (96.6%), *Mvm. psychrotolerans* (96.6%), *Mbc. tundripaludum*

(93.5%), and *Mgb. morosus* strain Kom1 (91.2%). NifH protein sequences showed associations with *Mvm. miyakonense*, *Mvm. psychrotolerans*, *Mbc. tundripaludum*, *Methylococcus oryzae* (100%), and *Mpf. sedimenti* (95.2%). To establish the affiliation of two or more strains to the same genus, a minimum of 95% identity for 16S rRNA gene sequences is required (Schloss and Handelsman, 2004; Adekambi *et al.*, 2008). 16S rRNA gene sequence similarities (Table S2) between strain TFB (Arctic thermal spring), strain M200 (*Sphagnum* mosses), and *Mvm. miyakonense* HT12<sup>T</sup> (forest soil) were 96.0–96.6%. This result suggests that strains TFB and M200 belong to the genus *Methylovulum*, or that they may denote two separate species, or even represent a new genus within Type Ia methanotrophs. Phylogenetic analyses of the 16S rRNA and *pmoA* genes of the strain TFB revealed that it, along with *Methylovulum* species (*Mvm. miyakonense* and *Mvm.*



**Fig. 4.** Molecular phylogenetic analysis (deduced amino acid sequences of the *pmoA* gene) of strain TFB (indicated in bold red) and other related Type Ia, Type Ib, and Type Ic gammaproteobacterial methanotrophs were inferred using the Maximum Likelihood method based on the Dayhoff matrix-based model. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. Bootstrap values (1,000 replicates) less than 40% are not shown. Evolutionary analyses were conducted in MEGA7. The Type Ib methanotroph, *Methylocapsa acidophila* B2<sup>T</sup> (AJ278727) of the class *Alphaproteobacteria* (in the family *Beijerinckiaceae*) was used as outgroup. Bar, 0.10 substitutions per amino acid position.

*psychrotolerans*) and the methanotrophic strain M200, most probably constitute a new cluster in the Type Ia methanotrophs of the class *Gammaproteobacteria* (Fig. 3, 4, S2, S3, S4, and S5). This phylogenetic assumption was facilitated using physiological characteristics (Table 1) and chemotaxonomic studies (Table 2). Furthermore, the 16S rRNA, *pmoA*, *mxoF*, and *nifH* sequences may contribute to the detection of related methane oxidizers from cold habitats and demonstrate how these cold-adapted bacteria are widely dispersed. Based on the 16S rRNA gene analysis, physiological properties, and fatty acid compositions, we propose that the colorless and neutrophilic methanotrophic strain TFB represents a new species of the genus *Methylovulum*. Strain TFB may be referred to as '*Methylovulum* sp. TFB'. However, the complete genus status of methanotrophic bacteria, namely *Mvm. miyakonense* HT12<sup>T</sup>, *Mvm. psychrotolerans* Sph1<sup>T</sup>, acid-tolerant methanotrophic strain M200, and the neutrophilic methanotrophic strain TFB require further investigations (*e.g.* comparisons including the whole genome) before decisions may be reached regarding the genus level.

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#### References

- Adekambi, T., Shinnick, T.M., Raoult, D., and Drancourt, M. (2008) Complete *rpoB* gene sequencing as a suitable supplement to DNA-DNA hybridization for bacterial species and genus delineation. *Int J Syst Evol Microbiol* **58**: 1807–1814.
- Bale, N.J., Rijpstra, W.I.C., Sahonero-Canavesi, D.X., Oshkin, I.Y., Belova, S.E., Dedysh, S.N., and Sinninghe Damste, J.S. (2019) Fatty acid and hopanoid adaption to cold in the methanotroph *Methylovulum psychrotolerans*. *Front Microbiol* **10**: 589.
- Baxter, N.J., Hirt, R.P., Bodrossy, L., Kovacs, K.L., Embley, T.M., Prosser, J.I., and Murrell, J.C. (2002) The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Arch Microbiol* **177**: 279–289.
- Bedard, C., and Knowles, R. (1989) Physiology, biochemistry, and specific inhibitors of CH<sub>4</sub>, NH<sub>4</sub><sup>+</sup>, and CO oxidation by methanotrophs and nitrifiers. *Microbiol Rev* **53**: 68–84.
- Berestovskaya, Y.Y., Vasil'eva, L.V., Chestnykh, O.V., and Zavarzin, G.A. (2002) Methanotrophs of the psychrophilic microbial community of the Russian arctic tundra. *Microbiol* **71**: 460–466.
- Bowman, J.P., Mccammon, S.A., and Skerratt, J.H. (1997) *Methylosphaera hansonii* gen. nov., sp. nov, a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. *Microbiol* **143**: 1451–1459.
- Conrad, R. (1996) Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, OCS, N<sub>2</sub>O, and NO). *Microbiol Rev* **60**: 609–640.
- Danilova, O.V., Kulichevskaya, I.S., Rozova, O.N., Detkova, E.N., Bodelier, P.L.E., Trotsenko, Y.A., and Dedysh, S.N. (2013) *Methylomonas paludis* sp. nov., the first acid-tolerant member of the genus *Methylomonas*, from an acidic wetland. *Int J Syst Evol Microbiol* **63**: 2282–2289.
- Dedysh, S.N., Berestovskaya, Y.Y., Vasylieva, L.V., Belova, S.E., Khmelena, V.N., Suzina, N.E., *et al.* (2004) *Methylocella tundrae* sp. nov., a novel methanotrophic bacterium from acidic tundra peatlands. *Int J Syst Evol Microbiol* **54**: 151–156.
- Deutzmann, J.S., Hoppert, M., and Schink, B. (2014) Characterization and phylogeny of a novel methanotroph, *Methyloglobulus morosus* gen. nov., spec. nov. *Syst Appl Microbiol* **37**: 165–169.
- Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S.B., *et al.* (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature* **450**: 879–882.
- Etiopie, G., and Klusman, R.W. (2002) Geologic emissions of methane to the atmosphere. *Chemosphere* **49**: 777–789.
- Fiebig, J., Woodland, A.B., Spangenberg, J., and Oschmann, W. (2007) Natural evidence for rapid abiogenic hydrothermal generation of CH<sub>4</sub>. *Geochim Cosmochim Acta* **71**: 3028–3039.
- Frindte, K., Maarastawi, S.A., Lipski, A., Hamacher, J., and Knief, C. (2017) Characterization of the first rice paddy cluster I isolate, *Methyloterricola oryzae* gen. nov., sp. nov. and amended description of *Methylomagnum ishizawai*. *Int J Syst Evol Microbiol* **67**: 4507–4514.
- Ghashghavi, M., Belova, S.E., Bodelier, P.L.E., Dedysh, S.N., Kox, M.A.R., Speth, D.R., *et al.* (2019) *Methylotriacoccus oryzae* strain C50C1 is a novel type Ib gammaproteobacterial methanotroph adapted to freshwater environments. *mSphere* **4**: e00631-18.
- Hanson, R.S., and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol Rev* **60**: 439–471.
- Hirayama, H., Fuse, H., Abe, M., Miyazaki, M., Nakamura, T., Nunoura, T., *et al.* (2013). *Methylomarinum vadi* gen. nov., sp. nov., a methanotroph isolated from two distinct marine environments. *Int J Syst Evol Microbiol* **63**: 1073–1082.
- Hirayama, H., Abe, M., Miyazaki, M., Nunoura, T., Furushima, Y., Yamamoto, H., and Takai, K. (2014) *Methylomarinovum caldicuralii* gen. nov., sp. nov., a moderately thermophilic methanotroph isolated from a shallow submarine hydrothermal system, and proposal of the family *Methylothermaceae* fam. nov. *Int J Syst Evol Microbiol* **64**: 989–999.
- Houghton, K.M., Carere, C.R., Stott, M.B., and McDonald, I.R. (2019) Thermophilic methanotrophs: in hot pursuit. *FEMS Microbiol Ecol* **95**: fiz125.
- Iguchi, H., Yurimoto, H., and Sakai, Y. (2010) Soluble and particulate methane monooxygenase gene clusters of the type I methanotroph *Methylovulum miyakonense* HT12. *FEMS Microbiol Lett* **312**: 71–76.
- Islam, T., Jensen, S., Reigstad, L.J., Larsen, Ø., and Birkeland, N.K. (2008) Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum. *Proc Natl Acad Sci U S A* **105**: 300–304.
- Islam, T., Larsen, Ø., Torsvik, V., Øvreås, L., Panosyan, H., Murrell, J.C., *et al.* (2015) Novel methanotrophs of the family *Methylococcaceae* from different geographical regions and habitats. *Microorganisms* **3**: 484–499.
- Islam, T., Torsvik, V., Larsen, Ø., Bodrossy, L., Øvreås, L., and Birkeland, N.K. (2016) Acid-tolerant moderately thermophilic methanotrophs of the class Gammaproteobacteria isolated from tropical topsoil with methane seeps. *Front Microbiol* **7**: 851.
- Islam, T., Gessesse, A., Garcia-Moyano, A., Murrell, J.C., and Øvreås, L. (2020) A novel moderately thermophilic type Ib methanotroph isolated from an alkaline thermal spring in the Ethiopian Rift Valley. *Microorganisms* **8**: 250.
- Kalyuzhnaya, M.G., Khmelena, V.N., Kotelnikova, S., Holmquist, L., Pedersen, K., and Trotsenko, Y.A. (1999) *Methylomonas scandinavica* sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. *Syst Appl Microbiol* **22**: 565–572.
- Kamagata, Y., and Mikami, E. (1991) Isolation and characterization of a novel thermophilic *Methanosaeta* strain. *Int J Syst Bacteriol* **41**: 191–196.
- Khalifa, A., Lee, C.G., Ogiso, T., Ueno, C., Dianou, D., Demachi, T., *et al.* (2015) *Methylomagnum ishizawai* gen. nov., sp. nov., a mesophilic type I methanotroph isolated from rice rhizosphere. *Int J Syst Evol Microbiol* **65**: 3527–3534.
- Kip, N., Ouyang, W.J., Van Winden, J., Raghoebarsing, A., Van Niftrik, L., Pol, A., *et al.* (2011) Detection, isolation, and characterization of acidophilic methanotrophs from Sphagnum mosses. *Appl Environ Microbiol* **77**: 5643–5654.
- Knief, C. (2015) Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on *pmoA* as molecular marker. *Front Microbiol* **6**: 1346.



- Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**: 1870–1874.
- Lauritzen, S.E., and Bottrell, S. (1994) Microbiological activity in thermoglacial karst springs, South Spitsbergen. *Geomicrobiol J* **12**: 161–173.
- Mesbah, M., Premachandran, U., and Whitman, W.B. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid-chromatography. *Int J Syst Bacteriol* **39**: 159–167.
- Murray, M.G., and Thompson, W.F. (1980) Rapid isolation of high molecular-weight plant DNA. *Nucl Acids Res* **8**: 4321–4325.
- Nazaries, L., Murrell, J.C., Millard, P., Baggs, L., and Singh, B.K. (2013) Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions. *Environ Microbiol* **15**: 2395–2417.
- Omelchenko, M.V., Vasileva, L.V., Zavarzin, G.A., Saveleva, N.D., Lysenko, A.M., Mityushina, L.L., et al. (1996) A novel psychrophilic methanotroph of the genus *Methylobacter*. *Microbiology* **65**: 339–343.
- Op Den Camp, H.J.M., Islam, T., Stott, M.B., Harhangi, H.R., Hynes, A., Schouten, S., et al. (2009) Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environ Microbiol Rep* **1**: 293–306.
- Orata, F.D., Meier-Kolthoff, J.P., Sauvageau, D., and Stein, L.Y. (2018) Phylogenomic analysis of the gammaproteobacterial methanotrophs (Order *Methylococcales*) Calls for the reclassification of members at the genus and species levels. *Front Microbiol* **9**: 3162.
- Oshkin, I.Y., Belova, S.E., Danilova, O.V., Miroshnikov, K.K., Rijkstra, W.I.C., Damsté, J.S.S., et al. (2016) *Methylovulum psychrotolerans* sp. nov., a cold-adapted methanotroph from low-temperature terrestrial environments, and emended description of the genus *Methylovulum*. *Int J Syst Evol Microbiol* **66**: 2417–2423.
- Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.A., and Hugenholtz, P. (2018) A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nat Biotechnol* **36**: 996–1004.
- Prior, S.D., and Dalton, H. (1985) Acetylene as a suicide substrate and active-site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS Microbiol Lett* **29**: 105–109.
- Rahalkar, M., Bussmann, I., and Schink, B. (2007) *Methylosoma difficile* gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral sediment of Lake Constance. *Int J Syst Evol Microbiol* **57**: 1073–1080.
- Reeburgh, W.S. (2007) Oceanic methane biogeochemistry. *Chem Rev* **107**: 486–513.
- Reigstad, L.J., Jørgensen, S.L., Lauritzen, S.E., Schleper, C., and Urich, T. (2011) Sulfur-oxidizing chemolithotrophic proteobacteria dominate the microbiota in high Arctic thermal springs on Svalbard. *Astrobiology* **11**: 665–678.
- Schloss, P.D., and Handelsman, J. (2004) Status of the microbial census. *Microbiol Mol Biol Rev* **68**: 686–691.
- Sharp, C.E., Smirnova, A.V., Graham, J.M., Stott, M.B., Khadka, R., Moore, T.R., et al. (2014) Distribution and diversity of Verrucomicrobia methanotrophs in geothermal and acidic environments. *Environ Microbiol* **16**: 1867–1878.
- Stoecker, K., Bending, B., Schoning, B., Nielsen, P.H., Nielsen, J.L., Baranyi, C., et al. (2006) Cohn's Crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc Natl Acad Sci U S A* **103**: 2363–2367.
- Tavormina, P.L., Hatzenpichler, R., Mcglynn, S., Chadwick, G., Dawson, K.S., Connon, S.A., and Orphan, V.J. (2015) *Methyloprofundus sedimenti* gen. nov., sp. nov., an obligate methanotroph from ocean sediment belonging to the 'deep sea-1' clade of marine methanotrophs. *Int J Syst Evol Microbiol* **65**: 251–259.
- Trotsenko, Y.A., and Khmelenina, V.N. (2005) Aerobic methanotrophic bacteria of cold ecosystems. *FEMS Microbiol Ecol* **53**: 15–26.
- Trotsenko, Y.A., and Murrell, J.C. (2008) Metabolic aspects of aerobic obligate methanotrophy. *Adv Appl Microbiol* **63**: 183–229.
- Van Teeseling, M.C.F., Pol, A., Harhangi, H.R., Van Der Zwart, S., Jetten, M.S.M., and Op Den Camp, H.J.M. (2014) Expanding the Verrucomicrobial methanotrophic world: Description of three novel species of *Methylococcoides* gen. nov. *Appl Environ Microbiol* **80**: 6782–6791.
- Vigliotta, G., Nutricati, E., Carata, E., Tredici, S.M., De Stefano, M., Pontieri, P., et al. (2007) *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic gamma-proteobacterium. *Appl Environ Microbiol* **73**: 3556–3565.
- Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F., and Dedysh, S.N. (2011) *Methyloferula stellata* gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. *Int J Syst Evol Microbiol* **61**: 2456–2463.
- Wartiainen, I., Hestnes, A.G., McDonald, I.R., and Svenning, M.M. (2006) *Methylobacter tundripaludum* sp. nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78° N). *Int J Syst Evol Microbiol* **56**: 109–113.
- Whittenbury, R., Phillips, K.C., and Wilkinso, J.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**: 205–218.