A novel Type I methanotroph *Methylolobus aquaticus* gen. nov. sp. nov. isolated from a tropical wetland

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**Abstract** A novel gammaproteobacterial methanotroph; strain FWC3 was isolated from a tropical freshwater wetland sample collected near a beach in Western India. Strain FWC3 forms flesh pink/peach colored colonies, is non-motile, and the cells are present as diplococci, triads, tetracocci and aggregates. Strain FWC3 grows only on methane and methanol. As the 16S rRNA gene of strain FWC3 showed low similarities with other Type I methanotrophs (less than 94.3%), it was further investigated for its novelty and characterisation by a polyphasic approach. ANI indices and DDH values deduced from the draft genome of strain FWC3 (SEYW00000000.1) with the other nearest type strains (*Methylocaldum marinum* S8T and *Methylococcus capsulatus* BathT) were ~ 70% and ~ 15%, respectively. The low level similarities indicated that strain FWC3 can belong to a new genus and species. Additionally, strain FWC3 showed a unique fatty acid profile with the dominance of C16:1 o7 and o6c, C16:0 and C16:1 o9c. During the characterisation of strain FWC3, a morphologically similar methanotroph, strain C50C1 was described (Ghashghavi et al. in *mSphere* 4:e00631–18, 2019) and named as ‘*Methylotetracoccus oryzae*’. We found that strain FWC3 and strain C50C1 belonged to the same genus but could belong to different species based on the ANI indices and dDDH values (~ 94% and ~ 55%, respectively). However, strain C50C1 has not been deposited in two culture collections and not been validly described. Also, the 16S rRNA gene of strain C50C1 is neither available on the database nor can it be retrieved from the genome assembly. Based on the polyphasic characterisation and comparison to the other type strains of *Methylococcaceae*, we propose strain FWC3 (= JCM 33786 T, = KCTC 72733 T, = MCC 4198 T) to be the type strain of a novel genus and species, for which the name *Methylolobus aquaticus* is proposed. Strain C50C1 (Ghashghavi et al. 2019) could represent another species (‘*Methylolobus oryzae*’).

**Keywords** Wetlands · Methanotrophs · India · Tropical · Novel genus · Type Ib · *Methylolobus*
**Introduction**

Aerobic methanotrophs are aerobic or micro-aerophilic bacteria that use methane, the second most important greenhouse gas, as the sole source of C and energy (Hanson and Hanson 1996). Methanotrophs play a vital role in the mitigation of wetland-derived methane (Conrad 2009). Methanotrophs are from the classes *Alphaproteobacteria* (Type II), *Gammaproteobacteria* (Type Ia and b) and recently have been reported from the phylum *Verrucomicrobia* (Dedysh and Knief 2018). Type Ib methanotrophs (earlier named Type X) are major players in wetland and freshwater habitats, however, very few members have been isolated (Ghashghavi et al. 2019). Except for *Methyloparacoccus* strains isolated from pond water sediments, representatives of most of the newly reported Type Ib genera have been isolated from rice fields, e.g. *Methyllogaea* (Geymonat et al. 2011), *Methylomagnum* and *Methyloterricola* (Frindte et al. 2017). Methanotrophs from freshwater wetlands and rice fields from tropical regions have been largely unexplored and these habitats are predicted to possess novel and unique methanotrophic taxa (Pandit et al. 2016). Culture independent data suggest that many novel Type Ib methanotrophs might exist in freshwater wetlands, small water bodies and rice fields (Ghashghavi et al. 2019). Type Ib methanotrophs show a high metabolic diversity, however, many of the clades lack isolates (Ghashghavi et al. 2019). In our pioneer work of isolation and description of novel taxa of methanotrophs from Indian rice fields and wetlands, we described a new genus (*Methylocucumis*) within Type Ia methanotrophs from a tropical rice field habitat in India. One new genus, *Methylocucumis oryzae* (Rahalkar et al. 2016; Pandit and Rahalkar 2018; Pandit et al. 2018; Oren and Garrity 2019; Pandit et al. 2016) and two new species within *Methylococcaceae* (Rahalkar and Pandit 2018; Rahalkar et al. 2019; Khatri et al. 2019b) have been reported by our group. This reflects that there is a high need for culturing novel methanotrophs from tropical wetlands.

In our present study we describe the enrichment and isolation of a Type Ib methanotroph, strain FWC3 which was isolated from a tropical wetland. Based on the 16S rRNA gene and genome based comparisons with type species, strain FWC3 showed a potential to be a member of a novel genus and species. During the course of characterisation and deposition of the strain FWC3 in culture collections, a morphologically similar methanotroph, strain C50C1 was described. Strain C50C1 was isolated from a temperate rice field in China and was tentatively named ‘*Methylotetraococcus oryzae*’ (Ghashghavi et al. 2019) due to its properties to form tetracocci. However, the strain C50C1 has not been deposited in two international culture collections and the name has not been validated till date (confirmed by personal communication). The purpose of this paper is to formally describe strain FWC3 as a novel member of a genus and species within the family *Methylococcaceae*. We would like to propose the name which was thought for this genus: *Methylolobus* (Khatri et al. 2019a) to keep it more general for inclusion of new species which might be isolated in the future instead of ‘*Methylotetraococcus*’ proposed by (Ghashghavi et al. 2019). To broaden the scope of the genus, the characters of strain C50C1 (Ghashghavi et al. 2019) have been used for comparison with strain FWC3. The genus would also represent an important lineage of *pmoA* sequences representing methanotrophs from freshwater lakes, sediments and rice fields (RPC1: Rice paddy cluster 1) (Knief 2015).

**Materials and methods**

**Sampling**

A mud sample was collected from a shallow freshwater patch of a wetland which was the end of a freshwater creek region, near Nagaon beach, Alibag, India (18°26’30” N 72°54’20” E) in September 2017. The top mud with some water was collected with a sterile 50 ml container and added to a sterile bottle. Water above the mud was also collected in the same bottle. The samples were brought to the laboratory on the same day and immediately used for enrichment experiments. Rest of the sample was stored at 4–8 °C.

Enrichment and isolation of methanotrophs

Enrichments for culturing methanotrophs were set up by serial dilutions in serum bottles by adding two...
millilitres of sample to 18 ml sterile dilute Nitrate Mineral Salts (dNMS) medium (Pandit et al. 2016). About 20% methane was added to the headspace by replacing 20% air and the bottles were incubated under static conditions at 25 °C for ~ 6 weeks. Methane oxidation was checked periodically using gas chromatography by measuring the decline in the headspace methane. This was followed by streaking 20 μl of the liquid positive enrichment culture on solid plates containing dilute NMS medium and 2% agarose as a solidifying agent. The plates were incubated in glass desiccators containing 20–30% methane and ~ 70–80% air in the headspace, for 2–3 weeks.

The isolation and purification of methanotrophs was done as described before (Pandit et al. 2016). For checking the purity of methanotrophs, the cultures were streaked on a nutrient agar plate. No growth on the plate confirmed the purity of the methanotroph. After extensive purification of the culture for ~ 4–6 months by streaking and sub culturing, pure culture of methanotrophs were obtained. Wet mounts from all single colonies appearing on plates incubated under methane were visualized under phase contrast microscope and microscopic images were taken. Gram staining was also done. The pure cultures were further subjected to phylogenetic analysis described further. The pure cultures were maintained by sub-culturing on solid and liquid medium with methane in the headspace, in serum bottles. A strain FWC3 was obtained and all the further experiments were performed using this strain.

Growth experiments

Utilisation of various carbon sources was studied in dNMS liquid medium (Pandit et al. 2016) with the following filter-sterilised or autoclaved carbon substrates (0.1% w/v): formate, formamide, formaldehyde, glucose, fructose, sucrose, arabinose, raffinose, lactose, maltose, xylose, succinate, pyruvate, glutamate and acetate was tested in microtitre plates. Growth of the strain on methanol was tested by the addition of 0.02–5% v/v methanol in liquid medium in serum bottles and also in glass flasks. The range of nitrogen sources utilised by strain FWC3 was tested by replacing KNO₃ from dNMS medium with one of the following nitrogen sources: NH₄Cl, urea, asparagine, aspartate, l-glutamic acid, glutamate, glycine, serine, valine, peptone or yeast extract. Growth without added nitrogen source was tested under micro-oxic and full oxic conditions (20% air, 20% methane, 60% molecular nitrogen in the headspace). A well grown culture (OD 0.3) grown on methane substrate was used as inoculum in the proportion 1:10 or 1:30. Additionally, growth on few of the substrates was checked in glass flasks: acetate, succinate, pyruvate, malate, glutamate, glucose, sucrose, maltose and fructose (0.1%) and incubated for a period of 1 month at 25 °C. Differences in the OD values and the presence or absence of visible growth were monitored in all cases. Wherever growth was observed in liquid media, one ml of the culture was removed, centrifuged and the supernatant was acidified using HCl followed by HPLC analysis.

The optimum pH and temperature ranges were evaluated in dNMS liquid medium. Citrate–phosphate buffer (pH 3–6.8) and glycine buffer (pH 8, 9 and 9.6) were used for buffering the medium. Growth was also checked without using any buffer, but using only HCl or NaOH for adjusting the pH from pH 3 to pH 10. All of the above experiments were performed in microtitre plates in triplicate. Strain FWC3 was grown at a temperature range from 10 to 40 °C. These experiments were done in triplicates in liquid media in sealed serum bottles with methane and air in the headspace. The ability of the culture to grow under varying oxygen concentrations was tested in serum bottles in the range from no air to complete air in the headspace. The media preparation was done by flushing the bottles with nitrogen gas before autoclaving and then adding the calculated percentage of filtered air (0, 5, 2, 50 and 80%) with 20% methane in all bottles.

DNA extraction, PCR amplification and sequencing

DNA extraction from the culture was done as described before (Pandit et al. 2016). Particulate methane monoxygenase β subunit (pmoA) gene amplification and the 16S rRNA gene amplification was carried out using A189f—mb661r primers and 27f and 1492r primers as described before (Pandit et al. 2016) using DNA extracted from a pure culture. The amplified products were sequenced using initially one and then with forward and reverse primers, and the sequencing was done by First Base Laboratories, Malaysia. The sequences obtained using both the
primers were aligned and assembled using SeqMan and were subjected to BLAST analysis.

Phylogenetic analysis

The complete 16S rRNA sequence of the isolate was subjected to nucleotide blast and the nearest neighbouring sequences were retrieved and aligned using MAFFT (https://mafft.cbrc.jp/alignment/server/). The alignment fasta file was used to construct phylogenetic tree using MEGA 7.0 (Kumar et al. 2016) using only the sequences of type strains of valid species (Tamura et al. 2013).

Phase contrast and scanning electron microscopy

For morphological examination the cells were observed under a phase contrast microscope (Nikon 80i, Japan microscope with a camera) with 40× and 100× magnifications. Cells were also observed by Scanning Electron Microscopy (SEM) (Zeiss model EVO-MA-15 SEM). Cells were prepared for SEM as described before (Pandit et al. 2018).

Whole genome sequencing of the novel genus (strain FWC3)

Genomic DNA of strain FWC3 was extracted from an axenic culture, grown on solid medium, using GenElute™ Bacterial Genomic DNA kit, Sigma-Aldrich. The genome was sequenced using Illumina HiSeq platform (2 × 150 bp) in Medgenome laboratories, Bangalore. The sequencing was carried out in HiSeq × 10 to generate 2 × 150 bp sequence reads at 100× sequencing depth. A minimum of 75% of the sequenced bases were of Q30 value. Sequenced data was processed to generate FASTQ files and uploaded on the FTP server for download and secondary analysis. Approximately 8 million high quality paired end reads were used for assembly with SPAdes (v 3.13.0) assembler. A total of 42 scaffolds of > 500 bp were constructed, with an N50 of 184 kb, the largest scaffold assembled measured 498.3 kb. BlastN annotation was performed to identify similarities with other known genomes. A blast based pairwise average nucleotide identity ANI-b was calculated using JSpecies (Sangal et al. 2016, Richter and Rosselló-Móra 2009). The prodigal software was used for Gene Prediction. RNAmmer was used for identifying tRNA genes. TRNAscan-SE was used for identifying tRNA genes. The Bio project id is PRJNA520977 and the accession number for the whole genome of strain FWC3 is SEYW0000000. The phylogenomic analysis including the phylogenomic tree construction was done using the PATRIC database (Wattam et al. 2017).

GC, HPLC and spectrophotometer

OD readings were taken by spectrophotometric analysis at 600 nm. GC measurements were done in a Chemito GC as described before (Pandit et al. 2016). HPLC was used to check if any products were formed after growth on methane and methanol. HPLC was performed in a Shimadzu HPLC equipped with a C-20AD micro-plunger. Samples were run with a 1000 ppm standard and concentrations were calculated in mM or ppm values. Standards of methanol, formate, lactate, malate and all the other sugars were run for determining the utilization of the substrate and products formed.

Results and discussion

Isolation of a novel gammaproteobacterial methanotroph, strain FWC3

In the current study, a methanotrophic strain (FWC3) was isolated on dilute nitrate mineral salts (NMS) agarose medium (Pandit et al. 2018) after an initial enrichment with methane and air in the headspace. The sample was the top sediment of a freshwater wetland on Nagaon beach, Alibag, India (18°26′30″ N 72°54′20″ E) in September 2017. After liquid enrichment for 8 weeks in the presence of methane and air, white to light pink turbidity was observed. A loopful of this cell material was streaked on solid agarose medium. After plating on dilute NMS agarose medium, several colonies were obtained and further isolations were carried out. Two of the strains (FWC1 and FWC2) showed bright pink to orange coloured colonies and were later identified to be Methylomonas strains (not described further). One of the other colonies had a unique flesh pink/peach colour. On phase contrast microscopic observation it showed the presence of cocci in pairs, tetracocci and small groups. This morphotype, arrangement of the cells and colour...
of the colony was not encountered in our earlier studies focusing on isolation of rice field methanotrophs from Indian rice fields (Pandit et al. 2016; Rahalkar et al. 2019), (Rahalkar, Monali and Pandit, Pranitha, data not shown). After streaking and re-streaking of the unique flesh pink colored colony, a pure culture was obtained and named as strain FWC3. strain FWC3 formed flesh pink colored to peach colored colonies on dilute NMS agarose (2% agarose) medium (Pandit et al. 2018) in methane: air environment (20:80) (Fig. 1a). In liquid medium, the culture grew as peach colored biofilm developing at the base (Fig. 1b). Under phase contrast microscope, the cells of strain FWC3 were seen to be coccoid to slightly oval-shaped with a length of 1.2–1.5 μm and breadth of ~ 1–1.2 μm. The cells could be seen as diplococci, tetra-cocci and small aggregates (Fig. 1c). In scanning electron micrographs, coccoid cells could be seen (Fig. 1d). The culture grew in liquid medium with methane and air in the headspace (20:80) with maximum OD values of 0.2–0.4 obtained within ~ 10–12 days at 25–35 °C temperature range in dNMS medium (Pandit et al. 2018). The growth in liquid as well as on solid was mucoid in nature and showed the typical peach color. Strain FWC3 grew in methanol-containing medium in a wide range of methanol concentrations (0.1–5% v/v). On further testing, we found that strain FWC3 could grow with very low methanol (0.02%). Other than methane and methanol, there was no visible growth in any of the

**Fig. 1** a Strain FWC3 growing on dil. NMS agarose plates in 20% methane environment. b Strain FWC3 growing in liquid NMS medium in the presence of 20% methane. c Phase contrast image of strain FWC3 growing on methane. The bar represents 5 μm. d Scanning electron micrograph of strain FWC3 growing on methane. The bar represents 1 μm
carbon sources. Faint growth was observed in formate and malate as the C source (OD increase less than 0.04). Strain FWC3 showed lesser growth under micro-oxic conditions (i.e. 4–5% oxygen) and showed a preference for aerobic growth. Strain FWC3 could also grow in the presence of N₂ without any bound nitrogen source under aerobic as well as micro-oxic conditions. During the phylogenetic characterisation, it was seen that the nearly complete 16S rRNA gene of strain FWC3 (MH789551.1) showed 94.3% similarity to Methylocaldum marinum S8T (nearest neighbour) and 90–94% to other Type Ib methanotrophs. This reflected that strain FWC3 could belong to a new taxon and further studies were done. (The 16S rRNA gene of strain FWC3 was deposited as MH789551.1, 1481 bp, and is available since August 2018).

Draft genome features of strain FWC3

The whole genome of strain FWC3 was sequenced for understanding its metabolic properties and for understanding the correct taxonomic position. The size of the strain FWC3 draft genome was 3.4 Mbp with a GC content of 63%. A total of 42 scaffolds of > 500 bp were constructed, with an N50 of 184 kb, the largest scaffold assembled measured 498.3 kb and the total coverage was ~700×. The genome contains 3013 genes (2975 protein coding genes, 35 tRNA and 3 rRNA genes). In total, 2831 proteins were annotated as per NCBI annotation and similar values were obtained using RAST. The draft genome of strain FWC3 is available as SEYW00000000.1 with the bioproject number PRJNA520977 since February 2019. The whole genome is in accordance with the minimal standards specified for the use of taxonomic data for prokaryotes with 600–700× coverage (Chun et al. 2018). The genome of strain FWC3 was compared with the nearest neighboring valid type strains Methylocaldum marinum S8T and Methylococcus capsulatus BathT using the average amino-acid identities (AAI) and average nucleotide indices (ANI). The AAI values were 59.2% and 60.7%, respectively and the average nucleotide indices (ANIIb) were ~70% well below the genus cut-off of 75% (Sangal et al. 2016). The digital DNA–DNA hybridization, (dDDH, http://ggdc.dsmz.de/) (Meier-Kolthoff et al. 2013) of strain FWC3 with Methylocaldum marinum S8T and Methylococcus capsulatus BathT were 13.20% and 13.70%, respectively. Additionally, the complete 16S rRNA sequence derived from the genome assembly MN080433.1 (1527 bp), showed 94.39% similarity to the closest valid species, Methylocaldum marinum S8T.

Phylogenetic analysis of strain FWC3 based on the draft genome, 16S rRNA gene and pMMO protein

Meanwhile, in July 2019, a strain C50C1 with very similar morphological features was described (Ghashghavi et al. 2019). This strain was tentatively named to be a member of ‘Methylotetracoccus oryzae’. The draft genome of strain C50C1 was available after July 2019 and it was found that strain FWC3 and C50C1 could be closely related. Therefore, strain C50C1 was included in our further phylogenetic analyses. The comparison showed us that the two-way AAI values as well as ANI-b value between strain FWC3 and strain C50C1 were close, reflecting that they represented a same genus (94.73% and 94%, respectively), but below the cut-off values of a species. The dDDH value comparing strain FWC3 with strain C50C1 was 55.7%, less than the 70% cut-off. In a phylogenomic tree, both the strains grouped closely with 100% bootstrap values and the closest genome was that of Methylococcus (Fig. 2). The 16S rRNA gene sequence of strain C50C1 could not be retrieved from the genome assembly and was obtained after request to the corresponding author (Ghashghavi et al. 2019). Also, it has not been separately submitted in the public database as per the minimum standards to propose novel taxa. In a 16S rRNA gene based maximum likelihood tree (Fig. 3a.) both the strains (strain FWC3 and strain C50C1) group with a 100% bootstrap. Also, these two strains and Methylocaldum species grouped together with a 58% bootstrap value, away from Methylophagaunum, Methyloparacoccus and Methylococcus. In a pMMO subunit B tree, (Fig. 3b) strain FWC3 and strain C50C1 proteins grouped together with 100% bootstrap value, away from Methylophagaunum, Methyloparacoccus and Methylococcus. In a pMMO subunit B tree, (Fig. 3b) strain FWC3 and strain C50C1 proteins grouped together with 100% bootstrap value, away from Methylophagaunum, Methyloparacoccus and Methylococcus.
Salient metabolic characteristics predicted from draft genome of strain FWC3

Strain FWC3 draft genome showed a complete methane oxidation pathway with a single set of pmoCAB (particulate methane monooxygenase genes) and two extra sets of pmoC gene. In contrast, strain C50C1 possesses exactly double number of pmoCAB (two sets) and four sets of pmoC. Also, strain FWC3 did not possess any alternative methane monooxygenases such as pmoA2 or pXMO or sMMO genes. Strain FWC3 showed three types of alcohol dehydrogenase, one zinc-binding alcohol dehydrogenase, one XOX-type PQQ dependent alcohol dehydrogenase and additionally an iron dependent alcohol dehydrogenase. On blast search, all three were also present in strain C50C1; however, the iron dependent dehydrogenase has not been mentioned in case of strain C50C1 (Ghashghavi et al. 2019). In total, 2831 proteins were annotated in strain FWC3 compared to 4018 proteins in strain C50C1 in reflecting that strain FWC3 genome was more streamlined. Instead of the classical formaldehyde dehydrogenase, a NADP-quinone oxidoreductase is present in strain FWC3, a similar feature seen in strain C50C1. Similar to strain C50C1, strain FWC3 has a tetrahydrofolate and a 5, 6, 7, 8-tetrahydromethanopterin H4MPT-linked C1 carrier pathways. In strain FWC3, formate oxidation occurs via formate dehydrogenase molybdenum dependent and also a nitrate-inducible formate dehydrogenase is seen. A complete RuMP pathway and majority of the enzymes of serine pathway enzymes are present which are responsible for the assimilation of formaldehyde, similar to strain C50C1. A simple respiratory chain consisting of a complex 1 (Na⁺ translocating NADH quinone oxidoreductase or NAD⁺-dehydrogenase), and a terminal cytochrome C oxidase and also a cytochrome bd terminal oxidase were present in the genome. Nitrogenase genes were detected in the genome of strain FWC3.

![Phylogenomic tree using PATRIC based ‘Codon Tree’](image-url)
Physiology and chemotaxonomy

Strain FWC3 used ammonium chloride, urea, glutamate, peptone, yeast extract and lysine as nitrogen sources and grew without bound nitrogen. Strain FWC3 was found to grow in the pH range of 3 to 9 under non-buffered conditions, and optimal growth between pH 5–8 (Table 1). Growth at pH 3 was minimal. Strain FWC3 was found to grow in the temperature range between 20 and 37 °C and maximum growth was detected in the range 25–35 °C. Strain C50C1 could grow from 4 to 30 °C with optimum around 18–25 °C. The overall characteristics of strain FWC3 are compared with the other Type Ib methanotroph genera (Table 1). During the comparison, characters of strain C50C1 have also been considered, as strain FWC3 and strain C50C1 represent the same genus. Strain FWC3 could be preserved and could be revived when cryo-preserved with 5% dimethyl sulfoxide (DMSO) as the cryo-preservation.
| Characteristics | Methyloparacoccus | Methyloparacoccus | Methylocaldum | Methylocaldum | Methylococcus | Methylococcus | Methyterricola | Methyterricola | Methylogaea | Methylogaea | Methyomagnum | Methyomagnum | Methylospira | Methylospira |
|-----------------|------------------|------------------|---------------|---------------|---------------|---------------|----------------|----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Isolation source/habitat | Wetlands, rice fields | Pond water | Marine sediments, wetlands | Thermal bath | Rice field | Rice field | Rice field | Sphagnum bogs |
| Growth temperature (°C) | 4–37 | 20–37 | 20–62 | 28–55 | 15–45 | 20–37 | 20–37 | 8–25 |
| pH | 3–9 | 5.8–9 | 5–9 | 5.5–9 | 4.6–7.5 | 5–8 | 5–9 | 4.2–6 |
| Tolerance to 1% NaCl | No | No | ND | Yes | No | No | No | No |
| Cell morphology | Cocci, diplo and tetracocci | Cocci | Coccobacilli | Cocci | Cocci-diplococci | Rods | Rods | Curved rods |
| Cell size (µm) | 1–1.5 by 1.2–1.8 | 0.8–1.5 | 0.6–1.2–1.0–1.8 | 0.8–1.5 by 1.0–1.5 | 1.6–1.9 by 1.2–1.4 | 0.5–0.7 by 2.0–2.2 | 1.5–2.0 by 2.0–4.0 | 1.0–1.5 by 2.0–2.5 |
| Major cell wall fatty acids | C16:1 ω7c, C16:1 ω6c, C16:1 ω9c, C16:0 | C16:1 ω7c | ND | C16:0, C16:1 ω7c | C16:0, C16:1 ω7c, C16:1 ω5c | C16:0 | C14:0, C16:0, C16:1 ω7c | ND |
| Pigmentation | Flesh pink, peach, white-brown | White | Brown | White to brown | White to slightly pink | White | White | White |
| G+C content % | 63.0 | 65.6 | 56.5–57.2 | 59–66 | 61.0 | 63.1 | 64.1 | 54.0 |

*ND* not detected
kept at − 80 °C. The salient differentiating characters comparing strain FWC3 and strain C50C1 are listed (Table 2). Strain FWC3 did not show any visible growth (difference in OD of 0.04 or above) on any of the sugars or organic acids tested after incubation of 1 month at 25 °C. The cells when grown in fructose showed the formation of yellow coloured minute aggregates, but no major increase in OD was seen. Also malate and formate showed faint growth with ~ 0.03–0.04 increase in OD. When the cells were grown in liquid medium with 10 mM methanol, very small quantities of formate were detected in the supernatant (~ 0.1 mM). Similarly, strain C50C1 has also been reported not to grow on multi-carbon substrates (Ghashghavi et al. 2019).

The cell wall fatty acids of strain FWC3 showed maximum amounts of 16:1 ω6c/16:1 ω7c (41%) followed by 16:0 (26%) and 16:1 ω9c (16%) (Table 3). The profile of strain FWC3 was distinct compared to other Type Ib methanotrophs. The fatty acid profile of strainC50C1 showed a dominance of 16:1 ω9c (33%) followed by 16:1 ω6c/16:1 ω7c (Table 3), and was different to that of strain FWC3.

Strain FWC3 has been deposited in two culture collections in two different countries (= JCM 33786 T, = KCTC 72733 T) and also in India (MCC 4198 T). We propose strain FWC3 to be the type strain of a novel genus and species for which the name Methylolobus aquaticus is suggested. Strain C50C1 (Ghashghavi et al. 2019) could be a member of a different species of the genus Methylolobus, and named as ‘Methylolobus oryzae’ strain C50C1 as per the new genus name in the phylogenetic trees. The genus Methylolobus is a new addition to the family Methylococcaceae (Type I) methanotrophs.

Table 2  Differentiating characteristics and information comparing strain FWC3 and strain C50C1 (Ghashghavi et al. 2019) representing the novel genus Methylolobus

| Characteristics                        | Strain FWC3 (this study) | Strain C50C1 (Ghashghavi et al. 2019) |
|----------------------------------------|--------------------------|--------------------------------------|
| Color of the colonies                  | Flesh pink or peach      | White to brown                       |
| Growth on methanol (range of concentration) | 0.02–5% (vol/vol)      | 0.1–4% (vol/vol)                     |
| pH range for growth                    | 3–9                      | 4.3–8.3                              |
| Optimum temperature range for growth   | 25–35 °C                 | 18–25 °C                             |
| Draft genome size                      | 3.4 Mbp                  | 4.8 Mbp                              |
| Copies of pMMO genes                   | 1                        | 2                                    |
| Deposition in culture collections with numbers | Yes, (= JCM 33786, = KCTC 72733, = MCC 4198) | No. not available                   |
| 16S rRNA gene (accession number), bp   | MN080433.1 (complete gene) | Not available                        |
| Genome (accession number)              | SEYW00000000.1           | SRSH00000000.1                       |
| G+C content                            | 63.0                     | 62.8                                 |
| Isolation source                       | Tropical wetland, India  | Rice field, China                    |
| Dominant cell wall fatty acid          | 16:1 ω6c/16:1 ω7c (summed feature) | 16:1 ω9c                             |
Methylocaldum, and Methylomagnum within the family Methylococcaceae in the class Gammaproteobacteria. Known habitats are freshwater ecosystems, such as paddy fields, freshwater creeks, wetlands and lake sediments. Methylolobus aquaticus is described as the type species of this genus. Another strain C50C1 (Ghashghavi et al. 2019) could represent a distinct species of this genus and tentatively named as 'Methylolobus oryzae'. However, strain C50C1 (Ghashghavi et al. 2019) has not been deposited in culture collections and would require formal description.

Description of Methylolobus aquaticus sp. nov

Methylolobus aquaticus sp. nov. (a.qua’ti.cus. L. masc. adj. aquaticus, belonging to or living in water). Most of the characters are as per the genus description, described below. The cells are cocccid to slightly oval-shaped with a length of 1.2–1.5 μm and breadth of ~ 1–1.2 μm. The cells could be seen in pairs, triplets or in tetra-cocci and larger aggregates. Methanol supports growth from 0.02 to 5%. Optimum growth occurs in the range 25–35 °C and pH 5–8. Can be cryo-preserved using 5% DMSO at –80 °C. Nitrogenase genes are present. The type strain FWC3 T was isolated from a freshwater wetland mud in Nagaon beach, near Alibag, India. The G+C content of the type strain is 63 mol% (genome sequence). The type strain of the novel species, Methylolobus aquaticus strain FWC3 T (= JCM 33786 T = KCTC 72733 T = MCC 4198 T) is described here. The DNA G+C content based on the draft genome of strain FWC3 T is 63.0 mol%. The GenBank/EMBL/DDBJ accession number for the complete 16S rRNA gene sequence of strain FWC T is MN080433.1. The whole genome shotgun project for the strain FWC3 has been deposited in GenBank database with the accession

Table 3 Cell wall fatty acids comparison of related Type Ib methanotrophs

| Fatty acids | Strain FWC3 | Strain C50C1 | Methylococcus | Methyloparacoccus | Methylocaldum | Methyloterricola | Methylomagnum |
|------------|-------------|--------------|----------------|-------------------|---------------|-----------------|---------------|
| 14:0       | 0.67        | 0.34         | 1–6            | 4.7               | 2–3           | TR              | 15.8          |
| 15:0       | –           | 1.12         | 1–13           | 3.2               | 2–4           | TR              | 1.56          |
| 15:1 ω8c   | –           | –            | 0.3            | –                 | –             | 0.22            |
| 16:1 ω9c   | 15.81       | 33.01        | 4–12           | 6.5               | –             | –               | –             |
| 16:1 ω9t   | –           | 3.91         | –              | –                 | –             | –               | –             |
| 16:1 ω6c/ 16:1 ω7c | 41.49 | 26         | 17–46          | 54.2 | – | 35.6 | 55.33 |
| 16:1 ω5c   | 6.32        | 5.95         | 4.2            | 0-TR              | 28.3          | –               | –             |
| 16:1 ω5t   | –           | 0.19         | 3–9            | 0-TR              | –             | –               | –             |
| 16:0       | 26.24       | 16.73        | 33–56          | 23.7              | 43–65         | 30.9            | –             |
| 16:1       | –           | 0.80         | –              | –                 | 12–47         | –               | –             |
| 16:0 3OH   | 2.46        | –            | 2.6            | 0-TR              | –             | 1.78            |
| 16: 1 ω10c | –           | –            | –              | –                 | 2.4           | –               | –             |
| 16: 1 ω11c | –           | –            | –              | –                 | 5.46          | –               | –             |
| 17:0       | –           | 0.41         | 0–15           | 3–9               | –             | –               | –             |
| 19:0 cyc   | –           | –            | 0–3            | –                 | –             | –               | –             |
| 17:1 ω7c   | –           | –            | 0–2            | –                 | –             | –               | –             |
| 17:1 ω8c   | –           | 0.16         | TR             | –                 | –             | –               | –             |
| 17:1 ω7t   | –           | 0–2          | –              | –                 | –             | –               | –             |
| 18:1 ω9c   | –           | 0–3          | –              | –                 | –             | –               | –             |
| 18:0       | –           | 0.53         | TR             | –                 | –             | –               | –             |
| 18:1       | –           | 0.11         | –              | –                 | 1.7           | –               | –             |
| 18:1 ω7c   | 0.62        | 0.93         | –              | –                 | 1.7           | –               | –             |
number NZ_SEYW00000001.1. The bioproject accession number is PRJNA52097. The link for the genome is https://www.ncbi.nlm.nih.gov/genome/?term=SEYW01.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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