Description and genome analysis of *Methylotetracoccus aquaticus* sp. nov. , a novel tropical wetland methanotroph, with the amended description of *Methylotetracoccus* gen. nov.

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Abstract

We enriched and isolated a novel gammproteobacterial methanotroph; strain FWC3, from tropical freshwater wetland, near Nagaon beach, Alibag, India. FWC3 is a coccoid, flesh pink/peach pigmented, non-motile methanotroph and the cells are present in pairs and as tetracocci. The culture can grow on methane (20%) as well as on a wide range of methanol from concentrations (0.02%-5%). Based on the comparison of genome data, FAME analysis, morphological characters and biochemical characters, FWC3 belongs to the tentatively and newly but not validly described genus '*Methylotetracoccus'* of which only a single species strain was described, *Methylotetracoccus oryzae* C50C1. The ANI index between FWC3 and C50C1 strains is 94%, and the DDH value is 55.7%, less than the cut-off values 96% and 70%, respectively. The genome size of FWC3 is smaller (3.4 Mbp) compared to that of C50C1 (4.8 Mbp). Additionally, the FAME profile of FWC3 shows differences in cell wall fatty acid profiles compared to *Methylotetracoccus oryzae* C50C1. Also, there are other differences on the morphological, physiological and genomic levels. We propose FWC3 to be a member of a novel species of the genus *Methylotetracoccus*, for which the name *Methylotetracoccus aquaticus* is proposed. Also, an amended description of the genus *Methylotetracoccus* gen. nov. is given here. FWC3 is available in two international culture collections with the accession numbers: MCC 4198 (Microbial Culture collection, India) and JCM 33786 (Japan Collection of Microorganisms, Japan).

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1. 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 [1]. Methanotrophs play a vital role in the mitigation of wetland derived methane [2]. Methanotrophs are from the classes Alphaproteobacteria (Type II), Gammaproteobacteria (Type Ia and b) and recently have been reported from the phylum Verrucomicrobia [3]. Type Ib methanotrophs (earlier named as Type X) are major players in wetland and freshwater habitats, however, very few members have been isolated. Except *Methyloparacoccus* strains isolated from pond water sediments, representatives of most of the newly reported Type Ib genera have been isolated from the rice fields, e.g. *Methylogaea* [4], *Methylomagnum* and *Methyloterricola* [5]. Wetlands and rice fields from tropical regions have been largely unexplored for culturable methanotrophic diversity and are predicted to possess novel and unique methanotrophic taxa studies [6] [7]. Culture independent data suggests that many novel Type Ib methanotrophs might be existing in freshwater wetlands, small water bodies and rice fields [8]. Type Ib methanotrophs show a high metabolic diversity, however, many of the clades lack isolates [8]. Additionally, very few methanotrophs isolated from wetlands, have been described. In our pioneer work of isolation and description of novel taxa of methanotrophs from Indian rice fields and wetlands, we described a new genus and species (*Methylocucumis*) within Type I methanotrophs from a tropical rice field habitat in India. *Methylocucumis oryzae* [6,9-12] and two new species each from the genera *Methylomonas* [13] (*Ca.* Methylomonas aquatic Kb3) and *Methylobacter (Ca.* Methylobacter oryzae*)* [14,15] were reported in a series of studies, reflecting that there is a scope for unraveling un-cultured methanotroph diversity from tropical regions.

Very recently, a strain C50C1, isolated from a temperate rice field in China represented a novel genus and species within Type Ib methanotrophs and tentatively named as

'*Methylotetracoccus oryzae*' [8]. In our present study we describe the enrichment and isolation of a methanotroph, strain FWC3, which could represent a putative new species of *Methylotetracoccus* [8]. FWC3 was probably isolated before C50C1, as the 16S rRNA gene sequence of our strain is available since August 2018 and the genome sequence since February 2019. In contrast, the genome of C50C1 is available since July 2019, and no 16S rRNA gene data is available. Additionally, *Methylotetracoccus oryzae* C50C1 has not been deposited in international culture collections and therefore not described validly, so far [8]. In the present paper, we compare the properties of FWC3 and C50C1 on the genomic, morphological and biochemical levels. We prove that FWC3 could be a distinct species of the genus *Methylotetracoccus* and thereby expand the description of the genus. Further, we also explain how both the members of *Methylotetracoccus* show uniqueness in their predicted metabolic capabilities as depicted from their genomes. We also describe the salient features of the genus *Methylotetracoccus* and of the novel species *Methylotetracoccus aquaticus* strain FWC3.

2. Materials and Methods

2.1 Sampling:

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 plastic 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 ºC.

2.2 Enrichment and isolation of methanotrophs:

Enrichments for culturing methanotrophs were set up by serial dilutions in serum bottles by adding two millilitres or 2 grams of sample to 18 ml sterile dilute Nitrate Mineral Salts

(dNMS) medium (Pandit et al 2016a). About 20% methane was added to the headspace by replacing 20% air and the bottles were incubated under static conditions at 25°C for 4-6 weeks. Methane oxidation was checked periodically using gas chromatography by measuring the decline in the headspace methane. This was followed by streaking 20µl of the liquid positive enrichment culture on solid plates containing dilute NMS medium and 2% agarose as a solidifying agent. Plates were incubated in glass desiccators containing 20-30% methane & air in the headspace for 2-3 weeks.

The isolation and purification of methanotrophs was done as described before [16]. The purity of methanotroph culture was checked by streaking the colony on dilute nutrient agar plus 0.1% glucose or 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, a pure culture of methanotroph was 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.

2.3 Growth Experiments:

Utilisation of various carbon sources was studied in dNMS liquid medium [16] supplemented with the following filter-sterilised or autoclaved carbon substrates (0.1% w/v): formate, formamide, arabinose, raffinose, lactose, maltose, xylose, glucose, fructose, sucrose, succinate, pyruvate, glutamate and acetate was tested in microtitre plates. The ability of the strain to grow on methanol was tested by the addition of 0.02%-5% v/v methanol in liquid medium in serum bottles. The range of nitrogen sources utilised by strain FWC3 was tested by replacing KNO_3 from dNMS medium with one of the following compounds: NH_4Cl, urea, glycine, serine, valine, asparagine, aspartate, L-glutamic acid, glutamate, peptone or yeast

extract. Growth without nitrogen source was tested under micro-oxic and full oxic conditions (20% air, 20% methane, 60% 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. Differences in the OD values and the presence or absence of visible growth were monitored in all the cases. Wherever growth was observed, 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. Growth at pH values in the range of 3 to 9.6 was checked after buffering the medium with citrate-phosphate buffer (pH 3 to 6.8) and glycine buffer (pH 8, 9 and 9.6). 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. Salt tolerance was tested with NaCl (0.1%, 0.5%, 1% and 2.0%, w/v) added to the mineral medium. Strain FWC3 was grown at a temperature range from 10°C to 40°C. These experiments were done in triplicates in liquid media in sealed serum bottles. The ability of the culture to grow under varying oxygen concentrations was tested in serum bottles by take 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.

2.4 DNA extraction, PCR amplification and sequencing:

DNA extraction from the culture was done as described before [6]. Particulate methane monooxygenase 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

[6] 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.

2.5 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 [17] using only the sequences of type strains of valid species [18].

2.6 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 40X and 100X magnifications. Cells were also observed by Scanning Electron Microscopy (SEM) (Zeiss model EVO-MA-15 SEM). Cells were prepared in the following manner for SEM as described before [11].

2.7 Whole genome sequencing of the novel genus (FWC3):

Genomic DNA of strain FWC3 was extracted from an axenic culture, grown on solid medium, using Gen Elute^{TM} Bacterial Genomic DNA kit, Sigma-Aldrich. The genome was sequenced using Illumina HiSeq platform (2 x 150 bp) in Medgenome laboratories, Bangalore. The sequencing was carried out in Hi Seq X10 to generate 2 X 150 bp sequence reads at 100X 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 *N*50 of 184 kb, the largest scaffold assembled measured 498.3 kb. BlastN annotation was performed to identify the similarity with other known genomes. A blast based pairwise average nucleotide identity ANI-b was calculated using JSpecies [19,20]. The prodigal software was used for Gene Prediction. RNAmmer was used for identifying rRNA genes. TRNAscan-SE was used for identifying tRNA genes. The Bio project id is PRJNA520977 and the accession number for the whole genome of FWC3 is SEYW00000000. The phylogenomic analysis was done using PATRIC database [21].

2.8 GC, HPLC and Spectrophotometer: OD readings were taken in a spectrophotometer at 600 nm. GC measurements were done in a Chemito GC as described before [6].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 sugars were run to check whether there is any utilization of the substrate and products formed.

3. Results and Discussion

3.1 Isolation of a novel gammaproteobacterial methanotroph, FWC3, a new member of the tentatively named genus *Methylotetracoccus* from a tropical wetland

In the current study, a methanotrophic strain (FWC3) was isolated on dilute nitrate mineral salts (NMS) agarose medium [11] after an initial enrichment with methane and air in the headspace. The sample was the top sediment of a fresh water 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 growth 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, tetrads and small groups. This morphotype, arrangement of cells and colour of the colony was not encountered in our earlier studies focusing on isolation of rice field methanotrophs from Indian rice fields [6,14], (Rahalkar, Monali and Pandit, Pranitha, unpublished data). After streaking and re-streaking of the unique flesh pink colored colony, a pure culture was obtained and named as FWC3. FWC3 formed flesh pink colored colonies on dilute NMS agarose (2% agarose) medium [11] in methane: air environment (20:80) (Fig. 1a). Under phase contrast microscope, the cells of 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 aggregates (Fig. 1b). In scanning electron micrographs, coccoid cells could be seen (Fig. 1c). 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 25ºC-35ºC temperature range in dNMS medium [11]. The growth in liquid as well as on solid was mucoid in nature. FWC3 grew in methanol containing medium in a wide range of methanol concentrations (0.1-5% v/v). On further testing, we found that FWC3 could grow in very low concentrations of methanol (0.02%). FWC3 showed lesser growth under micro-oxic conditions (i.e. 4-5% oxygen) conditions and showed a preference for aerobic growth. FWC3 could also grow under nitrogen free conditions without any additional nitrogen source under aerobic as well as micro-oxic conditions. During the phylogenetic characterization, it was seen that the nearly complete 16S rRNA gene of FWC3 (MH789551.1) showed 94.3% similarity to *Methylocaldum marinum* $S8^T$ (nearest neighbour) and 90- 94% to other Type Ib methanotrophs. This reflected that FWC3 could belong to a novel genus and further studies were done. (The 16S rRNA gene of FWC3 was deposited as MH789551.1, 1481 bp, and is available from August 2018).

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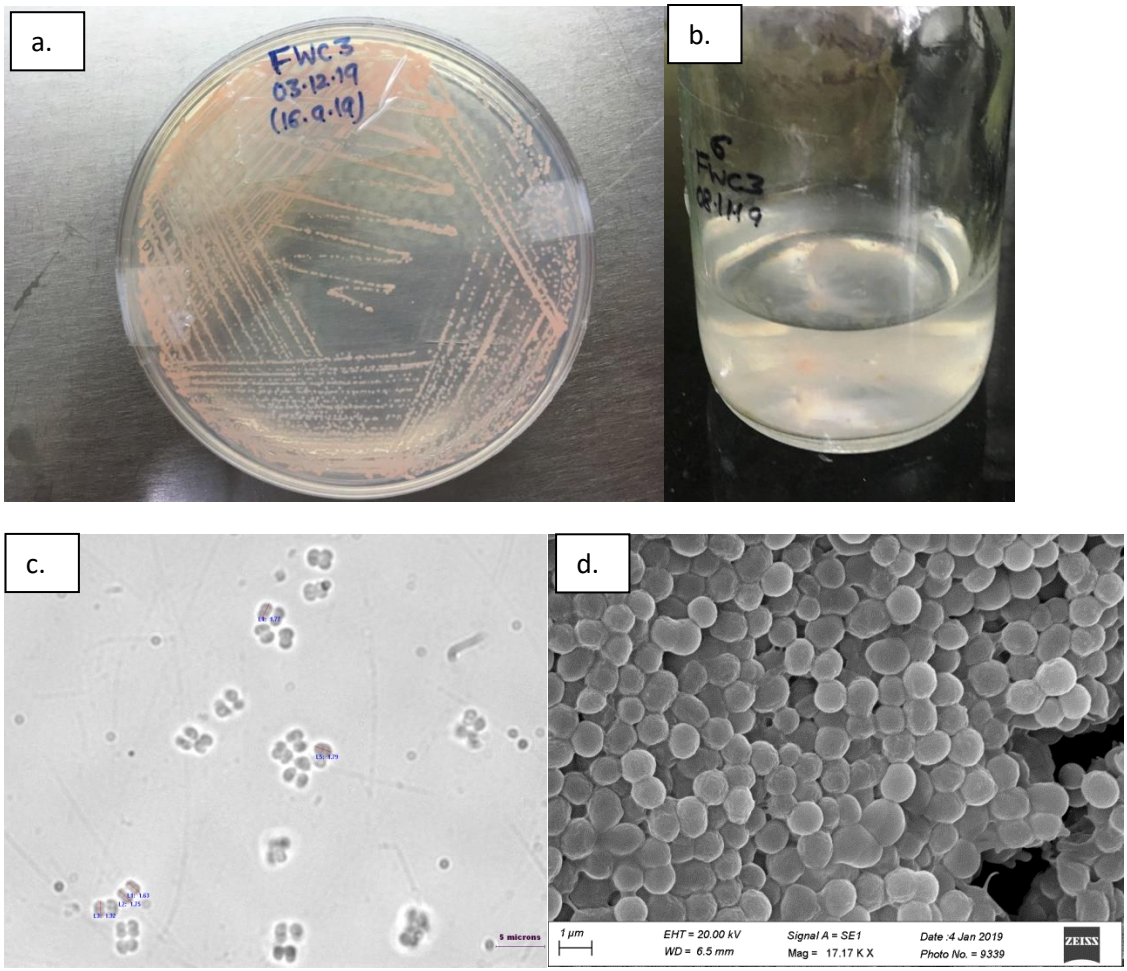

Fig.1: 1a. FWC3 growing on dil. NMS agarose plates in 20% methane environment. 1b. FWC3 growing in liquid NMS medium on 20% methane. 1c. Phase contrast image of FWC3 growing as tetra-cocci in the presence of 0.02% methanol, the bar represents 5 µm. 1d. Scanning electron micrograph of FWC3 growing on methane.

3.2.1 Draft genome features of FWC3

As FWC3 showed taxonomic novelty at the level of genus, the whole genome was sequenced for understanding the same in addition to understanding its metabolic properties. The size of the 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 700X. The genome contains 3,013 genes (2,975 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 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 600-700X coverage [22]. The genome of FWC3 was compared with the nearest neighbouring valid methanotrophs *Methylocaldum marinum* S8 and *Methylococcus capsulatus* Bath using the average amino-acid identities (AAI). The AAI values were 59.2% and 60.7%, respectively and the average nucleotide indices (ANI) were ~70% well below the genus cut-off of 75%. The digital DNA-DNA hybridization, DDH values of FWC3 with *Methylocaldum marinum* S8 and *Methylococcus capsulatus* Bath were 13.20% and13.70%, 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* S8^{T}.

3.2.2 Phylogenetic similarities between FWC3 and C50C1 based on genome

Meanwhile, in July 2019, a new genus *Methylotetracoccus oryzae* C50C1 with very similar morphological features was described [8]. Draft genome of C50C1 was available after July 2019 after which we compared it with that of FWC3. The genome size of the FWC3 is smaller (3.4 Mbp) compared to that of C50C1 (4.8 Mbp). The comparison showed us that the two-way AAI values as well as ANI-b value were below the cut-off, 94.73% and 94%, respectively, reflecting that FWC3 and C50C1 were closely related, but probably nor the same species. The DDH values comparing FWC3 with C50C1 was 55.7%, less than the 70% cut-off. Additionally, the draft genome of FWC3 was much smaller (3.4 Mbp) compared to that of C50C1 (4.84 Mbp). In a phylogenomic tree, both the strains grouped closely with 100% bootstrap values and the closest genome was of *Methylococcus* (Fig.2). The 16S rRNA gene sequence of C50C1 was obtained after request [8] as it is still not available in public database. Similarly, in a 16S rRNA gene based tree (Fig. 3a.) both the strains (FWC3 and

C50C1) group with a 100% bootstrap representing a single genus, *Methylotetracoccus*. Also, *Methylotetracoccus* and Methylocaldum species grouped together with a 58% bootstrap value, away from *Methylomagnum*, *Methyloparacoccus* amd *Methylococcus*.

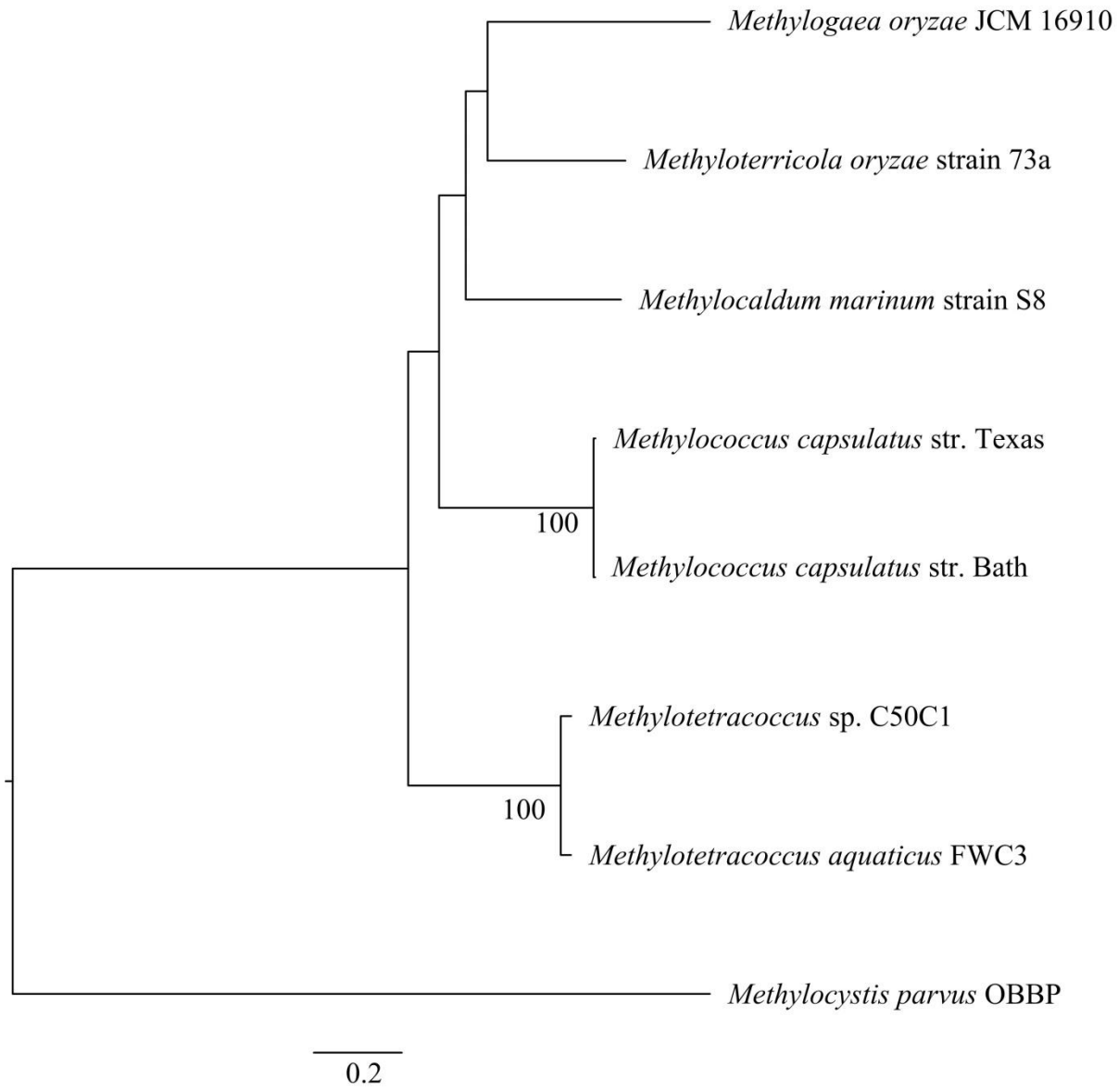

Fig.2: Phylogenomic tree using PATRIC based 'Codon Tree'; which selects single-copy PATRIC PGFams and analyzes aligned proteins and coding DNA from single-copy genes using the program RAxML. A total of 1000 genes were used.

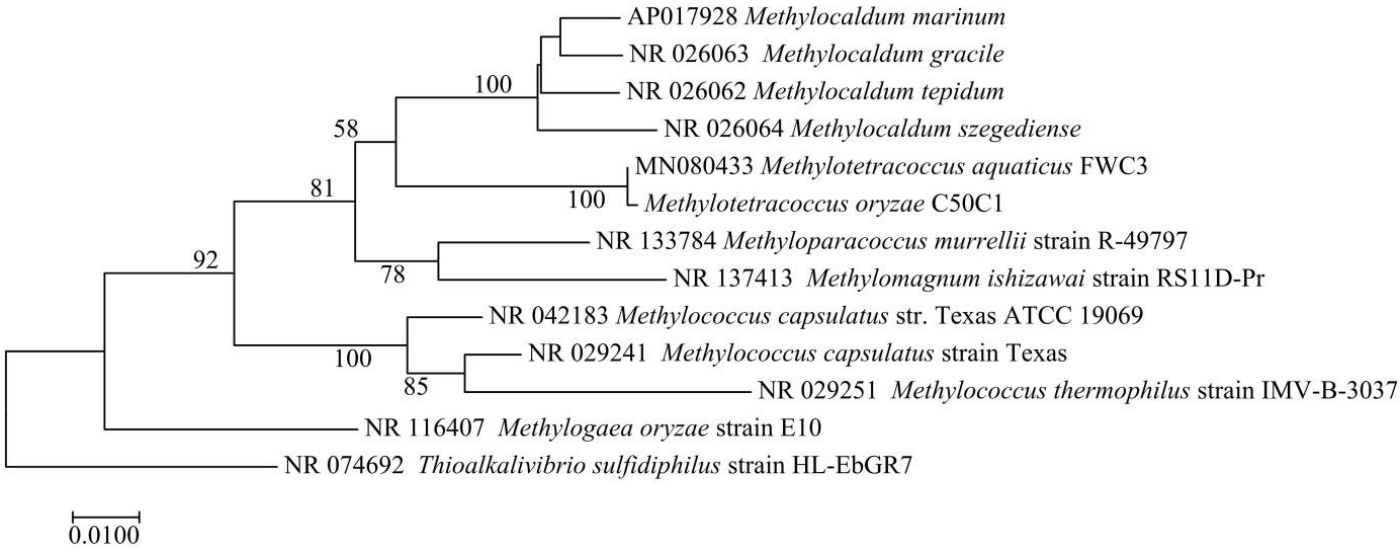

Fig.3a. Maximum-likelihood bootstrap tree of the 16S rRNA gene sequences of FWC3 in comparison with 16S rRNA of type strains of valid species. The evolutionary history was completed using the maximum-likelihood method based on the Tamura-Nei model and 1000 bootstraps. Bar represents 2% divergence. Phylogenetic tree was constructed using MEGA 7.

In a pMMO subunit B tree, (Fig. 3b) FWC3 and C50C1 proteins grouped together with 100% bootstrap. C50C1 has two copies of *pmo* operon, one of which showed 100% match to FWC3 and the other showed 97% match. Both of the pMMO protein from FWC3 and C50C1 branched close to that of *Methylococcus*.

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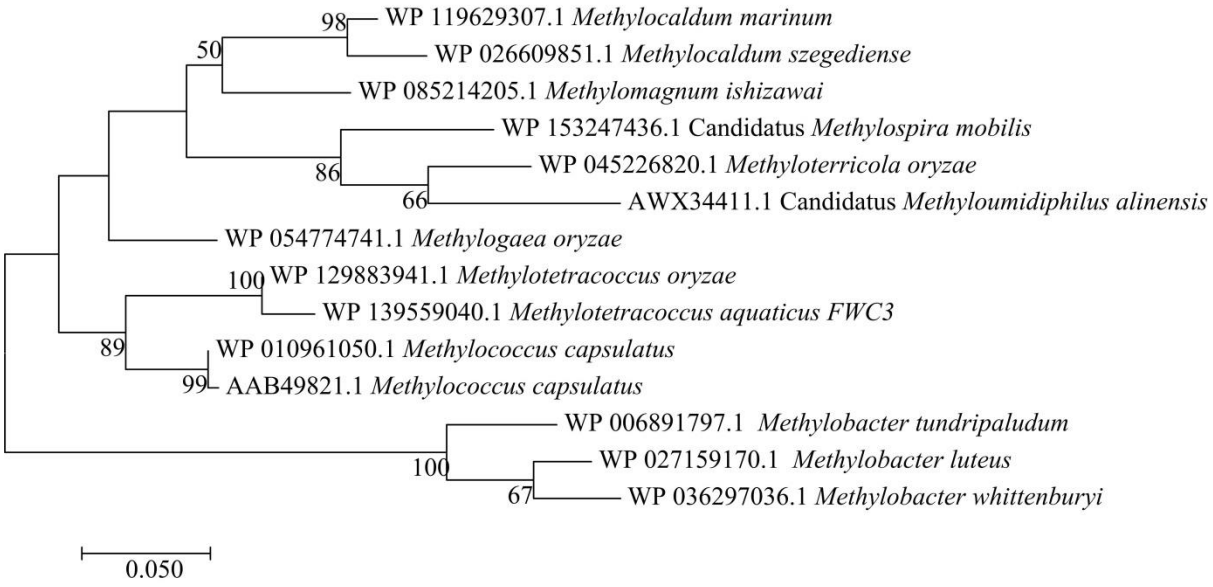

Fig.3b. Maximum-likelihood bootstrap tree of the pMMO B subunit protein sequences of FWC3 in comparison with the protein from type strains of valid species. The evolutionary history was completed using the maximum-likelihood method based on the Poisson correction model and 1000 bootstraps. Bar represents 2% divergence. Phylogenetic tree was constructed using MEGA 7.

3.3 Comparative genome features of *Methylotetracoccus* strains FWC3 versus C50C1 (

3.3.1 Methane oxidation pathway:

Both the strains of Methylotetracoccus, FWC3 and C50C1 showed very similar genome features. FWC3 draft genome showed a complete methane oxidation pathway with a single set of *pmo*CAB (particulate methane monooxygenase genes) and two extra sets of *pmo*C gene. In contrast, C50C1 possesses exactly double number of *pmo*CAB (two sets) and four sets of *pmo*C. Similar to C50C1 it does not possess any alternative methane monooxygenases such as pmoA2 or pXMMO or sMMO genes [8]. 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 the three were also present in C50C1; however, the iron dependent dehydrogenase

has not been mentioned in case of C50C1 [8]. In total, 4018 proteins were annotated in C50C1 in contrast to 2831 proteins in FWC3, reflecting that FWC3 genome was more streamlined.

Instead of the classical formaldehyde dehydrogenase, a NADP-quinone oxidoreductase is present in FWC3, a similar feature seen in C50C1. Similar to C50C1, FWC3 has a tetrahydrofolate and 5, 6, 7, 8-tetrahydromethanopterim H4MPT-linked C1 carrier pathways. In 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 C50C1.

3.3.2 Both the *Methylotetracoccus* strains/species show unique C metabolism genes

The p-blast similarities between FWC3 and C50C1 were above 90% in most of the cases (Supplementary Table 1 and 2). Nearly all of the unique genes in the FWC3 draft genome, showed closest similarities to that of C50C1 [8] but not to other methanotrophs (Supplementary Table 1). Instead, the second hits were from other phyla/ class (Supplementary Table 1) indicating that these genes were probably horizontally transferred. The highest genes were probably acquired by horizontal transfer from Betaproteobacteria followed by *Nitrospirae*, Gammaproteobacteria, *Firmicutes*, etc. The horizontally transferred proteins showed protein level similarity of ~50-75% (Supplementary Table 1).

Multiple ABC transporters

Several ABC sugar transporter kinases and permeases (Supplementary Table 1) including maltose/maltodextrin transporters (MalFGK complex) and other ABC sugar transporter cassettes were present (Supplementary Table 2). These were present in both FWC3 and C50C1 genomes. Methanotrophs do not usually show so many ABC transporters, and this is

one of the vital reasons why methanotrophs cannot utilize multi-carbon compounds [23]. Many of the ABC transporter permeases showed a probable horizontal transfer from other taxa (Supplementary Table 1). Fructokinase and phosphofructokinase appear to be horizontally transferred from Betaproteobacteria and *Lentisphaere*. The glycolytic pathway was complete in both, except the first enzyme glucokinase could not be detected in FWC3. Few of the other important enzymes of glycolysis and pentose phosphate pathway showed probable horizontal transfer, e.g. triose phosphate isomerase from Gram positive bacteria, glucose 6 phosphate dehydrogenase and 6-phosphogluconolactonase from sulfur oxidizing bacteria and Betaproteobacteria, etc., (Supplementary Table 1). Additionally, FWC3 also shows the presence of pyruvate formate lyase or formyl C-acetyl transferase, which catalyzes the formation of formate and acetyl CoA from pyruvate and CoASH. This enzyme appeared to be horizontally transferred from *Hydrogenispora* (Firmicutes) (~75-77% similarity). Amongst the other unique enzymes, malic enzyme (decarboxylating) converting malate to pyruvate and CO_2, phosphoenol pyruvate (PEP) carboxylase gene were exclusively found in FWC3 and *Methylotetracoccus oryzae* C50C1 and appear to be horizontally transferred from Betaproteobacteria (Supplementary Table 1). Few of the genes for pili related metabolism, such as prepilin and Type IV pili protein showed horizontal transfer (Supplementary Table 1). Type IV pili are also important in the horizontal transfer of genes, and thus have extra significance in FWC3. Also Vitamin B12 synthesis pathway seems to be absent in FWC3 and C50C1 [8]. Both of the genomes showed the presence of B12 binding domain protein, most similar to *Nitrospirae*. Enzymes like fructokinase, 6 phosphofructokinase, glycoside hydrolase family, trehalose pathway enzymes were found to be unique in FWC3 and C50C1 and were transferred horizontally, mostly from Betaproteobacteria. Trehalose is a solute produced for protection of the cells against desiccation and freezing and might explain how these bacteria protect themselves from drying and other environmental stresses. Trehalose

production is not observed in other methanotrophs, instead sucrose is produced in case of *Methylobacter* species [24].

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 was present in the genome. FWC3 lacks succinate dehydrogenase and hence the respiratory complex 2. On the contrary, C50C1 shows all of the complexes.

FWC3 shows capacity to produce rhamnose containing capsules and EPS as per the genome analysis. A complete pathway of rhamnose containing exopolysaccharides (rhamnose glycans) was also detected in FWC3. One of the genes in rhamnose EPS synthesis was absent in C50C1 and present only in FWC3. Fermentation genes in both FWC3 and C50C1 were most similar to those detected in *M. capsulatus* genome [25], indicating its possible fermentative lifestyle. We found that traces of formate were formed when the culture was grown on methanol. Formate has been one of the fermentation products in case of methanol grown cells [26].

FWC3 also shows the absence of ribulose-1, 5-bisphosphate carboxylase/oxygenase, similar to C50C1, and does not have an autotrophic lifestyle. The genome shows the possibility of producing starch, amylose and glycogen as storage molecules which can be converted to alpha, D- glucose and processed through glycolysis. This also could be useful in the metabolism of these compounds during starvation, as the genome shows the presence of multiple glycoside hydrolases (15, 16 and 57) (Supplementary Table 2).

In spite, of the fact that FWC3 showed multiple transporters for sugars, we did not detect visible growth (above OD 0.03-0.04) on any of the sugars or organic acids tested. Sucrose, glucose, maltose, fructose, acetate, pyruvate, succinate, glutamate and malate did not support

growth after 1 month of incubation. Similarly, C50C1 has also reported not to grow on multi-carbon substrates.

3.4 Dissimilar features (FWC3 versus C50C1)

FWC3 showed an incomplete tri-carboxylic acid (TCA) cycle with two consecutive enzymes missing, succinate dehydrogenase and fumarate hydratase, whereas C50C1 showed a complete one [8]. In FWC3, the TCA cycle was branched starting from oxaloacetate to succinate. Thus, two separate branches of TCA cycle, one from oxaloacetate to succinate and other from oxaloacetate to malate were seen to be present.

Glycoside hydrolase 16 and a gene in rhamnose EPS synthesis, were absent in C50C1 and present only in FWC3.

3.5 Physiological comparisons within *Methylotetracoccus*: FWC3 and C50C1

Strain FWC3 used ammonium chloride, urea, glutamate, peptone, yeast extract and lysine as nitrogen sources and grew on nitrogen-free medium. There is no detailed description about the nitrogen sources utilized for C50C1. However, it has been reported to grow on nitrate, ammonium and without any 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. C50C1 grew within a pH range of 4.3-8.3 and a similar optimum pH. Strain FWC3 was found to grow in the temperature range between 20ºC to 37°C and maximum growth was detected in the range 25-35°C. C50C1 could grow from 4-30ºC with optimum around 18-25ºC. As FWC3 was isolated from a tropical wetland (18ºN) and C50C1 was isolated from a temperate region (30ºN) and this was reflected in the growth temperatures. As the area from which FWC3 was isolated is close to the sea, the average temperatures are in the range 25-35ºC, whereas the temperature variation in the Cixi, China is in the range of 4-30ºC. The overall characteristics of FWC3 and C50C1 representing

Methylotetracoccus genus are compared with the other Type Ib methanotroph genera (Table1).

3.6 Chemotaxonomic characteristics and comparisons

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 2). In contrast, C50C1 shows a dominance of 16:1 ω9c (33%) followed by 16:1 ω6c/ 16:1 ω7c (Table 2).

Thus, FWC3 and C50C1 show similarities on the level of the genus. However, based on the genomic, morphological, chemotaxonomic and physiological differences, we propose that FWC3 warrants being a member of a new species of the genus *Methylotetracoccus*. FWC3 has been deposited in two international culture collections in two different countries with the accession numbers. Therefore, we propose FWC3 as the type strain of the species *Methylotetracoccus aqauticus*. We further also give the amended description of *Methylotetracoccus* gen. nov.

3.7 Description of *Methylotetraoccus aquaticus* sp. nov.

Most of the characters are as per the genus description, described below. The cells are 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 in pairs, triplets or in tetra-cocci and larger aggregates. Methanol supports growth from 0.03%-5%. Optimum growth occurs in the range 25-35ºC and pH 5-8. The type strain FWC3 was isolated from a freshwater creek mud in Nagaon beach, near Alibag, India. The G+C content of the type strain is 63 mol% (genome sequence). FWC3 is available in two international culture collections with the accession numbers: MCC 4198 and JCM 33786.

3.8 Amended description of *Methylotetracoccus* gen. nov. *Methylotetracoccus* [Me.thy.lo.tet.ra-coc=cus]. N.L. neut. n. *methylum* the methyl group *tetra*, four; N.L. masc. n.

coccus (from Gr. n. *kokkos*), a grain or berry; N.L. masc. n. *Methylotetracoccus*, a methyl-using organism with tetrad-forming coccoid cells.

Obligate methanotroph, which can utilise methane or methanol as the sole source of C and energy. Gram-stain negative, nonmotile cocci, which reproduce by binary fission and occur singly, in pairs, or as tetracocci or aggregates. Uses pMMO for methane oxidation. Gram-stain negative, aerobic, non-motile. Divides by binary fission. Can grow under neutral or a broader pH range. Mesophilic, some strains are psychrotolerant. Cells are capable of dinitrogen fixation. Belongs to the family *Methylococcaceae*. The major cell wall fatty acids are 16:1 ω6c/ 16:1 ω7c, 16:0 and 16:1 ω9c. The most closely related genera are *Methyloparacoccus*, *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. Two species, *Methylotetracoccus oryzae* C50C1 and *Methylotetracoccus aquaticus* FWC3 have been reported so far.

3.9 Database availability: The whole genome shotgun project for the strain FWC3 has been deposited in GenBank database under the accession number NZ_SEYW00000000.1 (released in Feb 2019) under the name, *Methylococcaceae* bacterium FWC3. The bioproject accession number is PRJNA52097. The link for the genome is https://www.ncbi.nlm.nih.gov/genome/?term=SEYW01.

Author contributions: Conceptualization: MCR and RAB Data Analysis: MCR and KK, Experimental work: Strain isolation, characterization, maintenance and growth experiments: KK, JM and PSP Fund acquisition: MCR Phylogenetic analysis and sample collection: MCR and RAB Supervision: MCR, Writing- original draft: MCR Writing-review and editing: MCR and RAB

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Figure Legends:

Fig.1: 1a. FWC3 growing on dil. NMS agarose plates in 20% methane environment. 1b. FWC3 growing in liquid NMS medium on 20% methane. 1c. Phase contrast image of FWC3 growing as tetra-cocci in the presence of 0.02% methanol, the bar represents 5 µm. 1d. Scanning electron micrograph of FWC3 growing on methane.

Fig.2: Phylogenomic tree using PATRIC based 'Codon Tree'; which selects single-copy PATRIC PGFams and analyzes aligned proteins and coding DNA from single-copy genes using the program RAxML. A total of 1000 genes were used.

Fig.3a. Maximum-likelihood bootstrap tree of the 16S rRNA gene sequences of FWC3 in comparison with 16S rRNA of type strains of valid species. The evolutionary history was completed using the maximum-likelihood method based on the Tamura-Nei model and 1000 bootstraps. Bar represents 2% divergence. Phylogenetic tree was constructed using MEGA 7.

Fig.3b. Maximum-likelihood bootstrap tree of the pMMO B subunit protein sequences of FWC3 in comparison with the protein from type strains of valid species. The evolutionary history was completed using the maximum-likelihood method based on the Poisson correction model and 1000 bootstraps. Bar represents 2% divergence. Phylogenetic tree was constructed using MEGA 7.

Table 1: Comparison of the growth and other characters of *Methylotetracoccus* to other Type Ib methanotrophic genera

Characteristics	*Methylo-tetracoccus*	*Methylo-paracoccus*	*Methylo-caldum*	*Methylo-coccus*	*Methylo-terricola*	*Methylo-gaea*	*Methylo-magnum*	*Methylo-spira*
Isolation source/ Habitat	Rice fields, wetlands	Pond water	Marine sediments, wetlands	Thermal bath	Rice field	Rice field	Rice field	Sphagnum bogs
Growth Temperature (ºC)	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	5.5-9	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 ω9c, C16:0	C16:1 ω7c	ND	C16:0, C16:1 ω 7c	C16:0, C16:1 ω 7c, C16:1 ω 5c	C16:0	C14:0, C16:0, C16:1 ω 7c	ND
Pigmentation	Flesh pink, peach, white-brown	white	brown	White to brown	White to slightly pink	white	White	ND
G+C content %	62.8-63.	65.6	56.5-57.2	59-66	61.0	63.1	64.1	54.0

ND: Not detected

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Table 2: Cell wall fatty acids comparison of related Type Ib methanotrophs

Fatty acids	*Methylo-tetraccoccus* FWC3	*Methylo tetraccoccus* C50C1	*Methylo-coccus*	*Methylo-paracoccus*	*Methylo-caldum*	*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					
18:1 ω7c	0.62	0.93	--	--	--	1.7	--