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Description and salient genome characteristics of a putative novel species Methylomonas strain WWC4, isolated from an Indian freshwater ecosystem

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Research Article

Keywords: Methylomonas, freshwater sediment, methanotrophs, India, novel species

Posted Date: April 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2614643/v1

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Abstract

Freshwater wetlands are interesting habitats for methane-oxidizing bacteria or methanotrophs. We isolated a methanotroph, strain WWC4, from the mud of a freshwater creek running close to a beach in the coastal regions of Western India, near Alibag town. Strain WWC4 was a strict methanotroph, thriving only on methane and marginally on methanol. It formed pink to slightly orange-colored round colonies and formed pinkish turbidity or surface pellicle in liquid culture, indicative of the *Methylomonas* genus. The strain is characterized by thick, short, and motile rods, 2.5-3 µm long and 0.8-1.2 µm wide in size, with a Gram-negative character. Based on the 16S rRNA gene sequence, the culture was classified as a *Methylomonas* strain, with the nearest type species being *Methylomonas koyamae* Fw12E-Y^T showing 97.81% sequence similarity. A comparison of the draft genomes indicated that *Methylomonas* sp. WWC4 genome showed only 74.45%, 75.72%, and 21.5% similarities of AAI, ANIb, and dDDH values, respectively, with its closest neighbor *Methylomonas koyamae* Fw12E-Y^T, indicating its taxonomic novelty at the level of species. The GC content of the genome was 55.9 mol%. The whole-genome shotgun project was deposited at DDBJ/ENA/GenBank, and the accession number is JAATWI01.1. The cell wall lipids indicated a unique fingerprint with 15:1 ω8c (21.09%), 16:0 3OH (15.7%), and 16:1ω5c (12.6%) being the primary fatty acids, dissimilar to any other related *Methylomonas* species, confirming its taxonomic novelty. The genome was further explored for carotenoid pathways and plant growth promotion genes for its biotechnological potential. Strain WWC4 did not withstand cryopreservation and could not be deposited in two international culture collections. The culture is part of our institutional, WDCM-approved culture collection as MCMB-1474, maintained live. Due to its taxonomical novelty, strain WWC4 is also proposed to be a member of a *Candidatus* species of the *Methylomonas* genus and named "*Candidatus* Methylomonas sedimenticola" strain WWC4.

Introduction

Wetlands occupy 3.8% of the Earth's land surface, amounting to about 20–40% of global CH_4 emissions. However, the wetlands' net emission results from the delicate balance between methanogenesis and methanotrophy. Methanotrophs are aerobic to micro-aerophilic bacteria that use methane as the sole carbon source for growth and energy [1, 2]. While various natural and anthropogenic activities emit methane, wetlands account for massive emitters and absorbers of methane gas [3]. Wetlands also harbor a rich community of methanotrophs which, with their methane monooxygenase enzyme activity, utilize over 18 to 90% of the methane [4] thus simultaneously establishing an equilibrium between methanogenesis and methanotrophy. Methane-oxidizing bacteria or methanotrophs are present in the oxic-anoxic interphase of wetlands and oxidize methane. There are very few studies based on methanotrophs isolated from tropical wetlands. Moreover, there are almost no cultured members isolated from wetlands in India except the newly described *Methylolobus aquaticus*, a novel genus and species isolated and described by us [5]. In the present study, we report the isolation of a strain of *Methylomonas* from a nearby habitat to this isolate. We have earlier documented the first novel genus and species of a methanotroph from rice fields in India: *Methylocucumis oryzae* [6, 7] and one putative novel species, '*Candidatus* Methylobacter oryzae' KRF1 [8]. We also reported the genome sequence and analysis of the novel genus *Methylocucumis* [9] and a novel isolate, *Methylomonas* strain Kb3 [10].

Methylomonas is one of the most well-known genera within methanotrophs, and the current nomenclature has nine validly described species. The first methanotroph isolated by Söhngen in 1906 from the aquatic plant material was also a *Methylomonas* named *Bacillus methanicus* and later modified *Methylomonas methanica* by Orla-Jensen in 1909 [11, 12]. *Methylomonas* belongs to Type I, gammaproteobacterial methanotrophs, grows between 10–40°C temperature range and is mesophilic. The morphological feature of the genus *Methylomonas* is to form pink or ochre-yellow coloured colonies on solid media [13]. The member of the genus *Methylomonas* can be found in diverse habitats, including wetland muds, rice fields, sediments of freshwater lakes and rivers, activated sludge and wastewater, and coal fields [13].

The purpose of the present study is to document the biochemical, morphological characteristics, and the salient genome features of the putative novel species of *Methylomonas* isolated from an Indian wetland. Further we also explore the genome for its biotechnological potential- carotenoid production and nitrogen fixation which is an attribute by which the methanotroph can promote plant growth in rice. Since the culture has to be maintained as a live culture and can be stored at 4°C for 3–4 months, but cannot be cryopreserved, it could not be deposited to two international culture collections. We have maintained it in our laboratory for the past five years since its isolation by sub-culturing and in our institutional WDCM approved culture collection, as MCMB-1474. We also propose it here as a novel species with *Candidatus* status and the proposed name for this species is '*Ca.* Methylomonas sedimenticola' sp. nov. strain WWC4.

Materials And Methods

Sampling

A mud sample was taken from a freshwater creek region near Nagaon beach, close to Alibag, Maharashtra, India (18º26'30" N 72º54'20" E) directly in a sterile plastic container. The creek was filled with water during peak monsoon season on August 30th 2017. The sample was processed for enrichment in methane and air-rich conditions and the remaining sample was stored at 4–8ºC for further experimentations.

Enrichment and isolation

The sample was diluted nine times in a sterilized sealed serum bottle containing diluted nitrate-mineral salts NMS liquid media and incubated in methane and air (20:80) as headspace gases [14]. On the 24th day of incubation of the enrichment bottle, a considerable reduction in the headspace methane was detected. The turbid liquid was streaked on solid NMS media plates, followed by incubation in a desiccator containing methane and air (20:80) as headspace gases. Different colored and sized colonies appeared after 3–4 weeks of incubation.

After visualization of growth, 20µl of enrichment broth was streaked on solid media petri-plates containing 2% agarose as a solidifying agent and incubated at 25°C in a desiccator with methane and air as headspace gas. Headspace gases were replaced weekly. Single colonies were picked using a sterilized toothpick and streaked on solid media petri-plates containing 2% agarose as a solidifying agent and incubated at 25°C in a desiccator with methane and air headspace gas.

Repetitively, single colonies were picked using a sterilized toothpick or wire loop and streaked on a solid media petri-plate to get the axenic culture of methanotrophs that contains single methanotrophs without any heterotrophic contamination.

Purity of axenic culture was carried out by streaking on 1/10th diluted nutrient agar plate containing 1% glucose and microscopic observation. Since methanotrophs can only grow on methane or methanol and cannot utilize multi-carbon or complex carbohydrates, usually no growth is seen on the nutrient agar plates. A dominant type of pink-colored colonies appeared and was named strain WWC4 after purification.

Morphological Characterization

Morphological analysis was done by wet-mount of culture under phase-contrast microscopy and Gram-staining under light microscopy to check the Gram character of isolates. Live cells of methanotrophs strain were observed under a phase-contrast microscope (Nikon 80i, Japan microscope with a camera) under 100X magnifications with oil emulsion. The Gram character of heat-fixed culture was determined using a standard Gram-staining protocol and observed under a bright-field microscope. The bacterial culture was fixed and processed to observe under a Scanning Electron Microscope (SEM) (Zeiss model EVO-MA-15 SEM). SEM sample preparation has been described before [15].

Biochemical Characterization

Various growth parameters were checked using liquid NMS media in triplicates. The utilization of different carbon sources was studied supplemented with one of the following autoclaved (at 10psi) or filter-sterilized carbon substrates (0.1%, w/v): formate, arabinose, lactose, xylose, fructose, formamide, raffinose, maltose, glucose, and sucrose using microtiter plates and incubated at 28ºC. Methanol utilization or tolerance was studied by growing the culture in a 0.2-5% range of methanol. The utilization of various nitrogen sources was studied using a microtiter plate containing liquid NMS (without KNO_3) supplemented with one of the following autoclaved (at 10psi) substrates (0.05%, w/v): NH4Cl, urea, glycine, serine, valine, asparagine, aspartate, L-glutamic acid, glutamate, peptone, and yeast extract. The culture was also grown in a range of pH with and without buffer conditions. Citrate–phosphate buffer (pH 3–6.8) and glycine buffer (pH 8, 9, and 9.6) were used for buffering the dNMS-medium. Without buffer conditions, only HCl or NaOH was used to adjust the pH from pH 3 to pH 10. The plate was incubated in glass desiccators containing around ~ 30% methane gas in the headspace as a carbon source and the rest air. The desiccators were incubated at 25ºC. The optimum temperature range (10–40ºC) was studied in sterilized sealed 35ml serum bottles containing 9ml dNMS media.

DNA Extraction and Sequencing

Particulate methane monooxygenase β subunit (*pmoA* gene) and 16S rRNA gene amplification were carried out using A189f-mb661r and 27f-1492r primers, respectively, as described earlier [15] and First Base Laboratories, Malaysia, sequenced the products. The sequences obtained were subjected to BLAST analysis. The alignment of sequences was done using the MAFFT alignment server

(https://mafft.cbrc.jp/alignment/server/). Based on the cut-off values of 98.65% for the 16S rRNA gene [16] and a corresponding cut-off value of 87% for the *pmoA* gene nucleotide sequence, the strains were designated to belong to putative novel species [17, 18]. DNA extraction of biomass was carried out using a modified Gram-negative process of GenElute™ Bacterial Genomic DNA Kit Protocol (Sigma Aldrich, USA) (https://www.sigmaaldrich.com/IN/en/product/sigma/na2110). The genome of WWC4 was sequenced in Medgenome laboratories, Bangalore. The genomic comparison of strain WWC4 with genomes of its closest members was analyzed to calculate the average nucleotide identity (ANIb-G, http://jspecies.ribohost.com/jspeciesws/#analyse), digital DNA–DNA hybridization (dDDH, http://ggdc.dsmz.de/) and the average amino-acid identity (AAI, http://enve-omics.ce.gatech.edu/aai/).

Genome sequencing

The genomes were sequenced by Medgenome, Bangalore, using Illumina sequencing technology, which produced paired-end reads. After low-quality bases and adapter sequence filtration, the high-quality paired-end reads were retained using Trimmomatic (v 0.35) and cutadapt (v 1.18). The assembly was optimized using an SSPACE-basic assembler to obtain high-quality genome assembly. High-quality reads were assembled using the Soapdenovo-127mer assembler (v 2.04). Scaffolds were constructed with an N_{50}. Genes were annotated using blastX.

Genome annotation:

NCBI (National Centre for Biological Information): NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used for genome annotation that combines alignment-based methods with methods of predicting protein-coding and RNA genes and other functional elements directly from the sequence (https://submit.ncbi.nlm.nih.gov/).

RAST: RAST (Rapid Annotation using Subsystem Technology) server was used for annotating complete or nearly complete bacterial and archaeal genomes (https://rast.nmpdr.org/). It provides high-quality genome annotations for these genomes across the whole phylogenetic tree. The annotation provides the mapping of genes to subsystems and metabolic reconstruction.

KEGG web server: The metabolic pathways were constructed using KEGG 2.2 webserver (https://www.kegg.jp/blastkoala/) using the amino acids FASTA file as an input. The pathway can also be reconstructed using KEGG Mapper (https://www.genome.jp/kegg/mapper/reconstruct.html) using the KEGG-annotation file. Pathways for carotenoid production and genes responsible for plant growth promotion, including nitrogen fixation pathways, were retrieved from the KEGG.

Genome comparison:

Digital DNA-DNA hybridization (dDDH) value was calculated using the webserver Genome-to-Genome Distance Calculator 3.0. The dDDH calculation can be performed by submitting the query genome against all the reference genomes (https://ggdc.dsmz.de/) [19]. The dDDH values of > 70% to identify strains within a species [20–22].

The average nucleotide identity (ANIb) value was calculated using the JSpeciesWS webserver. (http://jspecies.ribohost.com/jspeciesws/). A cut-off score of > 95% indicates that they belong to the same species [20–22]. Amino acid identity (AAI) value was estimated using the average amino acid identity of the genome. It is calculated by both best hits (one-way AAI) and reciprocal best hits (two-way AAI) between two genome datasets of proteins. The AAI was computed using a webserver (http://enve-omics.ce.gatech.edu/aai/index). A cut-off score of > 95% indicates that they belong to the same species [20–22]. ANI values of ~ 94% corresponded to the traditional 70% DDH standard of the current species definition [20–22]. A phylogenomic tree was constructed between whole/draft genomes of query and closed relative members using the following web servers: The PATRIC 3.6.12 webserver enables the construction of custom trees built (https://www.patricbrc.org/app/PhylogeneticTree). The Codon Tree method selects single-copy PATRIC PGFams, analyses aligned proteins, and codes DNA from single-copy genes using the RAxML program. It resulted in a Scaled Vector Graphics (SVG) image of the final tree and a Newick file which can be extracted in the interactive tree Viewer in PATRIC or downloaded and viewed in FigTree or other software [23, 24]. PATRIC was used to construct the phylogenomic relationship between the genome of the novel strain compared to the genomes of its closest members.

Long term preservation: Three methods were used for long-term preservation of the culture: 5% DMSO: Dimethyl sulfoxide (DMSO) [Chemical formula: $(CH_3)_2SO$] was added in a sterile 2ml screw-cap cryotube or microcentrifuge tube containing grown culture using a sterilized syringe under aseptic laminar airflow conditions. 1% trehalose in 1/10 diluted trypticase soy broth (1% TT) with 5% DMSO was prepared and added in a sterile 2ml screw-cap cryotube or microcentrifuge tube containing grown culture using a sterilized syringe under aseptic laminar airflow conditions. Glycerol stocks: 15% glycerol aqueous stock solution was prepared and sterilized by

autoclave. In a sterile 2ml screw-cap cryotube or microcentrifuge tube, 0.7ml of grown culture and 0.3 mL of sterile 50% of glycerol stock were added. The cultures were preserved at -80ºC. The revival of culture was checked after 1, 3, 6, and 12 months intervals. Preserved vials were replaced with fresh ones after one year.

Results And Discussion

Enrichment, Isolation, and Identification

Different colored colonies appeared on solid media plates after 3–4 weeks of incubation. A total of four methanotroph strains were cultivated and named with the designation WWC (wetland water creek/canal). Of the four, three: WWC1, WWC3, and WWC5, showed 99% 16S rRNA similarity with *Methylomonas koyamae* and were classified as *Methylomonas koyamae* after doing 16S rRNA gene sequencing and blast. Strain WWC4 showed only 97.81% similarity to *Methylomonas koyamae* Fw12E-Y^T and hence was further subjected to polyphasic characterization being a putatively novel species of *Methylomonas.*

Microscopic Identification

Strain WWC4 formed pink to slightly orange-colored colonies on the NMS media plate with 2% agarose as a solidifying agent (Fig. 1). The diameter of the colonies was 2–4 mm. The live cells were thick short motile rods 2.5-3 µm long and 0.8–1.2 µm (Fig. 1). This strain formed a thin pinkish-orange turbidity in liquid NMS media with methane and air (20:80) as headspace gas (Fig. 1). The cells showed Gram Negative character.

Phylogenetic and Phylogenomic Affiliation

The 16S rRNA gene of *Methylomonas* sp. WWC4 (MH764454.1) showed that *Methylomonas* sp. WWC4 showed the closest affiliation to *Methylomonas koyamae* Fw12E-Y^T with 97.61% similarity, followed by *Methylomonas rhizoryzae* GJ1^T 96.02% similarity, respectively, after nucleotide BLAST search. The maximum likelihood tree of the 16S rRNA gene of strain WWC4 showed a similar picture with three of these species as the closest neighbors (Fig. 2). The 16S rRNA gene of strain WWC4 and *Methylomonas* strain R-45383 shared 99.57% similarity, though this strain is not yet classified as a member of a new species. *Methylomonas* R-45383 was isolated from wetland (Ghent, Belgium) and has not been described and validated yet though the genome is described and available for comparison [25]. The *pmoA* gene of *Methylomonas* sp. WWC4 (MH806338) showed the closest affiliation to type strains; *Methylomonas koyamae* Fw12E-Y^T and *Methylomonas rhizoryzae* GJ1^T with 89.43% and 88.78% similarity, respectively, and this can be seen by its close phylogenetic grouping with these two species within the genus *Methylomonas* (Fig. 3). The genomic comparison of the genome of strain WWC4 with its closest members of the genus *Methylomonas* was carried out using the PATRIC webserver. The phylogenomic tree of strain WWC4 showed that it grouped with *Methylomonas rhizoryzae* GJ1^T and *Methylomonas koyamae* Fw12E-Y^T (Fig. 4).

Draft Genome Features:

The genome of strain WWC4 was sequenced using Illumina sequencing technology, which produced a total of 23,037,313 paired-end reads with average read length distribution of 141 bp and quality score distribution of 36. The 21,606,919 high-quality paired-end reads were retained after low-quality bases and adapter sequence filtration using Trimmomatic (*v 0.35*) and cutadapt *(v 1.18*). A total of 73 high-quality reads were assembled using the Soapdenovo assembler. A total of 56 scaffolds of > 500 bp were constructed, with an N50 of 223 kb. The largest scaffold assembled measured 691.7 kb. The G + C content of the draft genome was 55.9%, and the genome contains 4,672 CDS (Table 1). A total of 4,559 genes were annotated using BlastX (Table 1). These ~ 56 contigs, which belong to the genus *Methylomonas* were uploaded in RAST (http://rast.nmpdr.org/) and NCBI-PGAP for genome annotation (Table 1).

The nearest valid member of *Methylomonas* sp. WWC4 is *Methylomonas koyamae* Fw12E-Y^T with 74.45%, 75.72%, and 21.5 [19.3–23.9%] of AAI, ANIb, and dDDH values, respectively (Table 2).

Salient metabolic characteristics predicted from the draft genome

The complete details of the enzymes related to the carbon metabolism of strain WWC4 have been provided in Supplementary Table 1. Strain WWC4 contained two sets of particulate methane monooxygenase and one set of soluble methane monooxygenase for the oxidation of methane to methanol. The oxidation of methanol into formaldehyde was pursued by the presence of the methanol dehydrogenase enzyme present in the genome. Three copies of the PQQ-dependent dehydrogenase enzyme were present in the genome, out of which one of the enzymes was novel to methanotrophs and showed the closest similarity to *Gemmatimonadetes*

bacterium. One copy of each NAD(P)-, zinc, and iron-dependent methanol dehydrogenase was also found in the genome of stain WWC4. All sets of enzymes for formaldehyde to formate and formate to carbon-dioxide conversion were present in the genome (Supplementary Table 1). The delta subunit of formate dehydrogenase was novel to methanotrophs and showed the closest similarity to *Bradyrhizobium algeriense* RST91.

The details of nitrogen metabolism pathway genes present in the WWC4 genome has provided in Supplementary Table 1. The genes required for environmental nitrogen fixation were found in strain WWC4. The nitrogen fixation proteins NifT, X, Q, Z, W, and NifM, were found in the genome. The presence of classical nitrogenase molybdenum-iron proteins (NifH,D, K) was also detected in the genome. Within the nitrate reduction enzymes, nitrite reductase, nitrate reductase, and nitric oxide reductase were present in the genome (Supplementary Table 1).

Other important proteins involved in hemerythrin metabolism, and these hemerythrin genes are essential in oxygen scavenging conditions and helpful in transporting oxygen to particulate methane monooxygenase, Supplementary Table 1 [26–28].

Physiology and chemotaxonomy

Methane and methanol are the only substrates used by strain WWC4 as carbon sources. This strain cannot utilize multicarbon (0.1%) such as glucose, fructose, sucrose, maltose, xylose, arabinose, and raffinose. Neither formate nor formaldehyde was utilized by strain WWC4. It can tolerate up to 0.5% of methanol present in the growth media. The concentration of 0.05% of ammonium chloride, glutamate, peptone, yeast extract, and lysine can be used as a nitrogen source. It also can fix environmental nitrogen when growing optimally in nitrogen-free media. Strain WWC4 was a mesophilic methanotroph to grow in the 15–30ºC temperature range with an optimum 25ºC at a pH range of 3–9 under a buffered medium. It can tolerate up to 1% of NaCl-salinity in the medium. The comparisons of some of the significant characteristics of strain WWC4 with other members of the *Methylomonas* genus are enlisted in Table 3 [13, 29].

The cell wall fatty acids profile of strain WWC4 enclosed maximum amounts of 15:1 ω8c (21.09%), 16:0 3 OH (15.7%), and 16:1ω5c (12.6%). The detail of the cell wall fatty acids profile of strain WWC4 and type strains of species of the *Methylomonas* genus has provided in Table 4 [13, 29]. The FAME profile of WWC4 is unique as compared to the other *Methylomonas* species (Table 4). Strain WWC4 did not withstand cryopreservation and could not be deposited in two international culture collections. The culture is part of our institutional, WDCM-approved culture collection as MCMB-1474, maintained live, since its isolation (from 2018- till date). It can be stored in the fridge at 4–8°C for 3–4 months in liquid and has to be sub-cultured after intervals. Similar amount of survival is seen when maintained on agarose plates in methane-air environment.

Carotenoid pigment genes:

Genus *Methylomonas* has been reported to produce carotenoid pigment [30–32]. Strain WWC4 showed distinct pink color indicative of carotenoid pigment and therefore, the genome of strains was analyzed for the carotenoid production pathway. The production of carotenoid in *Methylomonas* strains is done by the following pathway and the enzymes were detected in the draft genome (Supplementary Fig. 1and 2). In this case, the strain produces carotenoids by utilizing two molecules of C_{15} farnesylpyrophosphate (FPP), resulting in C_{30} carbon skeleton formation (ref). Terpenoid is synthesised through the MEP pathway. The critical enzyme 1-deoxy-D-xylulose 5-phosphate synthase (dxs) genes of MEP/DOXP pathways was present in strain WWC4. MEP/DOXP pathways synthesise isopentenyl diphosphate (IPP) that were converted by farnesyl diphosphate synthase (FPPS/IspA) into farnesylpyrophosphate (FPP) and geranylpyrophosphate (GGPP). Geranaylgeranyl pyrophosphate synthase would convert IPP and FPP into GGPP, which will be sequentially converted into phytoene by the enzymatic activity of squalene/ phytoene synthase (crtB).

Plant growth promotion related genes:

As methanotrophs are associated with rice plant rhizospheres they can be applied as agents to promote plant growth (Rahalkar, Monali, unpublished data). The genome of WWC4 was explored for all different types of genes including the Plant growth promotion (PGP) genes: genes for IAA production, nitrogen fixation, phosphate solubilization. were analyzed. PGP-properties in a bacterium or facilitates the plant nutrient uptake from the surrounding environment [33]. The most essential nutrient for plants is nitrogen for its development [34]. Methanotrophs are present in large numbers near rice rhizospheres [35–37] and nitrogen fixation genes from methanotrophs are detected in the transcribed genes [38] The 2nd most important nutrient for plants after nitrogen is phosphorous, which is in an insoluble form [34]. Therefore, it becomes a major growth-limiting factor in agriculture [34]. Methanotrophs contain phosphate solubilising enzymes which make the soluble phosphates available to the plants for their development. The important PGP genes were also found

in the selected methanotrophic strains, such as glycoside hydrolase, alkaline phosphatase, phytase, and trehalose enzymes, to improve soil fertility and plant development (Supplementary Table 1).

Classical nitrogen fixation pathway genes (NifDKH operon) were detected in the genomes for environmental N_2-fixation. From the denitrification pathway, nitrite reductase *nir*BD genes and nitric oxide reductase *norB* and *norC* genes were present (Supplementary Fig. 3).

Description of " Candidatus **Methylomonas sedimenticola" strain WWC4**

A putative novel methanotroph strain WWC4 was isolated from a mud sample of a shallow freshwater part of a wetland of freshwater creek region, Alibag, near Nagaon beach, Maharashtra, India (18°26'30"N 72°54'20"E). Strain WWC4 showed 97.81% 16S rRNA and 89.46% *pmoA* gene similarity with *Methylomonas koyamae* Fw12E-Y^T. The genomic comparison showed 74.45%, 75.72%, and 21.5 [19.3–23.9%] of AAI, ANIb, and DDH values, respectively, with its closest member *Methylomonas koyamae* Fw12E-Y^T. The nearest strain is *Methylomonas* R45383 (genome accession number LUU01).

The genomic evaluation and phylogenetic analysis concluded the WWC4 strain represents a putative novel methanotroph species. This putative novel species was isolated from a muddy-water sample and was phylogenetically related to the *Methylomonas* genus; therefore, the name is tentatively proposed as a **"**Candidatus**Methylomonas sedimenticola"**sp. nov. strain WWC4. It shows 97.81% AAI and 97.01% ANIb and 77.3% DDH value with the not yet validly described strain: *Methylomonas* R45383, hence based on these closeness, the strain R45383 also could be a second member of the new Candidatus species.

Like almost all methanotrophs, this strain WWC4 can only utilize methane and methanol as carbon sources. The RuMP and serine pathway can be used for methane oxidation, and the presence of genes for the same was found in its genomes. While the nitrogen source, ammonium chloride, glutamate, peptone, yeast extract, and lysine can be used to grow the strain, the environmental nitrogen can be fixed by strain WWC4, which has been checked by laboratory experiments as well as the presence of genes in the genome.

Candidatus **Methylomonas sedimenticola"** strain WWC4 is a mesophilic methanotroph and grows in a 15–30°C temperature range at a 3–10 pH range. The optimum temperature and pH were 25°C and 6.8, respectively. The major fatty acids were 15:1 ω8c (21.09%), 16:0 3OH (15.7%), and 16:1ω5c (12.6%). The whole-genome shotgun project is deposited at DDBJ/ENA/GenBank, and the accession number is JAATWI0.1. The **"**Candidatus**Methylomonas sedimenticola"** strain WWC4 is maintained in our in-house WDCM-approved culture collection, (MACS collection of microorganisms), as a live culture, and designated as MCMB-1474.

Table 1: Draft genome details of strain WWC4

Draft genome traits	**WWC4**
NCBI Accession number	JAATWI0.1
Bio-project number	PRJNA520977
Bio-sample	SAMN10613771
Assembly	ASM1188220v1
Genome size	5.19 Mbp
G+C content	55.9%
N50	161,891 bp
Genes	4,560
Proteins	4,407
tRNA, nc RNA	39, 4
rRNA	2
Contigs	56

Table 2: AAI, ANIb, & DDH calculation of strain WWC4 with its closely related members			
Closely related members	**AAI value**	**ANIb value**	**dDDH value**
Methylomonas strain WWC4	-	-	-
Methylomonas sp. R-45383	97.18%	97.01%	77.3 [74.3 - 80%]
Methylomonas koyamae Fw12E-Y^T	74.45%	75.72%	21.5 [19.3-23.9%]
Methylomonas methanica S1^T	74.37%	74.77%	20.6 [18.4-23.1]
Methylomonas rhizoryzae GJ1^T	69.06%	72.58%	21.3 [19-23.7%]

Table 3: Comparative Table of *Methylomonas* species with strain WWC4. (Included strains: ***Methylomonas koyamae*** Type strain: Fw12E-Y, JCM 16701, NBRC 105905, NCIMB 14606; ***Methylomonas lenta*** Type strain: R-45377, LMG 26260, JCM 19378; ***Methylomonas methanica*** Type strain: ACM 3307, ATCC 35067, IMET 10543, NCIMB 11130, VKM B-2110).

Characteristics	**Strain WWC4**	***Methylomonas methanica***	***Methylomonas koyamae***	***Methylomonas rhizoryzae* GJ1**	***Methylomonas paludis***	***Methylomonas lenta***
Cell Morphology	Rods	Rods	Rods	Rods	Rods	Rods
Cell size	2.5-3 µm X 0.8-1.2 µm	0.5-3 µm X 0.5-1 µm	1.2-2.5 µm X 0.8-1.1 µm	0.6–1.0 µm X 0.7–2.3 µm	1-4 µm X 1-1.5 µm	1.3-2 µm X 0.6-0.9 µm
Motility	Yes	Yes	Yes	Yes	No	Yes
Surface pellicle	Yes	Yes	No	Yes	No	Not detected
Pigmentation	Pink to orange	Pink to orange	Pink to orange	Pink	Pale pink	White to pink sheen
Optimum growth temperature (°C)	25 (range 15-30)	25-30 (range 10-35)	30 (range 10-40)	25-33 (range 16-37)	20-25 (range 8-30)	20-25 (range 15-28)
Optimum pH	6.8 (range 3-10)	7 (range 5.5-9)	6.5 (range 5.5-7)	6-8 (range 5.5-8.5)	5-8-6.4 (range 3.8-7.3)	6.8-7.3 (range 6.3-7.8)
G+C Content	55.9%	51-54%	57%	53.87%	48-49%	47%
Major PLFAs	C15:1 ω8c, 16:0 3OH	C14:0, C16:1 ω8*c*	C14:0, C16:1 ω8*c*	C16:1, C14:0	C16:1 ω5*t*, C16:1 ω8*c*	C16:1 ω8*c*, C16:1 ω5*c*

Table 4: Comparison of fatty acid methyl esters (FAME) profiles in cell walls of various *Methylomonas* species with strain WWC4.

Fatty acids	Strain WWC4	*Methylomonas methanica* *Methylomonas aurantiaca* *Methylomonas fodinarum*	*Methylomonas koyamae*	*Methylomonas rhizoryzae* GJ1	*Methylomonas lenta*	*Methylomonas paludis*
9:0	0.81	-	-	-	-	-
10:0	1.24	-	-	-	-	-
11:0	1.52	-	-	-	-	-
12:0	3.20	-	-	0.53	0.6-2.5	-
12:0 aldehyde	1.91	-	-	-	-	-
13:0	2.12	-	-	0.05	0.6-0.9	-
14:0	9.99	18.9-24.6	23	20.2	6.4-9.8	11.8
15:0	-	0-1.2	1.2	-	5.3-5.8	0.5
15:0 iso	-	0-2.5	-	-	-	-
15:0 anteiso	-	0-2.4	-	-	-	-
14:1 ω5c	4.23	-	-	-	-	-
15:1 ω8c	**21.09**	-	-	-	1.0-2.3	-
15:1 ω6c	3.04	-	-	-	0.5-0.6	-
15:1 ω5c	7.34	-	-	-	1.0-1.1	-
16:0	5.22	4.3-8.7	7.7	6.37	5.0	5.6
16:1ω11c	-	-	-	-	-	-
16:1ω8c	-	18.7-41.3	39.4	-	40.8-42.4	22.1
16:1ω7c	-	7.7-15.3	4.35	-	9.1-10.5	13.9
16:1ω6c	-	4.5-13.3	-	-	-	5.0
16:1ω7c/ω6c (sum)	8.55	-	-	54.42	-	-
16:1ω5c	**12.60**	1.9-6.3	16.7	16.33	11.7-18.3	1.8
16:1ω5t	-	7.9-16.6	-	-	-	34.8
17:0 cyclo	-	0-2.1	-	-	-	-
17:1 ω8c	-	-	-	-	0-0.8	-
17:1 ω7c	-	0-0.7	-	-	-	-
17:1 ω7t	-	0-0.3	-	-	-	-
18:0	-	0-0.1	0.72	-	-	1.2
18:1 ω7c	-	0.2-2.5	-	-	-	-
18:1 ω6c	-	-	-	-	-	-
18:1 ω5c	-	0-0.2	1.7	-	-	-
19:0 cyclo	-	0.2-0.4	-	-	-	-
19:1 branched	-	0-0.5	-	-	-	-

10:0 3 OH	1.44	-	-	-	-	-
15:0 2 OH	-	-	1.43	-	-	-
16:0 3 OH	**15.70**	-	3.8	1.68	4.1-4.2	-

Declarations

Funding:

Monali C. Rahalkar acknowledges the Department of Science and Technology, DST, SERB (EMR/2017/002817) for the funds. Kumal Khatri acknowledges CSIR for junior research fellowship. Jyoti Mohite acknowledges SARTHI for junior research fellowship.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Conceptualization: Monali C. Rahalkar and Kumal Khatri Data Analysis: Kumal Khatri Experimental work: Strain isolation, characterization, maintenance and growth experiments: Kumal Khatri, Jyoti Mohite, Shubha Manvi and Kajal Pardhi Fund acquisition: Monali C. Rahalkar Writing of the manuscript: Kumal Khatri and Monali Rahalkar Writing-review and editing: Monali Rahalkar. Final version reviewed and approved by all the authors.

Data Availability

All the sequence data are available in the NCBI under the following accession numbers: Genome: JAATWI01.1 16S rRNA gene: MH764454.1 *pmoA* gene: MH806338.1. Culture deposited in the same institute's culture collection (MACS collection of microorganisms, Pune) as MCMB-1474 and available with the corresponding author.

There was no involvement of humans and/or animals in this study. And hence the following declarations are not applicable.

Ethics approval: Not applicable

Consent to participate: Not applicable

Consent to publish: Not applicable

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Figures

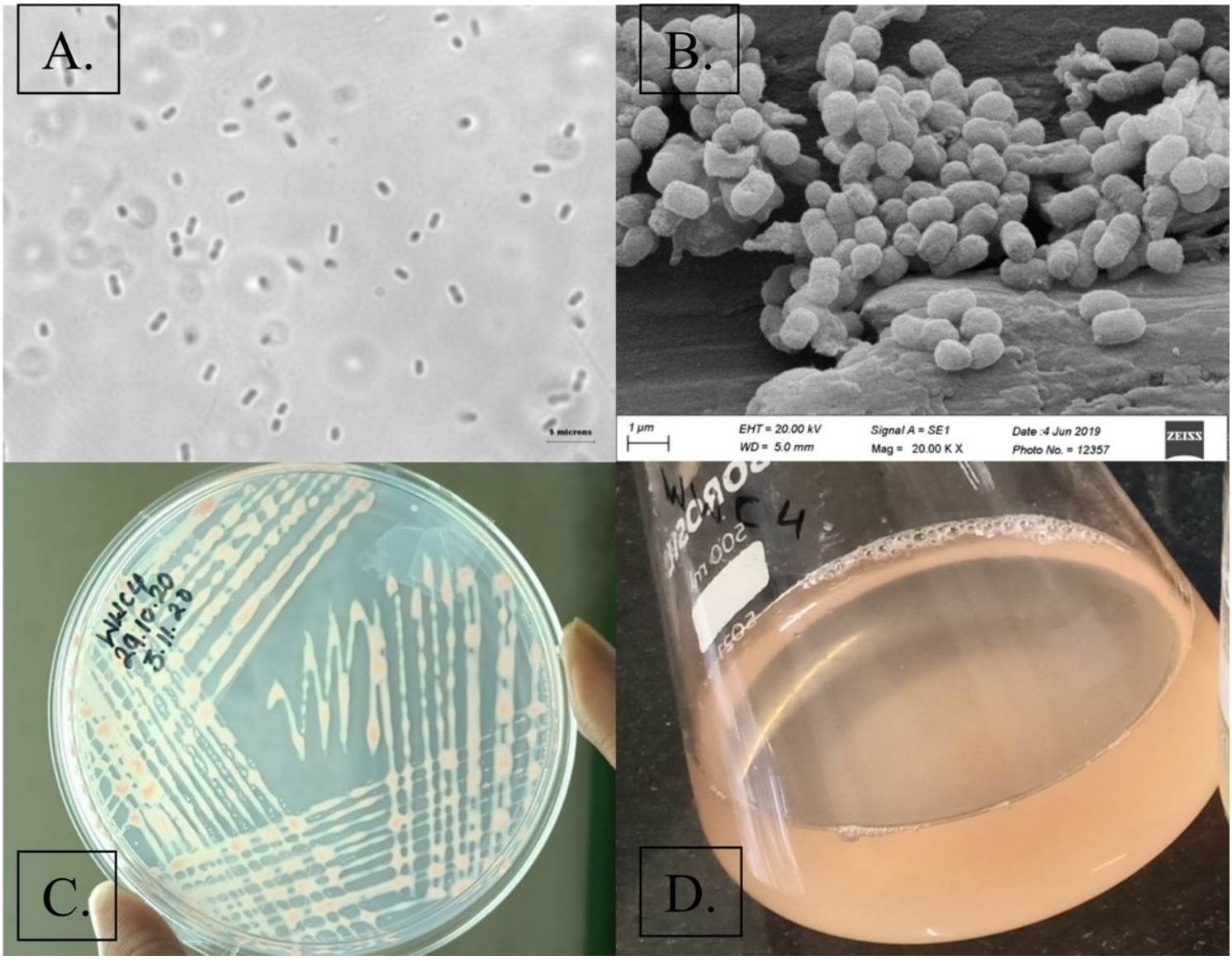

Figure 1

Morphological character of strain WWC4: A. Live cells were observed under a phase-contrast microscope (Nikon 80i, Japan microscope with a camera) under 100X magnifications with oil emulsion; B. fixed and processed culture were observed under a Scanning Electron Microscopy (SEM) (Zeiss model EVO-MA-15 SEM); C. colony morphology; D. Growth in liquid medium with methane and air in headspace. The culture shows light pink color.

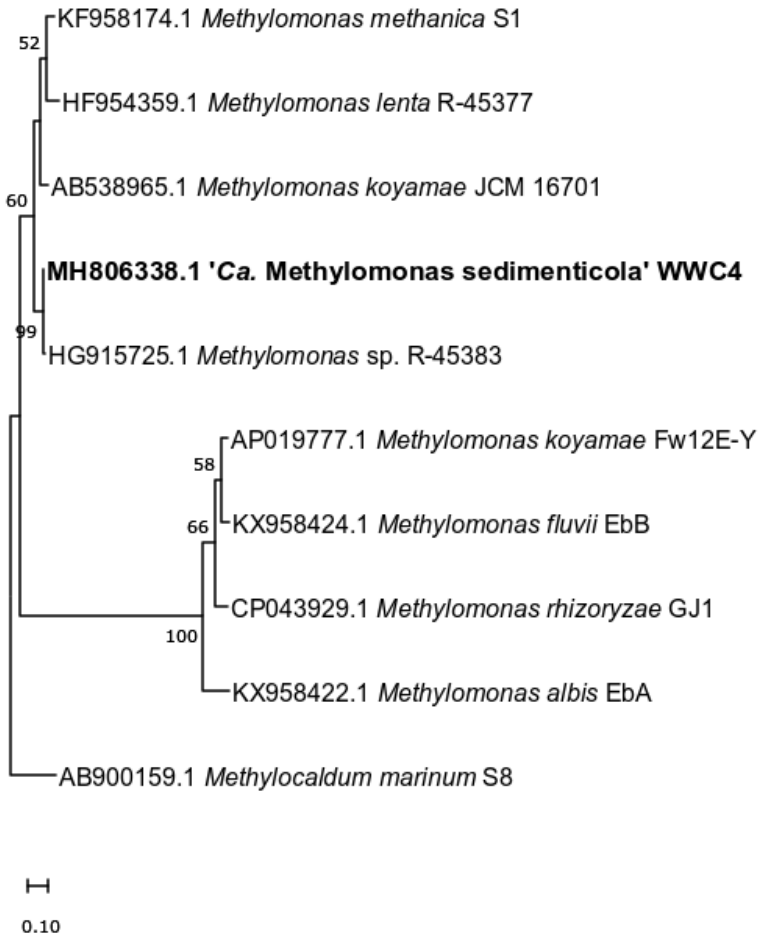

Figure 2

Functional *pmoA* gene-based phylogenetic tree of strain WWC4 with its closest members. The phylogenetic tree was constructed using the *pmoA*sequence of WWC4 in comparison with the *pmoA* gene using MEGA X software [39]. It was inferred by the Maximum Likelihood method and Tamura-Nei model [40]. The bar shows a 1% divergence.

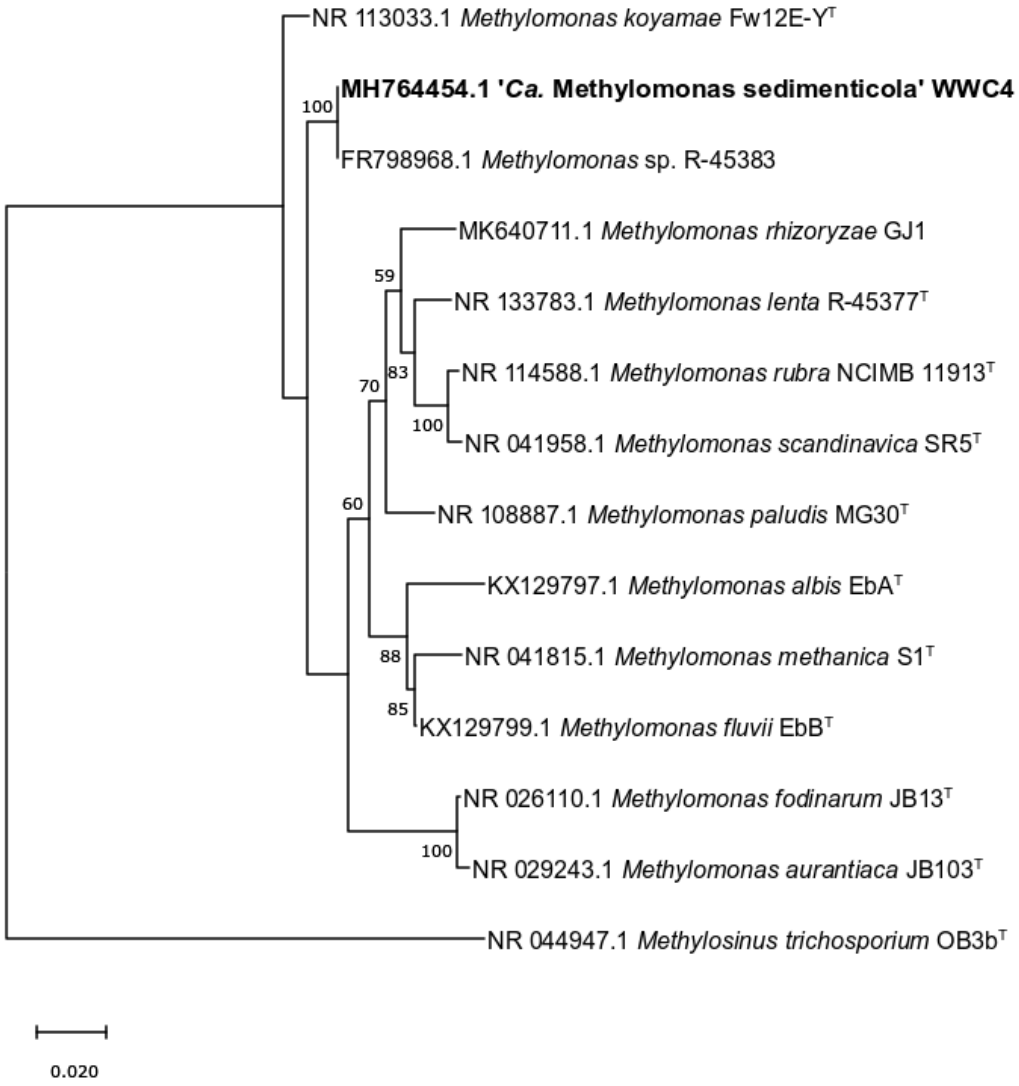

Figure 3

16S rRNA gene phylogenetic tree of strain WWC4 with its closest members. The phylogenetic tree was constructed using the 16S rRNA sequence of WWC4 in comparison with 16S rRNA of type strains of valid species using MEGA X software [39]. It was inferred by the Maximum Likelihood method and Tamura-Nei model [40]. The bar shows a 2% divergence.

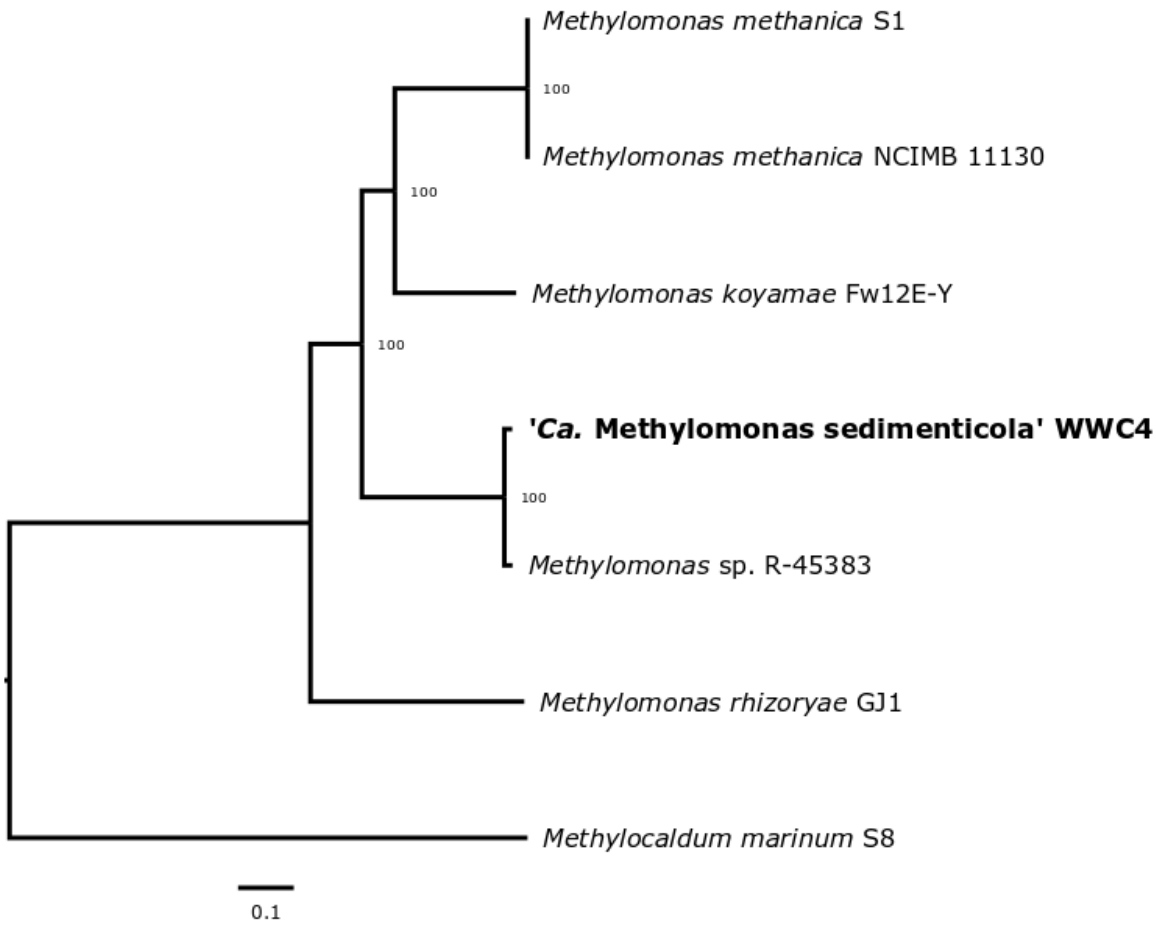

Figure 4

Phylogenomic tree of strain WWC4 with the genome of neighbour members. The tree was constructed using the PATRIC webserver (https://www.bv-brc.org/).

Supplementary Files

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