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ORIGINAL ARTICLE

Phylogenetic Study of Methanol Oxidizers from Chilika-Lake Sediments Using Genomic and Metagenomic Approaches

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Abstract Group-wise diversity of sediment methylotrophs of Chilika lake (Lat. 19°28′–19°54′N; Long. 85°06′–85°35′E) Odisha, India at various identified sites was studied. Both the culturable and unculturable (metagenome) methylotrophs were investigated in the lake sediments employing both *mxaF* and 16S rRNA genes as markers. ARDRA profiling, 16S rRNA gene sequencing, PAGE profiling of *Hae*III, *Eco*RI restricted *mxaF* gene and the *mxaF* gene sequences using culture-dependent approach revealed the relatedness of α-proteobacteria and *Methylobacterium*, *Hyphomicrobium* and *Ancyclobacter* sp. The total viable counts of the culturable aerobic methylotrophs were relatively higher in sediments near the sea mouth (S3; Panaspada), also demonstrated relatively high salinity (0.1 M NaCl) tolerance. Metagenomic DNA from the sediments, amplified using GC clamp *mxaF* primers and resolved through DGGE, revealed the diversity within the unculturable methylotrophic bacterium *Methylobacterium organophilum*, *Ancyclobacter aquaticus*, *Burkholderiales* and *Hyphomicrobium* sp. Culture-independent analyses revealed that up to 90 % of the methylotrophs were unculturable. The study enhances the general understandings of the metagenomic methylotrophs from such a special ecological niche.

Keywords ARDRA · Chilika · DGGE · Methylotroph · *mxaF* · Molecular markers · PAGE · RFLP

Introduction

Chilika (Lat. 19°28′–19°54′N; Long. 85°06′–85°35′E), a biodiversity hotspot of the country, is a simple cone-shaped, largest brackish water lake in Asia and second largest in the world, situated in humid tropical climatic coastal zone of Odisha (former Orissa), India, declared as a Ramsar site (listed as a wetland for intensive conservation and management by the ministry of Environment and Forests, Government of India) under the convention on 'Wetlands of international importance' [1]. Chilika, slowly but steadily transforming into a lagoon, is an attractive and unparalleled fusion of marine, river, and estuarine habitat that supports unique assemblage of marine, brackish water and freshwater microbes [2], presents a challenge to physiologists and biochemists [3]. Biological effects of siltation and salinity, overall biodiversity loss due to the changes in phytoplankton communities, and degradation of lake ecosystem owing to the freshwater invasive species thereby declining the overall productivity, have been profoundly documented in recent times [2, 3, 5, 7, 8].

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Bacterial assimilation of methane, methanol and methylamine is important in the detritus food chain. Methylotrophs are globally distributed and phylogenetically dispersed. These have been isolated from various environments, including soils, sediments, freshwater, marine sediments, seawater, acid peat bogs, hot springs and cold environments such as the Antarctic, and have attracted a great deal of interest due to their considerable commercial potential to produce bulk/fine chemicals and in bioremediation. Due to their specific properties, for instance, haloalkaliphilic methanotrophs could be of use in modern biotechnology [4]. Methylotrophs have been reported from diverse aquatic niches, such as, Washington Lake, USA [5], Mono Lake, USA [6], Lonar Lake, India [7], Weyerhaeuser [8], Warm pool, China [9], Colne Estuary, UK [10], Brackish marsh, Portugal [11], and as a marine symbiont [12]. There is increasing evidence for the striving methanotrophs in various ecogeographical regions including the saline and alkaline aquatic environments. Although the vast biodiversity of the brackish water system Chilika has been appreciated unquestionably [1–3], reports have been feeble.

These are being extensively studied in a range of environments due to their critical role in global methane cycle as well. PCR-based methods facilitate the methanotrophs ecology and diversity studies, viz., 16S ribosomal RNA technology and specific amplification of 'functional genes', such as those encoding unique enzymes in the organismal metabolism including methane monooxygenase and methanol dehydrogenase. Methanol-oxidizing bacteria play significant role in biogeochemical carbon cycling by facilitating incorporation of C_1 derivatives into biomass [13, 14] using methanol as the sole carbon and energy source. Cyto- and bio-chemical properties, such as, synthesis of osmoprotectants, accumulation of potassium ions, formation of glycoprotein S-layers on the outer cell wall surface, and modification of the chemical composition of their membranes, allow these specialized group (haloalkaliphilic methanotrophs) to adapt to saline and alkaline habitats [4, 15].

Global cycling of C_1 compounds affect important environmental phenomena related to climate change. Methylotrophs thriving on C-substrates like methane, methylated sulfur sp., methylated amines, halogenated methanes and methanol might play a crucial role in global warming and groundwater contamination, the two major environmental concerns. Alkalitolerant halophilic and type I alkaliphilic halotolerant methanotrophs utilize methane and methanol, to oxidize ammonium ions, and to transform various organic compounds even at 12 ‰ salinity and 5–11 pH [15]. Recent investigations reported methanol utilizing methylotrophs from salty water-bodies having impact on global warming and bioremediation of pollution by methanol and other C_1 compounds [15–18]. Microscopic and enzymatic evidence of the reducing sediments at hypersaline seeps in the abyssal Gulf of Mexico supported the hypothesis that methylotrophs capable of using reduced C_1 compounds as their carbon and energy sources occur as intracellular symbionts of the seep mussel and other benthic invertebrates resembling hydrothermal vent community assemblages [12]. These symbioses differed from those reported for bivalves from hydrothermal vents and reducing sediments (23 to −34 ‰ δC^{13}). The microbiology, the bioprocess of CH_4, N_2O, and CO_2 abatement, potential and limitations of the GHG biodegradation processes, technology niches and the knowledge gaps have been reviewed [19]. It is essential to mention that, methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2) emissions represent approximately 98 % of the global greenhouse gas (GHG) inventory.

The ecophysiological importance of methanotrophs in microbial communities inhabiting haloalkaline aquatic environments is due to their involvement in the global cycles of methane and major bioelements (such as, C, N, and S). The key microbial enzyme, methane monooxygenase (MMO; especially the soluble one), is remarkably substrate nonspecific. This unique capability, i.e., catalyzing reactions of environmental importance, has attracted great attention for applied microbiologists and bioengineers. Prudent biological technologies can become low-cost ecofriendly alternative to physicochemical methods for GHGs abatement. Molecular phylogenetic studies of microbial diversity based on the conserved functional gene sequences have greatly expanded our knowledge [9, 20–23]. The *mxaF* gene is supposed to be a phylogenetic chronometer for methylotrophs. This is also a conserved functional gene like 16S rRNA, primarily responsible for methylotrohy. With the functional methylotroph group as the target, this gene was used to map the diversity of the unculturable methanol oxidizers in Chilika Lake, which is slowly but steadily transforming into a lagoon. The *mxa* genes are well conserved among α-, β- and γ-proteobacteria classes as expressed gene sequences, suggesting a methylotrophic origin for methanol oxidation machinery encoded by these genes. The probes targeting the functional *mxaF* gene coding for MDH and 16S rRNA has been widely used to detect and analyze the methylotroph diversity within proteobacteria [24, 25]. Being a conserved gene, *mxaF* served dubiously as a genetic marker to detect methylotrophy in the environment [26, 27].

This study of summer 2010 isolated and characterized the aerobic methanol-utilizers from Chilika lake sediments, and analyzed their diversity using polyphasic approaches (ARDRA profiling, 16S rRNA sequencing, mxaF-RFLP, *mxaF* gene sequencing and real time quantification). Due to geographical vastness, the methylotrophs diversity of entire lake needs bigger comprehensive approach. None-the-less, the present study is a first such attempt.

Materials and Methods

Sampling Site

Samples were collected randomly by grab sampling method from the sediment surface, up to 10–20 cm deep, from diverse sites of Chilika lake (Lat. 19°28′–19°54′N; Long. 85°06′–85°35′E) from Balugaon, Bhusandapur, Panaspada, Nalaban island, and Breakfast island (Fig. 1). There was variation in the length of water column due to variation in the sea flow attached to the lake. At each of these identified sampling sites, one composite sample was prepared by pooling five sediment samples covering an area of 5 km^2, each sampling point being located at least 10 km distant from each other. In all, five composite samples S1 (Balugaon), S2 (Bhusandapur), S3 (Panaspada), S4 (Nalaban island), and S5 (Breakfast island) were made. Samples were transported to the lab on ice and stored at 4 °C until further analyses. A part of the samples was immediately stored at −80 °C for culture-independent studies. Samples were analyzed for various parameters, such as, pH, salinity, organic carbon, and aerobic heterotrophic (total) microbial counts following standard procedures [28]. The average mean values of these physiochemical and biological attributes are detailed sampling site-wise in Table 1.

Enrichment and Isolation

Nitrate mineral salt (NMS) medium was used for methylotrophs enrichment, a liter of which contained NaCl 5.0 g, Na_2HPO_4 0.72 g, KH_2PO_4 0.28 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, $CaCl_2{\cdot}2H_2O$ 0.02 g, $FeSO_4{\cdot}7H_2O$ 5.0 mg, $ZnSO_4{\cdot}7H_2O$ 70 μg, $MnCl_2{\cdot}4H_2O$ 30 μg, H_3BO_3 300 μg, $CoCl_2$ 200 μg, $NiCl_2{\cdot}6H_2O$ 20 μg, $Na_2MoO_4{\cdot}2H_2O$ 30 μg, $CuCl_2{\cdot}2H_2O$ 10 μg, Na_4-EDTA 1.0 mg, KNO_3 1.0 g, methanol 0.1 % v/v [29]. Antifungal cycloheximide (40 mg/ml) was added at the rate of 500 μl per liter, and pH was adjusted by inorganic (sodium; $NaHCO_3$–Na_2CO_3) buffer as per pH of the sample at sampling. The prepared sediment samples (10.0 g in 100 ml double distilled water) were placed in 500 ml Erlenmeyer flasks with 100 ml methanol-fortified (as above) NMS medium and shaker incubated (120 rpm; 30 °C) overnight. The samples were enriched following three transfers in the medium over 4–6 days, serially diluted, and plated on fortified-NMS agar for up to 24 h. After incubation colonies were picked and transferred on to the slants, flooded with glycerol and maintained as stocks.

Genomic DNA Extraction and PCR Amplification of 16S rRNA and *mxaF* Genes

Genomic DNA was isolated from the log phase broth cultures. Pelleted cells from 1.5 ml media were resuspended in

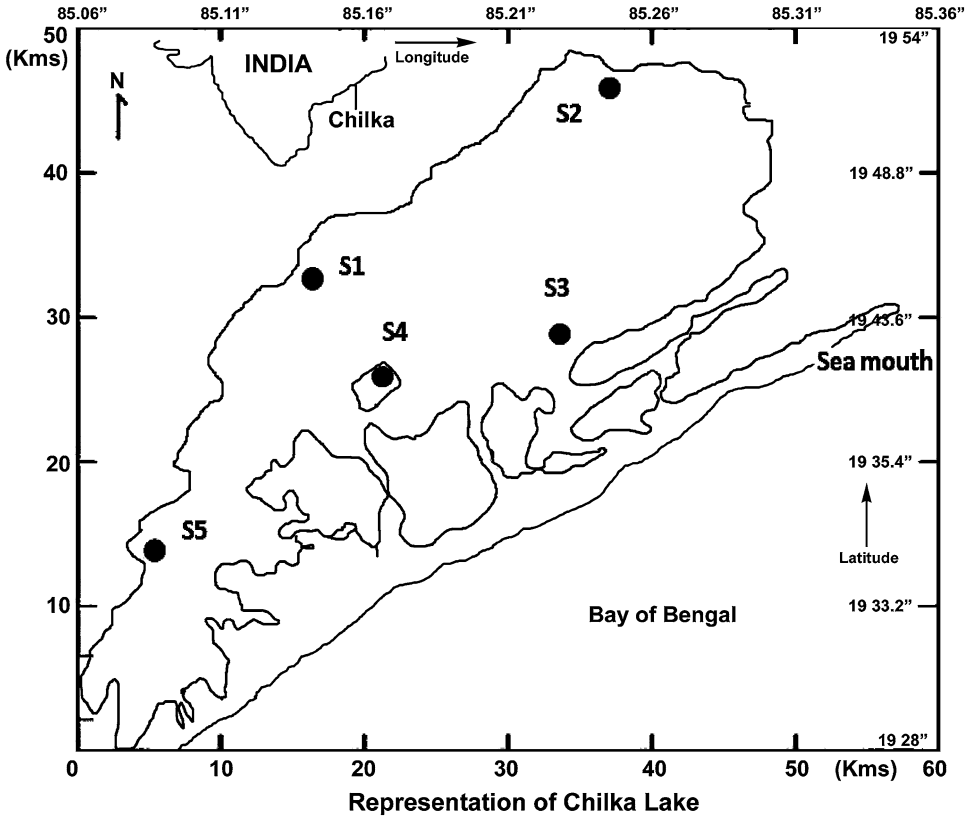


Fig. 1 Map of Chilika Lake showing sampling locations in black dots (*S1* Balugaon, *S2* Bhusandapur, *S3* Panaspada, *S4* Nalaban island, *S5* Breakfast Island)

Table 1 Mean average values of selected parameters at various sampling locations

Sample point	Location	Salinity, NaCl (mM)	pH	% Organic carbon	Log CFUs	*mxaF* gene copies (per gram sediment)
S1	Balugaon	80.0 ± 1.5	8.1 ± 0.2	0.8 ± 0.03	5.477 ± 0.35	4.9×10^6
S2	Bhusandapur	90.0 ± 1.6	7.8 ± 0.2	0.5 ± 0.02	5.792 ± 0.45	1.60×10^6
S3	Panaspada	100.0 ± 1.7	8.2 ± 0.3	1.5 ± 0.05	6.484 ± 0.51	1.25×10^7
S4	Nalaban	85.0 ± 1.1	8.1 ± 0.1	1.1 ± 0.03	5.991 ± 0.50	1.21×10^6
S5	Breakfast island	85.0 ± 0.9	8.8 ± 0.4	1.1 ± 0.04	6.305 ± 0.53	1.20×10^6

0.5 ml SET buffer (75 mM NaCl, 25 mM EDTA and 20 mM Tris) with 10 µl of lysozyme (10 mg/ml) and the genomic DNA was extracted [30]. The integrity and concentration of purified DNA was determined by ethidium bromide stained agarose gel (0.8 %) electrophoresis by comparing with the commercial DNA ladder concentration (Hyper ladder genetix). DNA from raw sediment samples were extracted using UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad). For PCR amplification, final genomic DNA concentration was adjusted to 50 ng/µl. 16S rRNA gene was partially amplified using primers pA (5′-AGAGTTTGATCCTGGCTCAG3′; *E. coli* position 8–27) and pH (5′-AAGGAGGTGATCCAGCCGCA3′; *E. coli* position 1525–1544) [31]. Amplification was carried out on a thermal cycler (Biorad PTC0220) in 100 µl volume by mixing 50–90 ng template DNA with polymerase reaction buffer (10×); 100 µM (each) dATP, dCTP, dTTP and dGTP; primers pA and pH (20 ng each) and 1.0 U *Taq* polymerase using following conditions: initial denaturation at 94 °C for 1.5 min; 35 cycles at 95 °C for 1.0 min, 55 °C for 1.0 min, 72 °C for 1.0 min; and final extension at 72 °C for 5 min.

The *mxaF* gene was used to identify/authenticate methanol-oxidizing population downstream [6]. The *mxaF* gene in the isolates was partially amplified using specific primers, mxaF-1003 (5′GCGGCACCAACTGGGGCTGGT3′; forward) and mxaR-1561 (5′GGGCAGCATGAAAGGGCTC CC3′; reverse) [24]. For environmental DNA, GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) attached at 5′ end with one of the primer was used to increase the bands separation in DGGE gel [32]. The PCR condition was similar to 16S rDNA amplification. PCR products were separated on ethidium bromide stained 1.5 % agarose gel, and documented in Alpha Imager TM1200 (Alpha InfoTech).

ARDRA and RFLP

Products were digested with selected restriction enzymes with different restriction sites after 16S amplifying rDNA. About 1 µg of PCR-amplified 16S rDNA fragment was restricted with three different endonucleases *Hae*III, *Msp*I and *Eco*RI (Fermentas) separately, incubated at 37 °C overnight and resolved on 2 % agarose gel. The *mxaF* gene PCR products were digested with *Hae*III and *Eco*RI and separated through PAGE (PolyAcrylamide Gel Electrophoresis). Banding patterns were visualized by ethidium bromide staining and analyzed in Alpha Imager TM1200 (Alpha InfoTech). Thus, amplified rDNA restriction analysis and restricted fragment length polymorphism were performed. Different phylotypes or operational taxonomic units were obtained by similarity and clustering analysis using NTSYSpc-2.02e. Similarities among isolates were calculated by Jaccard's coefficient [33], and dendrogram constructed using UPGMA method [34]. Since ARDRA is restriction digestion for (16S) ribosomal gene while RFLP is restriction digestion of mxaF gene, the aim and importance of ARDRA and RFLP in the investigation was to group the isolates based on structural and functional genes.

DGGE (Denaturing Gradient Gel Electrophoresis) Profiling

The *mxaF* PCR products were purified using SV-PCR purification kit (Promega). PCR products were separated on a 1.0 mm thick, vertical polyacrylamide gel (6.5 % w/v acrylamide:bisacrylamide::37.5:1, Bio-Rad) prepared with and electrophoresed in 1.0 × TAE, pH 7.4 (0.04 M Tris-base, 0.02 M sodium acetate, 1 mM EDTA) at 60 °C and a constant voltage of 150 V for 16 h [35]. A denaturing gradient of 100 % denaturant corresponded to 7 M urea plus 40 % v/v formamide. Gels were loaded with 30 µl of PCR product depending on the band intensity and electrophoresed on 1.5 % agarose gels. DGGE gels were stained for 20 min in water containing 0.5 µg/ml ethidium bromide, and the images were recorded in an Alpha Imager TM1200 (Alpha InfoTech) documentation analysis system. DNA bands migrating to the same position on the gel were assumed to be identical amplicons [32].

16S rRNA and *mxaF* Gene Sequencing

PCR amplified 16S rDNA products were purified with a Quaquick purification kit (Qiagen). DNA sequences were

double checked by sequencing both strands using primers pA and pH for forward and reverse reactions, respectively. Nucleotide sequences were dideoxy-cycle sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in a 3130×1 ABI prism automated DNA sequencer (Applied Biosystems). DGGE bands were excised from the gel using a sterile scalpel and incubated in 60 µl sterile Milli-Q purified water at 4 °C for 24 h. DNA was diffused out of the gel and the solution used as the template in a reamplification PCR, performed using the original primers but modified PCR regime (Table 2) and run on DGGE to confirm its identity. Only pure bands were used for sequencing by amplifying with primers without a GC clamp. PCR products for sequencing were purified and sequenced using ABI prism sequencer. Representatives of the dendrogram constructed from *mxaF*-RFLP PAGE patterns were also sequenced with fluorescent terminators (Big dye, Applied Biosystems) and run in the same DNA sequencer.

The sequences of 24 representative isolates are submitted to GenBank with accession numbers GQ281064-GQ281070, GQ281072-GQ281076, GQ354269-GQ354270, GQ411497-GQ411503, GQ411505, GQ227415 and GQ332407. Partial *mxaF* gene fragments sequences (HM765479–HM765503) and the excised DGGE bands sequences (HM628891–HM628901) have also been submitted to the GenBank.

Quantification Through RT PCR

The *mxaF* gene quantification was done in copies based on 'second derivative maximum method' (using LightCycler software 3.5; Roche diagnostics), wherein exponential phase of amplification curve was linearly related to a starting concentration of template DNA molecules. Each QC-PCR standard curve was generated by using six dilutions of standard DNA template from 10^2 to 10^6 copies of mxaF gene, and the standard DNA template was tenfold serial-diluted. Quantitative PCR using SYBR Green I technology [36] with the primers *mxaF* and *mxaR* was carried out amplifying five metagenome samples, negative control and five plasmid DNA standards. Mastermix [14 µl sterile water, 2 µl $MgCl_2$ (25 mM), 1 µl of each primer (20 pmol), 2 µl of SYBR Green master mix (20 pmol; Roche diagnostics) and 50 ng DNA] was prepared. Amplification started with a 10 min denaturation at 95 °C followed by a 40-cycles 4-segment amplification (denaturation for 15 s at 95 °C, annealing for 10 s at 55 °C, elongation for 20 s at 72 °C and appended for 5 s at 83 °C for possible primer-dimers through a single fluorescence measurement). The last step ensured the elimination of nonspecific fluorescence signal and ensured accurate quantification of the desired product. Finally, a systematic melting step (10 s at 95 °C, 10 s at 60 °C and slow heating at a rate of 0.1 °C per second up to 99 °C) with continuous fluorescence measurement was performed.

BLAST Search and Phylogeny Analysis

The partial 16S rDNA sequences, *mxaF* gene sequences of isolated strains and *mxaF* gene sequences from environmental DNA were compared with those available in the databases. Identification was based on sequence similarity of ≥97 % with that of public database sequences, by BLAST homology. The sequence alignment and comparison were performed using multiple sequence alignment program ClustalW2 [37] with default parameters, and data converted to PHYLIP format. Minor modifications were done manually on the basis of conserved domains, and columns containing more than 50 % gaps were removed. The phylogenetic trees were constructed on the aligned datasets using MEGA 4.0.2 [38] using the neighbor-joining method [39]. Bootstrap analysis [40] was performed on 1,000 random samples taken from the multiple alignments.

Results and Discussion

Methylotrophs are distributed in diverse environments from freshwater Lake [5], deep-sea sediments [9], hypersaline lake [24], chlorinated environments [41], plant

Table 2 Prevailing PCR and DGGE conditions for the various primers used

Primer[a]	Target gene	PCR		DGGE		
		Temperature profiles	Cycles	Gradient (%)	Time (h)	Voltage (V)
mxaFf1001/r1561 (with GC clamp)	α subunit of MDH	94 °C,1 min; 54 °C, 30 S; 72 °C,1 min	36	30–70	5	150
mxaFf1001/r1561 (without GC clamp)	α subunit of MDH	94 °C,1 min; 55 °C, 30 S; 72 °C, 1 min	30	–	–	–

[a] *mxaF*f1001: GCGGCACCAACTGGGCTGGT; *mxaF*r1561: GGGCAGCATGAAGGGCTCCC; GC clamp used: CGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCACGGGGGG

phyllosphere [42] to hot water effluent [43], suggesting their ubiquity. Present study focused on the aerobic methylotrophs covering 1,100 km^2 of the Chilika Lake sediments through established molecular markers (such as the functional gene *mxaF* and phylogenetic 16S rRNA gene probes) which demonstrated the presence of phylogenetically diverse aerobic methylotrophs. The total viable counts were relatively higher in sediments near the sea mouth (S3; Panaspada) as also viewed by Joshi et al. [44].

Enumeration of Methanol Oxidizing Bacteria

The bacterial counts were from 5.4 to 6.4 log CFUs/g sediment. A total of 80 isolates from the samples were selected for further study. Maximum methylotroph population was observed at relatively nearer to the sea mouth (at S3; Panaspada), followed by S5 (Breakfast island) down south, and minimum was in S1 (Balugaon), far away from sea mouth. All isolates were aerobic, catalase, urease positive and weakly oxidase positive. The halotolerance ability of the representative isolates was studied. *Methylobacterium* aerobically produces carotenoid pigment and bacteriochlorophyll A indicating that their ability to acquire ATP helps them to survive even in carbon deprivation [41]. Two *Methylobacterium* sp. isolated from the sediment samples from S3 (Panaspada) endured up to 1.0 M NaCl concentration (Table 3). The mixing of the sea water in the lake might be a reason for the relatively high halotolerance. Surface sediment was aerobic, whereas the oxygen typically depleted within millimeters below possibly due to the oxygen diffusional limitation across the column coupled with the active aerobic respirers [36].

Isolates growing on methanol, but not responding to the PCR amplification with *mxaF*-primers, may have alternate mechanism for primary methanol metabolism [26], or might be growing autotrophically. The lack of *mxaF* gene amplification in isolates CS3, CS8 and CS15 point to such alternate methylotrophy. Negative results suggest the role of some other alcohol dehydrogenase in methylotrophic metabolism.

The *mxaF* Gene Amplification

Results of the amplified gene *mxaF* coding for α-subunit of methanol dehydrogenase indicated that the C_1 compounds in the lake sediments may be derived from the organic sedimentation and degradation. *mxaF* gene quantification gives the real scenario of methylotroph biocoenose [9]. Most

Table 3 Characteristics of representative isolates based on the 16S rDNA phylogenetic resemblances

Isolate	Colony color	Morphology	Gram reaction	Salt tolerance (M)	Division	Phylogenetic similarities (%)
CS4	Pink	Rods	−ve	0–0.5	α-Proteobacteria	*M. radiotolerans* (99)
CS10	Pink	Rods	−ve	0–0.6	α-Proteobacteria	*M. radiotolerans* (99 %)
CS18	Pink	Rods	−ve	0–0.8	α-Proteobacteria	*M. radiotolerans* (99 %)
CS24	White	Rods	−ve	0–0.7	α-Proteobacteria	*Hyphomicrobium facile* (99 %)
CS28	Pink	Rods	−ve	0–0.9	α-Proteobacteria	*M. organophilum* (98 %)
CS34	Cream	Rods	−ve	0–0.6	α-Proteobacteria	*H. facile* (99 %)
CS38	Pink	Rods	−ve	0–0.5	α-Proteobacteria	*M. extorquens* (99 %)
CS41	Pink	Rods	−ve	0–1.0	α-Proteobacteria	*Methylobacterium* sp. (100 %)
CS44	Pink	Rods	−ve	0–0.6	α-Proteobacteria	*M. organophilum* (98 %)
CS49	Pink	Rods	−ve	0–0.9	α-Proteobacteria	*M. extorquens* (99 %)
CS62	Pink	Rods	−ve	0–0.90	α-Proteobacteria	*Methylobacterium hispanicum* (98 %)
CS65	Pink	Rods	−ve	0–1.0	α-Proteobacteria	*Methylobacterium* sp. (97 %)
CS67	White	Rods	−ve	0–0.8	α-Proteobacteria	*Methylobacterium* sp. (93 %)
CS70	Pink	Rods	−ve	0–0.9	α-Proteobacteria	*M. radiotolerans* (99 %)
CS72	Pink	Rods	−ve	0–0.5	α-Proteobacteria	*Methylobacterium* sp. (99 %)
CS74	Cream	Rods	−ve	0–0.7	α-Proteobacteria	*H. facile* (99 %)
CS75	Pink	Rods	−ve	0–0.3	α-Proteobacteria	*M. lusitanum* (99 %)
CS78	Pink	Rods	−ve	0–0.9	α-Proteobacteria	*M. zatmanii* (99 %)
CS80	Cream	Rods	−ve	0–0.4	α-Proteobacteria	*H. facile* (99 %)
CS12	Cream	Rods	−ve	0–0.4	β-Proteobacteria	*Methyloversatilis* sp. (85 %)
CS45	White	Rods	−ve	0–0.4	β-Proteobacteria	*Methylophilus methylotrophus* (99 %)
CS54	White	Rods	−ve	0–0.7	β-Proteobacteria	*Methyloversatilis universalis* (98 %)
CS20	Pink	Rods	−ve	0–0.6	γ-Proteobacteria	*Pseudomonas* sp. (99 %)
CS32	Cream	Rods	+ve	0–0.7	γ-Proteobacteria	*Mycobacterium brisbanense* (99 %)

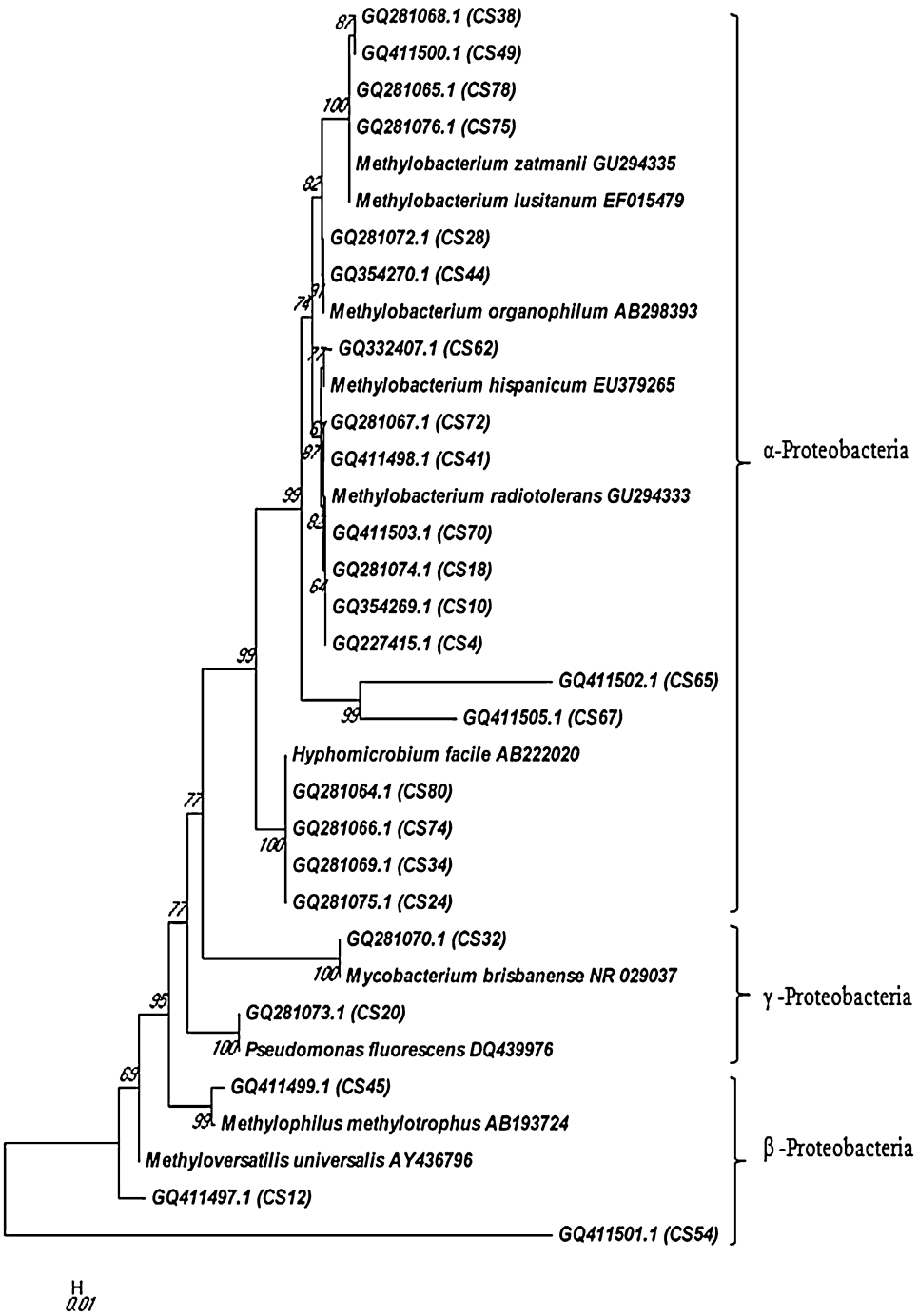


Fig. 2 Phylogenetic tree based on the 16S rRNA gene sequences of methylotrophs using neighbor-joining method. Data of all genera obtained are from GenBank database—sequence accession numbers are in parentheses, the *numbers* on the tree indicate the percentages of boot-strap sampling derived from 1,000 replicates, and the *bar* infers nucleotide substitutions

isolates gave a fine 550 bp partial gene amplification, which authenticated that all of them were methylotrophically active methanol oxidizers. However, a few (viz., CS3, CS15, CS8; Table 2) did not exhibit such amplification but still could grow on agar plates even after several streaks. The genus *Methylobacterium*, a pink pigmented facultative methylotroph follows Serine pathway to metabolize formaldehyde [41], also reported from sea water [45], was dominant in the explored diverse methylotroph communities.

Amplified Ribosomal DNA Restriction Analysis and RFLP

Restriction digestion of 16S rDNA gene (using *Hae*III, *Msp*I and *Eco*RI) and restriction digestion of *mxaF* gene (using *Hae*III and *Eco*RI) yielded distinct restriction patterns, with about two to five restricted fragments of varying sizes in each pattern. Cluster analysis of combined 16S rDNA restriction patterns based on the Jaccard's similarity index

grouped all 80 isolates in different groups (data not given). The majority of the isolates formed two (I—52.5 %; II—37.5 %) major clusters, whereas the remaining ones formed small clusters. A similar cluster analysis of the *mxaF* gene combined restriction patterns (data not provided) grouped the 77 isolates in two major groups, of which three isolates did not exhibit *mxaF* gene amplification.

16S rRNA Gene Sequence Analysis

All 24 pigmented and nonpigmented representative isolates with similar ARDRA pattern generated by three restriction enzymes were selected for sequence analysis, to study the species-level diversity of methylotrophs (Fig. 2). Based on 16S rDNA sequencing, the isolates identified were *Methylobacterium radiotolerans*, *M. extorquens*, *M. hispanicum*, *M. organophilum*, *M. lusitanum*, *M. zatmanii*, *Hyphomicrobium facile*, *Methyloversatilis* sp., *Mycobacterium brisbanense* and *Pseudomonas* sp. The prevalent genera were α-proteobacteria, *Methylobacterium* and *Hyphomicrobium*. CS11 showed distinctly high sequence similarity (100 %) with *Methylobacterium* sp. CS3, reportedly a novel strain from a brackish water environment, showed a lower similarity (85 %) with *Methyloversatilis universalis* (Table 3). Some reported gene sequences here matched with the gene sequences of active methylotrophs from Lake Washington [5] and from the deep sea [9].

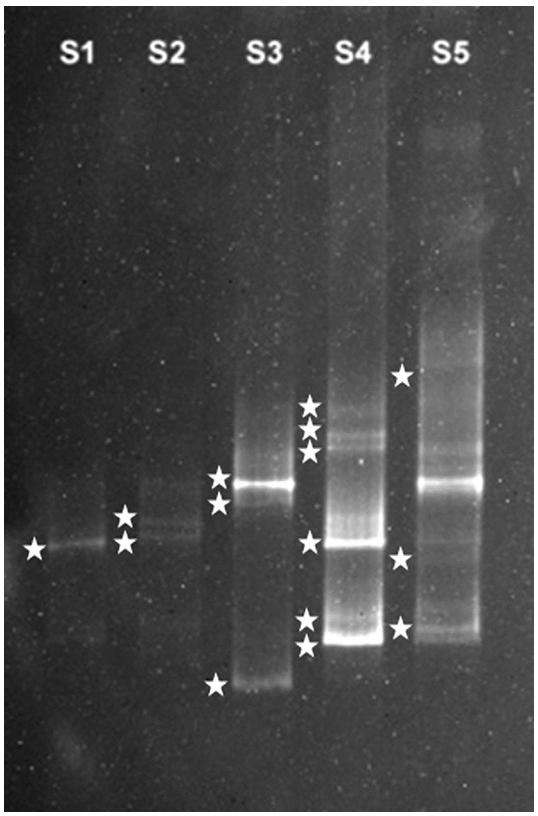


Fig. 3 DGGE pattern of the *mxaF* gene from different sites of the lake. *Star marks* represent the number of bands eluted

Fig. 4 Phylogenetic tree based on the *mxaF* gene sequences of culturable and unculturable methylotrophs using neighbor-joining method. Data of all genera obtained are from Genbank database. The *numbers* on the tree indicate the percentages of boot-strap sampling derived from 1,000 replicates, and the *bar* infers nucleotide substitutions ▶

mxaF Gene Sequence Analysis

From Culturable Ones

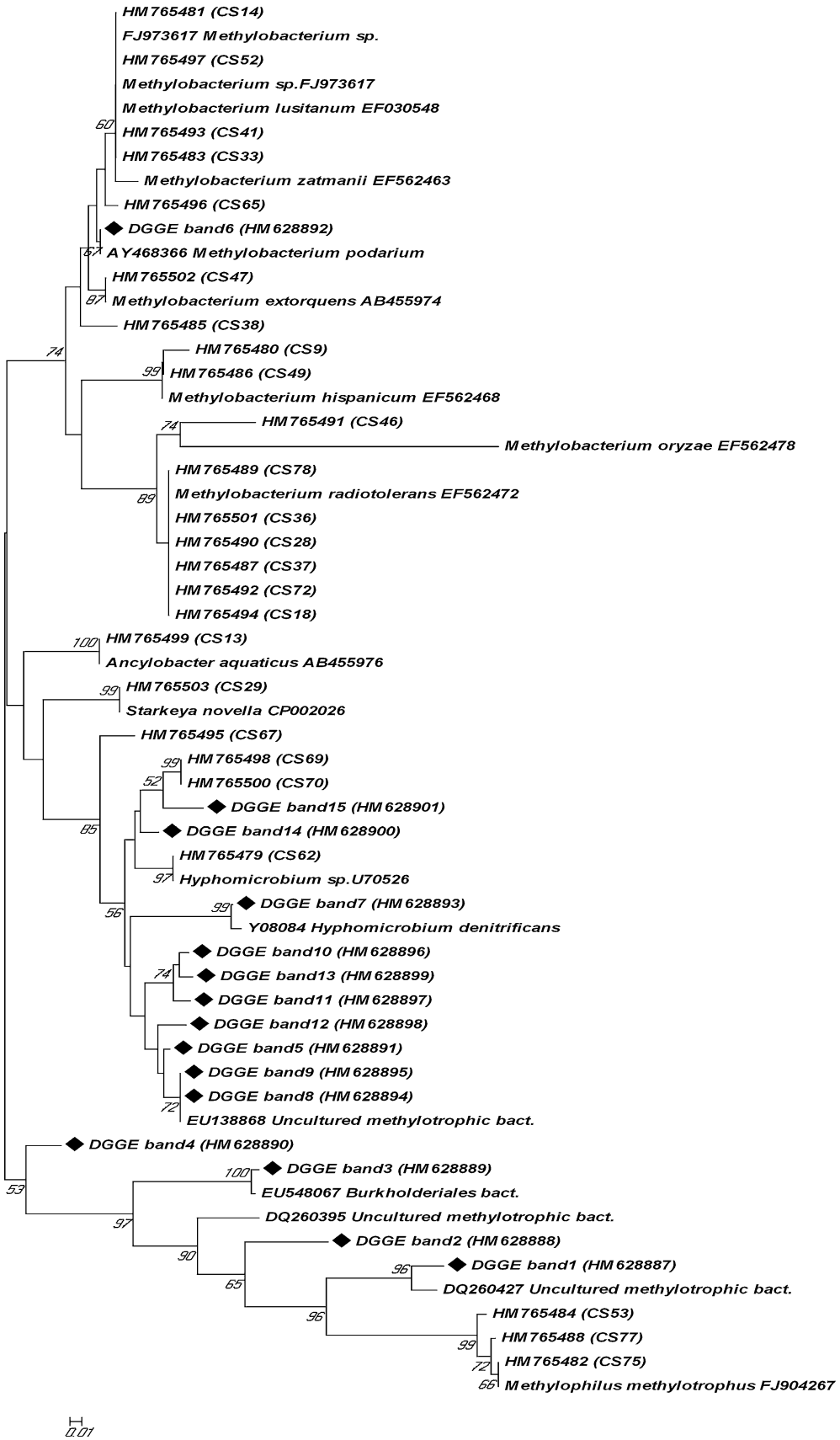
For sequence analysis, all 25 pigmenting and non-pigmenting representative isolates showing similar *mxaF*-RFLP pattern generated by restriction enzyme *Hae*III and *Eco*RI (data not presented) were selected. Genes coding for the larger subunit of methanol dehydrogenase were also sequenced and it was observed that the genus *Methylobacterium* was dominant followed by *Methylophilus* and *Hyphomicrobium* sp.

From mxaF Gene Sequences of Unculturable Ones

The *mxaF* gene amplified metagenome products gave distinct variation on DGGE profiling (Fig. 3). Culture-independent *mxaF* gene sequences revealed them as methylotrophs *Methylobacterium organophilum*, *Ancyclobacter aquaticus*, *Burkholderiales* and *Hyphomicrobium* sp. with 73.3 % unculturable methylotrophs, 6.6 % *Ancyclobacter aquaticus*, 6.6 % *Burkholderiales*, 6.6 % *Hyphomicrobium* and 6.6 % *Methylobacterium* sp. (Fig. 4).

Real Time PCR Quantification

Sensitivity of Lightcycler RT-PCR was evaluated using different starting amount of DNA and compared with the standard curve. SYBR Green I fluorescence determination at the elevated temperature 83 °C resulted in a reliable and sensitive environmental DNA quantification assay with high linearity (Pearson correlation coefficient 0.99) over five orders of magnitude from 10^2 to 10^6 standard *mxaF* cloned DNA start molecules (Fig. S1). All quantitative PCR reactions were performed in replicates. The corresponding specific *mxaF* gene fragment from different sediment samples were amplified, indicating the vast distribution of methylotrophs in brackish water lake sediment. The *mxaF* gene copy number per gram sediment was in the range of 4.9×10^6–1.25×10^7 (Table 1). The *mxaF* gene copy numbers were compared to the CFUs determined by


HM765481 (CS14)
FJ973617 Methylobacterium sp.
HM765497 (CS52)
Methylobacterium sp.FJ973617
Methylobacterium lusitanum EF030548
60
HM765493 (CS41)
HM765483 (CS33)
Methylobacterium zatmanii EF562463
HM765496 (CS65)
DGGE band6 (HM628892)
67
AY468366 Methylobacterium podarium
HM765502 (CS47)
87
Methylobacterium extorquens AB455974
HM765485 (CS38)
74
HM765480 (CS9)
99
HM765486 (CS49)
Methylobacterium hispanicum EF562468
74
HM765491 (CS46)
Methylobacterium oryzae EF562478
HM765489 (CS78)
89
Methylobacterium radiotolerans EF562472
HM765501 (CS36)
HM765490 (CS28)
HM765487 (CS37)
HM765492 (CS72)
HM765494 (CS18)
100
HM765499 (CS13)
Ancylobacter aquaticus AB455976
99
HM765503 (CS29)
Starkeya novella CP002026
HM765495 (CS67)
99
HM765498 (CS69)
52
HM765500 (CS70)
DGGE band15 (HM628901)
85
DGGE band14 (HM628900)
HM765479 (CS62)
97
Hyphomicrobium sp.U70526
99
DGGE band7 (HM628893)
56
Y08084 Hyphomicrobium denitrificans
DGGE band10 (HM628896)
74
DGGE band13 (HM628899)
DGGE band11 (HM628897)
DGGE band12 (HM628898)
DGGE band5 (HM628891)
DGGE band9 (HM628895)
DGGE band8 (HM628894)
72
EU138868 Uncultured methylotrophic bact.
DGGE band4 (HM628890)
100
DGGE band3 (HM628889)
53
EU548067 Burkholderiales bact.
97
DQ260395 Uncultured methylotrophic bact.
DGGE band2 (HM628888)
90
96
DGGE band1 (HM628887)
65
DQ260427 Uncultured methylotrophic bact.
96
HM765484 (CS53)
99
HM765488 (CS77)
72
HM765482 (CS75)
66
Methylophilus methylotrophus FJ904267
0.01

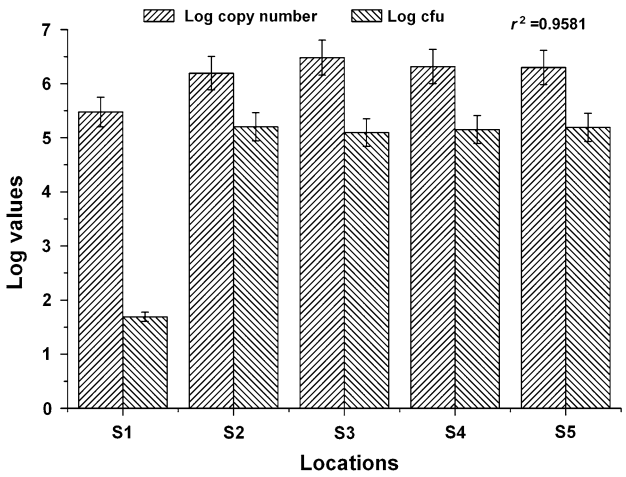


Fig. 5 Comparison of log CFU with log copy number of *mxaF* gene between samples (S1–S5) of the Chilika Lake

plate counts, and the calculated coefficient of correlation between the log copy number of *mxaF* gene and log CFU was 0.979 (Fig. 5). The quantification limit was 1.21×10^6 *mxaF* gene copies per gram sediment.

Of the total 16S rRNA based identified isolates, 62.5 % were close to *Methylobacterium* sp., 16.6 % were *Hyphomicrobium facile*, 4.1 % *Methylophilus* sp., *Mycobacterium brisbanense*, *Pseudomonas* sp. and 8.2 % *Methyloversatilis* sp., while *Methylobacterium* was dominant followed by *Methylophilus* and *Hyphomicrobium* sp. in *mxaF* based identification. The unculturable gene sequence analysis of *mxaF* reported the dominance of unculturable methylotrophic bacteria. PCR amplification of *mxaF* gene coding for α-subunit of methanol dehydrogenase indicate a methylotrophic metabolism. *Burkholderiales* was found through culture independent approach with 92 % *mxaF* gene sequence similarity. Bodrossy et al. [43] reported that *Burkholderiales* followed some other alcohol dehydrogenase for methanol assimilation, and thus, it might be one of the possible ecological adaptation reasons that *Burkholderiales* possesses the *mxaF* gene. Successful species-level identification and validation strategies for *Bacillus* [21], *Clostridium* [22], *Pseudomonas* [23], *Streptococcus* [48] based on the 16S rDNA (rrs) gene sequence through species-specific phylogenetic frameworks, 30–50 nucleotide long motifs (signature sequences) and (in silico) RE digestion patterns in the rrs have been reported. Kalia et al. [22] identified 84 novel Clostridia and advocated the approach to identify important 'food and healthcare' microbes.

High organic matter including lipids imparts toxic effects on the thriving microbial communities. Present study reports a more diversified methylotrophs group from saline sediments, like *Methylobacterium*, *Methyloversatilis*, *Pseudomonas*, *Hyphomicrobium* and *Mycobacterium*. *Methylophilus methylotrophus* could be a crop plants growth promoter under nitrogen stress conditions as it produced significant amount of low-viscosity extracellular polysaccharides from methanol in a chemostat culture under nitrogen limiting condition [46]. The bioengineering applications of methanotrophs are limited such as, the lack of suitable methanotrophic isolate, gas transfer limitation, competitive inhibition of methyl monoxygenase (MMO), regeneration of reducing equivalents for MMO and product toxicity [47]. *Hyphomicrobium facile*, an aerobic chemoorganotroph, plays an important role in denitrification to remove nitrate at drinking water treatment facilities [48], and sewage treatment plants [49]. *Methylobacterium* is a facultative methylotroph and cannot use methane, but is capable of utilizing methanol and some other C_1 compounds, as well as a wide range of multi-carbon substrates, as their sole carbon and energy source while *Hyphomicrobium* is a facultative methylotroph. Present study not only describes the methylotrophic diversity of the Chilika Lake but also indicates the richness of the sediments in terms of microbial wealth.

16S rDNA signature sequences for methylotrophs following different metabolic pathways were developed by Brusseau et al. [50]. Specific signature Serine pathway methylotrophy 5′-CCC-TGAGTT-ATT-CCG-AAC-3′ was found in isolate CS7 while isolate CS13 exhibited the signature RuMP pathway methylotrophy 5′-ATG-CAT-CTC-TGC-TTC-GTT-3′. As these were not found in the rest of the strains, they could be tentatively considered for novel species characterization.

Conclusion

Comparing the distribution and quantification of *mxaF* gene by qRT PCR method revealed higher methylotrophs population from S3 (Panaspada), suggesting a higher C_1 utilization rate. Higher *mxaF* gene copy numbers (about one log unit more) in all samples compared to the CFU counts may suggest that only up to 10 % of methylobacteria were culturable (detectable by the plating method). 16S rRNA, *mxaF*-RFLP, *mxaF* gene sequencings, *mxaF* gene quantification and ARDRA could classify and compare the species level communities. The methylotrophs were found to be predominantly α-proteobacteria. Up to a maximum 85 % similarity of CS3 with *Methyloversatilis* (GenBank sequences) suggests its novelty attributable to its evolution to adapt well to this unique environment, to confirm by polyphasic taxonomy approach. The phylogeny of the C_1-metabolism genes gave a better understanding of the origin of related enzymes and interactions between methylotrophs and non-methylotrophs. The present scientific reporting is one-of-its-kind of the specialized

methylotrophs comparing their culturable and metagenomic populations in 'sediment' niche covering a wider landscape of the Chilika Lake.

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References

1. Nayak BK, Acharya BC, Panda UC, Nayak BB, Acharya SK (2004) Variation of water quality in Chilika Lake, Orissa. Indian J Mar Sci 33:164–169
2. Rath J, Adhikary SP (2008) Biodiversity assessment of algae in Chilika Lake, East Coast of India. Monitoring and modelling lakes and coastal environments. Springer, Netherlands, pp 22–33
3. Behera PK (1999) Applied botany: biodiversity and biotechnology. UGC refresher course. Botany Department, Berhampur University, India, pp 1–92
4. Murrell JC, McDonald IR, Bourne DG (1998) Molecular methods for the study of methanotroph ecology. FEMS Microbiol Ecol 27:103–114. doi:10.1111/j.1574-6941.1998.tb00528.x
5. Nercessian O, Noyes E, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L (2005) Bacterial populations active in metabolism of C_1 compounds in the sediment of Lake Washington, a freshwater lake. Appl Environ Microbiol 71:6885–6899. doi:10.1128/AEM.71.11.6885-6899.2005
6. Nercessian O, Kalyuzhnaya MG, Joye SB, Lidstrom ME, Chistoserdova L (2005) Analysis of *fae* and *fhcD* Genes in Mono Lake, California. Appl Environ Microbiol 71:8949–8953. doi:10.1128/AEM.71.12.8949-8953.2005
7. Antony CP, Kumaresan D, Ferrando L et al (2010) Active methylotrophs in the sediments of Lonar Lake, a saline and alkaline ecosystem formed by meteor impact. ISME J 4:1470–1480. doi:10.1038/ismej.2010.70
8. Yu Z, Mohn WW (2001) Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analyses and 16S ribosomal DNA sequencing. Appl Environ Microbiol 67:1565–1574. doi:10.1128/AEM.67.4.1565-1574.2001
9. Wang P, Wang F, Xu M, Xiao X (2004) Molecular phylogeny of methylotrophs in a deep-sea sediment from a tropical west Pacific Warm Pool. FEMS Microbiol Ecol 47:77–84. doi:10.1016/S0168-6496(03)00252-6
10. Moussard N, Stralis-Pavese N, Bodrossy L, Neufeld JD, Murrell JC (2009) Identification of active methylotrophic bacteria inhabiting surface sediment of a marine estuary. Environ Microbiol Rep 1:424–433. doi:10.1111/j.1758-2229.2009.00063.x
11. De Marco P, Pacheco CC, Figueiredo AR, Moradas-Ferreira P (2004) Novel pollutant-resistant methylotrophic bacteria for use in bioremediation. FEMS Microbiol Lett 234:75–80. doi:10.1111/j.1574-6968.2004.tb09515.x
12. Cavanaugh CM, Levering PR, Maki JS, Mitchell R, Lidstrom ME (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325:346–348. doi:10.1038/325346a0
13. Chistoserdova L, Lidstrom ME (2013) Aerobic methylotrophic prokaryotes. In: Rosenberg E, DeLong EF, Thompson F, Lory S, Stackebrandt E (eds) The prokaryotes, 4th edn. Springer, pp 267–285
14. Vuilleumier S, Chistoserdova L, Lee M-C et al (2009) Methylobacterium genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources. PLoS One 4:e5584. doi:10.1371/journal.pone.0005584
15. Trotsenko YA, Khmelenina VN (2002) The biology and osmoadaptation of haloalkaliphilic methanotrophs. Microbiology 71:123–132. doi:10.1023/A:1015183832622
16. Sanseverino AM, Bastviken D, Sundh I, Pickova J, Enrich-Prast A (2012) Methane carbon supports aquatic food webs to the fish level. PLoS One 7:e42723. doi:10.1371/journal.pone.0042723
17. Yun J, Zhuang G, Ma A, Guo H, Wang Y, Zhang H (2012) Community structure, abundance, and activity of methanotrophs in the Zoige Wetland of the Tibetan Plateau. Microb Ecol 63:835–843. doi:10.1007/s00248-011-9981-x
18. Meena KK, Kumar M, Kalyuzhnaya MG, Yandigeri MS, Singh DP, Saxena AK, Arora DK (2012) Epiphytic pink-pigmented methylotrophic bacteria enhance germination and seedling growth of wheat (*Triticum aestivum*) by producing phytohormone. Antonie Van Leeuwenhoek 101:777–786. doi:10.1007/s10482-011-9692-9
19. López JC, Quijano G, Souza TS, Estrada JM, Lebrero R, Muñoz R (2013) Biotechnologies for greenhouse gases (CH_4, N_2O, and CO_2) abatement: state of the art and challenges. Appl Microbiol Biotechnol 97:2277–2303. doi:10.1007/s00253-013-4734-z
20. Rani A, Porwal S, Sharma R, Kapley A, Purohit HJ, Kalia VC (2008) Assessment of microbial diversity in effluent treatment plants by culture dependent and culture independent approaches. Bioresource Technol 99:7098–7107. doi:10.1016/j.biortech.2008.01.003
21. Porwal S, Lal S, Cheema S, Kalia VC (2009) Phylogeny in aid of the present and novel microbial lineages: diversity in *Bacillus*. PLoS One 4:e4438. doi:10.1371/journal.pone.0004438
22. Kalia VC, Mukherjee T, Bhushan A, Joshi J, Shankar P, Huma N (2011) Analysis of the unexplored features of rrs (16S rDNA) of the genus *Clostridium*. BMC Genom 12:18. doi:10.1186/1471-2164-12-18
23. Bhushan A, Joshi J, Shankar P, Kushwah J, Raju SC, Purohit HJ, Kalia VC (2013) Development of genomic tools for the identification of certain *Pseudomonas* up to species level. Indian J Microbiol 53:253–263. doi:10.1007/s12088-013-0412-1
24. McDonald IR, Murrell JC (1997) The methanol dehydrogenase structural gene *mxaF* and its use as a functional gene probe for methanotrophs and methylotrophs. Appl Environ Microbiol 63:3218–3224
25. McDonald IR, Kenna EM, Murrell JC (1995) Detection of methanotrophic bacteria in environmental samples with PCR. Appl Environ Microbiol 61:116–121
26. Kalyuzhnaya MG, Hristova KR, Lidstrom ME, Chistoserdova L (2008) Characterization of a novel methanol dehydrogenase in representatives of Burkholderiales: implications for environmental detection of methylotrophy and evidence for convergent evolution. J Bacteriol 190:3817–3823. doi:10.1128/JB.00180-08
27. Chistoserdova L, Kalyuzhnaya MG, Lidstrom ME (2009) The expanding world of methylotrophic metabolism. Annu Rev Microbiol 63:477–499. doi:10.1146/annurev.micro.091208.073600
28. APHA-AWWA-WPCF (2005) Standard methods for the examination of water and waste water, 20th edn. American Public Health Association, Washington
29. Patt TE, Cole GC, Bland JA, Hanson RS (1974) Isolation and characterization of bacteria that grow on methane and organic compounds as sole sources of carbon and energy. J Bacteriol 120:955–964
30. Pospiech A, Neumann B (1995) A versatile quick-prep of genomic DNA from gram positive bacteria. Trends Genet 11:217–218
31. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire

genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853
32. Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
33. Jaccard P (1912) The distribution of the flora in the alpine zone. New Phytol 11:37–50. doi:10.1111/j.1469-8137.1912.tb05611.x
34. Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273
35. Henckel T, Friedrich M, Conrad R (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase and methanol dehydrogenase. Appl Environ Microbiol 65:1980–1990
36. Pfaffl M (2001) Development and validation of an externally standardised quantitative insulin-like growth factor-1 RT-PCR using LightCycler SYBR Green I technology. Rapid Cycle Real-Time PCR. Springer, Berlin-Heidelberg, pp 281–291. doi:10.1007/978-3-642-59524-0_30
37. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving sensitivity of progressive multiple sequence alignments through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
38. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092
39. Saitou N, Nei M (1987) The neighbor-joining method a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
40. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evol 39:783–791
41. Hiraishi A, Shimada K (2001) Aerobic anoxygenic photosynthetic bacteria with zinc-bacteriochlorophyll. J Gen Appl Microbiol 47:161–180. doi:10.2323/jgam.47.161
42. Raja P, Balachandar D, Sundaram SP (2008) Genetic diversity and phylogeny of pink pigmented facultative Methylotrophic bacteria isolated from the phyllosphere of tropical crop plants. Biol Fertil Soils 45:45–53. doi:10.1007/s00374-008-0306-2
43. Bodrossy L, Murrell JC, Dalton H, Kalman M, Puskas LG, Kovacs KL (1995) Heat-tolerant methanotrophic bacteria from the hot water effluent of a natural gas field. Appl Environ Microbiol 61:3549–3555
44. Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Vani AA, Borgave SB, Sarnaik SS (2008) Cultivable bacterial diversity of alkaline Lonar Lake, India. Microbial Ecol 55:163–172
45. Wang X, Sahr F, Xue T, Sun B (2007) *Methylobacterium salsuginis* sp. nov. isolated from sea water. Int J Syst Evol Microbiol 57:1699–1703. doi:10.1099/ijs.0.64877-0
46. Jenkins O, Bryom D, Jones D (1987) *Methylophilus*: a new genus of methanol utilizing bacteria. Int J Syst Bacteriol 37:446–448. doi:10.1099/00207713-37-4-446
47. Jiang H, Chen Y, Jiang P, Zhang C, Smith TJ, Murrell JC, Xing X-H (2010) Methanotrophs: multifunctional bacteria with promising applications in environmental bioengineering. Biochem Eng J 49:277–288. doi:10.1016/j.bej.2010.01.003
48. Liessens J, Germonpre R, Kersters I, Beernaert S, Verstraete W (1993) Removing nitrate with a methylotrophic fluidized bed: microbiological water quality. J Am Water Works Assoc 85:155–161
49. Schmider F, Ottow JCG (1986) Characterization of denitrifying bacteria in the various compartments of a biological sewage plant. Arch Hydrobiol 106:497–512
50. Brusseau GA, Bulygina ES, Hanson RS (1994) Phylogenetic analysis and development of probes for differentiating methylotrophic bacteria. Appl Environ Microbiol 60:626–636