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RESEARCH ARTICLE

Identification of microbial communities involved in the methane cycle of a freshwater meromictic lake

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α-subunit of the particulate methane monooxygenase; α-subunit of the methyl-coenzyme M reductase; aerobic methanotrophy; methanogenesis.

Abstract

Lake Pavin is a meromictic crater lake located in the French Massif Central area. In this ecosystem, most methane (CH_4) produced in high quantity in the anoxic bottom layers, and especially in sediments, is consumed in the water column, with only a small fraction of annual production reaching the atmosphere. This study assessed the diversity of methanogenic and methanotrophic populations along the water column and in sediments using PCR and reverse transcription-PCR-based approaches targeting functional genes, i.e. *pmoA* (α-subunit of the particulate methane monooxygenase) for methanotrophy and *mcrA* (α-subunit of the methyl-coenzyme M reductase) for methanogenesis as well as the phylogenetic 16S rRNA genes. Although methanogenesis rates were much higher in sediments, our results confirm that CH_4 production also occurs in the water column where methanogens were almost exclusively composed of hydrogenotrophic methanogens, whereas both hydrogenotrophs and acetotrophs were almost equivalent in the sediments. Sequence analysis of markers, *pmoA* and the 16S rRNA gene, suggested that *Methylobacter* may be an important group actively involved in CH_4 oxidation in the water column. Two main phylotypes were characterized, one of which could consume CH_4 under conditions where the oxygen amount is undetectable.

Introduction

Methane (CH_4) is an important radiative trace gas responsible for about 20% of the anthropogenic additional greenhouse effect. Global CH_4 emissions from natural wetlands range from 100 to 237 Tg $year^{-1}$, corresponding to approximately 75% of all natural emissions. Lakes alone are believed to contribute 6–16% of these natural emissions (Bastviken, 2009). CH_4 production is a strictly anaerobic process restricted to environments with very low concentrations of alternative electron acceptors [manganese and iron oxides, oxygen (O_2), nitrate and sulfate] and high concentrations of common electron donors, particularly H_2 and acetate (Capone & Kiene, 1988). The two main pathways of production are acetotrophic (acetate dependent) and hydrogenotrophic (H_2 dependent) methanogenesis. In the former case, acetate is cleaved into CH_4 and carbon dioxide (CO_2). During hydrogenotrophic methanogenesis, H_2 reacts with CO_2 to produce CH_4 and H_2O (Bastviken, 2009). However, currently, there is little evidence of any significant rates of methanogenesis in anoxic water columns, supposedly due to the lower substrate concentrations than that in sediment (Bastviken, 2009). Stable syntrophic consortia could be more difficult to establish in water columns than sediments due to the greater transport processes involved. Water chemistry in meromictic lakes is another important factor in determining whether methanogenesis occurs in the water column and in the sediment (Winfrey & Zeikus, 1979).

Once produced in aquatic environments, CH_4 is consumed in both aerobic and anaerobic compartments. Thus, although from 45% to 100% of CH_4 produced in the water column of lakes or derived from benthic flux is oxidized (Bastviken, 2009), even today, little is known about the structure of methanotrophs in pelagic freshwater

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ecosystems. Aerobic oxidation by CH_4-oxidizing bacteria in surface sediments (Sweerts *et al.*, 1991) and in the water column (Utsumi *et al.*, 1998) is the main pathway controlling CH_4 escape into the atmosphere, making it a key process for the mitigation of CH_4 emissions from aquatic environments (Bastviken, 2009). In freshwaters, CH_4 oxidation is most active in the vicinity of the oxic–anoxic interface, where both CH_4 and O_2 are available.

The second process controlling CH_4 release is anaerobic oxidation of methane, which is thought to involve CH_4-oxidizing archaea in a consortium with sulfate-reducing bacteria (Boetius *et al.*, 2000). Although mainly observed in saline/alkaline anoxic continental habitats (Joye *et al.*, 1999), recent studies suggest that this pathway also occurs in freshwater environments through nonsulfate terminal electron acceptors (Raghoebarsing *et al.*, 2006).

Lake Pavin in the French Massif Central is a permanently stratified crater lake with layers of water that do not intermix. Hence, this meromictic lake has three water layers, an anoxic deep water layer (monimolimnion) separated from the oxygenated upper layer (mixolimnion) by an intermediate layer (mesolimnion) with strong chemical gradients. The sharp increase in the concentration of dissolved compounds within the mesolimnion leads to an increase in the density of the bottom water layers and consequently strengthens the stability of the physical stratification, despite a temperature increase of about 1 °C (Michard *et al.*, 2003). The isolation of the monimolimnion (by the mesolimnion) results in a significant CH_4 accumulation (over 4 mM at 90 m depth). This situation causes a strong CH_4 concentration gradient within the mesolimnion, but only a small amount (a 10^5 decrease in concentration from the bottom to the top of the lake) of this CH_4 actually reaches the atmosphere, as it is almost fully consumed in the water column.

The identification of organisms involved in methanotrophy and methanogenesis within Lake Pavin is a key step for a complete understanding of the CH_4 and carbon cycles in this ecosystem. In this study, the diversity and the vertical distribution of methanogens and methanotrophs were assessed along the entire water column and sediments of this lake using two functional gene markers [i.e. the α-subunit of the particulate methane monooxygenase (*pmoA*) for methanotrophy, the α-subunit of the methyl-coenzyme M reductase (*mcrA*) that catalyzes the last step in methanogenesis]. Additional studies with 16S rRNA gene analyses were also performed at two critical points in the water column. Our results provide insights into the substrate or niche adaptations of these communities.

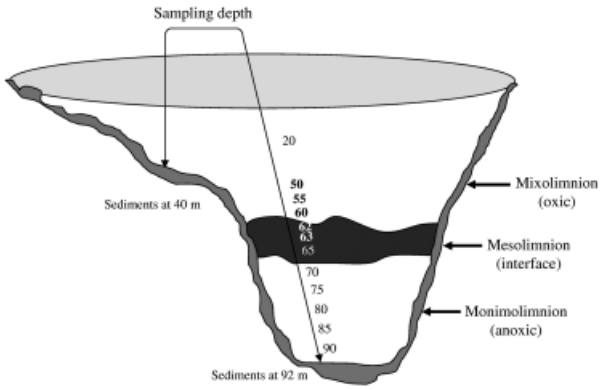

Fig. 1. Cross-section on Lake Pavin showing the location of sampling.

Materials and methods

Sampling site

Lake Pavin (45°29.74N, 2°53.28E) is a maar located at 1197 a.s.l. in the more recent volcanic area of the French Massif Central. It is divided into two permanent stratified layers (Fig. 1) (see Michard *et al.*, 2003). The surface stratum, or mixolimnion, includes three different layers according to variations in depth, temperature, pH and dissolved O_2 (the epilimnion, up to 5–15 m depth, the metalimnion, from the bottom of epilimnion up to 20 m depth and the hypolimnion from approximately 20 to 61 m depth). Seasonal dissolved O_2 depletion occurs at the base of the hypolimnion with a value close to zero between around 58 and 61 m depth. The mixolimnion is separated from the monimolimnion by the mesolimnion (∼60–70 m depth), which is characterized by a strong increase in specific conductivity. Finally, the monimolimnion, from ∼70 m depth to sediments, is not affected by spring or fall turnover. This layer is characterized by a steady state of the major physical and chemical parameters.

Analytical method

Conductivity, temperature and dissolved oxygen (DO) concentration were determined using a Seabird SBE 19 Seacat profiler (Sea-Bird Electronics Inc., Washington, DC) in July 2007 and May 2009. This CTD-O_2 probe was calibrated for DO with Winkler titrations in triplicate samples from 2 and 40 m depths (where the O_2 gradients are very weak). Profiles were acquired at a speed of descent as low as 5 cm s^{-1} in the O_2 gradient zone to allow the O_2 sensor to equilibrate.

Water samples for CH_4 concentration determination were collected in July 2007 using a custom-built automatic 1-L syringe sampler, which was built on a 1-L syringe Model S-1000 Hamilton basis, along a vertical profile near the center of the lake. The sampler was deployed from a stable platform, and the use of an electronic depth gauge allowed a 0.2 m depth precision and direct filtration from 1-L syringe just after retrieving on board without air contact (Luer

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connections and use of syringe filtration units). Water from the oxic mixolimnion (from the surface down to 55 m) was collected into 115-mL serum bottles filled to overflow and then sealed and poisoned with 1 mL of 0.5 g L^{-1} $HgCl_2$ until analysis. A 30-mL nitrogen headspace was then created before analysis, as described by Abril & Iversen (2002). In the CH_4-rich zone (from 55 to 90 m depth), a different sampling method was used in order to minimize CH_4 loss due to depressurization: 30 mL of water from the sampling syringe was rapidly transferred with a needle through the butyl cap into preweighed and N_2-preflushed sealed glass bottles. Thus, most of the depressurization occurred in the bottle, minimizing CH_4 loss. CH_4 concentrations were quantified using a gas chromatograph (Chrompack 438 gas chromatograph, Packard Instruments, Downers Grove, IL) equipped with a 2 m × 2 mm Haysep column and a flame ionization detector.

Sample collection

Water column samples were collected using an 8-L horizontal Van Dorn bottle along a vertical profile at nine depths (20, 50, 60, 63, 65, 70, 75, 80, 85 and 90 m) in July 2007 and three depths (65, 70 and 90 m) in June 2009 (Fig. 1). Immediately on collection, the water samples were transferred into sterile bottles and stored on ice until processing. The water samples (0.3–1 L) were filtered directly in the laboratory under vacuum, and collected onto a 47-mm TSTP Millipore filter (0.2-μm pore size). Filters were stored dry at −80 °C until nucleic acid extractions.

Supplementary water column samples (also 0.3–1 L) were collected using a 4-mm-bore PVC tube connected to a peristaltic pump and filtrated on-site through a 47-mm TSTP Millipore filter (0.2 μm) held in a Swinnex-47 (Millipore, Billerica, MA) filter holder connected to the tube, at 62 m depth in December 2007 and at four depths (50, 55, 60 and 63 m) in June 2009 (Fig. 1). This second sampling method was used, when possible, to reduce mRNA transcription modifications or their destruction, which could be induced by transport. Thus, filters were immediately placed into 2-mL sterile tubes (Eppendorf, Hamburg, Germany) and stored on dry ice until they were transferred at −80 °C to the laboratory for further nucleic acid extraction. Unfortunately, this sampling was not applied for depths below 63 m due to excessively high degassing in the anoxic zone.

Sediment samples were collected at 40 m depth (within the oxic zone) and at 92 m (within the anoxic zone) in June 2007 and only at 92 m in June 2009 using a gravitational Uwitec corer with a diameter of 90 mm and a length of 60 cm (Fig. 1). About 100 g of each sediment sample from the upper sediment horizon (first 20 cm) was separately placed in sterile containers and stored on dry ice until being transferred to the lab at −80 °C for further processing.

Nucleic acid extractions

Genomic DNA (gDNA) was extracted from samples collected in 2007, and total RNA was extracted from samples collected in 2009. Water column filters were placed in 900 μL of lysis buffer as described by Vetriani *et al.* (2003). Freeze–thaw cycles were replaced by the beat-beating method, and nucleic acids were extracted using a standard phenol–chloroform–isoamyl alcohol (50 : 49 : 1) method, followed by a second extraction with an equal volume of chloroform. Nucleic acids were then precipitated, washed in 70% ethanol and resuspended in nuclease-free water. For RNA samples, coextracted DNA was removed by digestion with 4 U of (RNase-free) DNase I (DNA-*free*, Ambion, Austin, TX) at 37 °C for 35 min following the manufacturer's instructions.

Frozen sediment aliquot (30 g) collected in June 2009 was mixed vigorously with 200 mL of buffer containing glucose, EDTA and Tris-HCl (Vetriani *et al.*, 2003) and centrifuged briefly at 200 ***g*** before filtering the supernatant onto a 47-mm TSTP Millipore filter (0.2 μm). The filter was then placed in 900 μL of lysis buffer. RNA was extracted as described above. DNA from frozen sediment aliquots (3 g) sampled in June 2007 was extracted using the protocol described by Zhou *et al.* (1996).

Nucleic acid concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop). The integrity of the extracted RNA was checked by analyzing an aliquot on a Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). DNA integrity was analyzed on an agarose gel.

cDNA synthesis

cDNA synthesis was performed starting with 100 ng RNA and using SuperScript III reverse transcriptase (Invitrogen Inc., Carlsbad, CA). To remove the secondary structure, the mixture containing RNA diluted with DEPC-treated water in a final volume of 12 and 0.5 μL of gene-specific primer (25 mM) was heated for 10 min at 70 °C and immediately placed on ice. Four microliters of 5 × buffer (supplied), 2 μL 0.1 M DTT, 0.5 μL dNTP mix (2.5 mM each), 0.5 μL (20 U) RNasIn$^+$ (Promega, Madison) and 0.5 μL SuperScript III reverse transcriptase (Invitrogen Inc.) were then added. The mixture was incubated for 2 h at 42 °C for cDNA synthesis. To check that the RNA extracts were uncontaminated by DNA, control reactions were prepared as described above, but without reverse transcriptase.

PCR and library construction

Table 1 summarizes the primers and cycling parameters used in this study. PCR amplifications were performed in tubes containing 25 μL (total volume) of a mixture using an

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iCycler thermal cycler (Bio-Rad, CA). Each PCR mix consisted of 1 × PCR buffer (Promega), 0.2 μM of forward and reverse primers, 250 μM of each dNTP (Invitrogen Inc.), 2 U of GoTaq DNA polymerase (Promega) and template DNA (5–20 ng of gDNA or 4.5 μL of cDNA). For all amplifications, a 5-min denaturation step (95 °C) was included at the beginning of the PCR run. This step was followed by cycles at the annealing temperatures given in Table 1, and a 10-min extension step (72 °C) was included at the end of the reaction. Negative controls were included in all PCR reactions to check for background contamination.

The PCR products were assessed on 1% w/v agarose gels. Amplicons of the correct size were purified using QIAquick spin columns (Qiagen, Chatsworth, CA) and cloned using the TOPO TA cloning kit (Invitrogen Inc.). Plasmids were screened for the correct size insert by digestion with EcoRI. DNA sequencing was performed using the Sanger method (Sanger *et al.*, 1977) on MWG DNA sequencing services (Ebersberg, Germany).

Sequence data and phylogenetic analysis

Sequences were processed and joined using the STADEN package program (Staden, 1996) and characterized using the BLASTN (16S rRNA genes) and BLASTX (functional genes) search tools (http://www.ncbi.nlm.nih.gov). The nucleic acid sequences encoding enzymes were translated using the EMBOSS TRANSEQ tool available at http://www.ebi.ac.uk/Tools/emboss/transeq/ and aligned using CLUSTALW to identify identical amino acid sequences (http://www.ebi.ac.uk/Tools/clustalw2/index.html). After alignment, we conserved only the different deduced amino acid sequences and then grouped them into operational taxonomic units (OTUs) at a cutoff level of 91% (Heyer *et al.*, 2002; Luton *et al.*, 2002; Kjeldsen *et al.*, 2007) using DOTUR software (Schloss & Handelsman, 2005). The input files for DOTUR were distance matrices generated by PROTDIST (http://mobyle.pasteur.fr/cgi-bin/portal.py). One representative of each OTU was subsequently chosen to build phylogenetic trees in MEGA version 4 (Tamura *et al.*, 2007) using the neighbor-joining method (Saitou & Nei, 1987) and then bootstrapped with 1000 trials. Closely related sequences identified by BLAST and available from GenBank (http://www.ncbi.nlm.nih.gov/) plus sequences of characterized species were included in the phylogenetic trees to decipher microbial community diversity.

Nucleotide sequence accession numbers

Nucleotide sequences without PCR primer sequences were deposited in the GenBank database under accession numbers GQ389796–GQ390089 for the *mcrA* gene, GQ390100–GQ390158 and JF811254–JF811322 for the *pmoA* gene, and GQ390159–GQ390243 and GU472558–GU472841 for 16S rRNA genes.

Results

Geochemistry

The investigations were carried out using water samples collected in Lake Pavin. Figure 2 shows the vertical profiles of DO, conductivity at 25 °C (C25), temperature and CH_4 concentration for July 2007 and May 2009 (except CH_4, which was not measured in May 2009). All parameters taken together, the profiles showed typical trends usually observed in Lake Pavin, i.e. O_2 depletion (detection limit $< 1\,\mu M$) and a sharp increase in C25 and CH_4 at around 60 m depth (Viollier *et al.*, 1997; Michard *et al.*, 2003). In July 2007, the maximum gradients for DO and C25 were located at 57 m depth (oxycline) and 62.1 m depth (mesolimnion), respectively. During this period, the maximum gradient for CH_4 was located at 62.5 m depth, i.e. almost superimposed with the mesolimnion. At 60 m depth, DO and CH_4 were $0.06\,mg\,L^{-1}$ and 0.3 μM, respectively, corresponding to a small overlap between the two parameters.

Methanogen abundance and distribution

PCR amplicons of *mcrA* were obtained both from gDNA and cDNA using a specific primer set (MM_01–MM_02) that enables the coverage of a large portion of methanogens (Mihajlovski *et al.*, 2008) and subsequently used for the construction of separate clone libraries. Ten clone libraries consisting of 306 clones were constructed from gDNA samples collected in 2007, eight from water column samples

Table 1. Primer sets and PCR conditions used to amplify *pmoA*, *mcrA* and 16S rRNA gene markers

Amplification	Primers	Sequence (5′–3′)	Annealing temperature (°C)	No. of cycles	Expected size (bp)	References
pmoA	pmoA189f	GGNGACTGGGACTTCTGG	55	40 (DNA)	~500	Costello & Lidstrom (1999)
	mb661r	CCGGMGCAACGTCYTTACC				
mcrA	MM_01	TAYATGTCIGGYGGTGTHGG	48	30 (cDNA)	~500	Mihajlovski *et al.* (2008)
	MM_02	ACRTTCATIGCRTAGTTIGG				
16S rRNA gene	27F	AGAGTTTGATCMTGGCTCAG	59	30 (DNA)	~1500	Lane (1991)
	1492R	AGGRTACCTTGTTACGACTT				

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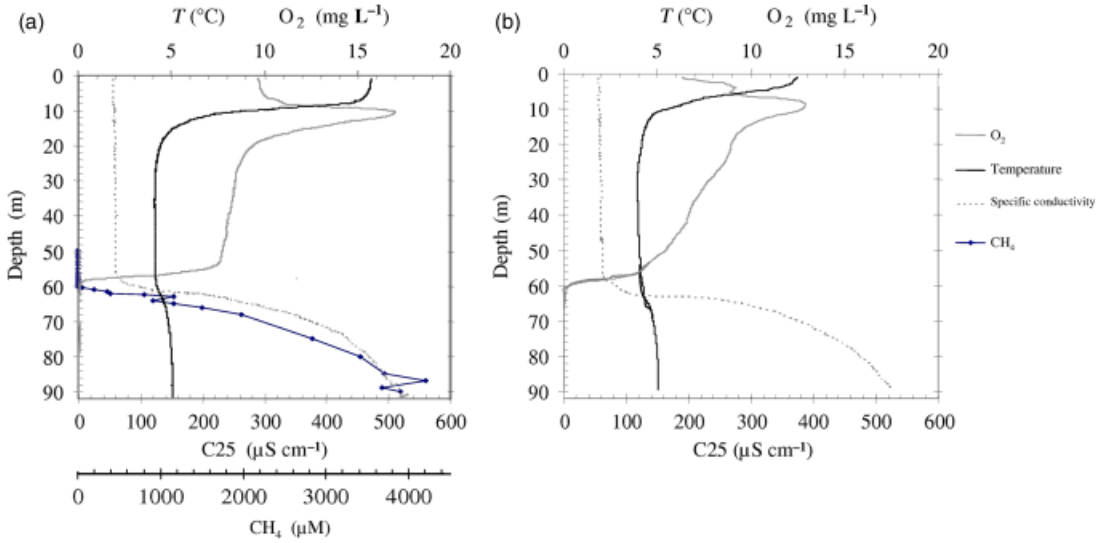

Fig. 2. Vertical profiles of DO, C25, temperature and CH_4 concentration along the water column of the Lake Pavin in July 2007 (a) and May 2009 (b).

(63, 65, 68 m and then every 5 m from 70 to 90 m) and two from sediments (40 and 92 m). Three additional clone libraries consisting of 67 clones were also constructed from cDNA samples collected in 2009 (70 m, 90 m and sediments at 92 m). No amplification of the *mcrA* gene could be achieved with gDNA collected from the water layer located above 63 m depth and with cDNA obtained from that located above 70 m depth. This suggested that the active methanogenic community was present only from nearly 70 m depth down to the sediments. Furthermore, considerable microdiversity was evident among the sequenced clones, as 303 unique *mcrA* nucleic acid sequences were identified that coded for 210 different polypeptides, taking into account all genetic code redundancy. In total, 20 distinct OTUs were detected among the inferred amino acid sequences of *mcrA* using an arbitrary 91% sequence identity threshold for grouping the retrieved environmental sequences. Nineteen OTUs were present in gDNA libraries and 10 in cDNA libraries. Comparisons at the amino acid sequence level of *mcrA* gene fragments revealed a number of associations. These OTUs covered three lineages encompassing *Methanomicrobiales*, *Methanosarcinales* and a putative third lineage.

Most of the *mcrA* sequences were closely related to the *Methanomicrobiales* order (52–100% of total gDNA clones and 58.6–92.3% of total cDNA clones, depending on the library). Two groups of sequences clustered within this order, where they formed a distinct branch (Fig. 3). One group (cluster I) included 12 OTUs (OTU1–12), which showed the strongest similarity to many environmental sequences (Luton *et al.*, 2002; Castro *et al.*, 2004; Galand *et al.*, 2005; Conrad *et al.*, 2008). The second group (cluster II) included only four OTUs (OTU13–16), which showed 83–84% similarity to only one recognized species recently described in an acidic peat bog, *Candidatus* Methanoregula boonei (Brauer *et al.*, 2006). Of the 16 OTUs, six included sequences both from gDNA and cDNA libraries, nine included only sequences from gDNA libraries and one (OTU12) consisted of only one cDNA sequence (Fig. 3). Both gDNA and cDNA libraries were dominated by a small number of OTUs, the most abundant being OTU2 (Fig. 4). Moreover, the spatial distribution of these OTUs showed clear differences between water column and sediments, but also between sediments in contact with oxygenated water (40 m) and those in contact with anoxic water (92 m). Obviously, OTU7, OTU8, OTU10 and OTU11 dominated sediments collected at 92 m while they were poorly represented (in clone number) or absent in the water column (Fig. 4a). Similarly, OTU16 dominated sediments collected at 40 m, while it was not retrieved from those collected at 92 m. All these findings suggested that certain OTUs could be adapted to different environmental niches (organic matter content, microbial interactions, substrate availability). This hypothesis was reinforced by the analysis of *mcrA* transcripts with OTU2 and OTU4 from cluster I mainly expressed in the water column and OTU13 from cluster II preferentially expressed in the bottom of the water column and in sediments (Fig. 4b).

The second most abundant group of *mcrA* sequences was closely related to the *Methanosarcinales* order (2.7–12.5% of the total gDNA clones and 7.7–37.9% of the total cDNA clones, depending on the library). All sequences grouped into two distinct OTUs (OTU17 and OTU18), both converging on a monophyletic group that included the acetoclastic species *Methanosaeta concilii* (Fig. 3). This order, therefore, displayed a lower diversity than that of *Methanomicrobiales*.

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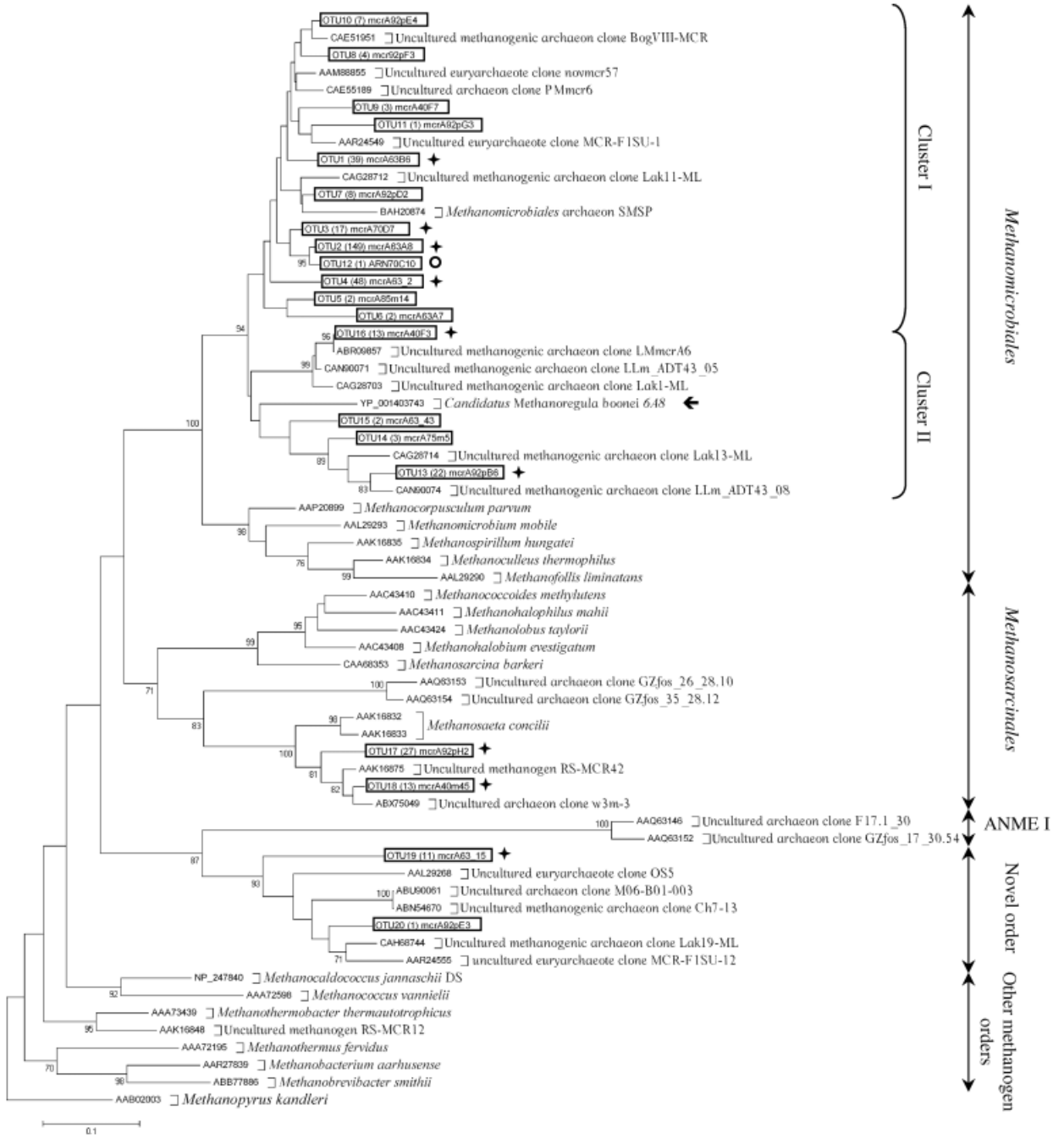

Fig. 3. Evolutionary distance tree showing the phylogenetic relationship of the deduced McrA amino acid sequences including the representative sequences derived from this study and sequences of isolates and uncultivated organisms. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). Evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 127 positions in the final dataset. Bootstrap values > 70% derived from 1000 replicates are indicated at the nodes. The number of clones assigned to each OTU is given in brackets together with the name of the representative clone used in this study. *Methanopyrus kandleri* sequence (AAB02003) was used as an outgroup. Arrow indicates the unique closest cultured methanogen species to environmental *Methanomicrobiales* sequences. Arrow indicates the species *Candidatus* Methanoregula boonei, which is the unique cultured species closely related to environmental sequences identified in this study. ✦, Phylotype clustering gDNA and cDNA sequences; ○, phylotype with only a cDNA sequence.

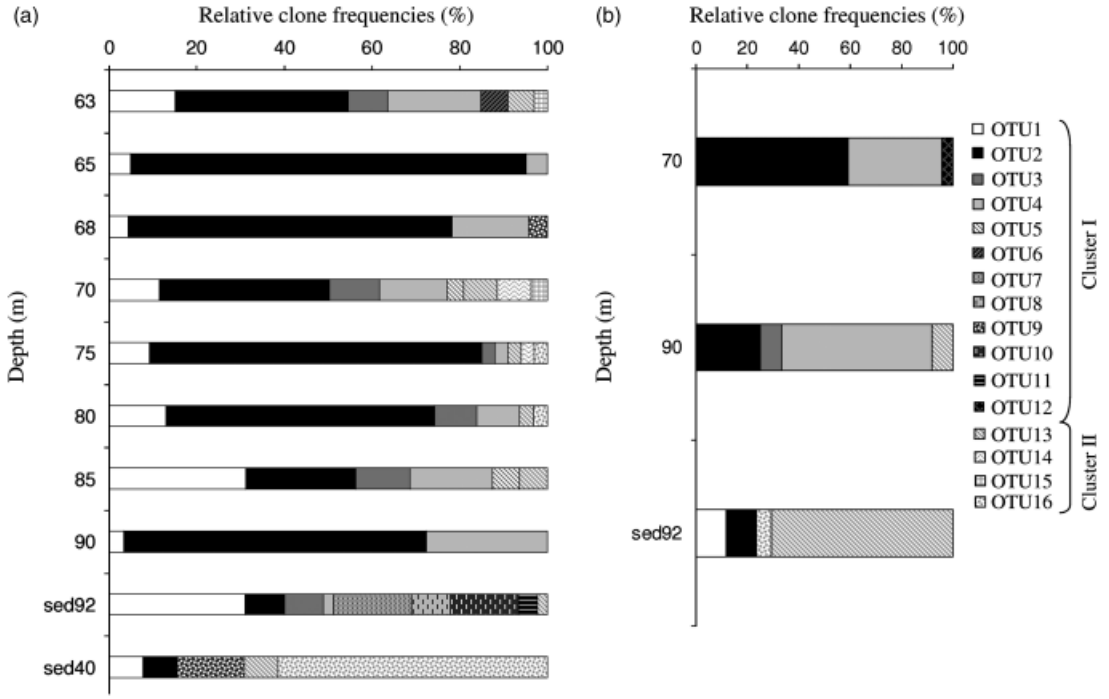

Fig. 4. Distribution pattern of the different OTUs related to the *Methanomicrobiales* order along the water column and in sediments. (a) Sequences retrieved from gDNA libraries. (b) Sequences retrieved from cDNA libraries.

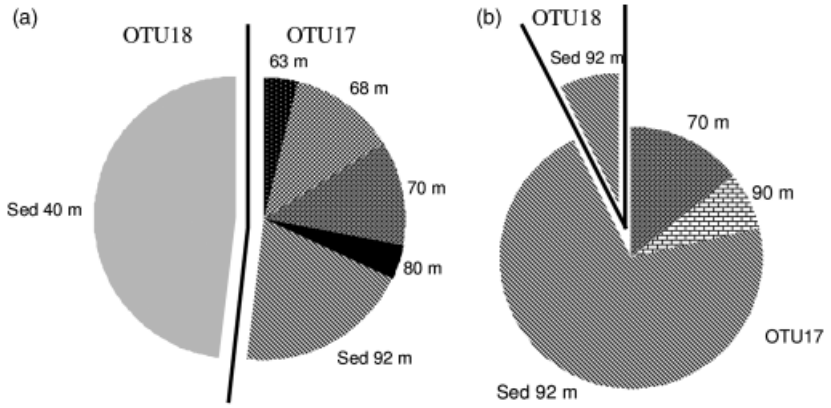

Fig. 5. Distribution of sequences affiliated with the two OTUs related to the *Methanosarcinales* for each library. (a) Sequences retrieved from gDNA libraries. (b) Sequences retrieved from cDNA libraries.

For these OTUs, the largest number of clones was retrieved from sediments (Fig. 5). Furthermore, they showed a clearly different distribution profile, with OTU18 retrieved mainly from sediments in contact with oxygenated water (40 m), whereas OTU17 was retrieved exclusively from those in contact with anoxic water (92 m) and the water column (Fig. 5). These results were consistent with those obtained from mRNA samples. Hence, the shift in *Methanosaetaceae* assemblage could be correlated with changes in environmental conditions (i.e. pressure, O_2 and microbial interactions).

The third lineage clustered only 12 clones (11 in OTU19, 1 in OTU20) that fell outside any described methanogenic order and formed a deep-branching cluster separate from the other five methanogen orders (Fig. 3). The evidence suggests that this cluster may represent a novel methanogenic lineage. These OTUs showed the highest similarity (80–83%) to *mcrA* sequences from remotely different habitats including wetland ecosystems (Juottonen *et al.*, 2005), landfill (Luton *et al.*, 2002), drainage water (Castro *et al.*, 2004) and human gut (Mihajlovski *et al.*, 2008), whose phylogenetic affiliation remained doubtful. The fact that this lineage is recovered from various independent ecosystems suggests that it is widely distributed in the environment. In addition, transcripts for this lineage were detected

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in both the water column and the 92 m sediment. The closer relative of this lineage is the anaerobic methane oxidizer ANME-1 cluster (Fig. 3), which is characterized by a cysteine-rich (CCX4CX5C) stretch in its *mcrA* sequence (Hallam *et al.*, 2003; Shima & Thauer, 2005). Because of the absence of this signature in *mcrA* sequences affiliated to the novel lineage, its involvement in methanogenesis or anaerobic methanotrophy remains unclear.

Methanotroph abundance and distribution

The planktonic methanotroph assemblages were investigated by *pmoA* gene amplification using a specific primer set (Costello & Lidstrom, 1999) from fewer sample points along the water column. PCR amplicons of *pmoA* were subsequently used for the construction of separate clone libraries. Seven clone libraries consisting of 120 clones were constructed from gDNA samples, five from the oxic layer (20, 50, 60, 62 and 63 m) and two from the anoxic layer (70 and 75 m). Five additional clone libraries consisting of 119 clones were also constructed from cDNA samples (50, 55, 60, 63 and 65 m). Only one clone of the six sequenced for gDNA sample collected at 75 m was *pmoA*; therefore, amplicons obtained from greater anoxic depths were considered as false positives and not cloned. No reverse transcription (RT)-PCR products were achieved with cDNA from the water layer located below 65 m depth, suggesting that this gene was only expressed up to this zone, where O_2 was below the detection threshold. As for the *mcrA* gene, a large microdiversity was also observed for *pmoA* because 128 unique *pmoA* nucleic acid sequences were identified that coded for 94 different polypeptides. In total, six distinct OTUs were detected among the inferred *pmoA* amino acid sequences ($>$ 91% sequence identity threshold); only two were present in cDNA libraries (Fig. 6).

The most abundant *pmoA* sequences, distributed into three OTUs (OTU1–3), were from type I methanotrophs closely related to the *Methylobacter* genus. These sequences were dominant at all the depths tested (Fig. 7). OTU1, which clustered the majority of clones both from gDNA and cDNA libraries, was more closely related to *Methylobacter psychrophilus*, whereas the last two OTUs were readily distinguishable from the known *Methylobacter* sp. and formed a separate branch within this group, clustering with environmental clones (Fig. 6). *pmoA* transcripts were detected only for OTU1 and OTU2, suggesting that they were the predominant active groups in this ecosystem. Figure 8 showed, subsequently, that transcripts for each OTU had a distinct distribution profile along the water column, OTU2 being mainly expressed in the upper part of the water column up to 55 m depth and OTU1 being most actively present below 55 m depth. These findings could suggest that OTU2 activity would be more sensitive to O_2 deprivation than OTU1, with an almost total disappearance of this gas being observed around 60 m depth.

The last three OTUs (OTU4–6) were only retrieved from gDNA libraries. They were only marginal (i.e. in terms of the relative clone abundance). Their distribution along the water column was different (Fig. 7), suggesting a vertical shift in their assemblage that could be linked to CH_4 or O_2 concentration (Fig. 2). Both OTU4 (seven clones) and OTU5 (15 clones) were associated with members of the *Methylococcaceae*, the first being closely related to *Methylosarcina lacus* (AAG13081) in type I methanotrophs and the second to the clone group B6 (Pester *et al.*, 2004) in type X methanotrophs. Obviously, only OTU6 (five clones) was associated with type II *Methylocystaceae* methanotrophs and was closely related to *pmoA* from *Methylocystis parvus* (AAQ10310), with one of the deduced amino acid sequences (clone 20A2) sharing 100% amino acid sequence identity with the *M. parvus* sequence.

Methanotroph community analysis from the 16S rRNA gene biomarker

16S rRNA gene fragments were PCR amplified from gDNA extracted at two critical points of the water column, for example at 62 m depth corresponding to the oxic–anoxic interface and at 70 m depth in the anoxic water layer, in order to confirm the *pmoA* results. In summary, 135 clones were sequenced, which clustered into 42 distinct OTUs (threshold $\geq$97% sequence similarity). The relative abundance of dominant bacterial varied vertically, with *Beta-*, *Epsilon-* and *Gammaproteobacteria* highly abundant at the oxic–anoxic interface and both *Actinobacteria* and *Verrucomicrobia* increasing sharply with decreasing O_2 concentration at 70 m (Fig. 9). Most of the *Gammaproteobacteria* clones (79%) were related to type I methanotroph sequences, which were distributed into three distinct OTUs and matched most closely with the type strains of *M. psychrophilus* (97–99% similarity) and *Methylobacter tundripaludum* (95% similarity).

Discussion

Methanogenesis in Lake Pavin

In this study, we confirm that methanogens present in the water column of Lake Pavin are metabolically active (Figs 4 and 5). This finding is in agreement with Lopes' model (Institut de Physique du Globe de Paris, pers. commun.), which suggests that this compartment could contribute to 20% of the total CH_4 production. Although the same trend was described by Winfrey & Zeikus (1979) and Iversen *et al.* (1987) for Knaack Lake and Big Soda Lake, respectively, CH_4 produced by lakes seems mainly due to the CH_4 flux from the sediment. The absence of sulfate and nitrate in the

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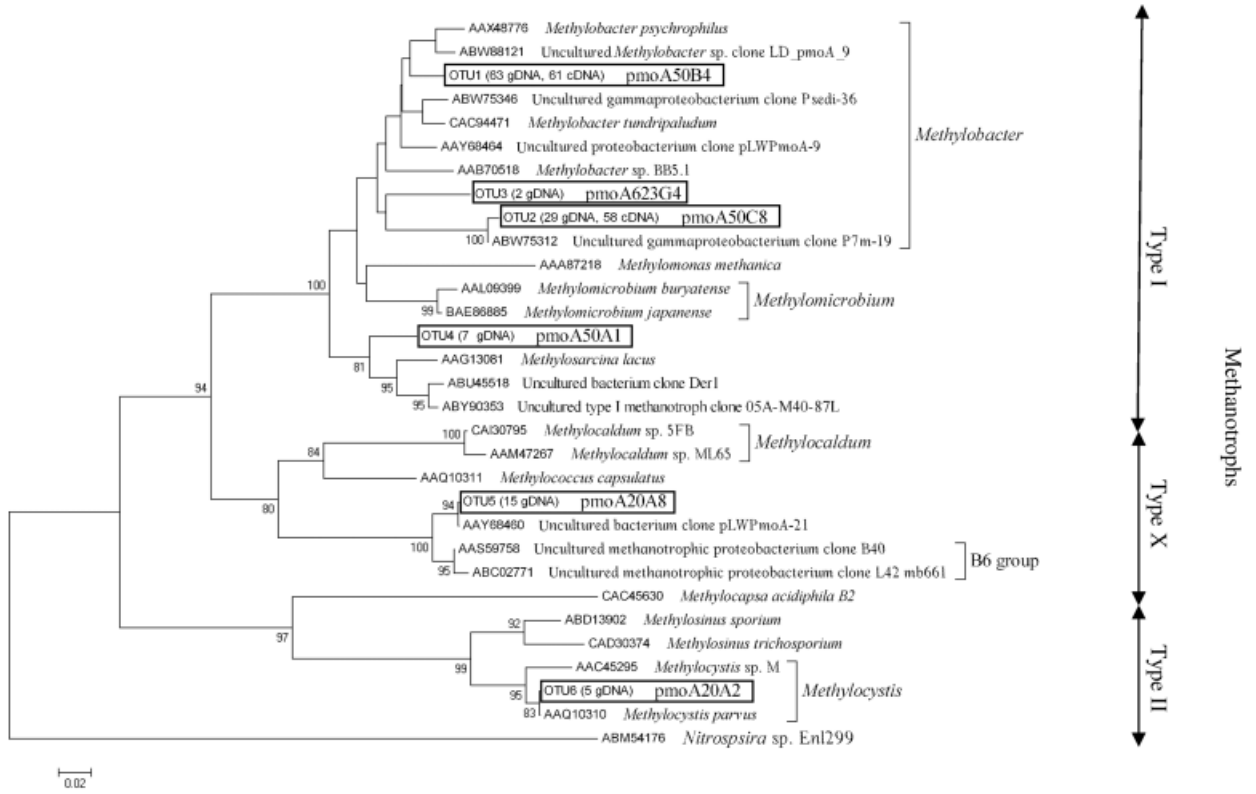

Fig. 6. Evolutionary distance tree showing the phylogenetic relationship of the deduced PmoA amino acid sequences including representative sequences derived from this study and sequences of isolates and uncultivated organisms. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 139 positions in the final dataset. Bootstrap values $>$ 70% derived from 1000 replicates are shown at nodes. The number of gDNA and cDNA clones assigned to each OTU is given in brackets, together with the name of the representative clone used in this study. *Nitrospira* sp. Enl299 sequence (ABM54176) was used as an outgroup. On the right, the name of the representative clone used for each phylotype is indicated.

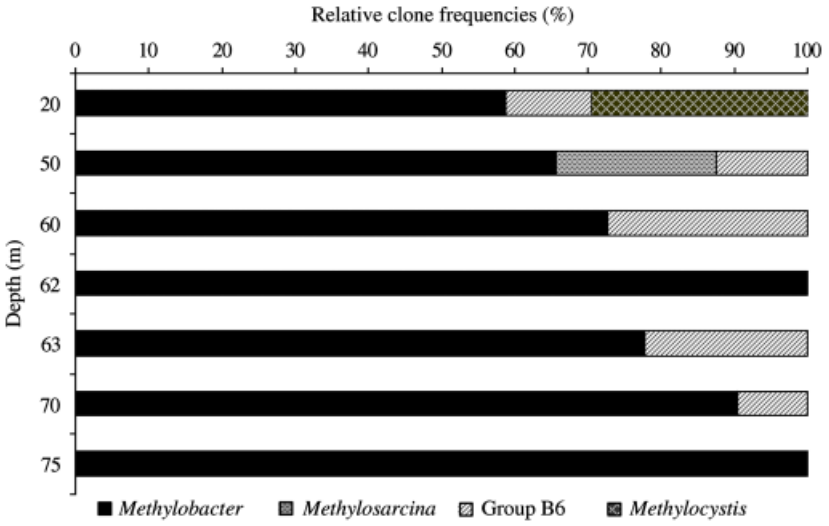

Fig. 7. Relative composition of *pmoA* clone libraries generated from gDNA samples at different depths along the water column of Lake Pavin. The sequences related to *Methylobacter* sp. and the sequences related to group B6 were broadly distributed through the water column, whereas *Methylocystis* and *Methylosarcina* sp. were specific to certain layers. The percentage of clones calculated for each depth is presented.

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bottom waters of Lake Pavin corroborates this assumption (Michard *et al.*, 1994).

Most of the *mcrA* OTUs identified in this study belonged to the *Methanomicrobiales* order. They were widely dominant in all the water samples tested and highly diverse compared with those belonging to the *Methanosarcinales* order. Additionally, as the closest relative of *Methanomicrobiales* sequences is the hydrogenotrophic species, *C.* Methanoregula boonei, it is likely that phylotypes present in Lake Pavin are hydrogenotrophic. However, this assumption remains unconfirmed (Fig. 3). The hydrogenotrophic methanogenesis could be, therefore, the main CH_4 production process in the water column. In contrast, in sediments, the number of clones related to *Methanomicrobiales* and *Methanosarcinales* was almost equivalent. As suggested by Schwarz *et al.* (2007), members of these two orders could be efficient syntrophic partners in the complete degradation of organic biomass in freshwater sediments.

Several studies report a greater involvement of hydrogenotrophic methanogens in CH_4 production as compared with acetoclastic methanogens (Schwarz *et al.*, 2007; Nettmann *et al.*, 2008). Although our study supports this observation, this contrasts with studies based on 16S rRNA gene that suggested that the acetoclastic methanogens dominated the water column and the sediment–water interface of Lake Pavin (Lehours *et al.*, 2005, 2007). This difference could be explained by a bias in the PCR where some sequences are amplified in preference to others. Bias in PCR has been observed when amplifying 16S rRNA genes where a single universal primer is used, leading to significant underestimation of true community diversity at all levels of taxonomic hierarchy (Jeon *et al.*, 2008; Hong *et al.*, 2009). The acetoclastic methanogenesis pathway is also supported by a study using carbon isotope fractionation of acetate (Whiticar, 1999). Goevert & Conrad (2008) showed, however, that the sulfate reducer *Desulfobacca acetoxidans*, which uses the acetyl-CoA pathway, had a similar fractionation as acetoclastic methanogens using the same pathway. Both types of microorganisms were identified in the water column of Lake Pavin (Biderre-Petit *et al.*, 2010). Thus, carbon isotope fractionation of acetate in Lake Pavin may also be strongly dependent on the sulfate-reducing community oxidizing acetate.

The third lineage with two OTUs formed a deep branch with the highest similarity to environmental *mcrA* sequences from various methanogen ecosystems. The species carrying these sequences are metabolically active, suggesting an involvement of this lineage in the CH_4 cycle. Although their closest relative is the anaerobic methane oxidizer ANME-1, no specific signature for this cluster was identified.

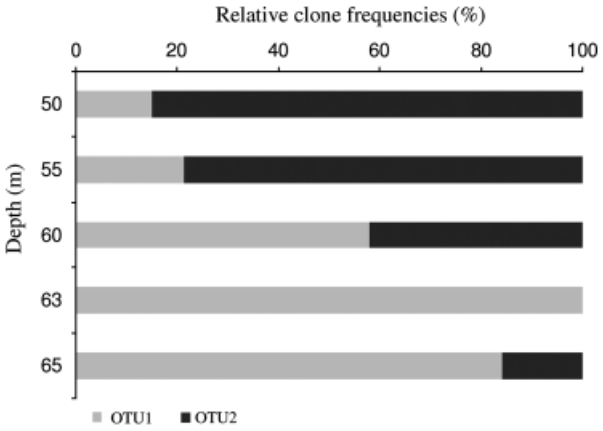

Fig. 8. Distribution pattern of transcripts along the water column for OTU1 and OTU2, both related to the *Methylobacter* genus. OTU2 was dominant up to 55 m and OTU1 from 55 to 65 m.

Aerobic methanotrophy in Lake Pavin

In this study, we show that aerobic methanotrophy occurs at the oxic–anoxic interface both with functional and with phylogenetic biomarkers. This finding is also in good agreement with Lopes' model (pers. commun.), which suggests that aerobic CH_4 oxidation is the main CH_4 sink in the water column of Lake Pavin.

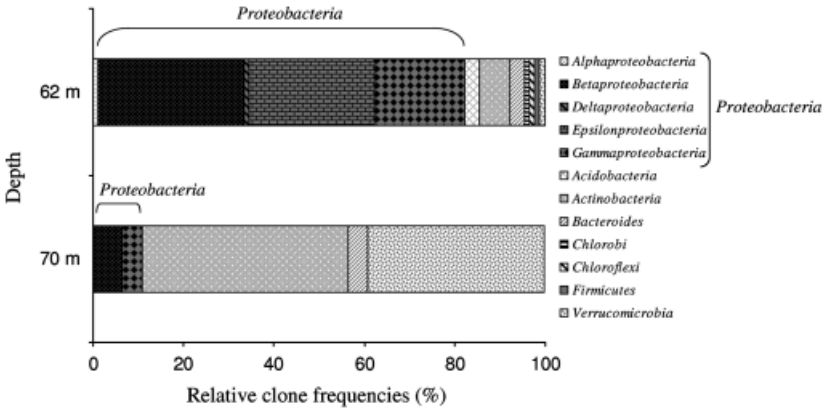

Fig. 9. Percentage contribution of 16S rRNA gene sequences in the water column of Lake Pavin assigned to the main bacterial phyla.

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The planktonic methanotroph assemblages investigated by *pmoA* gene analysis showed that the oxic zone of the water column lodges methanotrophic bacterial species that fall into type I, II and X methanotrophs (Fig. 6). Most of the clones obtained in this study were closely related to *Methylobacter* sp., particularly *M. psychrophilus*. This is consistent with results from different marker analyses in a wide variety of ecosystems (Wartiainen *et al.*, 2003; Pester *et al.*, 2004; Nercessian *et al.*, 2005; Rahalkar & Schink, 2007; Kojima *et al.*, 2009; Liebner *et al.*, 2009). These methanotrophs could be the key players in CH_4 oxidation regardless of selection pressure due to changes in O_2 and CH_4 concentrations. Moreover, in the present study, both 16S rRNA gene and *pmoA* biomarkers allowed the identification of three distinct phylotypes closely related to the genus *Methylobacter*: one more closely related to *M. psychrophilus* and two constituting a separate group within this cluster. These findings suggest that *Methylobacter* forms a microdiverse community at the species level in this ecosystem. These results were confirmed by the RT-PCR approach used to characterize *pmoA* gene expression. Only *Methylobacter pmoA* transcripts were detected in the water column up to 65 m. This suggests that it was the predominant active group in this ecosystem (Fig. 8). However, at 65 m, no O_2 could be measured, its concentration being below the detection limit ($< 1\,\mu M$) (Fig. 2). As *Methylobacter* sp. has not been conclusively demonstrated to adapt to anaerobic conditions, an explanation may be that the microaerophilic microorganisms, among which *Methylobacter* sp., could immediately consume the low amount of O_2 available in this zone by making it undetectable. Moreover, transcripts for OTU1 and OTU2 showed distinct distribution profiles along the water column, suggesting differences in their assemblage structure that could be correlated to substrate availability or changes in physicochemical parameters. This finding led us to speculate that the *Methylobacter* phylotype identified in the lower surface layer (OTU1) could be less sensitive to the O_2 concentration.

In terms of relative abundance, the number of sequences of *Methylosarcina* sp.-related type I methanotrophs, group B6-related type X methanotrophs and *Methylocystis*-related type II methanotrophs was only marginal. This is consistent with the results obtained by Rahalkar & Schink (2007) and Nercessian *et al.* (2005) from Lake Constance and Lake Washington, respectively. However, to date, the B6 group and *Methylosarcina*-related sequences were only described in freshwater sediments and not in the pelagic water column (Pester *et al.*, 2004; Nercessian *et al.*, 2005; Rahalkar & Schink, 2007). Concerning type II methane oxidizers, it is generally assumed that they thrive in environments with high CH_4 and low O_2 concentrations (Hanson & Hanson, 1996). This is challenged by our results and those of several recent studies (Knief & Dunfield, 2005; Mohanty *et al.*, 2006). In our study, *Methylocystis* sp. was only recovered at 20 m, a zone that is O_2 rich, but almost entirely CH_4 depleted. Therefore, this finding may reflect a limited activity of *Methylocystis* sp. in CH_4 oxidation in this ecosystem. Moreover, the distribution of all these phylotypes along the oxic water column did not appear to be random, with their disappearance at the same time as the decrease in the O_2 concentration (Fig. 7). Finally, unlike *Methylobacter*, no transcript was detected for these phylotypes. This suggests that either the *pmoA* genes of these methanotrophs were not expressed or that their transcripts were present at levels below the detection limit of RT-PCR. A third explanation could be that these populations used another carbon source such as methanol or acetate (Dunfield *et al.*, 2010; Belova *et al.*, 2011) and did not therefore express *pmoA*.

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