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ORIGINAL ARTICLE

Active methylotrophs in the sediments of Lonar Lake, a saline and alkaline ecosystem formed by meteor impact

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Lonar Lake is a unique saline and alkaline ecosystem formed by meteor impact in the Deccan basalts in India around 52 000 years ago. To investigate the role of methylotrophy in the cycling of carbon in this unusual environment, stable-isotope probing (SIP) was carried out using the one-carbon compounds methane, methanol and methylamine. Denaturing gradient gel electrophoresis fingerprinting analyses performed with heavy ^{13}C-labelled DNA retrieved from sediment microcosms confirmed the enrichment and labelling of active methylotrophic communities. Clone libraries were constructed using PCR primers targeting 16S rRNA genes and functional genes. *Methylomicrobium*, *Methylophaga* and *Bacillus* spp. were identified as the predominant active methylotrophs in methane, methanol and methylamine SIP microcosms, respectively. Absence of *mauA* gene amplification in the methylamine SIP heavy fraction also indicated that methylamine metabolism in Lonar Lake sediments may not be mediated by the methylamine dehydrogenase enzyme pathway. Many gene sequences retrieved in this study were not affiliated with extant methanotrophs or methylotrophs. These sequences may represent hitherto uncharacterized novel methylotrophs or heterotrophic organisms that may have been cross-feeding on methylotrophic metabolites or biomass. This study represents an essential first step towards understanding the relevance of methylotrophy in the soda lake sediments of an unusual impact crater structure.
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Introduction

Bacterial assimilation of the one-carbon (C_1) compounds methane, methanol and methylamine constitutes an important component of microbe-driven food web chains in many ecosystems. Methylotrophic bacteria, phylogenetically distributed across diverse phyla, contribute significantly towards the biogeochemical cycling of carbon by facilitating the incorporation of C_1 compound-derived carbon into biomass (Anthony, 1982; Chistoserdova *et al.*, 2009). The global cycling of methane and related C_1 compounds further affects important environmental phenomena related to climate change. Methanotrophs are a specialized group of methylotrophs that use methane as the sole carbon and energy source. These are distributed among *Gammaproteobacteria* (type I methanotrophs), *Alphaproteobacteria* (type II methanotrophs) (reviewed in Trotsenko and Murrell, 2008), filamentous methane oxidizers (Stoecker *et al.*, 2006; Vigliotta *et al.*, 2007) and *Verrucomicrobia* (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). Methanotrophs oxidize methane to methanol by the enzyme methane monooxygenase (MMO), present either as the particulate form (pMMO) in all characterized methanotrophs (except in the genus *Methylocella* (Dedysh *et al.*, 2000)) or as the soluble form (sMMO) in some methanotrophs (Trotsenko and Murrell, 2008). Methanol dehydrogenase (MDH) catalyzes the conversion of methanol to formaldehyde in methylotrophs (Trotsenko and Murrell, 2008, Chistoserdova *et al.*, 2009). Probes targeting *pmoA*, *mmoX* and *mxaF,* genes that encode the 27 kDa subunit of pMMO, the active site subunits of sMMO and MDH, respectively, have been widely used for the detection of methanotrophs/methylotrophs in environmental samples (reviewed in McDonald *et al.*, 2007). One pathway

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by which methylamine is used by methylotrophic bacteria contains methylamine dehydrogenase, but alternative pathways may also be present (Anthony, 1982; Latypova *et al*., 2009). Some of the marine methylotrophs involved in the metabolism of methylamine have been identified using PCR primers targeting the structural gene (*mauA*) encoding the small subunit of methylamine dehydrogenase (Neufeld *et al*., 2007c). Relatively few studies have focused on isolation of methylotrophs from saline and alkaline environments (Khmelenina *et al*., 1996; Sorokin *et al*., 2000; Doronina *et al*., 2001, 2003a, b; Kaluzhnaya *et al*., 2001), and the active organisms (Lin *et al*., 2004, 2005) and enzymes involved are poorly characterized.

Lonar crater (centred at 19°59′ N and 76°31′ E) is a simple, bowl-shaped, near-circular crater formed by meteor impact (Fredriksson *et al*., 1973) around 52 000 years ago (Sengupta *et al*., 1997) in the Deccan volcanic flood basalts in Maharashtra, India. Being the only well-preserved terrestrial crater to be formed entirely on basalt, it provides an excellent analogue for studying basaltic impact crater structures that are common on the surfaces of other terrestrial planets such as Mars (Hagerty and Newsom, 2003) and the Moon (Fudali *et al*., 1980). The crater has an average rim diameter of 1830 m and a rim-to-floor depth of about 150 m (apparent depth) (Fredriksson *et al*., 1973). A saline (NaCl ~0.9%) and alkaline lake (pH ~10) occupies most of the crater floor (Surakasi *et al*., 2007). Microbiological studies using culture-dependent and -independent strategies have identified and characterized both bacterial (Kanekar *et al*., 1999, 2002; Nilegaonkar *et al*., 2002; Wani *et al*., 2006; Joshi *et al*., 2008) and archaeal (Thakker and Ranade, 2002; Surakasi, 2007; Surakasi *et al*., 2007) communities in the Lonar Lake water and sediments. A culture-independent study that assessed archaeal diversity in the sediments reported that most of the retrieved euryarchaeotal sequences were related to methanogens (Wani *et al*., 2006). Enrichment of methanogens resulted in the isolation of *Methanosarcina*, *Methanocalculus* and *Methanoculleus* strains (Thakker and Ranade, 2002; Surakasi *et al*., 2007). However, no studies have focused on the identification of active methylotrophic bacteria in sediments of the Lonar crater lake.

DNA stable-isotope probing (DNA-SIP) can reveal phylogenetic identity of previously unknown and uncultivated organisms that are metabolically active in a particular ecosystem (Radajewski *et al*., 2000; Dumont and Murrell, 2005). DNA-SIP successfully identified active methanotrophs and methylotrophs in Transbaikal soda lake sediments (Lin *et al*., 2004), Washington fresh water lake sediments (Nercessian *et al*., 2005), Colne estuary sediments (Moussard *et al*., 2009) and alkaline soils (pH 9) from a Chinese coal mine (Han *et al*., 2009). To our knowledge, DNA-SIP experiments have not been used to characterize methylotrophs utilizing methanol and methylamine in soda lakes. In this study, we explore the diversity of active methylotrophic bacteria in saline and alkaline sediments of Lonar Lake by C_1 substrates-based DNA-SIP.

Materials and methods

Sediment sampling

Surface sediment samples (top 8–12 cm) were collected in October 2008 from Lonar Lake at a depth of 6 m. Samples were stored in sterile tubes in ice and transported to the laboratory within 24 h. The surface temperature of sediment samples was determined on site to be 27 °C. The pH values measured *in situ* and *ex situ* were 10.0 and 9.5 ± 0.2, respectively.

Analysis of sediment chemical parameters

The chemical parameters (TDS, TOC, TKN, total phosphorus as PO_4^{3-}, NO^{3-}, NaCl, CO_3^-, Cl^-, NH_3, SO_4^{2-}, Ca, Co, Ni, B, Mg, K, Fe and Cu) of wet sediment samples were analysed at a certified chemical testing laboratory (Accurate Analytical Laboratory Pvt. Limited, Pune, India) using standard methods (APHA, 1998).

Stable-isotope probing

Time-course SIP incubations were carried out in triplicate microcosms (two containing ^{13}C substrate and one containing ^{12}C substrate). Five grams of sediment were placed in sterile 120 ml serum vials, which were then sealed with butyl rubber stoppers and injected with $^{13}CH_4$ (99% ^{13}C atom enriched; Linde gases) to yield headspace concentrations of 1% (v/v). Similarly, labelled methanol- and methylamine-based microcosms were set up with 25 mM $^{13}CH_3OH$ (Cambridge Isotope Laboratories, Hook, UK) and $^{13}CH_3NH_2HCl$ (Sigma, Poole, Dorset, UK). Microcosms set up with ^{12}C substrates served as control for SIP incubations. Substrate uptake was not detected in methylamine SIP microcosms (data not shown). To facilitate the active utilization of the substrate, separate methylamine SIP incubations were supplemented with 10% modified nitrate mineral medium (Kaluzhnaya *et al*., 2001) containing ($g\,L^{-1}$): KNO_3, 0.5; NH_4Cl, 0.5; KH_2PO_4, 0.35; $Na_2HPO_4.12H_2O$, 0.65; NaCl, 7.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02. Added trace elements ($g\,L^{-1}$) were: disodium EDTA, 5; NaOH, 0.1; $ZnSO_4$. $6H_2O$, 0.1; $CaCl_2.2H_2O$, 0.073; $MnCl_2.5H_2O$, 0.025; $CoCl_2.6H_2O$, 0.005; $FeSO_4.7H_2O$, 0.075; $CuSO_4.5H_2O$, 0.002; and ammonium molybdate pentahydrate, 0.005. The pH was adjusted to 9.5 by the addition of 50 ml 2 M $NaHCO_3$ and 10 ml 1 M Na_2CO_3 to 1 l medium. All incubations were carried out in the dark at 28 °C. Methane and methanol consumption was measured by gas chromatography (Agilent, CA, USA). Methylamine consumption was quantified on the basis of the methods of Fearon (1942) and Ormsby and Johnson (1950). A volume of 3.5 ml of the solution under test was mixed with 0.25 ml 80 mM lactose

solution and 0.1 ml 5 M sodium hydroxide solution and incubated at 70 °C for 30 min. The solution was cooled to room temperature and allowed to incubate for a further 60 min. A_{545} was measured and the concentration of methylamine was derived on the basis of a millimolar extinction coefficient for the pigmented imine product of 1.26 $mM^{-1} cm^{-1}$. SIP incubations were terminated after the consumption of ≈100 μmol of $^{13}CH_4$ per gram sediment; ≈65 μmol of $^{13}CH_3OH$ per gram sediment; and ≈22 μmol of $^{13}CH_3NH_2$ per gram sediment.

Community DNA extraction and density gradient fractionation
After completion of SIP incubations with labelled CH_4, CH_3OH and CH_3NH_2, total community DNA was extracted from the respective sediment samples using a FastDNA SPIN Kit (Qbiogene Inc., Carlsbad, CA, USA). DNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer. DNA fractionation and precipitation were subsequently carried out as described previously (Neufeld *et al.*, 2007b). The buoyant density of each fraction was estimated by determining the refractive index (nD) of CsCl solutions with a digital refractometer (Reichert AR200, Reichert Inc., NY, USA).

PCR amplification of 16S rRNA and functional genes
Aliquots comprising ≈30 ng of ^{13}C or ^{12}C DNA pooled from microcosms representing each substrate were used as template in PCRs employing 16S rRNA and functional gene primers. Denaturing gradient gel electrophoresis (DGGE) and clone library analyses based on 16S rRNA genes were performed using PCR products amplified with primer sets GC341F/907R (Muyzer *et al.*, 1998) and 27F/1492R (Weisburg *et al.*, 1991), respectively. PCR amplifications were also carried out with primers specific for the functional genes *mxaF*, 1003f and 1555r (Neufeld *et al.*, 2007c); *pmoA*, A189f and mb661r (Costello and Lidstrom, 1999); *mmoX*, 206F and 886R (Hutchens *et al.*, 2004); and *mauA*, mauAf1 and mauAr1 (Neufeld *et al.*, 2007c). All PCR reactions were carried out in a total volume of 50 μl in 0.5 ml tubes. Each PCR mix consisted of 1.5 mM $MgCl_2$, 250 μM dNTPs, 50 pmol of each primer, 0.75 μl (3.75 U) Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada), 5 μl 10 × PCR buffer, 0.07% bovine serum albumin (BSA) and ≈30 ng DNA. With the exception of the PCR for *pmoA*, all reactions were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing (55 °C with 27F/1492R, GC341F/907R and 1003f/1555r; 60 °C with 206F/886R; and 48 °C with mauAf1/mauAr1) for 1 min and at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. For PCR with A189f/mb661r, the following touchdown conditions were used: 94 °C for 5 min, then 11 cycles of 1 min at 94 °C, 1 min at 62 °C (−1 °C per cycle for 10 cycles), 1 min at 72 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. All PCR products were checked for size and purity on 1% (w/v) agarose gels.

DGGE analysis of 'heavy' and 'light' DNA
PCR products generated from 'heavy' and 'light' DNA fractions after SIP were resolved by DGGE on an 8% acrylamide:bisacrylamide (37.5:1) gel with a denaturing gradient ranging from 30 to 70%. Denaturant of 100% is 7 M urea and 40% deionized formamide. Electrophoresis was carried out on a DCode universal mutation detection system (BioRad, Hercules, CA, USA) at 80 V for 16 h at 60 °C. The gel was run in 1 × TAE buffer and stained with Sybr Gold (Invitrogen, Paisley, UK). The most prominent bands from the DGGE gel were sequenced as previously described (Han *et al.*, 2009).

Construction of clone libraries for 16S rRNA and functional genes
PCR products were purified using the QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK), cloned into the pGEMT easy vector (Promega, Southampton, UK) and then transformed into *E. coli* JM109 (Promega) following the manufacturer's instructions. A total of 100 clones (from each 16S rRNA gene library) and 50 clones (from each functional gene library) were picked for direct colony PCR, with M13F/M13R primers targeting the flanking vector sequences. PCR products were run on agarose gels with DNA ladder to confirm the correct size of the cloned inserts, and subsequently purified by PEG-NaCl precipitation (Sambrook *et al.*, 1989) before sequencing.

DNA sequencing and phylogenetic analysis
Sequencing was performed on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the ABI Big-Dye version 3.1 sequencing kit as per the manufacturer's instructions, with both M13F and M13R primers for all functional gene library-based PCR products and with only M13F for 16S rRNA gene library-based products (partial sequencing). The generated sequences were analysed using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html) and compared with the current database of nucleotide sequences at GenBank and Ribosomal Database Project (RDP). Reference sequences were chosen on the basis of BLASTn similarities. All 16S rRNA gene sequences were checked for possible chimeric artefacts using the Pintail program (Ashelford *et al.*, 2006) in conjunction with Bellerophon (Huber *et al.*, 2004). Functional gene sequences were inspected for chimeras by BLASTn analysis. Multiple sequence alignments of 16S rRNA gene sequences were performed with Clustal W, Version 1.8 (Thompson

et al., 1994) and were edited manually using DAMBE (Xia and Xie, 2001) to obtain an unambiguous sequence alignment. Nucleotide distance matrices were constructed with DNADIST from PHYLIP version 3.61 (Felsenstein, 1989) using the Kimura two-parameter model (Kimura, 1980). OTUs were generated using the DOTUR program (Schloss and Handelsman, 2005) at 97% sequence similarity cutoff (for 16S rRNA gene sequences) and 94% sequence similarity cutoff (for functional gene sequences) with the furthest neighbour algorithm. A Bayesian method was used for the construction of phylogenetic tree. Before Bayesian inference analysis, a DNA substitution model for the complete data set was selected using MrModeltest2 (Posada and Crandall, 1998) and the Akaike information criterion (AIC). The model selected for the Bayesian approach for the phylogenetic tree was GTR + G with a log likelihood ratio (-lnL) = 2567.2607 and Akaike information criterion (AIC) = 5152.5215. The Markov chain Monte Carlo chains were started from a random tree and run for three million generations (MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003)). Trees were sampled every 100 generations and a consensus tree was built over all trees with the exclusion of the first 1200 trees (burn-in). Posterior probabilities were determined by constructing a 50% majority-rule tree of all trees sampled. Three separate runs were performed using the above parameters because the Bayesian approach is known to result in inflated levels of nodal support. 16S rRNA, *pmoA*, *mmoX* and *mxaF* gene sequences obtained in this study were deposited in GenBank under accession numbers GU363876–GU363923.

Results

The chemical properties of Lonar Lake sediment samples used in SIP incubations are presented in Supplementary Table S1 (See Supplementary Information). Methane, methanol and methylamine uptake rates of the sediments were calculated to be 3.3 μmol CH_4 day^{-1} g^{-1} wet sediment, 8.3 μmol CH_3OH day^{-1} g^{-1} wet sediment and 3 μmol CH_3NH_2 day^{-1} ml^{-1} sediment enrichment medium, respectively (Figure 1). Fractionation of sediment community DNA from labelled methane-, methanol- and methylamine-based SIP incubations yielded 'heavy' or ^{13}C fraction and 'light' or ^{12}C fraction with buoyant densities of 1.725 and 1.707 g ml^{-1}, respectively. The DGGE analyses of bacterial 16S rRNA gene PCR products (~606 bp) amplified from heavy and light DNA fractions were used to confirm the success of SIP incubations. Banding patterns associated with all of the heavy fractions were distinct from those of light fractions (Figure 2), implying assimilation of each of the labelled C_1 substrates by active methylotrophic populations in the sediment samples. Analysis of unlabelled (^{12}C) substrate controls further confirmed the enrichment of specific organisms in the ^{13}C-exposed samples (data not shown).

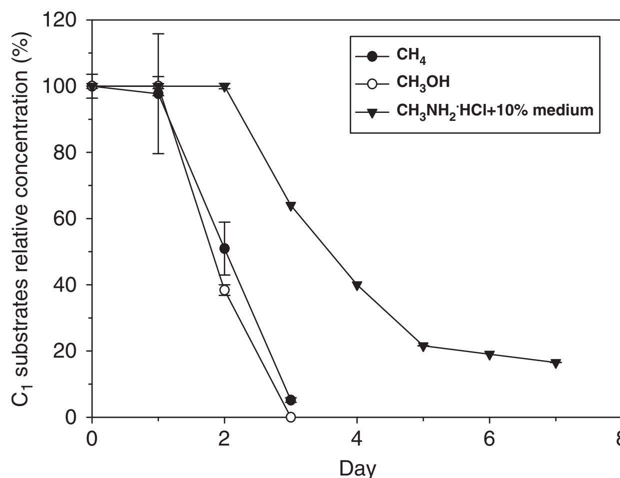


Figure 1 C_1 substrate utilization by Lonar Lake sediment samples. The values shown are the mean of triplicate microcosm experiments (two ^{13}C and one ^{12}C). Standard error bars are indicated. Methane microcosms were injected with 1.2 ml of CH_4 (10 080 ppmv); methanol microcosms were set up with 25 mM CH_3OH and methylamine microcosms were set up with 25 mM CH_3NH_2.HCl, in addition to 10% nutrient medium. All incubations were carried out in the dark at 28 °C. Substrate utilization rates were calculated by measuring the disappearance of each substrate during incubation.

Characterization of active methane utilizers

PCR product of the expected size (~1.4 kb) was obtained from the methane SIP heavy DNA fraction using universal bacterial 16S rRNA gene-specific primer set 27F/1492R. Cloning of PCR product and subsequent partial sequencing (~700 bp) of inserts generated 78 good-quality sequences. DOTUR analysis of the clone sequences identified 10 operational taxonomic units (OTUs) phylogenetically affiliated with *Gammaproteobacteria* (4 OTUs), *Betaproteobacteria* (1 OTU), *Deltaproteobacteria* (2 OTUs), *Firmicutes* (2 OTUs) and *Verrucomicrobia* (1 OTU) (Figure 3). BLASTn analysis showed OTUs CH_4_A9, CH_4_A7 and CH_4_A8 (representing around 57% of the library) to be most closely related (98% identity) to the 16S rRNA gene sequences of '*Methylomicrobium buryatense*' (AF096093), *Methylomicrobium japanense* (D89279) and an unpublished soda lake isolate *Methylomicrobium* sp. ML1 (DQ496231), respectively. The sequences of CH_4_A10 and CH_4_A6 (representing around 13% of the library) were related to the 16S rRNA genes of methylotrophs *Methylophaga* sp. AM3Q (EU001739; 96% identity) and *Methylophilus leisingeri* (AB193725; 92% identity), respectively. For CH_4_A4, CH_4_A3 and CH_4_A1 sequences, closest cultivated neighbours were *Paenibacillus* sp. xw-6-66 (FJ862051; 93% identity), *Symbiobacterium thermophilum* (AB004913; 90% identity) and *Kofleria flava* (AJ233944; 93% identity), respectively. The rest of the OTU sequences, CH_4_A5 and CH_4_A2, showed maximum affiliation with 16S rRNA genes of

uncultured representatives of *Verrucomicrobia* (AF454310; 93% identity) and *Deltaproteobacteria* (EU283460; 95% identity), respectively. From the 'heavy' fraction DGGE profile of methane SIP incubations, seven prominent bands (CH1–CH7), representing partial bacterial 16S rRNA gene products (~560 bp), were excised and sequenced (Figure 2). Sequencing DGGE bands yielded sequences that were also well represented in the 16S rRNA gene clone library (Figure 3).

Primer set A189f/mb661r amplified a 472 bp fragment of *pmoA* gene and primer set 206F/886R amplified a 719 bp fragment of *mmoX* from the 'heavy' DNA fraction of methane SIP experiment. The *pmoA* and *mmoX* gene-based clone libraries (~45 sequences from each) generated two OTUs (PM1 and PM2) and a singleton OTU (MM1), respectively. PM1 and PM2 sequences were most closely related to the *pmoA* genes of *Methylomicrobium japanense* (AB253367; 95% identity) and '*Methylomicrobium buryatense*' (AF307139; 91% identity), respectively. The MM1 sequence was most closely related to the *mmoX* gene of *Methylomicrobium japanense* (AB253366; 96% identity).

Characterization of active methanol utilizers

A total of 79 sequences were obtained with the 16S rRNA gene-based clone library and three prominent bands were sequenced after DGGE fingerprint analysis of the methanol SIP 'heavy' DNA fraction. One chimeric OTU was detected and removed from the clone library sequences. Of the 10 OTUs identified, five OTUs affiliated with *Gammaproteobacteria* and the rest affiliated with *Alphaproteobacteria*, *Deltaproteobacteria*, *Spirochaetes*, *Bacteroidetes* and *Actinobacteria* (Figure 3). The sequences of OTUs CH_3OH_B9 and CH_3OH_B10 (representing 81% of the library) and DGGE band OH1 showed maximum identity to the 16S rRNA genes of *Methylophaga* spp. (EU001739; NR_026313; 94% identity). DGGE band OH2 sequence and CH_3OH_B8 sequence from the library showed maximum identity of 98% to the 16S rRNA gene of *Methylomicrobium* sp. 4G (AF194539). The CH_3OH_B6 sequence shared 97% identity with the 16S rRNA gene of *Rhodobacter* sp. EL-50 (AJ605746). The CH_3OH_B1 sequence lacked cultivated affiliates in the database and was most closely related to the 16S rRNA gene of uncultured *Myxococcales* bacterium (AB265925; 93% identity).

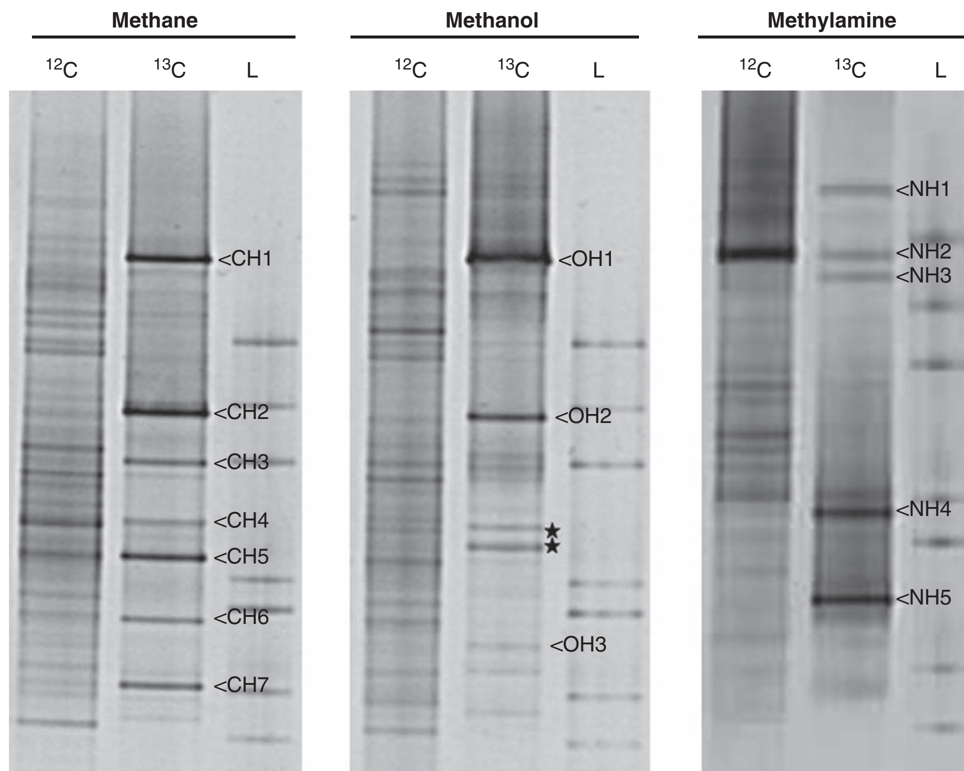
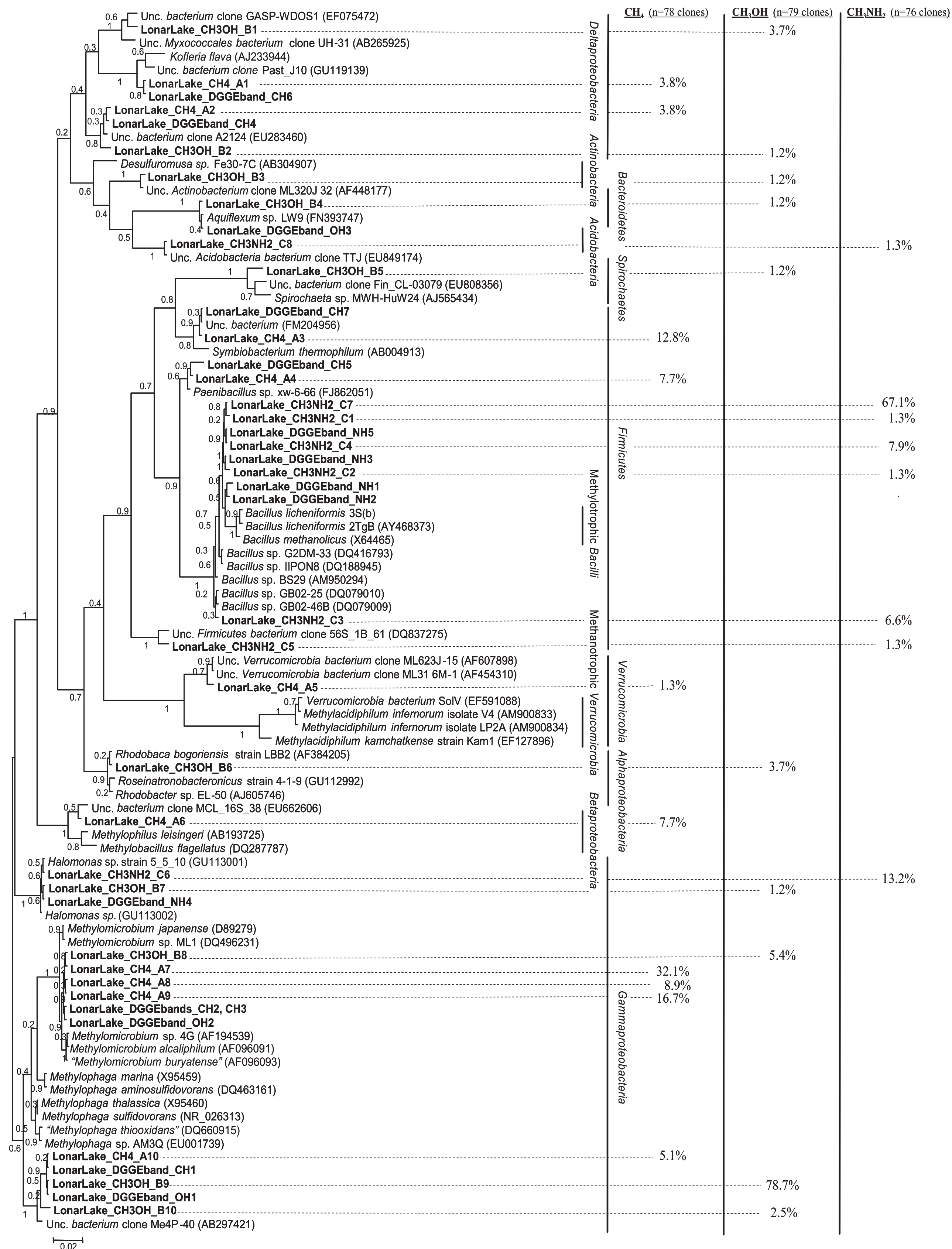


Figure 2 DGGE fingerprint profiles for ^{12}C (fraction 11) and ^{13}C DNA (fraction 7) from the ^{13}C-methane, methanol and methylamine SIP incubations. Bands that were successfully sequenced are indicated and those that failed are assigned a star. L indicates DGGE ladder.

Figure 3 Bayesian phylogenetic tree showing the relationship between 16S rRNA gene sequences recovered from clone libraries constructed with the 'heavy' DNA from ^{13}C-methane, methanol and methylamine SIP incubations and reference sequences obtained from the NCBI database. 16S rRNA gene sequences obtained from DGGE fingerprint profiles (indicated on Figure 2) are also included. One sequence per OTU is shown and GenBank accession numbers of reference sequences are given in brackets. Bayesian posterior probabilities (based on the mean of three separate analyses) are shown. The scale bar represents 2% substitution per site. The percentage values indicate the relative abundance of each OTU in the respective clone libraries.


CH4 (n=78 clones)
CH3OH (n=79 clones)
CH3NH2 (n=76 clones)
Unc. bacterium clone GASP-WDOS1 (EF075472)
LonarLake_CH3OH_B1
Unc. Myxococcales bacterium clone UH-31 (AB265925)
Kofleria flava (AJ233944)
Unc. bacterium clone Past_J10 (GU119139)
LonarLake_CH4_A1
LonarLake_DGGEband_CH6
LonarLake_CH4_A2
LonarLake_DGGEband_CH4
Unc. bacterium clone A2124 (EU283460)
LonarLake_CH3OH_B2
Desulfuromusa sp. Fe30-7C (AB304907)
LonarLake_CH3OH_B3
Unc. Actinobacterium clone ML320J 32 (AF448177)
LonarLake_CH3OH_B4
Aquiflexum sp. LW9 (FN393747)
LonarLake_DGGEband_OH3
LonarLake_CH3NH2_C8
Unc. Acidobacteria bacterium clone TTJ (EU849174)
LonarLake_CH3OH_B5
Unc. bacterium clone Fin_CL-03079 (EU808356)
Spirochaeta sp. MWH-HuW24 (AJ565434)
LonarLake_DGGEband_CH7
Unc. bacterium (FM204956)
LonarLake_CH4_A3
Symbiobacterium thermophilum (AB004913)
LonarLake_DGGEband_CH5
LonarLake_CH4_A4
Paenibacillus sp. xw-6-66 (FJ862051)
LonarLake_CH3NH2_C7
LonarLake_CH3NH2_C1
LonarLake_DGGEband_NH5
LonarLake_CH3NH2_C4
LonarLake_DGGEband_NH3
LonarLake_CH3NH2_C2
LonarLake_DGGEband_NH1
LonarLake_DGGEband_NH2
Bacillus licheniformis 3S(b)
Bacillus licheniformis 2TgB (AY468373)
Bacillus methanolicus (X64465)
Bacillus sp. G2DM-33 (DQ416793)
Bacillus sp. IIPON8 (DQ188945)
Bacillus sp. BS29 (AM950294)
Bacillus sp. GB02-25 (DQ079010)
Bacillus sp. GB02-46B (DQ079009)
LonarLake_CH3NH2_C3
Unc. Firmicutes bacterium clone 56S_1B_61 (DQ837275)
LonarLake_CH3NH2_C5
Unc. Verrucomicrobia bacterium clone ML623J-15 (AF607898)
Unc. Verrucomicrobia bacterium clone ML31 6M-1 (AF454310)
LonarLake_CH4_A5
Verrucomicrobia bacterium SolV (EF591088)
Methylacidiphilum infernorum isolate V4 (AM900833)
Methylacidiphilum infernorum isolate LP2A (AM900834)
Methylacidiphilum kamchatkense strain Kam1 (EF127896)
Rhodobaca bogoriensis strain LBB2 (AF384205)
LonarLake_CH3OH_B6
Roseinatronobacteronicus strain 4-1-9 (GU112992)
Rhodobacter sp. EL-50 (AJ605746)
Unc. bacterium clone MCL_16S_38 (EU662606)
LonarLake_CH4_A6
Methylophilus leisingeri (AB193725)
Methylobacillus flagellatus (DQ287787)
Halomonas sp. strain 5_5_10 (GU113001)
LonarLake_CH3NH2_C6
LonarLake_CH3OH_B7
LonarLake_DGGEband_NH4
Halomonas sp. (GU113002)
Methylomicrobium japanense (D89279)
Methylomicrobium sp. ML1 (DQ496231)
LonarLake_CH3OH_B8
LonarLake_CH4_A7
LonarLake_CH4_A8
LonarLake_CH4_A9
LonarLake_DGGEbands_CH2, CH3
LonarLake_DGGEband_OH2
Methylomicrobium sp. 4G (AF194539)
Methylomicrobium alcaliphilum (AF096091)
"Methylomicrobium buryatense" (AF096093)
Methylophaga marina (X95459)
Methylophaga aminosulfidovorans (DQ463161)
Methylophaga thalassica (X95460)
Methylophaga sulfidovorans (NR_026313)
"Methylophaga thiooxidans" (DQ660915)
Methylophaga sp. AM3Q (EU001739)
LonarLake_CH4_A10
LonarLake_DGGEband_CH1
LonarLake_CH3OH_B9
LonarLake_DGGEband_OH1
LonarLake_CH3OH_B10
Unc. bacterium clone Me4P-40 (AB297421)
0.02
Deltaproteobacteria
Actinobacteria
Acidobacteria
Spirochaetes
Methylotrophic Bacilli
Methanotrophic Verrucomicrobia
Betaproteobacteria
Bacteroidetes
Firmicutes
Verrucomicrobia
Alphaproteobacteria
Gammaproteobacteria
3.7%
3.8%
3.8%
1.2%
1.2%
1.2%
1.3%
1.2%
12.8%
7.7%
67.1%
1.3%
7.9%
1.3%
6.6%
1.3%
1.3%
3.7%
7.7%
13.2%
1.2%
5.4%
32.1%
8.9%
16.7%
5.1%
78.7%
2.5%

Primer set 1003f/1555r targeting *mxaF* yielded a PCR product of 552 bp when 'heavy' DNA from methanol SIP was used as template. The subsequent clone library constructed generated 45 good-quality sequences that grouped into two OTUs (MX1 and MX2). The MX1 and MX2 sequences were most closely related to the *mxaF* genes of *Methylomicrobium japanense* (AB432885; 92% identity) and *Methylophaga alcalica* (EU001862; 83% identity), respectively.

Characterization of active methylamine utilizers
No methylamine uptake was detected in the methylamine SIP incubations without added nutrients (data not shown). Consumption of labelled methylamine was initiated when the microcosm sediments were supplemented with 10% nitrate mineral salts medium modified on the basis of sediment chemical properties (Supplementary Table S1). Four DGGE band sequences (Figures 2 and 3) and 76 clone library sequences were obtained from the 'heavy' DNA fraction of methylamine SIP. The library sequences grouped into a total of eight OTUs, out of which six were associated with *Firmicutes* and two were associated with *Acidobacteria* and *Gammaproteobacteria* (Figure 3). The majority of the DGGE band sequences (NH1, NH2, NH3 and NH5) and OTU sequences (CH_3NH_2_C1, CH_3NH_2_C2, CH_3NH_2_C3, CH_3NH_2_C4 and CH_3NH_2_C7) representing over 84% of the library showed maximum identity of 96–98% to the 16S rRNA genes of extant *Bacillus* spp. (DQ188945, DQ416793, DQ079010, DQ079009 and AM950294) (Figure 3). Ten sequences representing OTU CH_3NH_2_C6 along with DGGE band NH4 sequence shared 100% identity with the 16S rRNA gene of *Halomonas* sp. (GU113002).

The primer set mauAf1/mauAr1 targeting *mauA* did not yield amplicons from the 'heavy' DNA fraction of methylamine SIP, despite the use of PCR additives such as BSA and successful amplification of appropriate positive controls (data not shown).

Discussion

Lonar Lake represents an extreme environment with high pH and moderate salinity. Iron and magnesium concentrations were particularly high (21.9 g kg^{-1} sediment and 10.9 g kg^{-1} sediment, respectively) (Supplementary Table S1). This may be due to the Fe- and Mg-rich composition of the basalt bed rock and to meteorite iron (Schoonen *et al.*, 2004). High total organic carbon (TOC) levels in Lonar Lake sediments could be attributed to the high primary productivity rates (up to 10 g m^{-1} per day) in soda lakes that often exceed other aquatic ecosystems (Jones *et al.*, 1998). Exceptionally high total P levels may be explained by the basaltic origin of the sediments and fertilizer runoff from agricultural fields close to the crater. The Lonar crater is the only known depression in the region and hence may serve as a drain for excess runoff from anthropogenically influenced surrounding areas. However, the contribution of such natural or anthropogenic factors towards elevated phosphate and nitrate levels in the lake sediments warrants further investigation. The detected level of carbonates was relatively low but analysing the extent of contribution of other natural components towards alkalinity was beyond the scope of this study. Lonar Lake water is green throughout the year because of dense cyanobacterial blooms dominated by *Arthrospira* (Surakasi *et al.*, unpublished). Decomposition of cyanobacterial biomass in soda lakes is likely to produce high quantities of methane, methanol, methylamine and dimethylsulfide (Jones *et al.*, 1998). Organisms in soda lakes intracellularly accumulate osmolytes such as betaine and dimethylsulfoniopropionate, and their degradation by methanogens (Zavarzin *et al.*, 1999) is likely to enrich the pool of methylated compounds. Methanotrophs and methylotrophs in such environments oxidize the C_1 compounds produced, returning carbon to the food web. Methane oxidation rates are at least two-fold higher than the rates of methane formation in some soda lakes of the southern Transbaikal region (Doronina *et al.*, 2003a). C_1 intermediates (methanol, formaldehyde and formate) excreted by methanotrophs might also drive alkaline methylotrophy (Trotsenko and Khelenina, 2002).

In this study, experiments with $^{13}CH_4$ identified phylotypes closely related to the type I methanotroph *Methylomicrobium* (Fuse *et al.*, 1998; Kaluzhnaya *et al.*, 2001; Eshinimaev *et al.*, unpublished). *Methylomicrobium* spp. have been isolated from several terrestrial and marine samples (Bowman *et al.*, 1993, 1995; Sieburth *et al.*, 1987; Fuse *et al.*, 1998) and soda lake sediments (Kalyuzhnaya *et al.*, 1999, 2008; Khmelenina *et al.*, 1997, 2000; Sorokin *et al.*, 2000; Kaluzhnaya *et al.*, 2001). Interestingly, the haloalkaliphilic/-tolerant *Methylomicrobium* isolates were resistant to heat and desiccation despite the absence of cysts (Kaluzhnaya *et al.*, 2001). Protection from such extreme conditions is mediated by the intracellular accumulation of the compatible solute ectoine (Khmelenina *et al.*, 1997, 2000; Trotsenko *et al.*, 2005). Methane-based DNA-SIP experiments with Transbaikal soda lake sediments identified the dominant methanotrophs as *Methylomicrobium* spp. (Lin *et al.*, 2004). *Methylomicrobium* spp. have also been detected in the sediments of a fresh water lake (Lake Washington) through reverse-transcription-PCR amplification of *pmoA* and *fae* transcripts (Nercessian *et al.*, 2005).

Methylotroph sequences related to *Methylophaga thalassica* and *Methylophilus* sp. were also retrieved in methane DNA-SIP experiments. These organisms are known to use methanol in saline and alkaline environments (reviewed in Trotsenko *et al.*, 2007) and may have cross-fed on methanol produced by ^{13}C-labelled methanotrophs. Some *Methylophaga*

strains exhibit high growth rates on methanol (De Zwart *et al.*, 1996) and this might have led to the rapid assimilation of labelled methanol by phylotypes related to *Methylophaga* spp. in the sediment microcosms. Surprisingly, ~29% of 16S rRNA gene clone library sequences and a number of DGGE band sequences had no phylogenetic affiliation with extant methanotrophs or methylotrophs. One explanation here would be the potential cross-feeding by these organisms on some labelled component from active methylotrophs. A relatively long incubation period (12 days) was necessary to permit sufficient incorporation of ^{13}C-methane and this may have led to enrichment of 'cross-feeders' (reviewed in Neufeld *et al.*, 2007a). The 16S rRNA gene sequences discussed above shared low phylogenetic identities with that of nearest cultivated neighbours in the database (90–93%). Therefore, it is difficult to determine whether these phylotypes have been labelled by cross-feeding or these sequences represent uncharacterized methanotrophs. One of them showed maximum identity to the 16S rRNA gene of an uncultured *Verrucomicrobium* clone obtained from alkaline Mono Lake (Humayoun *et al.*, unpublished). This sequence did not, however, cluster with that of thermo-acidophilic *Verrucomicrobia* methanotrophs (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008) (Figure 3).

16S rRNA gene sequences from methanol SIP experiments were dominated by sequences related to *Methylophaga* sp. retrieved from a marine methanol SIP study (Neufeld *et al.*, 2007c). Another possibly methylotrophic OTU (CH3OH_B10) was affiliated with the 16S rRNA gene sequence of *Methylophaga sulfidovorans*, a methylotroph isolated from a microbial mat using dimethylsulfide as substrate (De Zwart *et al.*, 1996). Sequences related to *Methylophaga alcalica*, a haloalkaliphilic methylotroph isolated from sediments of an East Mongolian soda lake (Doronina *et al.*, 2003b), were recovered from the *mxaF* clone library. *Methylophaga* are aerobic, moderately halophilic, non-methane using methylotrophs, mostly isolated from marine (Janvier *et al.*, 1985; Doronina *et al.*, 1997; Kim *et al.*, 2007) and soda lake ecosystems (Doronina *et al.*, 2003a, 2003b). 16S rRNA and *mxaF* gene sequences related to the alkaline environment isolate *Methylomicrobium* sp. 4G (Kaluzhnaya *et al.*, 2001) and *Methylomicrobium japanense*, respectively, were recovered from ^{13}C DNA. High methanol concentrations ranging from 5 to 7% v/v are known to support the growth of soda lake *Methylomicrobium* isolates (Kaluzhnaya *et al.*, 2001). The presence of *Rhodobacter*-related clone sequences is not surprising, as *Rhodobacter* spp. are capable of growth on methanol (Wilson *et al.*, 2008). The detection of a singleton OTU (CH_3OH_B7) clustering closely with *Halomonas* sp. (Figure 3) may be a result of cross-feeding, as some *Halomonas* strains are known to metabolize C_1 intermediates such as formaldehyde and formate (Azachi *et al.*, 1995). The rest of the OTUs related to *Aquiflexum* sp., *Spirochaeta* sp. and uncultured representatives of *Actinobacterium* and *Myxococcales* again did not affiliate with known methylotrophs.

Absence of PCR amplicons for *mauA* confirmed the findings of DGGE fingerprinting and clone library analysis, as none of the bacteria represented by the OTUs identified (Figure 3) are known to assimilate methylamine by the methylamine dehydrogenase pathway. Methylamine can be metabolized by other pathways containing methylamine–oxidase or methylamine–glutamate *N*-methyl-transferase (Anthony, 1982; Chistoserdova *et al.*, 2009; Latypova *et al.*, 2009). The majority of sequences in the methylamine SIP 16S rRNA gene library and DGGE fingerprint profile were related to *Bacillus* spp. (Figure 3). *Bacillus* strains growing on methylamine, methanol and dimethylsulfide have been characterized (Dijkhuizen *et al.*, 1988; Arfman *et al.*, 1989; Anesti *et al.*, 2005). Though the methylamine degradation pathway in the genus *Bacillus* is poorly characterized, all Gram-positive methylotrophs studied to date use the methylamine oxidase pathway (Chistoserdova *et al.*, 2009). Methylamine SIP sequences from our study clustered with the 16S rRNA gene sequences of both methylotrophic *Bacillus* strains (Arfman *et al.*, 1992; Anesti *et al.*, 2005) and strains isolated from contaminated soils (Desai *et al.*, 2009; Stobdan *et al.*, unpublished) and marine sediments (Dick *et al.*, 2006; Sass *et al.*, 2008) (Figure 3). This is the first SIP study to identify methylamine-utilizing *Bacillus* spp. directly from environmental samples. However, it may be noted here that these results may not entirely represent active participants in methylamine metabolism *in situ*. *Methylophylaceae* were implicated as active consumers of labelled methylamine in Lake Washington sediment microcosms (Nercessian *et al.*, 2005). Although *Methylophilus*-related 16S rRNA gene sequences were retrieved from our methane SIP heavy fraction, no such phylotypes were detected in the methylamine SIP microcosms. Methylamine SIP studies carried out with sea water (Neufeld *et al.*, 2007c) and estuarine sediments (Moussard *et al.*, 2009) identified *Methylophaga* spp. as the dominant methylamine utilizers. Clone library and DGGE band sequences closely related to *Methylophaga* spp. were recovered from our methane and methanol SIP heavy fractions, but were not detected in the methylamine SIP heavy fraction. This may be due to the lack of suitable microcosm conditions for *Methylophaga* spp. to utilize methylamine or due to distinct substrate preferences developed as a result of competition for C_1 substrates among bacterial communities of the extreme Lonar Lake environment.

In conclusion, SIP enabled the identification of *Methylomicrobium*, *Methylophaga* and *Bacillus* spp. as the predominant utilizers of methane, methanol and methylamine, respectively, in Lonar Lake sediments. We also detected a number of uncultured

organisms associated with C_1 metabolism and these data will assist the design of future culture-based studies to isolate novel methylotrophs from Lonar Lake.

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