Molecular diversity of methanogens and identification of *Methanolobus* sp. as active methylotrophic *Archaea* in Lonar crater lake sediments

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**Abstract**

Soda lakes constitute extreme aquatic ecosystems with remarkably high primary productivity rates, but information on the diversity and activity of methanogens in such environments is sparse. Using 16S rRNA and functional genes, we investigated the diversity of methanogens in the sediments of Lonar Lake, a unique saline and alkaline ecosystem formed by meteorite impact in the Deccan basalts. Although domain and phylum level 16S rRNA gene libraries were dominated by phylotypes related to *Halobacteriales*, sequences related to potentially novel *Archaea* within the orders *Methanosarcinales* and *Methanomicrobiales* were obtained together with a significant fraction of sequences representing uncultivated *Euryarchaeota* [Correction added after online publication 16 April 2012: orders ‘*Methanosarcina* and *Methanomicrobiaeae*’ changed to ‘*Methanosarcinales* and *Methanomicrobiales*’]. To identify the active methylotrophic *Archaea* involved in methanogenesis, mRNA transcripts of *mcrA* were retrieved from methanol consuming and methane emitting sediment microcosms at two different time points. Reverse-transcription PCR, qPCR, DGGE fingerprint, and clone library analysis showed that the active *Archaea* were closely related to *Methanolobus oregonensis*. To our knowledge, this is the first study identifying active methylotrophic methanogens in such an environment.

**Introduction**

Methanogenesis plays a pivotal role in the cycling of carbon in many ecosystems. The process is mediated by phylogenetically diverse methanogenic *Archaea* that span six orders of the phylum *Euryarchaeota* namely *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales*, and *Methanocellales*. Methanogens can be divided into three groups based on their catabolic pathway for methanogenesis: methylotrophs that utilize C1 compounds, acetoclasts that utilize acetate, and hydrogenotrophs that utilize H2:CO2 (Zinder, 1993). The terminal step of methanogenesis is catalyzed by the methyl coenzyme M reductase enzyme (Mcr). Primers targeting the gene encoding the α-subunit of the enzyme (*mcrA*) coupled with/independent of primers targeting 16S rRNA genes have been used to investigate methanogen diversity across several habitats such as peat (Hales et al., 1996; Juottonen et al., 2006), landfill (Luton et al., 2002), rice paddy soils (Ramakrishnan et al., 2001), aquatic sediments (Banning et al., 2005; Dhillon et al., 2005; Webster et al., 2006; Conrad et al., 2010), and mud volcanoes (Kormas et al., 2008; Lazar et al., 2011).

Soda lakes are among the most extreme aquatic environments on Earth and are characterized by high pH (9.0–12.0) and salinity (up to 35% w/v) (Grant, 2006). Owing to high temperatures, high light intensities, and unlimited supplies of CO2, the primary productivity rates in such lakes often exceed 10 g cm⁻² day⁻¹ (Melack & Kilham, 1974). Decomposition of biomass at the anoxic bottom layers of soda lakes can generate several substrates that favour methanogenesis (Grant, 2006). However, the identity and activity of methanogenic *Archaea* in saline and alkaline environments is poorly understood. Scholten et al. (2005) were unsuccessful in obtaining PCR amplification for the *mcrA* gene from Mono Lake water samples. Barring a few studies that cultivated methanogens from soda lakes (Boone et al., 1986; Mathrani et al., 1988; Liu et al., 1989), the diversity of methanogens in soda lakes was poorly understood.
et al., 1990; Kevbrin et al., 1997; Thakker & Ranade, 2002) and profiled rates of methanogenesis (Oremland et al., 1982a; Namsaraev et al., 1999), a detailed molecular survey of methanogen diversity in a soda lake environment, has not thus far been undertaken.

Lonar Lake, located at Buldhana district, India, is an extreme ecosystem characterized by high pH (≈ 10) and moderate salinity (NaCl ~ 1% w/v) (Surakasi et al., 2007). Situated at the centre of a ~ 52 000-year-old meteorite impact crater formed entirely on basalt (Fredriksson et al., 1973; Sengupta et al., 1997), the lake represents a unique geomicrobiological environment. Presently, the lake is separated from the impact breccia by an overlying sediment column of ~ 100 m and a water column of ~ 6 m (Louzada et al., 2008). A clone library prepared from the lake sediments using universal Archaeal 16S rRNA gene-specific primers retrieved euryarchaeotal sequences mostly related to methanogens (Wani et al., 2006). Previous culture-based studies have isolated Methanosarcina, Methanocalculus, and Methanoculleus spp. from Lonar Lake sediment enrichments supplemented with acetate, trimethylamine, and hydrogen plus carbon dioxide (Thakker & Ranade, 2002; Surakasi et al., 2007). The first representatives of obligately methylotrophic methanogens – Methanolobus tindarius and Methanoococcales methylutens – were isolated from coastal marine sediments (Konig & Stetter, 1982; Sowers & Ferry, 1983). Subsequently, many moderately or extremely halophilic obligate methylotrophs were isolated from various saline environments (Zhilina, 1983; Mathrani & Boone, 1985; Paterek & Smith, 1985). Obligately methylotrophic methanogens seem to have a distinct ecological advantage over other groups of methanogens as their absolute requirement for one carbon (C\textsubscript{1}) compounds such as methanol and methylated amines avoids competition with sulfate reducing bacteria (Oremland & Polcin, 1982; Oremland et al., 1982b). Methylotrophic methanogenesis has accounted for the bulk of methane produced from salt marsh sediments (Oremland & Polcin, 1982), and about half of the culturable methanogen population of salt marsh sediments is reported to consist of obligate methylotrophs (Franklin et al., 1988). Obligately methylotrophic methanogens belonging to the genera Methanolobus and Methanosaltsim have been isolated from several soda lake environments (Mathrani et al., 1988; Liu et al., 1990; Kevbrin et al., 1997), and methanogenesis from C\textsubscript{1} substrates was found to be the predominant form of methanogenesis in sediment slurries from Big Soda Lake, USA (Oremland et al., 1982a). The identity of active methylotrophic Archaea responsible for methanogenesis from C\textsubscript{1} substrates such as methanol has never been characterized in the Lonar Lake sediments or any other environment. Here, we explore the diversity of methanogenic archaea in Lonar Lake sediments by analyzing mcrA and 16S rRNA gene-based clone libraries constructed using multiple primer sets. The identity of active methylotrophic Archaea in the lake sediments was characterized by time-course-based retrieval and molecular analyses of mcrA mRNA transcripts from sediment microcosms.

### Materials and methods

#### Sediment DNA extraction and construction of 16S rRNA gene and mcrA gene clone libraries

Sediment samples (pH 9.88 ± 0.01) were collected from Lonar Lake (19°59′ N and 76°31′ E), India, in April, 2010. DNA was extracted from the samples using the PowerSoil DNA Isolation Kit (MoBio) as per manufacturer’s instructions. The quality of DNA obtained was analyzed on an agarose gel (1% w/v) and Nanodrop ND-1000. Subsequently, the sediment community DNA (~ 50 ng) was used as template in PCR employing 16S rRNA gene and mcrA gene-specific primer sets. For Archaeal 16S rRNA gene library construction, PCR was performed with the universal primer set 20F/915R (Stahl & Amann, 1991) according to conditions described previously (Mason et al., 2007); for Euryarchaeotal 16S rRNA gene library, PCR was performed with the primer set 21F/Eury498 (DeLong, 1992; Burggraf et al., 1994) according to conditions described previously (Steinberg & Regan, 2008). For the methanogen 16S rRNA gene library, PCR was performed with the primer set 335F/1068R according to conditions described previously (Banning et al., 2005); for mcrA gene library, PCR was performed with the ML primer set as described previously (Luton et al., 2002). All PCR reactions were performed in triplicate and were pooled, purified, cloned, sequenced, and analyzed as described previously (Antony et al., 2010). BLASTN similarities of all sequences were determined by NCBI nucleotide database search.

#### Phylogenetic analysis

All 16S rRNA gene sequences were checked for possible chimeric artifacts using the Pintail program (Ashelford et al., 2006). Functional gene sequences were inspected for chimeras by BLASTN analysis. Operational taxonomic units (OTUs) were generated by using the DOTUR program (Schloss & Handelsman, 2005) at 96% sequence similarity cut off (for 16S rRNA gene sequences) and 88% sequence similarity cut off (for mcrA gene sequences) as recommended by Steinberg & Regan (2008). Translated nucleotide sequences for McrA were edited and aligned with relevant sequences obtained from GenBank by using the CLUSTALW function of MEGA 4 (Tamura et al., 2007) and by visual
inspection to maximize sequence similarity. To estimate evolutionary distances between unambiguously aligned sequences, Poisson correction was used. By using these estimates, a bootstrap-supported (1000 re-samplings) phylogenetic tree was constructed from the resulting distance matrix by the NJ method with the MEGA 4 package.

Microcosm experiments

Five grams of the lake sediment was transferred under aseptic conditions to four sterile 120-mL serum bottles, crimp sealed with rubber stoppers and flushed with nitrogen to maintain anaerobic conditions. Methanol consumption and methane emissions were monitored daily on a gas chromatograph (GC). Incubations of duplicate microcosms were terminated at day 5 and day 9 by freezing at −80 °C to facilitate RNA recovery.

Sediment RNA extraction and reverse-transcription PCR

Sediment community RNA was extracted in duplicate using the PowerSoil Total RNA Isolation Kit (MoBio) according to manufacturer’s instructions. DNA was further treated twice with 1 unit RNase-free DNase I (Qiagen) followed by purification with the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The yield and quality of RNA obtained was analyzed on a Nanodrop ND-1000. The absence of carry-over DNA was confirmed by PCR using 16S rRNA gene and mcrA gene-specific primer sets. RNA from duplicate microcosms sampled at each time point was pooled for use as template in cDNA synthesis. cDNA of mcrA was prepared with the reverse ML primer using SuperScript II One-Step RT-PCR Kit (Invitrogen) according to the manufacturer’s instructions. The control reaction for cDNA synthesis consisted of a reaction mixture containing all the required reagents except the reverse transcriptase enzyme.

Denaturing gradient gel electrophoresis analysis

PCR amplicons of mcrA genes obtained from the sediment community DNA and cDNA were resolved by DGGE in a 8% acrylamide/bis acrylamide (37.5 : 1) gel with a denaturant gradient from 30% to 70%. (100% denaturant is 7 M urea and 40% deionized formamide). Electrophoresis was carried out on a DCode Universal Mutation Detection System (Bio-Rad) at 80 V for 16 h at 60 °C. The gel was run in 1× TAE buffer and stained with Sybr Gold (Invitrogen). The most prominent bands from the DGGE gel were sequenced as previously described (Han et al., 2009).

Realtime PCR

The conditions for the realtime PCR-based quantitation of mcrA transcript abundance were as described previously (Freitag et al., 2010).

Nucleotide sequence accession numbers

The sequences obtained in this study were deposited in the GenBank database with the following accession numbers: 16S rRNA gene sequences JN185017-JN185089 and mcrA gene sequences JN185004-JN185016.

Results and discussion

Archaeal 16S rRNA gene library

By employing a universal Archaeal 16S rRNA gene-specific primer set, the Archaeal community composition in the sediment DNA was determined. Forty-eight unique OTUs representing the orders Halobacteriales, Methanosarcinales, Methanomicrobiales, and the phylum Crenarchaeota (Fig. 1) were obtained from 319 sequences. Halobacteriales-related sequences constituted a major fraction (86.5%) of the library, which is in agreement with a recent report of Archaeal diversity in a similar soda lake in the Kenyan Rift valley (Mwirichia et al., 2010). BLASTN analysis showed seven sequences within the order Methanosarcinales to be most closely related (98% gene identity) to Methanoseta sp. (AJ133791), a phylotype detected in aquatic sediment enrichments (Zengler et al., 1999). Methanoseta spp. are distinguished from other Methanosarcinales by their obligate requirement for acetate as sole substrate for methane genesis and their rod-shaped cells (Ma et al., 2006). Two sequences showed maximum identity (96% gene identity) to Methanocalculus sp. AMF2 (HM053969), a haloalkaliphilic methanogen isolated from soda lakes in Kulunda Steppe, Russia (Sorokin & Muyzer, unpublished). Methanocalculus spp. are halotolerant hydrogenotrophic methanogens that have been isolated from variety of habitats such as oil wells, waste disposal sites, estuaries, and marine fish ponds (Lai et al., 2004). Methanocalculus-related sequences have also been retrieved earlier from Lonar Lake sediment enrichment cultures (Surakasi et al., 2007) as well as an Archaeal 16s rRNA gene-specific clone library (Wani et al., 2006). Around 10% of the library sequences affiliated with Euryarchaeota but lacked cultivated representatives in the database (Fig. 1).

Euryarchaeotal 16S rRNA gene library

Twenty-one unique OTUs were detected from 154 sequences, which mainly spanned across the orders
Halobacteriales, Methanosarcinales, Methanococcales, and Methanomicrobiales (Fig. 1). Halobacteriales-related clones were again found to dominate around 78% of the library sequences. Twenty-one 16S rRNA gene sequences affiliated with the Methanosarcinales order again showed maximum identity (98% gene identity) to the 16S rRNA gene of Methanosaeta sp. (AJ133791). A singleton OTU showed maximum phylogenetic identity of 94% to the 16S rRNA gene of Methanoculleus sp., a hydrogenotrophic methanogen detected in aquatic sediment enrichments (Zengler et al., 1999). Surakasi et al. (2007) also isolated Methanoculleus spp. from Lonar Lake sediment enrichment cultures. Two 16S rRNA gene sequences within the Methanococcales order showed very low phylogenetic identities (82%) to its nearest cultivated neighbor in the database – Methanococcus aeolicus (U39016), a coccoid hydrogenotrophic methanogen isolated from marine sediments (Keswani et al., 1996). A fraction of sequences again affiliated with uncultivated Euryarchaeota (Fig. 1).

**Methanogen 16S rRNA gene library**

Only four unique OTUs were retrieved from 147 sequences generated from the methanogen 16S rRNA gene library. Seventy-six sequences showed maximum identity (97% gene identity) to the 16S rRNA gene of Methanocalculus sp. LA2 (DQ987525), a haloalkaliphilic hydrogenotrophic methanogen cultivated earlier from Lonar Lake sediments (Surakasi et al., 2007). Sixty clone sequences closely affiliated (98% gene identity) with the 16S rRNA gene of Methanosaeta harundinacea (AY970347), an acetoclastic methanogen isolated from an upflow anaerobic sludge bed (UASB) reactor (Ma et al., 2006), and seven clone sequences affiliated (95% gene identity) with the 16S rRNA gene of Methanoculleus bourgensis (AY196674), a hydrogenotroph isolated from a tannery by-product enrichment culture (Ollivier et al., 1986). Just four sequences showed maximum identity (98% gene identity) to the 16S rRNA gene of Methanospirillum hungatei (AB517987), a hydrogenotrophic methanogen isolated from sewage sludge (Ferry et al., 1974).

Overall, the diverse OTUs captured by the three 16S rRNA gene-specific libraries differed in their relative abundances (Fig. 1), but this may be due to differences in the number of clones sequenced from each library and primer bias.

**mcrA gene library**

Eight unique OTUs were detected out of 136 sequences from the mcrA gene library. Seventy-five percent of the clone sequences showed 84% gene identity to the mcrA gene from Methanolinea sp. TNR (AB496719) (Figs 1 and 2), a novel methanogen isolated from rice paddy soil (Sakai et al., unpublished). The genus Methanolinea currently comprises of only one valid taxon – Methanolinea...
tarda, a hydrogenotrophic methanogen capable of growth on hydrogen and formate (Imachi et al., 2008). Sequences with low phylogenetic identity (73%) to Methanosarcina thermophila (AB353225) constituted the second major fraction of sequences in the library (Fig. 1). Five sequences within Methanosarcinaceae closely affiliated (92% and 96% gene identity) with mcrA from Methanoseta harundinacea (HQ188223) and Methanolobus oregonensis (U22242). Methanolobus oregonensis is a haloalkaliphilic obligately methylotrophic methanogen isolated from a saline aquifer near Alkali Lake, USA (Liu et al., 1990). The rest of the OTUs within Methanomicrobiales showed maximum identities of mcrA genes (77–87%) to those of Methanoculleus palmolii (AB300784), Methanospirillum lacunae (AB517988), Methanoculleus bourgensis (AB300785), Methanoculleus palmoli (AB300782) and Methanospirillum hungatei (AF313805). Methanoculleus bourgensis is a haloalkaliphilic obligately methylotrophic methanogen isolated from a saline aquifer near Alkali Lake, USA (Liu et al., 1990). The rest of the OTUs within Methanomicrobiales showed maximum identities of mcrA genes (77–87%) to those of Methanoculleus palmolii (AB300784), Methanospirillum lacunae (AB517988), Methanoculleus bourgensis (AB300785), Methanoculleus palmoli (AB300782) and Methanospirillum hungatei (AF313805). These results interestingly point towards the possible presence of novel methanogenic lineages within the Lonar Lake sediments (Figs 1 and 2).

Overall, the clone libraries recovered some unique OTUs that were not commonly shared and thus reiterated the need to use multiple primer sets while analyzing total prokaryotic diversity in environmental samples. High coverage values were obtained for all the libraries prepared (Archaeal 16S rRNA gene: 85%; Euryarchaeotal 16S rRNA gene: 86.4%; Methanogen 16S rRNA gene: 97.3%; mcrA: 94.1%). Rarefaction analysis of methanogen-specific 16S rRNA gene and mcrA gene libraries showed flattening curves (Supporting information, Fig. S1), further confirming that our sequencing efforts gave adequate coverage of major groups of methanogens in this environment.

**Characterization of active methylotrophic Archaea**

To identify active methylotrophic methanogens, Lonar Lake sediment microcosms were set up in duplicate and supplemented with ~27 mM methanol. Gas chromatography estimation of the headspace showed a reduction in methanol concentration to 22mM by day 5 and to 5mM by day 9. Corresponding methane amounts of 4.2 l mol (day 5) and 48.4 μmol (day 9) were produced during the incubation of these microcosms (Fig. 3). As we intended to specifically target methylotrophic methanogens, microcosm incubations were not continued after 9 days to avoid undesired cross-feeding or stimulation of hydrogenotrophic or acetoclastic methanogens in the sediments. mRNA was extracted from duplicate microcosms sampled at day 5 (total yield = 250 ng) and day 9 (total yield = 1.3 μg) and used for the RT-PCR amplification of mcrA genes. No mRNA could be extracted from non-enriched sediment samples (data not shown). Relative quantitation of mcrA transcript abundance by real time PCR showed the levels at day 9 to be 8.6-fold higher than that at day 5.
Fig. 3. Graph showing methanol uptake and methane emission rates for anaerobic Lonar Lake sediment microcosms incubated for 9 days at 30 °C. Filled squares and triangles indicate the methanol consumption and methane emission curves, respectively. The values shown are mean of duplicate microcosm measurements. Standard error bars are indicated. RNA extractions from sediment at day 5 and day 9 are indicated by a square and triangle on the x-axis, respectively.

at day 5, thereby confirming enhanced metabolic rates and/or enrichment of methylotrophic Archaea in sediment microcosms. DGGE fingerprinting and sequencing of prominent bands (B1 and B2) from the mcrA gene RT-PCR profiles of both the time points (Fig. S2) showed the active methanogens to be closely affiliated (96% mcrA gene identity) to M. oregonensis (U22242). Sequencing of 76 clones from a mcrA gene clone library prepared from pooled RT-PCR products of both time points also retrieved only sequences (CMCR10) related to M. oregonensis (Fig. 2). Methanolobus oregonensis is capable of utilizing methanol, trimethylamine, or dimethylsulfide as substrates for methanogenesis, but the growth rate on methanol was observed to be one-fourth of that on trimethylamine (Liu et al., 1990). However, no Methanolobus-related phylotypes were recovered from previous Lonar Lake sediment enrichment cultures supplemented with 20 mM trimethylamine. All the phylotypes retrieved were related to Methanoculleus spp. (Surakasi et al., 2007). The interesting possibility of niche differentiation among methylotrophic methanogens due to competition for C1 substrates in the extreme Lonar Lake environment needs further exploration. 16S rRNA gene sequences related to M. oregonensis have been retrieved by PCR-DGGE fingerprint analysis of methanethiol (MT) degrading communities in a UASB reactor (pH-10) inoculated with sediments from the Wadden Sea (Netherlands), Soap Lake (USA), and Russian soda lakes (van Leerdam et al., 2008). Methanolobus-related phylotypes in the Lonar Lake environment could be dependent on methanol and other C1 substrates that are derived from the decomposition of cyanobacterial mats that extend across surface layers of the water column (Surakasi et al., 2010) and/or dead plant matter (Warneke et al., 1999). A thick forest cover of tropical deciduous trees and shrubs surrounds the periphery of the lake. C1 compounds may also be produced by the degradation of osmolytes such as betaine and ectoine that are accumulated intracellularly by haloalkaliphilic bacteria (Jones et al., 1998). A novel haloalkaliphilic methylorophic bacterium accumulating up to 197 μg ectoine per mg cell dry wt has been recently isolated from the Lonar Lake sediments (Antony et al., 2011). Interestingly, phylotypes related to Methanolobus sp. were not detected in any of our 16S rRNA gene-based libraries. In silico analyses did not reveal any mismatches between the three primer sets used and the 16S rRNA gene sequences of Methanolobus spp. in the database (data not shown). It is likely that Methanolobus species are relatively low in abundance but play a significant role in methanogenesis from C1 compounds in Lonar Lake. Methylotrophic methanogenesis has been shown to be an important biogeochemical component of carbon cycling in soda lakes such as Big Soda Lake, USA (Oremland et al., 1982a). Our chemical analysis of Lonar Lake sediment samples revealed the concentration of sulfates to be ~10.4 mM (data not shown). Methylotrophic methanogenesis is known to be favoured over hydrogenotrophic and acetoclastic methanogenesis in sulfate containing sediments because of competition from sulfate reducing bacteria for hydrogen and/or acetate (Oremland & Polcin, 1982; Oremland et al., 1982b; King, 1984). Further studies employing more methylogenic substrates at varying concentrations are warranted to better understand the extent of methylotrophic methanogenesis and the ecophysiology of methylotrophic Archaea in Lonar Lake sediments.

In conclusion, we characterized the molecular diversity of methanogens in the Lonar Lake sediments. Although the diversity of active methylotrophic Archaea was rather low, the total methanogen diversity was characterized by several phylotypes that were not represented by sequences of extant methanogens in current databases. These findings will direct future culture-based strategies to isolate novel methanogens from the Lonar Lake. Importantly, studies need to be extended to other soda lakes across the globe to fully understand methanogenesis in saline and alkaline habitats.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Rarefaction curves inferred from DOTUR analysis of 16S rRNA gene and *mcrA* gene library sequences obtained from community DNA extracted from the Lonar Lake sediments.

**Fig. S2.** Sybr Gold stained gel image of the DGGE fingerprint profile for: (a) *mcrA* gene PCR amplicon from non-enriched sediment community DNA; (b) *mcrA* gene RT-PCR amplicon from mRNA transcripts extracted at day 5 of microcosm incubations and (c) *mcrA* gene RT-PCR amplicon from mRNA transcripts extracted at day 9 of microcosm incubations.

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