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Research Letter

Title: Identification of active aerobic methanotrophs in plateau wetlands using DNA stable isotope probing

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# **Abstract**

Sedge-dominated wetlands on the Qinghai-Tibetan Plateau are methane emission centers. Methanotrophs at these sites play a role in reducing methane emissions, but relatively little is known about the composition of active methanotrophs in these wetlands. Here we used DNA stable isotope probing (SIP) to identify the key active aerobic methanotrophs in three Sedge-dominated wetlands on the plateau. We found *Methylocystis* species were active in two peatlands, Hongyuan and Dangxiong. *Methylobacter* species were found to be active only in Dangxiong peat. Hongyuan peat had the highest methane oxidation rate and cross-feeding of carbon from methanotrophs to methylotrophic *Hyphomicrobium* species was observed.Owing to a low methane oxidation rate during the incubation, the labeling of methanotrophs in Maduo wetland samples was not detected. Our results indicate that there are large differences in the activity of methanotrophs in the wetlands of this region.

**Keywords:** active methanotrophs, *pmoA*, SIP, wetland

**Introduction**

The Qinghai-Tibetan Plateau is the Earth’s highest (average ~ 4500 m a.s.l.) plateau and has its unique geographical and ecological characteristics (Huddleston *et al*. 2003). Natural wetlands, which cover a large area on this plateau (about 13.3×l04 km2), are suggested to be the main sources of methane (CH4) emission (Ding and Cai, 2007). The annual CH4 emission in these wetlands was estimated to be 0.56-1 Tg (Jin *et al*. 1999) resulting from anaerobic respiration by methanogens. In addition to CH4 production, natural wetlands are important sites of CH4 consumption. Aerobic methanotrophs, at the anaerobic-aerobic interface of wetlands, consume CH4 before it reaches the atmosphere and are responsible for most of the biological CH4 oxidation (Murrell, 2010). Recently, anaerobic oxidation of methane (AOM) in natural wetlands is also reported to be a previously overlooked biological CH4 sink (Hu *et al*. 2014; Segarra *et al*. 2015). However, for wetland soils on Qinghai-Tibetan Plateau, neither CH4 consumption rates nor the identities of the functionally active methanotrophs have been well characterized.

Methanotrophs can use CH4 as a sole carbon and energy source. They belong to the phylum Proteobacteria (Bowman 2000), and recently have also been found within the Verrucomicrobia(Op den Camp *et al*. 2009) and the candidate phylum NC10 (Ettwig *et al*. 2010). Methanotrophic Proteobacteria are commonly designated type I (Gammaproteobacteria) and type II (Alphaproteobacteria) and methanotrophic Verrucomicrobia referred to as type III (Knief, 2015). The genera *Methylobacter, Methylomonas, Methylomicrobium, Methylosarcina*, *Methylococcus* and *Methylocaldum* are all typical type I methanotrophs. Type II methanotrophs are divided into type IIa (*Methylocystis and Methylosinus* in the family *Methylocystaceae*) and type IIb (*Methylocella,* *Methyloferula* and *Methylocapsa* in the family *Beijerinckiaceae*) (Lüke and Frenzel, 2011). The methane monooxygenase (MMO) enzyme converts CH4 to methanol, which is the key step in CH4 oxidation pathway (Hanson and Hanson, 1996). The well-conserved *pmoA* gene encoding a subunit of the particulate methane monooxygenase (pMMO) is a powerful functional marker for detecting methanotrophs (Dumont and Murrell 2005a).

Stable-isotope probing (SIP) can identify active consumers of a substrate within an environmental sample (Dumont and Murrell 2005b). 13CH4-DNA-SIP has been shown to be an effective method to identify functionally active methanotrophic populations in various environments, such as acidic forest soil (Bengtson *et al*. 2009), cave water (Hutchens *et al*. 2004), coal mine soil (Han *et al*. 2009), freshwater and marine sediments (Moussard *et al*. 2009; Dumont *et al*. 2011). By employing the 16S rRNA or *pmoA* genes as molecular markers, the diversity of active aerobic methanotrophs has been extensively studied in a large variety of natural wetlands (Chen *et al*. 2008a; Graef *et al*. 2011; Gupta *et al*. 2012; Kip *et al*. 2011; Morris *et al*. 2002); however, most of the studies were conducted in acidic peatlands at the high latitudes of the Northern Hemisphere, especially *Sphagnum*-covered peatlands (Kip *et al*. 2011) and *Calluna*-coveredwetlands (Chen *et al*. 2008a). In the present study we used DNA-SIP to identify the methanotrophs active in high altitude wetland soils on the Qinghai-Tibetan Plateau. The results indicated that each site is composed of unique assemblages of CH4-assimilating populations.

**Materials and methods**

## Site description and soil sampling

Two bogs, Dangxiong (DX, 4290 m a.s.l., pH 6.1) and Riganqiao/Hongyuan (HY, 3459 m a.s.l., pH 6.2), and a fen, Maduo (MD, 4229 m a.s.l., pH 8.4) were chosen for this study. These three wetlands were Sedge-dominated and located on the Qinghai-Tibetan Plateau. The description of soil properties of these wetlands has been previously published (Deng *et al*. 2014). Surface soil cores (0-5 cm in depth) were collected from two random hummocks in each of these wetlands in August 2011. Soils were stored in the laboratory at 4°C for one month until SIP incubations were initiated.

## 13CH4 labeling and gradient separation

For DNA-SIP, 10 g soils from two replicates of each wetland were mixed with 10 ml sterile deionized water and incubated in 120-ml serum vials capped with black butyl stoppers. The labeled (>99.9% [13C]-CH4) or unlabeled CH4 was injected into each vial. The volume of CH4 added represented 10% of the vial headspace. Soils were incubated on a shaker (100 rpm) in the dark at 25°C (average air temperature at sampling time) for 5 days. Headspace CH4 was measured daily using a gas chromatograph with a flame ionization detector.

DNA was extracted immediately after the incubation using NucleoSpin® Soil kit (Macherey-Nagel). The purified DNA was centrifuged in CsCl gradients (Neufeld *et al*. 2007) with an average density of 1.725 g ml-1 prepared by mixing 4.8 ml of a 1.85 g ml-1 CsCl stock solution with 1 ml of gradient buffer (GB, 100 mM Tris-HCl, pH 8.0; 100 mM KCl; 1 mM EDTA), including up to 5 µg DNA. Density gradient centrifugation was performed in 6-ml polyallomer UltraCrimp tubes in a TV865 vertical rotor (Sorvall). Centrifugation conditions were 20°C and >36 h at 177 000 × g(av). The heavy and light DNA fractions were retrieved from the gradient as described previously (Dumont *et al*. 2011). The density of each collected fraction was determined using an AR200 refractometer (Reichert). DNA was precipitated from CsCl gradient fractions with polyethylene glycol (Neufeld *et al*. 2007) and dissolved in 30 µl of elution buffer (Qiagen).

## Real-time PCR, T-RFLP and clone analysis of gradient fractions

A189f-mb661r primers were used to quantify the *pmoA* gene copies in different DNA gradient fractions (Kolb *et al*. 2003). Quantitative PCR was carried out with SYBR Green JumpStart Taq ReadyMix (Sigma) with an iCycler instrument (Bio-Rad).

Terminal restriction length polymorphism (T-RFLP) of bacterial 16S rRNA genes was performed from the heavy andlight DNA fractions using primers 27F-FAM and 907r (Lueders *et al*. 2004). PCRs were performed in a total volume of 50 μl and contained 10 × PCR Buffer (Peqlab), 0.2 mM dNTPs (Fermentas), 0.5 μM each primer, 10 μg of BSA, 5 U of Taq polymerase (Peqlab) and 1 μl of template. PCR was performed with an initial DNA denaturation step (94°C, 3 min), followed by 32 cycles of denaturation (94°C, 30 s), annealing (52°C, 45 s), elongation (72°C, 1 min) and a final extension at 72°C for 10 min performed on a Eppendorf Mastercycler instrument. PCR products were then puriﬁed using GenElute PCR Clean-up Kit (Sigma). Approximately 1 μg of PCR product was incubated for 30 min at 30°C with 5 U of mung bean nuclease (New England Biolabs) and 10 μl of 10 × reaction buffer in a total volume of 100 μl. The digestions were also purified using GenElute PCR Clean-up Kit (Sigma). Approximately 100 ng purified product was digested in a 10-µl volume containing 1 µl Tango Buffer with BSA (Fermentas) and 5 units of *Msp*I enzyme (Fermentas) and incubated at 37°C overnight. Reactions were subsequently desalted using SigmaSpin™ Post-Reaction Clean-up Columns (Sigma) and 3 µl of the desalted fragments were mixed with 11 µl of Hi-Di™ Formamide (Applied Biosystems), 0.3 µl of DNA fragment length standard (MapMarker 1000 + 30, 40; Bioventures), denatured for 3 min at 94°C and cooled on ice. The purified terminal restriction fragments were separated using a 3130 Genetic Analyzer (Applied Biosystems).

The taxonomy of the labeled methanotrophs present in DX and HY peatlands was determined by the 16S rRNA gene clone libraries constructed from the heavy fractions (ca. 1.75 g ml-1). 16S rRNA fragments were targeted with primers 27F and 907r (Lueders *et al*. 2004) using the same PCR program described above for T-RFLP. PCR products were ligated into the pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109 competent cells according to the manufacturer’s instructions. 16S rRNA clone sequences were aligned against the SILVA ref database (Pruesse *et al*. 2007) and checked manually. A Neighbor-joining phylogenetic tree was constructed in ARB (Ludwig *et al*. 2004). All DNA sequences obtained were deposited in Genbank under accession numbers KT793042-KT793125.

***Statistical analysis***

Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarities of T-RFLP community profiles were generated in the R package phyloseq 1.14.0 (McMurdie and Holmes, 2013). Analyses of similarity (ANOSIM) were performed to test whether the differences between various fractions and wetlands in the NMDS ordinations were statistically significant (Clarke 1993). Significant differences of methanotrophic abundance between different wetlands were further evaluated by ANOVA followed by Tukey’s HSD post hoc test. All statistical analyses and NMDS plot were implemented in *R* (version 3.2.3 for MacOS).

**Results**

Using *pmoA* gene copies as a proxy, the abundance of methanotrophs differed between the three sites: HY 1.49±0.21×108, DX 8.85±3.89×106 and MD 2.04±0.8×105 copies g-1 fresh soil. Methanotrophic abundance in HY wetland was significantly higher than DX and MD wetlands (P<0.001). SIP experiments were performed to detect the active methanotrophs in each wetland soil. CH4 consumption was detected immediately, with highest rates in HY and lowest in MD (Figure 1). Incubation of DX and HY soils with 13CH4 resulted in the appearance of ‘heavy’ *pmoA* genes in both replications (density > 1.74 g ml-1); however, the MD soils with 13CH4 incubation only showed a peak in the light fraction (density < 1.74 g ml-1) indicating that the methanotrophs were not labeled (Figure 2).

NMDS of 16S rRNA T-RFLP profiles was used to visualize differences in bacterial community structure between the different wetland soils and the effect of labeling (Figure 3). NMDS clearly showed a separation between heavy fractions of DX soils and those of HY soils. ANOSIM statistics also indicated significant differences between them (R=0.7604, P=0.033); however, no significant difference was detected between the light fractions of HY and DX soils (R=0.2292, P=0.177). Within HY soils, there was an obvious separation (R=0.9127, P=0.006) between communities in heavy and light/control fractions. Similar separation was observed in DX soils (R=0.8452, P=0.004), but the separation between MD soils was less evident (R=0.2421, P=0.079).

In total, 43 16S rRNA gene clones obtained from heavy fractions of DX and 37 from HY were analyzed. Among them, 32/43 clones from DX and 11/37 from HY peat were closely related to sequences of cultivated methanotrophs and these sequences were in good agreement with the T-RFLP profiles in the heavy fractions (Figure 4). The proportion of non-methanotroph sequences was greater in HY peat, including organisms belonging to various Proteobacteria, Actinobacteria and Acidobacteria. Relatively high (12%) and low (3%) relative abundances of *Hyphomicrobium*-related sequences (436 bp T-RF, Figure 4) were found in HY and DX soils, respectively.

Thirty percent of the 16S rRNA clone sequences from DX soils were most closely related to the type I methanotroph *Methylobacter tundripaludum* (Figure 5), a species originally isolated from an Arctic wetland in Norway (Wartiainen *et al*. 2006). This relatively high proportion of labeled *Methylobacter* in DX peatwas evident from the high abundance of a 442 bp T-RF in heavy fractions (Figure 4). In contrast, only one *Methylobacter* 16S rRNA clone sequence wasdetected in the HY peat and no corresponding T-RFs were identified in the heavy fractions. In addition to *Methylobacter*,another two sequences were obtained from DX soils that were most closely related to *Methylovulum* (Figure 5).

16S rRNA genes from type II methanotrophs made up 40% and 27% of the 16S rRNA genes retrieved from heavy DNA fractions from DNA SIP experiments with samples from DX and HY wetlands respectively (Figure 5). Most of these 16S rRNA gene clones (26/27) clustered together with *Methylocystis* and *Methylosinus*-like sequences. The analysis of the sequence data allowed the assignment of the 143 bp, 146 bp and 147 bp 16S rRNA T-RFLP fragments to *Methylocystis* and *Methylosinus*-like clusters (Figure 5). The relative abundance of these T-RFs in light and control fractions was not high, indicating that their elevated abundance was not an artifact (Figure 4). T-RFLP profiles from gradient DNA fractions for MD soil incubated with 13CH4 was performed and no significant community differences were obtained from heavy and light DNA fractions, confirming that detectable labeling of this soil did not occur (Figure S1).

**Discussion**

In this work, we used DNA-SIP to detect the active methanotrophs that consumed CH4 in wetlands on the Qinghai-Tibetan Plateau. The soils exhibited different CH4 oxidation rates (Figure 1), which were consistent with the copy number of *pmoA* genes detected in each soil. The relatively low *pmoA* gene abundance and CH4 oxidation rate of MD soil compared with the others indicates that methanotrophs are not highly active in this soil. MD soil had 2.04±0.8×105 *pmoA* gene copies per gram fresh soil, which is ~50-fold lower than DX and ~750-fold lower than HY. Methanotrophs in MD soil may have required a longer incubation with 13CH4 to be detected by SIP.

NMDS analysis revealed a different bacterial community structure in the heavy DNA fractions of HY and DX soils. Although both of these are peatlands, HY has higher soil organic carbon and total nitrogen (390±14 g kg-1, 13.9±1.9 g kg-1) than DX (134±7 g kg-1, 7.6±2.3 g kg-1) (Deng *et al*. 2014), which provide different conditions for the microbial communities. The clear separation of heavy and light fractions compared with the controls indicated the successful 13C-labeling without a severe effect of DNA G+C content on the results. The NMDS also showed that labeling was not detected with MD soil, which might be owing to the relatively low content of soil organic carbon and total nitrogen (38±7 g kg-1, 3.2±0.3 g kg-1).

The observation that the methanotroph T-RFs (143bp, 146bp and 147bp) increased in the heavy DNA fractions (Figure 4) was corroborated with the high abundance of the *Methylocystis* and *Methylosinus*-like methanotrophs in the clone libraries (Figure 5). This is in agreement with our previous studies showing *Methylocystis* sp. are the dominant methanotrophs in the original HY and DX peat soils (Deng *et al*. 2013; Deng *et al*. 2014). In addition, *Methylocystis* sp. were also found to be dominant in six of seven studied European peatlands (Chen *et al*. 2008b) and were detected as active methanotrophs in the peatlands of England, North and South America (Chen *et al*. 2008b; Gupta *et al*. 2012; Kip *et al*. 2012). *Methylocystis* members are capable of forming resting cells, surviving on multicarbon compounds and using CH4 at both high and low concentrations (Ho *et al*. 2013). These abilities will likely help them survive different conditions and may explain their high activity and abundance in the Qinghai-Tibetan peat and other peat ecosystems.

*Methylobacter* were found to be active in DX and much less active in HY soils. The ecophysiology of methanotrophs that could explain this difference is not clear. A difference in temperature optimum is one possibility. *Methylobacter* species have been reported to prefer cold environments such as the active layer of arctic permafrost (Liebner *et al*. 2009), high arctic wetlands (Graef *et al*. 2011) and lake sediments (He *et al*. 2012; Oshkin *et al*. 2015). Compared with HY peat, the DX site is located at higher altitude and has lower air and ground temperatures.

SIP also has the potential to show cross-feeding of carbon from methanotrophs to other soil microorganisms. Previous studies have demonstrated that methanotrophs can supply carbon sources for other non-methanotrophic organisms (Hutchens *et al*. 2004; Qiu *et al*. 2009; Oshkin *et al*. 2015). The first two steps in the oxidation of CH4 by methanotrophs are CH4 to methanol, followed by methanol to formaldehyde. *Hyphomicrobium* could potentially remove excess methanol and formaldehyde in the vicinity of active methanotroph cells, preventing a toxic accumulation that could inhibit methanotrophic activity (Morris *et al*. 2013). The detection of a high abundance of *Hyphomicrobium* in HY peat labeled with 13CH4 suggests the cross-feeding between methanotrophs and methylotrophs within this microcosm.

Other putatively non-methanotrophic organisms in the phylum Actinobacteria and Acidobacteria were also detected in the heavy fractions. Actinobacteria were previously shown to be abundant in wetlands on the Qinghai-Tibetan Plateau (Deng *et al*. 2014). Sequences of Acidobacteria were previously obtained from a 13C-methanol labeled acidic soils (Radajewski *et al*. 2002); however, until now there is only limited evidence of methylotrophic growth among the Acidobacteria (Pankratov *et al*. 2008). One possible explanation for labeling on one-carbon compound might be by growth on slime-producing methylotrophs (Pankratov *et al*. 2008). The labeling of these non-methanotrophic organisms suggests there is a cooperating functional group of both methylotrophic and non-methylotrophic microorganisms involved in methane consumption (Oshkin *et al*. 2015). More research is needed to investigate the ecological significance of carbon cross-feeding and the factors regulating the extent to which it occurs in various habitats.

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**Conflict of interest:** None declared.

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**Figure Legends**

**Figure 1**. CH4 concentrations in the headspace of bottles with DX, HY and MD wetland soils during the SIP labeling. Error bars represent standard deviations (n=3).

**Figure 2**. Relative *pmoA* gene abundances of both 13CH4 and unlabeled (control)CH4 incubations recovered from CsCl gradient fractions. The top panel corresponds to the gradients containing nucleic acids from incubations of DX soils, the middle panel of HY soils and the lower panel of MD soils. Quantities were determined by qPCR for *pmoA* genes. The *y*-axis indicates the relative abundance at each gradient fraction, with the total quantity detected from a gradient equal to 1.0. Dashed lines indicate the boundaries between what were considered ‘light’ and ‘heavy’ fractions.

**Figure 3**. Nonmetric multidimensional scaling (NMDS) plot (Bray-Curtis distance matrix) of 16S rRNA T-RFLP data reflecting bacterial community structure. Each community profile is labeled with the corresponding wetland name with heavy, light or unlabeled control fraction.

**Figure 4**. Bacterial 16S rRNA gene T-RFLP analysis of DNA recovered from CsCl gradients. The density is listed on the right of each panel. Densities between 1.740 and 1.762 g ml-1 correspond to heavy fractions (labeled DNA) and density of 1.73 g ml-1 correspond to light fractions (unlabeled DNA). The top two panels represent two replications (DX-1 and DX-2) of 13C-labeled DX soils and the panels below them are the unlabeled controls of DX (DX-CK). The results of HY 13C-labeled soils (HY-1 and HY-2) and the corresponding controls (HY-CK) are shown on the lower half of the figure. The number legend indicates the T-RF length (bp), from which some representative sequences can be found in the 16S rRNA gene phylogenetic tree (Figure 5).

**Figure 5**. Neighbor-joining tree illustrating 16S rRNA gene sequences amplified from heavy (13C-DNA) gradient fractions. Sequences were ~845 bp excluding the primer regions. Short sequences (<844 bp) were added to the tree by maximum-parsimony. Clones obtained from DX and HY wetlands are prefixed ‘HY-’ and ‘DX-’, respectively. The numbers after the sequences indicate their T-RF sizes.

**Figure S1**. Bacterial 16S rRNA gene T-RFLP analysis of CsCl gradient fractions with MD wetland soil. The density is listed on the right of each panel. Densities between 1.74 and 1.78 g ml-1 correspond to heavy fractions (labeled DNA) and those between 1.72 and 1.74 g ml-1 correspond to the light fractions (unlabeled DNA).



Figure 1



Figure 2



Figure 3



Figure 4



Figure S1