Revealing the community and metabolic potential of active methanotrophs by targeted metagenomics in the Zoige wetland of the Tibetan Plateau

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Summary

The Zoige wetland of the Tibetan Plateau is one of the largest alpine wetlands in the world and a major emission source of methane. Methane oxidation by methanotrophs can counteract the global warming effect of methane released in the wetlands. Understanding methanotroph activity, diversity, and metabolism at the molecular level can guide the isolation of the uncultured microorganisms and inform strategy-making decisions and policies to counteract global warming in this unique ecosystem. Here we applied DNA stable isotope probing using ^{13}C-labeled methane to label the genomes of active methanotrophs, examine the methane oxidation potential, and recover metagenome-assembled genomes (MAGs) of active methanotrophs. We found that gmmaproteobacteria of type I methanotrophs are responsible for methane oxidation in the wetland. We recovered two phylogenetically novel methanotroph MAGs distantly related to extant *Methylobacter* and *Methylovulum*. They belong to type I methanotrophs of gammaproteobacteria, contain both *mxa*F and *xox*F types of methanol dehydrogenase (MDH) coding genes, and participate in methane oxidation via H_4MPT and RuMP pathways. Overall, the community structure of active methanotrophs and their methanotrophic pathways revealed by DNA-SIP metagenomics and retrieved methanotroph MAGs highlight the importance of methanotrophs in suppressing methane emission in the wetland under the scenario of global warming.

Introduction

Global warming has accelerated the breakdown of soil organic carbon into either carbon dioxide or methane over the past few decades, particularly in cold ecosystems like the Tibetan Plateau (Schuur et al., 2009). Methane has a global warming potential 34 times greater than carbon dioxide (Ciais and Sabine, 2013). Wetlands are the largest natural methane emission source (Aronson et al., 2013). Wetland soils across the Tibetan Plateau store vast amounts of organic carbon held at low temperature, and are facing the risk of becoming a net methane source under continuous global warming. With the thawing soil layer becoming deeper, carbon loss induced by continuous global warming will potentially turn the Tibetan Plateau into a net carbon source instead of a carbon sink by 2100 (Wang et al., 2020).

Methane emissions from wetlands are mainly controlled by the activities of methane-producing archaeal methanogens and aerobic methane-oxidizing bacterial methanotrophs, which are two specialists involved in the biological methane cycle. Methanotrophs can oxidize up to 95% of the greenhouse gas methane before it escapes to the atmosphere (Frenzel et al., 1990; Le Mer and Roger, 2001; Bastviken et al., 2008), thereby greatly reducing methane emissions from wetlands under global warming scenarios. Methanotrophs are abundant at the aerobic and anaerobic interface of wetland environments (Kalyuzhnaya et al., 2019), and have been reported to be responsible for ameliorating methane release from permafrost ecosystems (Liebner et al., 2009; Blazewicz et al., 2012).

Extant methanotrophs are affiliated to the alpha- and gamma- *Proteobacteria* (Hanson and Hanson, 1996), the *Verrucomicrobia* (Op den Camp et al., 2009), and members of NC10 phylum (Ettwig et al., 2010). The particulate monooxygenase (pMMO) and soluble methane monooxygenase (sMMO) are two structurally and biochemically distinct forms of MMO which catalyze the first step of methane oxidation (Dalton, 2005; Trotsenko and Murrell, 2008; Smith and Murrell, 2009). Some methanotrophs also possess *pxm*A that encodes a subunit of a highly divergent monooxygenase of undetermined function (pXMO) (Tavormina et al., 2011). The environmental distribution of aerobic methanotrophs is typically investigated, using cultivation-independent molecular techniques, with the gene *pmo*A, encoding a subunit of pMMO. This has been frequently used as a key marker gene (functional gene probe) to study the environmental distribution of methanotrophs (Héry et al., 2008; Jiang et al., 2010; Boden and Murrell, 2011; Kumaresan et al., 2011; Yun et al., 2012; Yun et al., 2015; Singleton et al., 2018). Different from pMMO, *Methyloferula* and *Methylocella* within the *Beijerinckiaceae* only possess sMMO, so the *mmo*X gene, encoding subunit A of the sMMO, is used as a complementary marker gene (Vorobev et al., 2011). However, sMMO genes are less widespread in the environment and therefore limited studies were reported (Auman et al., 2000; Horz et al., 2001).

Methanol, produced from the oxidation of methane by methane monooxygenase, can be further oxidized by methanol dehydrogenase (MDH). There are two types of MDH, the calcium-containing MxaF system and the lanthanum-dependent XoxF enzyme system (Keltjens et al., 2014; Vekeman et al., 2016). There are two major pathways by

which methanotrophs assimilate carbon into cellular material: the ribulose monophosphate (RuMP) and the serine pathway (Kalyuzhnaya et al., 2019). Some methanotrophs can also assimilate carbon at the level of CO_2 using ribulose bisphosphate carboxylase (RuBisCO) (Khadem et al., 2011). The pathways of carbon metabolism in methanotrophs have been described in detail (Trotsenko and Murrell, 2008).

The Zoige wetland of Tibetan Plateau (102°52′E, 33°56′N) forms one of the largest high-altitude wetland in the world (Xiang et al., 2009), is one of the biggest regions for methane emission in China and has an average annual temperature of around 1°C (Xiang et al., 2009; Chen et al., 2013). The community structure and abundance of the methanotrophs of the Zoige wetland has been preliminarily analyzed based on *pmo*A genes clone libraries and real-time PCR methods, finding that novel uncultivated gammaproteobacterial methanotrophs are responsible for the methane oxidation in this wetland environment (Yun et al., 2012). Recently, the denitrifying anaerobic methane oxidation (DAMO) has also been reported (Xie et al., 2020), indicating a complementary methane oxidation pathway in the Zoige wetland.

Knowledge of methanotrophs in this high altitude wetland in the Tibetan Plateau is limited to information obtained by retrieval of *pmo*A genes from soil samples (Yun et al., 2012) and very little is known about the activity and the metabolic potential of methanotrophs in this environment. Difficulties in isolating these methanotrophs make a study of the metabolism of methanotrophs in this environment tricky. However, targeted metagenomics, combining DNA stable isotope probing (DNA-SIP) techniques

with analysis of ^{13}C-labeled DNA by high throughput DNA sequencing allows us to study the activity and metabolic potential of specific groups of microbes in these unusual environments (Hutchens et al., 2003; Héry et al., 2008; Avrahami et al., 2011; Khadem et al., 2011; Grob et al., 2015; Esson et al., 2016; Jameson et al., 2017; Carrión et al., 2020). The technology recovers partial or complete genomes from the available metagenomic data. The information gained from these focused metagenomic approaches enables capture of partial and in some cases, near complete genomes of target organisms and analysis of metagenome-assembled genomes (MAGs) can help to predict pathways and metabolism of the specific groups of microbes, which in turn greatly improves the chances of isolating them using targeted isolation approaches (Grob et al., 2015; Coyotzi et al., 2016; Crombie et al., 2018; Kumaresan et al., 2018; Larkemejia et al., 2019).

In this study, active methanotrophs and their methane oxidation potential were analyzed by labeling methanotrophs in soil samples from the Zoige wetland with ^{13}C-methane using DNA-SIP. Key functional genes involved in methane oxidation were retrieved to examine methanotrophs diversity, and the metabolic potential encoded by methanotroph MAGs retrieved from metagenomes was analyzed. The characterization of methane oxidation potential, the diversity and the genomic potential of active methanotrophs in the Zoige wetland is an important step in elucidating the role of methanotrophs in the Tibetan Plateau under global warming, and uncovers clues that explain their role in the global carbon cycle.

Results

Comparative study design

To exploit the activity, community structure, and the metabolic characteristics of the methanotrophs in the Zoige wetland, we carried out a comparative study as shown in Fig. 1. Firstly, representative soil samples from the Zoige wetland were collected to establish microcosms to be exposed to either $^{13}CH_4$ for DNA-SIP, or $^{12}CH_4$ as control. Then, the original samples and the methane-consuming microcosms were subjected to DNA extraction, ^{13}C-labeled DNA fractionation, and metagenomic sequencing (Fig. 1A). Afterwards, sequencing data were processed to identify the active methane-oxidizing members in the microbial community and uncover the detailed metabolic pathways of functionally active methanotrophs in the Zoige wetland (Fig. 1B).

Methane oxidation potential and methanotrophic bacterial communities

Microcosms were established for the measurement of methane oxidation potential and the identification of methanotrophic microbial populations in the wetland samples. All microcosms completely oxidized 125,000 ppm methane within five days (Fig. 2A), corresponding to an average methane oxidation rate of 26.7 μmol day^{-1} g^{-1} (wet weight soil) at 25°C. After incubation, total DNA was extracted to enrich ^{13}C labeled DNA of active methanotrophs from the microcosms exposed to $^{13}CH_4$. The SIP experiment was successful as indicated by the density shift based on qPCR quantification of *pmo*A genes for fractionated layers (Fig. 2B). Compared to the $^{12}CH_4$ control, the peak of

*pmo*A genes shifted to the heavy layers in microcosms exposed to $^{13}CH_4$, indicating that assimilation of the ^{13}C into cell biomass had occurred for active methanotrophs.

Metagenomic sequencing of the original samples and SIP microcosms was carried out to identify the active methanotrophic populations. The read-based MetaPhlAn2 v2.5.0 (Truong rt al., 2015) and the assembly-based BLASTp v2.9.0 (Camacho et al., 2009) methods were used to analyze the sequencing data. The total community composition analysis shows 18 groups of archaeal and bacteria at class level belonging to ten phyla (Fig. 2C). Bacteria communities belong to *Proteobacteria* (alpha-, beta-, delta-, gamma-) constitute more than 50% and more than 80% of total bacteria community before and after incubation, respectively. The *Methanomicrobia* of *Euryarchaeota* archaea decreased dramatically after incubation. Other bacteria belong to *Actinobacteria* and *Acidobacteria* were also abundant bacteria communities in the wetland, and their relative abundance did not fluctuate with the microcosm incubation. *Deinococci*, *Chlorobia* and *Opitutae* constitute only minor parts of the total community, and indicate limited variation before and after microcosm incubation (Fig. 2C).

With respect to methanotrophs, the relative abundance indicate a discrepancy in the abundance of methanotrophs by different analysis methods, especially for the relative abundance of type II methanotrophs (Fig. 2D, Supplementary Fig. S1, S2). Before $^{13}CH_4$ labeling (replicates $t_{0\text{-}1}$ and $t_{0\text{-}2}$), the total relative abundance of methanotrophs in t_0 samples was rather low (5.46% with MetaPhlAn2; 0.57% with BLASTp). After $^{13}CH_4$ labeling ($t_{1\text{-}1}$ and $t_{1\text{-}2}$), methanotrophs were highly enriched. The most enriched methanotrophs were: type I methanotrophs (gammaproteobacteria) of unclassified

Methylomonas (3.78%) and unclassified *Methylobacter* (2.44%), type II methanotrophs (alphaproteobacteria) belong to *Methylocystis rosea* (5.72%) and unclassified *Methylocystis* (6.75%). Methanotrophs were enriched after $^{13}CH_4$ labeling; however, the methanotroph diversity was not significantly changed during the incubation, indicating the labeling incubation experiments did not influence the overall composition of the methanotrophs present. However, according to BLASTp method, type I methanotrophs were much more abundant than type II methanotrophs (Supplementary Fig. S1). Besides, there was a higher diversity (more genera detected) of type I methanotrophs than type II methanotrophs in the wetland samples. Type II methanotrophs were limited to two genera, *Methylocystis and Methylocella.* Type I methanotrophs of the genera *Methylobacter*, *Methylomonas*, *Methylovulum*, etc., were identified (Supplementary Fig. S1).

Besides methanotrophs, the relative abundance of other biochemical metabolic microbial groups also shifted in SIP microcosms (Supplementary Fig. S2). For example, methylotrophic *Methylotenera*, *Sulfuricella denitrificans*, *Nitrosospira* phyla members, and *Actinoplanes* genus members were considerably enriched for t_1, with relative abundance ranged from ~2.54% to ~7.40%.

Phylogenetic analysis of methane monooxygenase genes

To further characterize the methanotrophs identified from the wetland samples, we retrieved and performed phylogenetic analyses of *pmo*A gene fragments (>400 bp) from

the metagenomic co-assemblies. Phylogenetic analysis revealed that all *pmo*A genes obtained belonged to type I methanotrophs, including the *Methylobacter*-, *Methylomonas*- and *Methylovolum*-like genera (Fig. 3A). The type I *pmo*A genes obtained exhibited high similarity to the *pmo*A genes of methanotroph strains from cold environments, such as *Methylobacter tundripaludum* (GCF_000190755) and the *Methylovulum psychrotolerans* (GCF_002209385). Novel *pmo*A genes distantly related to *Methylococcus capsulatus* were also retrieved (Fig. 3A). However, no extant *pmo*A gene of type II methanotroph was found, which is consistent with the population identification of active methanotrophs (Supplementary Fig. S1).

The phylogenetic diversity of *mmo*X genes, encoding one subunit of the soluble methane monooxygenase (sMMO) (Sullivan et al., 1998; Auman and Lidstrom, 2002), were also retrieved from the metagenomic data and showed a different trend to *pmo*A genes (Supplementary Fig. S3). Except for recovery of *mmo*X genes with high sequence identity to extant type I methanotrophs, *mmo*X genes affiliated to type II methanotrophs *Methylocella* and *mmo*X fragments distantly related to uncultured type II methanotrophs were also detected.

To examine the relative abundance of MMOs that catalyze the methane oxidation's first step, the relative abundance of *pmo*A and *mmo*X gene fragments in the phylogenetic tree (Fig. 3A and Supplementary Fig. S3) and the total relative abundance of *pmo*A, *pxm*A, and *mmo*X genes in each sample were analyzed (Fig. 3B). Both analyses revealed similar trends in the relative abundance of methanotrophic groups: the most abundant methanotrophs are from the *Methylococcaceae* family (Fig. 3B),

especially those from *Methylobacter* and *Methylovulum* genera (Fig. 3A); a much lower abundance of the *Methylocystaceae* across the metagenomes of all samples, with only a few sequence reads were found (Fig. 3B and Supplementary Fig. S3). The relative abundances of genes encoding methanol dehydrogenase (MDH) that catalyze the second step of methane oxidation, including *mxa*F and *xox*F (Keltjens et al., 2014), were also analysed (Supplementary Fig. S4). Not surprising, MDH gene sequences from *Methylococcaceae* (gammaproteobacterial methanotrophs) were again found to be more abundant than those who belong to the *Methylocystaceae* family of type II methanotrophs.

Recovery of methanotroph MAGs and genomic phylogenetic analysis

MAGs were recovered from metagenome of all samples. A total of 200 partial MAGs were recovered. Two *pmo*A-containing MAGs with a completeness >70% and contamination of <10% were selected for genomic analysis. According to the genomic phylogenetic analysis, the two MAGs were clustered with *Methylobacter* (bin_199) and *Methylovulum* (bin_110), both belong to the type I methanotrophs (Fig. 4). One MAG (bin_199) has an ANI of 82.4% to *Methylobacter tundripaludum SV96* (GCF_000190755), the other MAG (bin_110) has an ANI of 80.0% to *Methylovulum psychrotolerans* Sph1 (GCF_002209385). The low ANI indicates that the two MAGs differ from the known methanotrophs, and as such, probably represent novel methanotrophs in the wetland. The two MAGs constitute 0.06% (bin_110) and 0.11%

(bin_199) total bacteria in the original soil samples (t_0), and about 0.14% (bin_110) and 0.24% (bin_199) in the $^{13}CH_4$-labeled samples (t_1), respectively (Fig. 4). The increasing abundance of the two MAGs alone with the *pmo*A gene abundance of them (Fig. 3A) before and after DNA-SIP implies they represent two key active methanotrophs in this wetland environment. Genomic properties of the two MAGs are listed in Table 1.

Metabolic potential analysis of MAGs

To further understand the metabolism characteristics of the methanotrophs in the Zoige wetland. The detailed metabolic pathway of methane oxidation was constructed from the two MAGs (Fig. 5). Both MAGs possess the complete pMMO (*pmoC*AB) gene cluster. Neither the distinct *pxm*ABC encoding pXMO nor genes for sMMO were identified in two MAGs. The analysis of two MAGs also showed the presence of both *mxa*F- and *xox*F-type methane dehydrogenase genes. The presence of the RuMP pathway was confirmed by the presence of genes encoding 3-hexulose-6-phosphate synthase (*hps*) and 6-phospho-3-hexuloisomerase (*phi*). The H_4MPT pathway was confirmed by the existence of genes encoding the tetrahydromethanopterin hydro-lyase (*fae*), methenyltetrahydromethanopterin cyclohydrolase (*mch*) and methylene-tetrahydromethanopterin dehydrogenase (*mtd*B) (Fig. 5, Supplementary Table S2). In addition to the RuMP and H_4MPT pathways that are vital for methanotrophic metabolism, both MAGs encode major pathways that are found in methanotrophs, including the tricarboxylic acid cycle (TCA), the Embden-Meyerhof-Parnas pathway

(EMP) pathway, the Enter-Doudoroff pathway (ED pathway) and the tetradehydrofolate (H_4F) pathway. Interestingly, both MAGs encode several enzymes of the serine pathway, which is the major formaldehyde assimilation of type II methanotrophs, such as genes encoding malyl-CoA lyase (*mcl*), and malate dehydrogenase (*mdh*) (Oshkin et al., 2016; Mateos-Rivera et al., 2018).

For nitrogen metabolism, both MAGs lack genes involved in nitrogen fixation (*nif*H). The presence of *nar*, *nir*, and genes encoding putative nitrite and nitrate transporters indicates the dependence on nitrogen assimilation denitrification (Fig. 5, Supplementary Table S2). No genes involved in sulfur oxidation (*sox*ABCDXYZ) were detected in both MAGs, implying that these methanotrophs do not oxidize sulfur. The detailed metabolism characterization of the two MAGs can also be found in Supplementary Table S2.

Discussion

The Zoige wetland of the Tibetan Plateau is one of the biggest methane emission centers in China (Chen et al., 2013). The accelerated global warming may switch the Tibetan Plateau from a carbon sink to a carbon source in the next decades (Wang et al., 2020). Methanotrophs in the wetland play as a bio-methane filter that greatly relief methane emission. The activity, diversity, and the metabolic potential of methane utilizing bacteria in low temperature need to be clarified. As such, our study here gives the first detailed description of the activity, diversity, the identity, and the methane metabolism

potential of methanotrophs in the Zoige wetland. Our report provides essential insight into the methane oxidation metabolisms, which will guide the cultivation of valuable methanotroph isolates and wetland protection policies under increased global warming.

Methane oxidation potential and methanotroph communities in the Zoige wetland

The methane oxidation rate measured by $^{13}CH_4$ methane isotope labeling is about 26.7 μmol day^{-1} g^{-1} (wet weight soil) at 25°C (Fig. 2A). We have previously measured a methane oxidation rate of 15.5 μmol day^{-1} g^{-1} wet weight soil (data unpublished) at 10°C, which is the average temperature of the sampling time. The higher methane oxidation rate under both low and moderate temperature suggests a flexible range of methane oxidation capacity of the wetland soil and the high potential of steady methane oxidation capacity under low temperature.

The methanotrophic communities variation before and after labeling indicate a discrepancy by using the read-based MetaPhlAn2 (Truong et al., 2015) analysis and the assembly based BLASTp method (Camacho et al., 2009), especially for the relative abundance of type II methanotrophs (Fig. 2C, Supplementary Fig. S1, S2). Both types I and II methanotrophs were found with high abundance according to the MetaPhlAn2 analysis. However, a predominance of type I methanotrophs was recovered by BLASTp. The discrepancy on the observed abundance of type I and type II by two methods reflects their differences: the MetaPhlAn2 method uses the quality trimmed sequencing reads, and the BLASTp uses the metagenomic assembly and predicted coding

sequences (CDS). The decreased abundance of methanotrophs observed with the BLASTp method might be due to the reads lost during the metagenome assembly. The unclassified methanotrophs indicated that novel species likely constitute a large proportion of the methane oxidizing bacterial populations.

Besides methanotrophs, the metagenomic sequencing also revealed a relative abundant presence of other biochemical metabolic activity of the bacterial community, including methanol (Lv et al., 2020), sulfur (Kojima and Fukui, 2010), nitrogen metabolisms (Jiang and Bakken, 1999; Lourenço et al., 2018), and antibiotic productions (Vobis, 2006). The co-existence of these distinct physiological microbial groups suggests their importance in biogeochemical cycling in the Zoige wetlands.

The diversity of functional genes confirms the abundance of type I methanotrophs

The *pmo*A gene encodes a key subunit of pMMO which catalyzes the oxidation of methane to methanol and has been used widely in profiling methanotrophs communities in environmental samples (Héry et al., 2008; Jiang et al., 2010; Boden and Murrell, 2011; Kumaresan et al., 2011; Yun et al., 2013; Yun et al., 2015; Singleton et al., 2018). Phylogenetic analyses of *pmo*A and *mmo*X genes indicate the higher diversity of type I methanotrophs in the wetland (Fig. 3, Supplementary Fig. S3). Despite phylogenetic diversity of MMO encoding genes, the relative abundance of *pmo*A, *mmo*X, and *pxm*A genes in metagenome datasets also indicate the absolute dominance of type I methanotrophs (Fig. 3, Supplementary Fig. S3).

The relative abundances of MDH (*mxa*F and *xox*F) genes from *Methylococcaceae* of type I methanotrophs were also found to be more abundant than those from the type II *Methylocystaceae*. The very high abundance of *xox*F genes affiliated to the *Methylophilaceae* suggests that methanol produced by methanotrophs may well be consumed by this group of methylotrophs. MDH genes affiliated to the *Hyphomicrobiaceae,* also methanol utilizers, were abundant in this wetland environment (Supplementary Fig. S4), indicating that bacteria of this group also contribute to the aerobic C_1 cycle in this environment and are potentially new methanotrophic species. It has been suggested that the *Hyphomicrobiaceae* should be added to the recognized type I methanotrophs because they might be potential methanotrophs that participate in methane oxidation (Singleton et al., 2018). Another possibility is that the *Hyphomicrobiaceae* observed might also be cross-feeding off methanol produced by methanotrophs.

When considering methanotrophs diversity and abundance, the analysis confirms that type I methanotrophs dominate in the Zoige wetland (Fig. 3, Supplementary Fig. S3, S4). The predominance of type I methanotrophs in the low-temperature environments has been repeatedly reported in previous studies (Liebner and Wagner, 2007; Liebner et al., 2009; Graef et al., 2011; Yun et al., 2012; Yun et al., 2013; Yun et al., 2015; Singleton et al., 2018). Type I methanotrophs dominate and active in cold areas such as Arctic land and high-altitude alpine wetlands. Within the active layer of cold wetlands, the temperature is the most important environmental factor in shaping methanotrophic communities (Liebner and Wagner, 2007; Liebner et al., 2009; Graef

et al., 2011). The mechanisms underpinning the success of cold-adapted type I methanotrophs are unclear. However, there is clearly an environmental and ecological selection for methanotrophic species in the extreme environments (Liebner et al., 2009): for instance, the dominant of type II methanotrophs in the acidic peatlands (Dedysh et al., 2000; Dedysh et al., 2002; Dedysh et al., 2004), the consumption of atmospheric methane by the uncultivated UCSγ cluster distantly from type I and type II methanotrophs (Horz et al., 2005; Kolb et al., 2005; Pratscher et al., 2018). All these environments selected specific methanotrophs groups with low diversity.

Another explanation for the presence of cold adaptive methanotrophs is based on the different metabolic pathways. Type I methanotrophs have more efficient RuMP carbon assimilation pathways, whereas type II methanotrophs use the serine pathway for carbon assimilation (Hanson and Hanson, 1996; Macalady et al., 2002). Type I methanotrophs have been found to grow between 5ºC and 15 ºC, such as in the Arctic, and have a low DNA G + C content (43–54 mol %) (Kalyuzhnaya et al., 2019). Furthermore, methanotrophs abundance is high in regions of high dissolved methane or oxygen availability due to oxygen transportation via plant roots (Singleton et al., 2018). Unlike the Arctic and high altitude area, our study site is an alpine wetland, thriving with emergent plants with extensive, highly developed root systems, which can provide oxygen for methane oxidation. Therefore oxygen may not be a factor that constrains the diversity of methanotrophs in this wetland.

Theories for the dominance of type I methanotrophs are not consistent in all studies. However, there is one trend in common: the low methanotroph diversity in the cold

regions. The prevalence of type I methanotrophs might be a combination of several factors, such as the low temperature and the methane/oxygen availability. The low diversity of methanotrophs suggests a particular vulnerability of these communities, therefore their role in methane cycling deserves increased attention.

Genomic analysis and metabolic reconstruction of cold-adaptive methanotrophs indicate environmental selection

To obtain MAGs with higher genome quality, different assembly strategies were used here. First, each metagenomic data was assembled separately. Then the four metagenomic data were co-assembled together. Finally, the MGAs quality of the two assembly strategies were compared based on the CheckM estimation (Parks et al., 2015). The two MAGs, bin_110 and bin_199, retrieved from the co-assemblies were chosen for the genomic analysis and metabolic reconstruction because they showed higher genome quality. Despite higher genome quality, these two MAGs were also retrieved in each metagenomic data both before and after $^{13}CH_4$ labeling, but with smaller genome size. Therefore, we suppose they are prevalent in the study site and are key players in the methane consumption.

Genomic phylogenetic analysis identified the closest relatives of the two MAGs recovered here are both cold-adaptive type I methanotrophs (Fig. 4). However, the low ANI values further indicate these methanotrophs are potentially novel species in this cold environment. Although the relative abundance of these MAGs based on the current

results is relative low (Fig. 4), combining the results of community, the functional gene abundance of the two dominant genera, we suppose the two MAGs represented bacteria are key members in methane consumption in the wetland. Their importance can be further proofed by the available isolates or transcriptomic data.

Genome-scale metabolic reconstruction of the two MAGs confirmed the typical lifestyle of type I methanotrophs (Fig. 5). Firstly, the genome analyses indicated that these two key methanotrophs from the Zoige wetland only use pMMO to oxidize methane. Both MAGs possess the complete pMMO (*pmoC*AB) gene cluster. Genome analysis also confirmed the absence of sMMO coding genes, which is consistent with extant *Methylobacter* strains (Dalton, 2005; Wartiainen et al., 2006; Smith and Murrell, 2009; Smith et al., 2018). Although the sMMO gene was present in the genome of the first reported isolate of *Methylovulum miyakonense* (Iguchi et al., 2011), the closest extant methanotroph genome to the MAG bin_110, *Methylovulum psychrotolerans* HV10-M2, also did not contain sMMO (Oshkin et al., 2016). Genes encoding the alternative pMMO operon of some type I methanotrophs *pxm*ABC was also absent (Tavormina et al., 2011).

Next, the analysis of MAGs confirmed the presence of genes encoding the calcium-dependent MDH (*mxa*F-encoded) (Keltjens et al., 2014) and the lanthanide-dependent (*xox*F-encoded) MDH (Vekeman et al., 2016), which catalyzes the second step in methane conversion. The co-existence of *mxa*F and *xox*F genes suggests that methanol can be oxidized by both types of MDH. However, *xox*F may not be the major route of methanol utilization in methanotrophs. Based on *xox*F gene abundance, *xox*F was

mostly from methylotrophs such as *Methylophilaceae* and *Hyphomicrobiaceae* (Supplementary Fig. S4). Methanol is potentially secreted by methanotrophs to the environment and consumed by the syntrophic partners, eg, cross-feeding between methanotroph and methylotroph (Krause et al., 2017). Both MAGs also contain *fds*, the gene encoding formate dehydrogenase that catalyzes the last step in the methane oxidation pathway from formate to carbon dioxide (Yoch et al., 1990; Kalyuzhnaya et al., 2019).

Furthermore, the two MAGs recovered here contain genes encoding both ribulose monophosphate (RuMP) and the tetrahydromethanopterin (H_4MPT) pathways, which are the two major pathways of formaldehyde metabolism in type I methanotrophs (Kalyuzhnaya et al., 2019). The present of the RuMP pathway was confirmed by the presence of genes encoding 3-hexulose-6-phosphate synthase (*hps*) and 6-phospho-3-hexuloisomerase (*phi*). The H_4MPT pathway was confirmed by the existence of genes encoding tetrahydromethanopterin-dependent formaldehyde-activating enzyme (*fae*), methylene-tetrahydromethanopterin dehydrogenase (*mtd*B), and methenyltetrahydromethanopterin cyclohydrolase (*mch*) (Fig. 5, Supplementary Table 2).

In addition to the RuMP and H_4MPT pathways that are vital for methane metabolism, both MAGs also encode key metabolic pathways that are regularly found in methanotrophs, including the Tricarboxylic Acid cycle (TCA), the Embden-Meyerhof-Parnas (EMP) pathway, the Enter-Doudoroff pathway (ED pathway) and the tetradehydrofolate (H_4F) pathway. Interestingly, both MAGs encode several enzymes

of the serine pathway, which is the major formaldehyde assimilation of alphaproteobacterial type II methanotrophs, such as genes encoding malyl-CoA lyase (*mcl*), and malate dehydrogenase (*mdh*) (Oshkin et al., 2016; Mateos-Rivera et al., 2018). The presence of genes encoding serine pathway enzymes has also been reported in *Methylobacter* tundripaludum and *Methylovulum* (Svenning et al., 2011; Mateos-Rivera et al., 2018). However, the serine pathway does not function in these methanotrophs because of the lack of key genes of the pathway. For example, genes encoding for the phosphoenolpyruvate carboxylase encoding genes (*ppc*) are missing in both MAGs recovered.

Meanwhile, some important genes might not have been recovered in the two partially complete MAGs. The absence of *nif*DKH genes in the *M. tundripaludum* related bin_199, as well as some other genes, such as phosphogluconate dehydratase (*edd*) in the Enter-Doudoroff pathway (Fig. 4, Supplementary Table 2), indicate that these genes might not have been recovered in these partially complete genomes (lacking 21.6% for bin_110 and 28.07% for bin_199, Table 1). It has been suggested that *M. tundripaludum* could employ the NAR and NIR enzymes in denitrifying anaerobic methane oxidation, utilizing a pathway similar to that of *Candidatus M. oxyfera* and are capable of growing anaerobically by denitrification (Ettwig et al., 2010; Tveit et al., 2013). This might explain *M. tundripaludum* appears to be abundant in water-saturated peat bogs or freshwater wetlands similar to our study.

The metabolic pathways reconstructed from the MAGs indicate that methane oxidation pathways of the methanotrophs in the wetland are comparable to those type I

methanotrophs from other cold environments such as permafrost and Arctic regions (Tveit et al., 2013; McCalley et al., 2014; Kits et al., 2015; Mateos-Rivera et al., 2018; Rainer et al., 2020). However, their genomic properties are phylogenetically distantly related to those methanotrophs from other environments. Alone with The low AAI values suggest that these are novel methanotrophs which act as effective methane bio-filters in the Zoige wetland, which could enhance methane oxidation in response to rising global temperatures.

Overall, our findings suggest that methane emission in the Zoige wetland of the Tibetan Plateau is effectively mitigated by methanotrophs, which are adapted to low temperatures. By incorporating metagenomic computational methods into DNA-SIP experiments, we characterized the functional activity, phylogenetic identity, and metabolic potential of key microbial populations involved in methane oxidation. We discovered that type I methanotrophs of gammaproteobacteria were the major populations responsible for methane oxidation. Importantly, two MAGs of methanotrophs that genetic distantly related to *Methylovulum psychrotolerans* and *Methylobacter tundripaludum* were recovered, indicating that novel cold-adaption strategies are prevalent in this environment. These findings will assist in formulating enrichment strategies to isolate these novel methanotrophs, and also highlight the major contribution of methanotrophs in reducing methane emissions from the great carbon sink of the Tibetan Plateau.

Experimental procedures

Sampling site description The sampling site was located at the Flower Lake National Nature Reserve (Chen et al., 2009). Aquatic plants, *Carex muliensis*, dominate this freshwater lake and the study site. Two lake soil cores (10 cm in diameter and ~10 cm in depth) were sampled as previously described (Yun et al., 2013) in early August 2016, the two soil cores were about 50 meters away from each other along the lake walkway, the standing water depth was about 30 cm for both sampling location. Samples were kept in sterile bags and stored in ice during transportation back to the laboratory. Each soil core was thoroughly mixed in the lab. We stored 100 g sample at -80ºC before metagenomic sequencing and the remainder at 4ºC until used in SIP experiments and soil physiocochemical analysis.

Soil physiocochemical analysis

The soil pH and temperature was measured *in situ* using a portable multi-parameter pH meter (Fischer Scientific, Wilmington, DE, USA). Ammonium (NH_4^+) and nitrate (NO_3^-) were determined with an AA3 continuous flow analytical system (Seal, German). Moisture content (MC) was measured by drying soil samples at 105ºC for 24 h. Total organic carbon (TOC) and total nitrogen (TN) were measured with an elemental analyzer (vario MACRO cube, Elementar, Germany).

Microcosms preparation

We assembled three soil microcosms for the SIP experiments (Radajewski et al.,

2000; Cai et al., 2016), with two replicates exposed to $^{13}CH_4$ and one exposed to $^{12}CH_4$ as the control, a mixture of two soils were used for the three microcosms. Each contained 5 g fresh soil samples sealed in a 120 mL serum vial capped with a butyl rubber stopper, and then injected with 0.6 mmol (15 mL) CH_4 (99.99% purity, AP BAIF Gases, Beijing, China). The microcosms were incubated at 25°C in the dark. Headspace methane concentrations were monitored daily for six days using a Shimadzu GC12-A Gas Chromatograph with Flame Ionization Detector (GC-FID).

DNA extraction, pmoA gene quantification, and sequencing

Replicated DNA extractions from original soil samples ($t_{0\text{-}1}$, $t_{0\text{-}2}$), the $^{13}CH_4$ labeled samples ($t_{1\text{-}1}$, $t_{1\text{-}2}$) and $^{12}CH_4$ controls were carried out by using the FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA) following the manufacturer's instructions.

DNA extraction of each microcosm was spatially resolved by cesium chloride density gradient centrifugation and fractionated into 15 density layers with their density determined by an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). These DNA fraction layers were recovered and purified by PEG-6000 precipitation and resuspended in 30 μL TE buffer (tris(hydroxymethyl) aminomethane (Tris) 10 mM, ethylenediaminetetra-acetate (disodium salt) (EDTA) 1 mM, pH 8.0), and the quantity of *pmo*A genes in each layer were then determined by real-time PCR (qPCR) to identified whether the labeling was succeed. Four DNA samples, including

two original soil samples ($t_{0\text{-}1}$, $t_{0\text{-}2}$), and two $^{13}CH_4$ labeled samples ($t_{1\text{-}1}$, $t_{1\text{-}2}$) were sequenced on an Illumina HiSeq X10 platform (2×150 bp). The DNA-SIP fraction layers of heavy DNA (the peak layer and the two adjacent layers) were pooled for targeted sequencing of $^{13}CH_4$ labeled DNA. 120 Gb raw reads data were obtained in total with 30 Gb for each DNA sample.

Metagenome analysis, MAGs assembly, and annotation

All the sequencing reads were first trimmed using Trimmomatic v0.38 (Bolger et al., 2014) to obtain clean reads. The co-assembly of data of all samples (t_0 and t_1) was used to generate high-quality fragments. MEGAHIT v1.0 (Li, 2012) was used to perform the co-assembly of data of all samples. The relative abundances of microbial communities was estimated by using two methods, the MetaPhlAn2 v2.5.0 (Truong et al., 2015) and the BLASTp v2.9.0 (Camacho et al., 2009) methods. The MetaPhlAn2 method was performed using quality trimmed data and the BLASTp method was performed based on the MEGAHIT output co-assemblies. First, coding sequences (CDS) were predicted using Prodigal v2.6.3 (Hyatt et al., 2010). The taxonomic assignment of each CDS was based on BLASTp v2.9.0 (Camacho et al., 2009) best hit against NCBI non-redundant protein database (NR database) downloaded in September 2018 using DIAMOND v0.9.10.111 (Buchfink et al., 2015). Microbe abundance was calculated using the formula reported previously (Qin et al., 2012; Zheng et al., 2020).

To obtain MAGs of active methanotrophs, we mapped sequencing reads of all

samples to the co-assemblies using Bowtie2 v0.12.7 (Langmead and Salzberg, 2012). Assemblies of all samples were binned into MAGs using differential coverage binning with METABAT2 v2.12.1 (Kang et al., 2015). The completeness, and contamination of the MAGs were estimated using CheckM v1.0.12 (Parks et al., 2015). MAGs in which *pmo*A genes were found by GraftM v0.13.0 (Boyd et al., 2018) were selected for metabolism analysis. Ribosomal RNA (rRNA) coding regions (16S, 23S) of MAGs were predicted with Barrnap (https://github.com/tseemann/barrnap). Genome annotation were based on BLASTp (Camacho et al., 2009), PROKKA v1.14.5 (Seemann, 2014), and the KEGG server (BlastKOALA) were used to annotate protein functions, the KO assignments and the pathway map of methanotrophic MAGs. The average amino acid identity (AAI) and average nucleic acid identity (ANI) were used to determine the evolutionary distance comparisons between the assembled MAGs from the soil samples and extant genomes of closely related methanotroph strains (Rodriguez-R and Konstantinidis, 2016; Medlar et al., 2018).

Phylogenetic analysis of genes encoding methane monooxygenases

The fragments of *pmo*A and *mmo*X genes were extracted from all assemblies using BLASTn (Camacho et al., 2009), and then aligned with reference sequences downloaded from the NCBI database using MUSCLE v3.8.1551 (Edgar, 2004). A phylogenetic tree was built using MEGA7 (Kumar et al., 2016). The relative abundance of *pmo*A and *mmo*X fragments used in the phylogenetic tree in each sample was

estimated by using contig option in CoverM v0.4.0 (https://github.com/wwood/CoverM). GraftM v0.13.0 (Boyd et al., 2018) were used to search and count key genes that are involved in methane oxidation including *pmo*A, *pxm*A, *mmo*X, *max*F, and *xox*F (Boyd et al., 2018). The abundance of these genes was calcuated according to the ratio of their gene reads against the total sequencing reads in each sample. All heatmaps were built in R (Kolde et al., 2019).

Phylogenetic analysis of MAGs

Phylogenetic analysis of the two MAGs recovered in this study was inferred based on a concatenated set of maker proteins using GTDB-Tk v0.3.3 (Chaumeil et al., 2020). A total of 120 marker proteins were identified and aligned using the identify and align commands in GTDB-Tk with default parameters. The maximum-likelihood phylogenomic tree was then constructed with FastTree v.2.1.10 using the WAG+GAMMA module (Price et al., 2010) and visualized with Mega7 (Kumar et al., 2016). The relative abundance of each MAG in each sample was estimated using the genome option CoverM v0.4.0 (https://github.com/wwood/CoverM) and visualized using the pheatmap package in R (Kolde, 2019).

The reconstruction of the pathway for methane Metabolism in MAGs

The metabolic pathway of methane in MAGs was constructed based on annotaton results from PROKKA v1.14.5 (Seemann, 2014) and KEGG online annotation server BlastKOALA (Kanehisa et al., 2016).

Data availability

The raw metagenomic data in this study are available from the NCBI BioProject under accession number PRJNA644254. The MAGs information is listed in Supplementary Table 2. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JACXTQ000000000 and JACXTR000000000. The version described in this paper is version JACXTQ010000000 and JACXTR010000000.

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Conflict of Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Author contributions

JLY, JCM, YFW and WBD designed research. JLY, YFC, ZJJ performed research. JLY, ATC, MFU-H, JW and XWZ analyzed data. All the authors contributed in writing the manuscript.

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Table 1 Genome statistics of two metagenome-assembled genomes (MAGs) of methanotrophic bacteria from the Zoige wetland.

Parameter	bin_110	bin_199
Collection site	The Zoige wetland (China)	The Zoige wetland (China)
Recovered from	co-assembly	co-assembly
Total length (bp)	2807698	4040813
No. of contigs	403	543
No. of coding regions	2665	3746
No. of rRNA gene	1	None
No. of tRNA gene	31	36
No. of 16S rRNA gene	None	None
N50 (bp)	8072	9337
GC content (%)	49.13	48.35
Completeness[a] (%)	78.4	71.9
Contamination[a] (%)	3.47	8.77

[a] The completeness and contamination are based on the CheckM estimations.

Figure legends

Fig. 1 Workflow of DNA-SIP (Stable Isotope Probing) targeted metagenomic study of methanotrophs in the Zoige wetland. **A.** Soil cores under the water level were sampled from the Zoige wetland at sites with *Carex muliensis* as the dominant plants; soil microcosms were incubated with $^{13}CH_4$ for SIP and with $^{12}CH_4$ as control**;** total DNA was extracted from both original samples and SIP labeled heavy DNA for metagenomic sequencing. **B.** The metagenomic computational analysis covers community structure shift before and after DNA-SIP, assembly of metagenome-assembled genomes (MAGs), and genome-scale metabolic reconstruction of the active methanotrophs.

Fig. 2 Methane oxidation potential and CH_4-utilizing communities. A. Methane oxidation potential measured by microcosms exposed to CH_4. B. After CsCl centrifugation, density shifts of DNA-SIP exposed to $^{13}CH_4$ compare with $^{12}CH_4$ control were measured by Real-time PCR of *pmo*A genes for fractionated layers. Heavy DNA layers (the peak layer and the adjacent two layers) used for metagenomic were labeled with open blue circles. C. Relative abundance of community composition before and after ^{13}C labeling at the class level. D. Relative abundance of methanotrophic species before and after ^{13}C labeling estimated using Metaphlan2.

Fig. 3 Phylogenetic tree of methane monooxygenases genes obtained in this study and their abundances. A. The Maximum Likelihood tree of particulate methane monooxygenase alpha subunit (*pmo*A) was built, showing the genetic distances of the obtained *pmo*A genes (bold) and NCBI references (regular). The *pmo*A genes were

extracted from metagenomic assemblies (co-assemblies from all metagenome data), 133 amino acids sequences with a bootstrap value of 1000 replicates were used. Bootstrap >80% were shown. The scale bar represents 20% sequence divergence. The bin_110 and bin_199 represent *pmo*A genes recovered from the two MAGs, and relative abundance of MAGs in metagenomes was shown before ($t_{0\text{-}1}$, $t_{0\text{-}2}$) and after ($t_{1\text{-}1}$, $t_{1\text{-}2}$) DNA-SIP. B. Heatmap of the relative abundance of methanotrophs displayed according to the proportion of particulate methane monooxygenase (pMMO, *pmo*A, and *pxm*A) and soluble methane monooxygenase (sMMO, *mmo*X) in each sample. The abundance was calculated by dividing the number of each MMO gene to the total reads number of each metagenome dataset. Abundances are indicated by the colored scale (from white to blue and red) after each *pmo*A gene with the order $t_{0\text{-}1}$, $t_{0\text{-}2}$, $t_{1\text{-}1}$ and $t_{1\text{-}2}$. The tree was inferred using Mega7 with 1000 replicates.

Fig. 4 Phylogenetic tree showing the placement of the two MAGs within the gamma- and alpha-proteobacteria. The maximum likelihood phylogeny of representative reference genomes and the two MAGs from this study was generated using a set of 120 concatenated marker proteins. Bootstrap values were calculated from 100 replicates. Scale bar equals 0.1 amino acid substitutions per site. The relative abundance of MAGs before and after DNA-SIP samples was shown in heatmap after each MAGs in the order of $t_{0\text{-}1}$, $t_{0\text{-}2}$, $t_{1\text{-}1}$ and $t_{1\text{-}2}$.

Fig. 5 Metabolic reconstruction of the two MAGs, including bin_110 and bin_199, recovered from the co-assembly of all metagenomic data. Green and pink represent genes in bin_199 (*M. tundripaludum* like) and bin_110 (*M. psychrotolerans* like)

MAGs, respectively, blue indicates the communal genes that were found in both genomes. The pathways were drawn based on KEGG map files and KO assignments. A dashed line indicated the absence of the gene in both MAGs.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Soil physio-chemical properties of the two original sediment cores

Table S2. KEGG Orthology (KO) annotations and gene copy numbers for the two MAGs.

Fig. S1 Relative abundance of methanotrophic communities before and after $^{13}CH_4$ labeling based on the abundance of Coding sequences (CDS) belonging to each microbe. CDS predicted from the co-assemblies of metagenome data of all samples were used, the taxonomic assignment of each CDS was the result of BLASTp best hit against NCBI nr database (downloaded in Sep. 2018) using Diamond (Buchfink et al., 2015). Methanotroph abundance in each sample was calculated using the formula reported previously (Qin et al., 2012; Zheng et al., 2020).

Fig. S2 The 25 bacterial/archaeal OTUs with the highest relative abundance before and after ^{13}C-CH_4 labeling in each sample. The relative abundances and the taxonomy assignments were analyzed by using quality controlled metagenomic data with MetaPhlAn2.

Fig. S3 Maximum Likelihood tree of soluble methane monooxygenase genes (*mmo*X) extracted from metagenomic data. *mmo*X genes were extracted from metagenome assemblies (co-assemblies from all metagenome data), 233 amino acids sequences with bootstrap value of 1000 replicates were used. Bootstrap values of >80% are shown. Sequences in bold represent the *mmo*X genes obtained in this study. Others are reference sequences obtained from NCBI database. The scale bar represents 20% sequence divergence. The scaled heatmap after each gene indicates the relative abundance of each genes in samples before and after labeling, in the order of $t_{0\text{-}1}$, $t_{0\text{-}2}$, $t_{1\text{-}1}$ and $t_{1\text{-}2}$.

Fig. S4 The abundance of methanol dehydrogenase gene (*max*F, *xox*F) containing bacteria in the metagenome data. *max*F and *xox*F genes were retrieved from each metagenome data by using gene specific packages in GraftM. The heatmap showing the relative abundance of bacteria was based on the proportion of functional gene reads to the total number of metagenomic reads in that sample. Abundance is indicated by the coloured scale (from white, to red).