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Ultra-deep pyrosequencing of *pmoA* amplicons confirms the prevalence of *Methylomonas* and *Methylocystis* in *Sphagnum* mosses from a Dutch peat bog

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Summary

Sphagnum **peatlands are important ecosystems in the methane cycle. Methanotrophs in these ecosystems have been shown to reduce methane emissions and provide additional carbon to** ***Sphagnum*** **mosses. However, little is known about the diversity and identity of the methanotrophs present in and on** ***Sphagnum*** **mosses in these peatlands. In this study, we applied a** ***pmo*****A microarray and high-throughput 454 pyrosequencing to** ***pmo*****A PCR products obtained from total DNA from** ***Sphagnum*** **mosses from a Dutch peat bog to investigate the presence of methanotrophs and to compare the two different methods. Both techniques showed comparable results and revealed an abundance of** ***Methylomonas*** **and** ***Methylocystis*** **species in the** ***Sphagnum*** **mosses. The advantage of the microarray analysis is that it is fast and cost-effective, especially when many samples have to be screened. Pyrosequencing is superior in providing** ***pmo*****A sequences of many unknown or uncultivated methanotrophs present in the** ***Sphagnum*** **mosses and, thus, provided much more detailed and quantitative insight into the microbial diversity.**

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Introduction

Methane is an important greenhouse gas and as all other greenhouse gases its concentration has been rising rapidly since pre-industrial times (Forster *et al*., 2007). Methanotrophs can act as a sink for methane and occur in many different ecosystems like rice paddies, soils, volcanic areas and peat bogs (Hanson and Hanson, 1996; Conrad, 2009; Op den Camp *et al*., 2009; Kip *et al*., 2010). In 2005, it was shown that methanotrophs also occur in the hyaline cells of *Sphagnum* mosses (Raghoebarsing *et al*., 2005). Moreover, using a *pmo*A microarray, we recently detected a wide variety of methanotrophic symbionts in and on the mosses, showing the global prevalence of this symbiosis (Kip *et al*., 2010). Acidic peat bogs are the most extensive type of wetlands, occupying about 3% of the world's total land area and storing about 30% of carbon in soils globally. Methanotrophs present in these peatlands can act as a filter for methane, thereby reducing methane emissions from these wetlands and recycling carbon within the ecosystem (Raghoebarsing *et al*., 2005; Kip *et al*., 2010; Larmola *et al*., 2010).

Aerobic methanotrophs occur in several monophyletic lineages within the *Proteobacteria* and one lineage within the *Verrucomicrobia* (Conrad, 2009; Op den Camp *et al*., 2009; Vorobev *et al*., 2010). Gammaproteobacterial type I methanotrophs use the ribulose monophosphate pathway for formaldehyde fixation and have disc-shaped intracellular membranes (ICMs) that occur throughout the cell. The type II methanotrophs belong to the *Alphaproteobacteria*, comprise two lineages which use the serine pathway for formaldehyde fixation and have ICMs along the periphery of the cell. These methanotrophs include the *Methylocystis–Methylosinus* genera and the acidophilic methanotrophs of the genera *Methylocella*, *Methylocapsa* and *Methyloferula*. The recently described extremely acidophilic methanotrophic members of the *Verrucomicrobia* phylum isolated from

volcanic areas do not show intracellular membrane structures and still need to be characterized further (Op den Camp *et al*., 2009).

Peat bogs are harsh environments for microbes to live in because of the low pH between 3.8 and 4.8 and a low nutrient content. The isolation of methanotrophs from these ecosystems is still a challenge. After using very diluted oligotrophic media, several methanotrophs could be isolated from peatlands (Dedysh *et al*., 1998; 2000; 2002; 2004) all belonging to the *Alphaproteobacteria*, but molecular tools have shown that many more remain uncultivated. This exemplifies that molecular tools are necessary to gain better insight into these microbial communities and to identify new species. Methanotrophic communities can be studied using general taxonomic markers like 16S rRNA, or using functional markers of methanotrophs like the genes encoding subunits of the methane monooxygenase enzymes, *pmo*A or *mmo*X. The *pmo*A gene is the most commonly used marker. It encodes a subunit of the particulate (membrane bound) methane monooxygenase, and is present in all aerobic methanotrophs except *Methylocella* spp. and *Methyloferula stellata* (Vorobev *et al*., 2010). The *mmo*X gene is only present in some methanotrophs (including *Methylocella* spp.) and encodes a subunit of the soluble methane monooxygenase (McDonald *et al*., 2008). A *pmo*A-based microarray (Bodrossy *et al*., 2003) was developed to rapidly screen the composition of methanotrophic communities. The microarray probes are based on *pmo*A genes from known methanotrophic isolates and from environmental samples. This molecular screening tool quickly provides qualitative information about the diversity of the methanotrophic communities in an ecosystem. Conversely, *pmo*A clone libraries are labour intensive and hardly quantitative. Nowadays, next-generation high-throughput sequencing is an attractive alternative, because of the large amount of sequence data per run (max. 600 Mb per plate) and sufficient read length of pyrosequencing (currently ~500 nt). Although 454 technology is more expensive than microarray technology, it is expected that the technology will become affordable on a routine basis in the near future. Several *pmo*A-based PCR primer sets used for preparing clone libraries (Bourne *et al*., 2001) result in a PCR product between 450 and 500 nt, which make these products suitable for pyrosequencing.

Here, we compare 454 pyrosequencing of a *pmo*A PCR product with a *pmo*A microarray to investigate the composition of a methanotrophic community in *Sphagnum* mosses. Since microarray analysis showed the presence of both type I and type II methanotrophs, the more quantitative pyrosequencing approach was used to measure their abundances and phylogenetic classification in much greater detail.

Results and discussion

We investigated methanotrophic communities living in and on *Sphagnum* mosses from a Dutch peat bog, located in the Nature Reserve the Hatertse Vennen. *Sphagnum* mosses from the studied peat ecosystem were tested for methane oxidation activity by incubating the thoroughly washed *Sphagnum* mosses with methane. All samples showed methane oxidation rates between 20 and 40 μmol (g DW)$^{-1}$ day^{-1} at 20°C, comparable to rates reported in previous studies (Raghoebarsing *et al*., 2005; Kip *et al*., 2010).

Methanotrophic community in Sphagnum *investigated using a* pmoA *microarray*

We analysed the diversity of the methanotrophic communities in two independent *Sphagnum* moss samples from the same site with a *pmo*A microarray. DNA was isolated from non-incubated *Sphagnum* mosses and a nested PCR based on general *pmo*A primer sets, A189-A682r followed by A189-Mb661r (Holmes *et al*., 1995; Costello and Lidstrom, 1999), was performed. Sample HV 1.1 was obtained starting from DNA extracted before (Kip *et al*., 2010) and HV 1.2 represents a new sample. These PCR products were hybridized to the microarray (Fig. 1A) (Bodrossy *et al*., 2003; Kip *et al*., 2010). The microarray showed a high diversity of methanotrophs belonging to *Alpha-* and *Gammaproteobacteria*. Other studies that applied the same microarray to peat soils (Chen *et al*., 2008a), rice fields (Vishwakarma *et al*., 2009) and upland soils (Cébron *et al*., 2007) revealed a much less diverse methanotrophic community in comparison to our microarray results. Nevertheless, the diversity was similar to previously analysed *Sphagnum* mosses from Siberia and Patagonia (Kip *et al*., 2010). The microarray showed an abundance of *Methylobacter*, *Methylomonas*, *Methylomicrobium*, *Methylocystis* and *Methylosinus* genera. The presence of gammaproteobacterial methanotrophs, e.g. *Methylomonas* and *Methylobacter*, is surprising, since other studies showed a very low abundance of these methanotrophs in peatlands (Dedysh, 2002). Therefore, a high-throughput approach was used to get a deeper insight into the methanotrophic community in which *pmo*A PCR products obtained from mosses from the same peat bog were sequenced.

High-throughput sequencing

To investigate the methanotrophic community two PCRs were performed using genomic DNA extracted from *Sphagnum* mosses as a template and using the primer sets A189-Mb661 and A189-A650 for amplification (Holmes *et al*., 1995; Bourne *et al*., 2001). This resulted in PCR

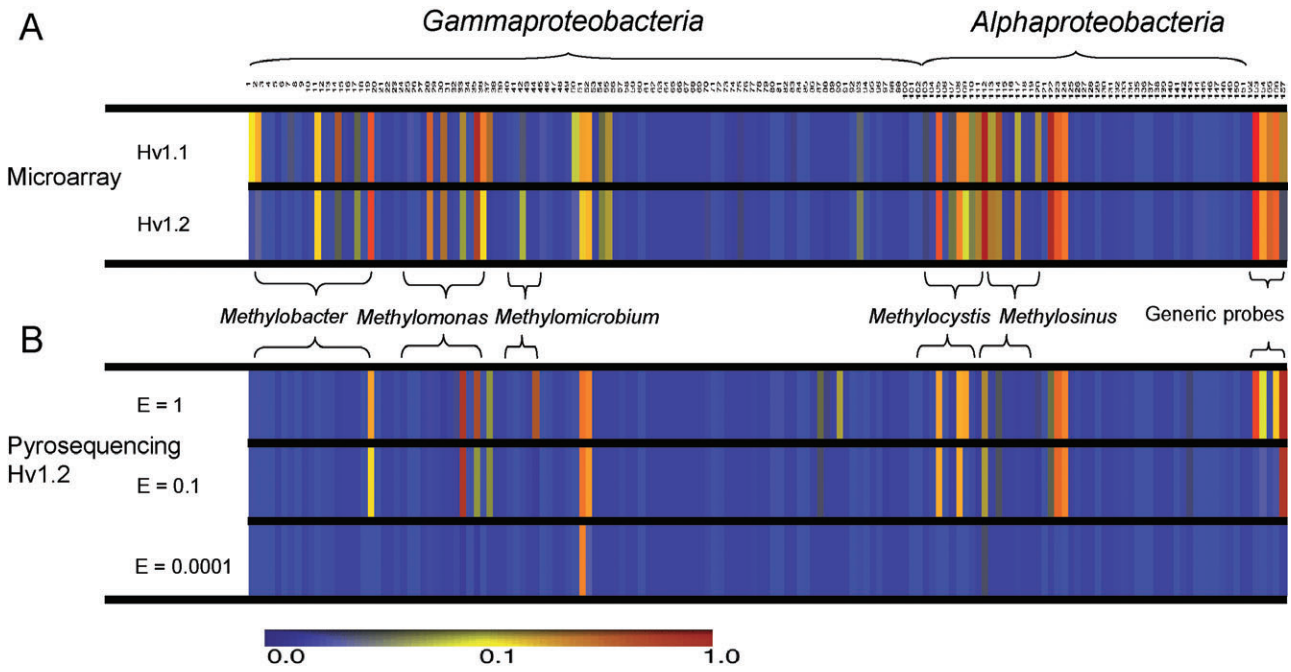

Fig. 1. A. *pmo*A microarray based analysis of methanotrophic microbial community of two different mosses, Hv1.1 and Hv1.2.
B. BlastN mapping of the *pmo*A microarray probes on the 454 titanium sequencing reads of Hv1.2 using different *E*-value cut-offs. The colour coding bar represents achievable signal per probe (1 indicates maximum signal obtained, 0.1 indicates that 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). Hv1.1 and Hv1.2 represent two independent samples. The exact numbers of the mapping are listed in Table S1 and the probes are listed in Table S3.

products suitable for the 454 GL FX technology (≤500 bp). These PCR products were mixed in a 1:1 ratio and were sequenced by pyrosequencing (ROCHE 454 GS GLFX titanium). A problem that is known to occur when pyrosequencing highly similar sequences (e.g. a PCR product) is simultaneous flashing of the same incorporated pyronucleotides, which leads to blinding of the camera (A. Hoischen, pers. comm.). To reduce this problem, we combined the *pmo*A PCR product with 16S rRNA PCR products from a non-related experiment. After sequencing, we extracted a total of 134 330 *pmo*A reads by BLAST searches against a large *pmo*A and *amo*A database containing 3849 unique sequences. These included the *pmo*A sequences from isolates and environmental sequences that were used to design the probes for the *pmo*A microarray (Bodrossy *et al*., 2003), supplemented with novel sequences, e.g. from more recent literature. Using an *E*-value cut-off of 0.1, allowed us to exclude all 16S rRNA reads.

Comparison of pmoA *microarray and high-throughput sequencing*

The probes present on the microarray are between 17 and 26 nucleotides long. A rapid analysis of these sequences was done by querying them by BlastN against all 454 pmoA reads. This approach allows a direct comparison of the pyrosequencing reads with the microarray results in terms of the probes. The number of reads that mapped to each probe was recorded at different *E*-value cut-offs and compared with the hybridization intensity of the microarray (Fig. 1B). We required high *E*-value cut-offs due to the short length of the reads, the lowest found *E*-value for each probe is indicated in Table S1. An *E*-value cut-off of one resulted in perfectly matching of the probes with the sequencing reads for several probes. Figure 1B shows that a high signal intensity on the microarray correlates well with the number of 454 sequencing reads identified as hits in the BlastN search. Using both methods we identified the presence of Type I methanotrophs, especially *Methylomonas*, *Methylomicrobium* and *Methylobacter* species and type II methanotrophs *Methylocystis* and *Methylosinus* species in the *Sphagnum* mosses. Type II methanotrophs are commonly found in peatlands and these sequencing data confirm the high abundance of type I methanotrophs, which were previously considered to represent only a very low percentage of the total methanotrophic community in peat ecosystems (Dedysh, 2009). It should be noted that the studies summarized by Dedysh (2009) were performed on peat soils from Siberia and not on living *Sphagnum* mosses as in the present study. Additionally, the microar-

ray analysis in Kip and colleagues (2010) showed also a much lower abundance of type I methanotrophs in North and West Siberian mosses.

The microarray and the probes mapped to the deep sequencing reads showed a rather similar pattern, although differences were found. These differences might be due to the different PCR protocols used to amplify the *pmo*A sequences for the microarray and for pyrosequencing. While the primers we employ have been extensively used to characterize methane oxidizing communities (Henckel *et al*., 2000), PCR inherently biases the results in any study. The primer combination with A682r is also able to amplify the homologous *amo*A gene of ammonia oxidizers, and was shown to recover mainly *amo*A genes (Bourne *et al*., 2001). However, a nested PCR of 189f/Mb661r on the 189f/682r PCR product, as performed for the microarray, can specifically retrieve the *pmo*A sequences. Primers Mb661r, used in both methods, and A650r, only used in the pyrosequencing method, are more specific in amplifying *pmo*A genes, but Mb661r misses the cluster RA21 (Bourne *et al*., 2001) and certainly the rice clusters taxonomically located between *pmo*A and *amo*A (Lüke *et al*., 2010). Moreover, A650r showed biases in recovering mainly *Methylococcus capsulatus pmo*A sequences and none related to *Methylomonas* or *Methylomicrobium* (Bourne *et al*., 2001). As all three approaches showed the abundance of *Methylomonas* and *Methylocystis*, we conclude that this overall result cannot be attributed to a bias in any of the primers. What remains is a possible influence of the T7-tag needed for the microarray procedure. It has been shown that a GC clamp can influence PCR results (Bourne *et al*., 2001) and as a T7-tag consists of seven nucleotides, its effect on hybridization might be considerable.

Phylogenetic analysis of the methanotrophic community in Sphagnum

We determined the phylogenetic distribution of the community by mapping the pyrosequencing reads to the *pmo*A database. Each pyrosequencing read was then assigned to its most similar *pmo*A gene sequence in this database, and conversely, for each database sequence, we counted the number of reads that could thus be mapped to it. In case of multiple equally scoring top hits we divided the read equally. The *Gammaproteobacteria* represented a total of 78 488 reads (58%) of which 80% were closely related to *Methylomonas* spp. The *Alphaproteobacteria* represented 53 378 reads (40%) and almost all of them (95%) were closely related to *Methylocystis* spp.

We constructed a maximum likelihood phylogenetic tree from those database sequences that mapped at least 10 reads (98% of all reads are represented), plus several reference sequences (Fig. 2). For visualization, we collapsed several of the branches, the details are presented in supplementary materials Table S1. The tree clearly shows the abundance of one or just a few species of *Methylomonas* and *Methylocystis* attached on and in the tested *Sphagnum* mosses of the Hatertse Vennen. However, it should be noted that amplicon sequencing (and microarray) relies on PCR, but 454 pyrosequencing may allow a quantitative measure of the diversity and relative abundances of *pmoA* phylotypes in a given PCR amplicon. To count gene copy numbers a quantitative real-time PCR approach would be needed. *Methylocystis* sp. are commonly found in any ecosystem where methane is present (Dedysh *et al*., 2007; Chen *et al*., 2008b) and active *Methylomonas* species have been detected in peat ecosystems (Chen *et al*., 2008a), however not in the high abundance we report here. The highest number of reads was found to relate to a cluster of environmental clones, detected with microarray probe peat 1_3–287 and *Methylomonas* strain M5, which was recently isolated from a Dutch peat bog and cannot be detected with any current specific microarray probe (N. Kip, unpubl. data). The second highest numbers of reads relate to *Methylocystis* species H2s and F10V2 and many environmental clones, which can be detected with microarray probe peat264 and Mcy522, which both showed high hybridization in the microarray. Verrucomicrobial *pmo*A reads were not found, since the used primers have more then five mismatches (Pol *et al*., 2007) this was to be expected.

Because *Methylocella* sp., a common methanotroph in Northern peatlands (Dedysh *et al*., 2000; 2004), does not possess a *pmo*A gene, it cannot be detected by the *pmo*A microarray. Therefore, we assessed the abundance of *Methylocella* sp. separately using a *mmo*X PCR clone library.

mmoX *clone library*

Because the molecular tools outlined above are based on the *pmo*A gene, they cannot detect methanotrophs of the *Methylocella* genus, which does not possess this gene (Dedysh *et al*., 2000). Still, *Methylocella* sp. is commonly found in peat ecosystems, so we documented their presence in the *Sphagnum* mosses using a *mmo*X-based PCR with different primer sets (see supplementary methods in *Supporting information* for PCR protocols). The results indicated that *Methylocella* sp. is either not abundant in this ecosystem or that the primers do not capture the indigenous *mmo*X genes. A neighbour-joining tree of sequences obtained after a nested PCR demonstrated that 21 out of 23 sequences clustered with known *mmo*X genes of *Methylocella* spp. (Fig. S1). This analysis shows the presence of some *Methylocella* spp. in or

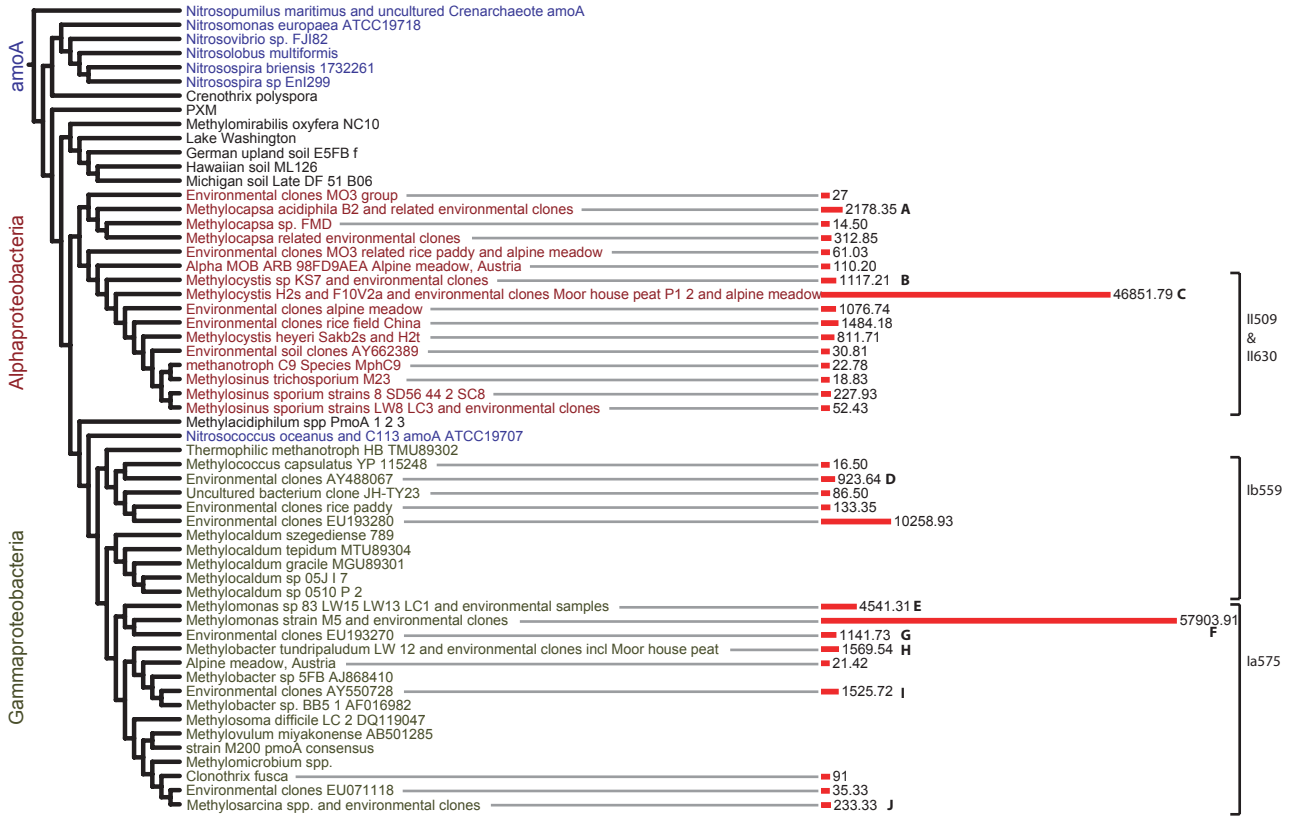

Fig. 2. Phylogenetic tree showing the *pmo*A reads mapped to *pmo*A and *amo*A sequences from methanotrophs and environmental samples. We translated the DNA sequences into protein, and determined the correct frame of translation, as the database contained the reverse complement of the *pmo*A gene in some cases. Then, these protein sequences were aligned using ClustalW 2.0.12 with default parameters (Larkin *et al*., 2007). The amino acids in the alignment were then translated back into the original codons of DNA, which was used as input for PhyML with the following parameters: approximate likelihood ratio test (SH-like branch supports), HKY85 model, estimated Ts/tv ratio, estimated proportion of invariable sites (5.38%), 4 substitution rate categories, estimated gamma distribution parameter, mean of rate class, maximum likelihood nucleotide equilibrium frequencies, optimized tree topology, NNIs tree topology search, BioNJ starting tree optimized branch lengths and substitution model parameters (Guindon *et al*., 2009). Microarray probes with a 100% sequence match are indicated behind the branches, with letters or in the figure. Probe letters: A: B2 and B2rel probes; B: Mcy270 and Mcy413; C: Peat264, Mcy522; D: FW1 probes; E: Mm531, Mm275, Mm-Es543, Mm-Es546; F: Peat1_3_287; G: Alp7-441, Mb460, Mb271, Mb380; I: Mb267, MbA486, MbA557, Mmb562. Red bars indicate the total number of reads found for that branch. Accession codes and sequences per branch can be found in Table S2. H: Mb460, Mb271, Mb380; J: Mmb562.

attached on the *Sphagnum* mosses, but they cannot be quantified easily. Since not many methanotrophs contain the *mmo*X gene, this gene is not very suitable as a functional marker to test the abundance among the total number of methanotrophs. Very recently, a method was developed to quantify *Methylocella mmo*X genes using new *mmo*X primers (Rahman *et al*., 2011).

Final conclusions

Overall, the results of the pyrosequencing and the microarray are consistent and show the abundance of both gammaproteobacterial as well as alphaproteobacterial genera in this acidic peat ecosystem. By comparing the microarray with the probes mapped to the deep sequencing reads, we obtained quite a similar pattern. We conclude that both approaches can be used to map the methanotrophic diversity in this ecosystem, although pyrosequencing gives a more quantitative answer. Most studies thus far have shown that peat ecosystems are dominated by alphaproteobacterial clades. The high diversity of methanotrophs in and on *Sphagnum* mosses is striking, and might suggest that these mosses provide different subniches suitable for different types of methanotrophs. These results call for more detailed analyses into the role of each of the methanotrophs, and their contribution to the *Sphagnum* peat bog ecosystem.

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Supporting information

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

Fig. S1. Neighbour-joining tree of the *mmo*X gene, showing the relationship of the clones to the *mmo*X gene of selected methanotrophs (500 bootstrap replicates). Bootstrap values >60% are indicated at the node of the branch. The evolutionary distances (scale bar: 0.02 substitutions per site) were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004).

Table S1. BlastN results based on mapping the 454 pyrosequencing reads on the microarray probes. Results are represented in Fig. 1.

Table S2. *pmo*A sequences per branch of the phylogenetic tree represented in Fig. 2.

Table S3. Probes used for microarray analysis. Order corresponds to the order in which the probes are arranged on the microarray analysis shown in Fig. 1.

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