pmoA Primers for Detection of Anaerobic Methanotrophs[▽]

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Published *pmoA* primers do not match the *pmoA* sequence of “*Candidatus Methyloirabilis oxyfera*,” a bacterium that performs nitrite-dependent anaerobic methane oxidation. Therefore, new *pmoA* primers for the detection of “*Ca. Methyloirabilis oxyfera*”-like methanotrophs were developed and successfully tested on freshwater samples from different habitats. These primers expand existing molecular tools for the study of methanotrophs in the environment.

Aerobic methane oxidation is performed by several specialized groups of bacteria that are widely distributed in the environment (6). The first step of aerobic methane oxidation is the conversion of methane to methanol, catalyzed by either soluble or particulate methane monooxygenase (pMMO). The alpha subunit of pMMO, encoded by the *pmoA* gene, is highly conserved (5) and often used as a functional marker for analyzing methanotrophs in the environment (14). The first oligonucleotide primers targeting the *pmoA* gene, A189 and A682, have been used extensively in environmental studies (14). As the *pmoA* gene phylogeny is largely comparable to the 16S rRNA gene phylogeny (7, 12), *pmoA* primers provide a useful tool for obtaining simultaneous functional and taxonomic inventories of methanotrophs in the environment (10).

Recently, new *pmo*-like (*pxm*) genes in cultured, methanotrophic *Gammaproteobacteria* (21) and new groups of aerobic methane oxidizers with divergent *pmo* genes were discovered. Most noteworthy are the proteobacterial *Crenothrix polyspora* (19) and three acidophilic verrucomicrobial *Methyloirabilis* species (16). The presence of a complete *pmo* gene cluster in the genome of the anaerobic, methanotrophic, nitrite-reducing bacterium “*Candidatus Methyloirabilis oxyfera*” (3) further expanded the array of *pmo* gene diversity. “*Ca. Methyloirabilis oxyfera*,” a member of the uncultured NC10 phylum (17), forms a novel taxonomic group of bacterial methanotrophs (reviewed in reference 24). The presence of *pmo* genes in an anaerobe was explained by the unusual metabolism of “*Ca. Methyloirabilis oxyfera*”: molecular oxygen was produced

from nitric oxide, an intermediate of denitrification, and then used to oxidize methane via the complete aerobic pathway starting with pMMO (3).

It was not possible to amplify *pmoA* genes with the most commonly used forward primer A189 and the reverse primers A682 (7), mb661 (2), and A650 (1), even from “*Ca. Methyloirabilis oxyfera*” enrichment cultures. The alignment of the primers with the *pmoA* sequence of “*Ca. Methyloirabilis oxyfera*” revealed several critical mismatches especially with the reverse primers (Fig. 1), explaining the failure to obtain a PCR product. Based on this alignment, new *pmoA* primers were developed. We changed one nucleotide to a wobble base in the forward primer A189, resulting in a general degenerate primer, A189_b (Fig. 1), matching most methanotrophs in the GenBank database with the exception of the genus *Methyloirabilis*. The reverse primer A682 differed in eight nucleotides from the *pmoA* sequence of “*Ca. Methyloirabilis oxyfera*.” Therefore, the newly developed reverse primer designated cmo682 specifically targets “*Ca. Methyloirabilis oxyfera*”-like bacteria. Also, a second set of primers for a nested-PCR approach specific for nitrite-dependent anaerobic methane-oxidizing bacteria was developed based on this alignment. These primers were named cmo182 and cmo568 (Fig. 1) and were used in a nested approach after the amplification with A189_b and cmo682. The newly designed primers were first tested with DNA extracted from Ooijpolder drainage ditch sediment, which had previously been used to obtain an enrichment culture performing nitrite-dependent anaerobic methane oxidation (4). The same approach was then used to screen samples from several oxygen-limited freshwater habitats for the presence of “*Ca. Methyloirabilis oxyfera*”-like bacteria: an alpine peat bog (China), wastewater treatment plants (WWTP) (Netherlands), and contaminated aquifers (United States and Netherlands) (Table 1). There is circumstantial evidence for the occurrence of anaerobic methane oxidation in the contaminated aquifers (18, 22).

To extract DNA from the samples from Ooijpolder, Banisveld, Rotterdam, and Lichtenvoorde (Table 1), biomass was collected by centrifugation. The pellets were resus-

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Nucleotide position on <i>M. capsulatus</i> str. Bath	A189b		cmo182		cmo568		cmo682	
	136	153	182	199	549	568	649	666
AE017282 <i>Methylococcus capsulatus</i> str. Bath	ATGGGTGACTGGGACTTCTGGTGG	GGGTACCGGTGACCCGATCGTAC	CATCCAGGGTTACAACTATGTGGTA	TCGGCATTTCTTCTCGGCTTCATG				
AB253367 <i>Methylomicrobium japanense</i> str. NI	GGCGGTGACTGGGACTTCTGGACT	GGGTAACTGTAGCGCAATCGTTT	CCTTCAAGGTTACCACTATGTAGAA	TCAGCGTTCTTCTCTGGTTTCGTA				
AB275418 <i>Methylolaldum</i> sp. T 025	ATGGGTGACTGGGACTTCTGGGCG	GGGTACCGGTGACGCGATTTGTGT	CATTCAAGGTTACCACTATGTAGAA	TCGGCGTTCTTCTCGGCTTCATG				
AB501285 <i>Methylovulum miyakonense</i> str. HT12	GGGGACTGGGACTTCTGGGTT	GGGTACCGGTAGTTCGATTTGAT	TATTATGGGTTACCAATACGTCGGA	TCAGCTTTCTTCTCGGTTGGTGA				
DQ119048 <i>Methylosinus sporium</i> str. LC3	GGGGACTGGGACTTCTGGGTC	GGCGACCGGTATCCCGATCCTGG	TCTGATCGGCTCCCACTCGTCGCA	GCCGCTTCTTCTCGGCTTC				
BX649604 <i>Methylocystis</i> str. SC2	GCCGCGGACTGGGACTTCTGGATC	GGCGACCGGTGACGCGATCGTCG	CCTCATCGGCTTCAACTACGTCGCA	GCCGCTTCTTCTCGGCTTCATT				
DQ379514 Uncultured <i>Methylocystis</i> sp. GSC357	GCGGGGACTGGGACTTCTGGGTC	GGCGACCGGTATTCGATCCTGG	TCTGATCGGCTTCACTTCGTCGCA	GCCGCTTCTTCTCGGCTTCGTC				
EF623812 UB Schoehsee sediment Germany Ssedi43	GGTGACTGGGACTTCTGGGTC	GGGTACCGGTACACCGATTTGAT	TTATATGGGTTACGATATGTCGTA	TCAGCTTCTTCTCGGCTTC				
EU722433 <i>Methylomonas methanica</i> S1	GGCGATTGGGACTTTTGGGTC	GGCCATTGGTACACCGCTTAATCG	TTTGTTCGGTTTCAATATATCGTA	GCGGCTTCTTCTCGGCTTCGTA				
EF175100 <i>Nitrosospira</i> sp. EnI299	GCAGGCGACTGGGACTTCTGGCTG	GGCGGTAGTGACCCGATCCTGG	CTACACCGGCTTCTGATGTCGCA	GCCCTATTCTTCTCGGCTTCGTC				
AF042171 <i>Nitrosolobus multififormis</i> str. Nm24C	GCGGGAGACTGGGACTTCTGGCTT	GGCGGTGCTGACCCGATCGTAG	CTACACCGGCTTTTGTATGTCGCA	GCCGCTTCTTCTCGGCTTCGTC				
AL954747 <i>Nitrosomonas europaea</i> str. ATCC 19718	GCAGGTGACTGGGACTTCTGGATG	GGCGGTGCTGACCCGATCGTAG	TTACATGGGACATCTGTATGTCGTA	GCAGCTTCTTCTCGGCTTCGTA				
DQ295901 <i>Crenothrix polyspora</i> 3	GGACACTGGGACTTCTGGCTC	GGCGGTGCTGACCCGATTCACC	CTACATGGGCTTCACTGATGTCGTA	TCGGCTTCTTCTCGGCTTC				
AF047705 <i>Nitrosococcus oceanii</i> ATCC19707	ATGGGGGACTGGGACTTCTGGGTT	GGCCACCGGTAGTTCGATTTGAT	TATTATGGGTTACCAATACGTCGGA	TCAGCTTCTTCTCGGCTTTTGT				
EF591085 <i>Methylacidiphilum fumariolicum</i> PmoA1	GCCGGGACTGGGCTTCTGGATC	GGCCAGTGTGTCGCTCATCATGG	CCTGATTCAATATCAGTATCGGAA	TCGGCTTCTTCTCGGCTTCGTC				
EF591086 <i>Methylacidiphilum fumariolicum</i> PmoA2	GCTGGGATTTGGTCAATTTGGACC	GGCCAGTGTGTCGCTCATCATGG	TCTCATCCAGTATGAATACATCGGA	TCCGCTTCTTCTCGGCTTTTGT				
EF591087 <i>Methylacidiphilum fumariolicum</i> pmoA3	GAAGGAGACTGGGACTTCTGGATA	TTCCAAATTTAGGAGTATCTCCT	TTACATCGGATTTTCCATGTCGCTT	GCATATTTCTTCTCGGCTTCGTA				
FJ462791 <i>Methylacidiphilum kamchatkense</i> PmoA4	GCTGGAGACTGGGCTTTTGGATC	GGCCAGTGTGTCGCTCATCATGG	TCTTATTCATATCAGTATATTCGAA	TCGGCTTCTTCTCGGCTTTTGT				
DQ367738 UB Sediment Lake Washington pmoA3	GGGGACTGGGACTTCTGGACC	GGCTGCTGCTGACGCGGCTCTGC	CCTCAGGGTTCCAGTATATTCGGA	TCGGCTTCTTCTCGGCTTCGTA				
EU723734 UB forest soil Hawaii ML22	GGAGACTGGGACTTCTGGACC	GGCTGCTGCTGACGCGGCTCTGC	CTTGCAAGGTTCCAGTATATTCGCA	TCGGCTTCTTCTCGGCTTCGTA				
EU723750 UB forest soil Hawaii CCRd431	GGGGACTGGGACTTCTGGGTC	GGCTGCTGCTGACGCGGATCTGC	CCTTCAGGGTTCCAGTATATTCGCA	TCGGCTTCTTCTCGGCTTCGTA				
FP565575 <i>Candidatus Methyloiraxia oxyfera</i>	GTTGGCGACTGGGACTTCTGGGTC	GGGTACCGGTGACGCGGATCCTGT	TCTGATGGGGATGAGATATGCGCT	TCCTCGTCTTCTCGGCTTCGTA				
New pmoA primers for "Ca. M. oxyfera"	GGNGACTGGGACTTCTGGGTC	TCACGTTGACGCGGATCC	GATGGGGATGAGATATGTCG	TCGTTCTTCTCGGCTTCGTA				
pmoA primers (Holmes et al. 1995)	GGNGACTGGGACTTCTGGGTC	TCACGTTGACGCGGATCC	GATGGGGATGAGATATGTCG	TCGTTCTTCTCGGCTTCGTA				

FIG. 1. Alignment using MEGA4 of newly developed primers for the alpha subunit of particulate methane monooxygenase (*pmoA*) of "Candidatus Methyloiraxia oxyfera" and nucleotide sequences encoding the *pmoA* genes of different methanotrophic *Gammaproteobacteria* (GenBank accession no. AE017282, AB253367, AB275418, AB501285, DQ295901), methanotrophic *Alphaproteobacteria* (DQ119048, BX649604, DQ379514, EF623812), and *Verrucomicrobia* (EF591085, EF591086, EF591087, FJ462791); different ammonia monooxygenase (*amoA*) sequences of nitrifying *Betaproteobacteria* (EF175100, AF042171, AL954747); *amoA* sequences of nitrifying *Gammaproteobacteria* (AF047705); *pmoA* sequences of methanotrophic *Gammaproteobacteria* (EU722433); and *pmoA* sequences of different uncultured methanotrophs of unknown affiliation (DQ367738, EU723734, EU723750) (20). Identical nucleotides are shaded in gray. cmo568 and cmo682 are shown as the reverse complementary sequence.

pended in 120 mM sodium phosphate buffer, glass beads (~0.3 g, 0.25-mm diameter) were added, and bead beating (Retsch MM301) was performed for 60 s at 30 Hz. DNA was extracted and purified according to reference 13. For the nitrogen-contaminated Cape Cod aquifer (15), 2 liters of groundwater from a depth of 8.9 m (relative to mean sea level) at sampling site F168 was filtered using a Sterivex cartridge filter (0.2- μ m membranes; Millipore). The membrane filter was used for DNA extraction. DNA from Cape Cod and Zoige soil (0.25 g) was extracted using the Power-Soil DNA isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol.

To amplify the *pmoA* gene fragment, the primers A189_b and cmo682 were used for the first PCR, and cmo182 and cmo568 were used for the second PCR. For both PCRs, thermal cycling was performed with an initial melting step for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing using a temperature gradient from 50 to 60°C (Zoige sample, 53 to 63°C) for 1 min, and elongation at 72°C for 1.5 min. Finally, an elongation step at 72°C for 10 min was performed. After PCR, amplicons of all annealing temperatures were pooled to minimize random polymerase errors and

the effects of primer mismatches. All PCRs were performed with PerfeCTa SYBR Green FastMix (Quanta BioSciences, Inc., Gaithersburg, MD).

PCRs with the primers A189_b and cmo682 on DNA from Ooijpolder sediment yielded faint and multiple PCR bands, whereas the nested PCR using the pooled amplicons of the first PCR with primers cmo182 and cmo568 resulted in a strong and single band on the gel of the correct size (389 bp). The PCR product mix was ligated into the pGEM-T Easy cloning vector and cloned according to the manufacturer's instructions (Promega). Plasmids were isolated from randomly selected clones for each PCR product with the GeneJET miniprep kit (Fermentas, Lithuania). The plasmids were sequenced using the M13 forward primer. Sequences were checked for quality with Chromas Lite 2.01, erroneous sequences were removed, and BLAST search analysis was performed (<http://www.ncbi.nlm.nih.gov/Blast.cgi>). Sequences matching *pmoA* were aligned to a data set of different *pmoA*, *pmoA*, and *amoA* sequences using the MEGA4 software package and the Clustal W algorithm (20). A phylogenetic tree with the *pmoA* sequences of *Methylacidiphilum* as an outgroup was calculated with the neighbor-joining method, and tree to-

TABLE 1. Overview of sampling locations

Site abbreviation	Sampling location	Geographic coordinate	Environment ^a	Reference
Ooijpolder	Nijmegen, Netherlands	51°50'N, 5°54'E	Ditch	Ettwig et al. (4)
Zoige	Tibetan plateau, China	33°56'N, 102°52'E	Peat bog	Zhang et al. (25)
Cape Cod	Massachusetts, United States	41°41'N, 70°17'W	Aquifer, well F168	Smith et al. (18); Miller and Smith (15)
Banissveld	Oirschot, Netherlands	51°33'N, 5°17'E	Aquifer, 3.2-m depth	van Breukelen and Griffioen (22)
Lichtenvoorde	Lichtenvoorde, Netherlands	51°59'N, 6°34'E	WWTP	WWTP Lichtenvoorde ^b
Rotterdam	Rotterdam, Netherlands	51°55'N, 4°28'E	WWTP	van der Star et al. (23)

^a WWTP, wastewater treatment plant.

^b http://www.waterstromen.nl/onze_activiteiten/afvalwaterbehandeling/afvalwaterzuivering_royal_dutch_tanneries_lichtenvoorde.aspx.

FIG. 2. Phylogenetic tree of *amoA*, *pmoA*, and *pxmA* nucleotide sequences, including the sequences obtained in this study with *Methylacidiphilum* as the outgroup. The tree was calculated using the neighbor-joining method. Bootstrap support values greater than 60% ($n = 1,000$) are indicated at the nodes.

pology robustness was tested by bootstrap analysis of 1,000 replicates (Fig. 2).

Similar to the results with the Ooijpolder samples, the direct PCR on the different environmental samples using the primer combination A189_b and cmo682 yielded multiple PCR products. Therefore, the same nested approach was applied as described above. From all the different environments analyzed (Table 1), *pmoA* sequences closely related to “*Ca. Methyloirabilis oxyfera*” (at least 85.5% nucleotide identity and 92% protein identity) were retrieved, most of which cluster according to location (Fig. 2). Together they form a distinct group affiliated with the *pmoA* genes of aerobic methanotrophs (Fig. 2). The closest related sequences obtained with PCR using the

conventional *pmoA* primers (8, 9, 11) were not targeted by the new primers (Fig. 1). All newly obtained sequences share the insertion of a proline at position 131 (3), a distinct feature compared to the sequences of known methane or ammonium monooxygenases. The fact that they are not very divergent from each other may reflect functional conservation relative to the oxygenic pathway.

This study provides a rapid and robust method for the amplification of NC10 phylum *pmoA* genes and enables the screening of different environments.

Nucleotide sequence accession numbers. Representative sequences from each location were submitted to GenBank under accession numbers HQ698926 to HQ698937.

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