pmoA Primers for Detection of Anaerobic Methanotrophs


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Published pmoA primers do not match the pmoA sequence of “Ca. Methylophilus mirabilis oxyfera,” a bacterium that performs nitrite-dependent anaerobic methane oxidation. Therefore, new pmoA primers for the detection of “Ca. Methylophilus 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 monoxygenase (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 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 Methylacidiphilum species (16). The presence of a complete pmo gene cluster in the genome of the anaerobic, methanotrophic, nitrite-reducing bacterium “Ca. Methylomirabilis oxyfera” (3) further expanded the array of pmo gene diversity. “Ca. Methylomirabilis 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. Methylomirabilis 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. Methylomirabilis oxyfera” enrichment cultures. The alignment of the primers with the pmoA sequence of “Ca. Methylomirabilis 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 Methylacidiphilum. The reverse primer A682 differed in eight nucleotides from the pmoA sequence of “Ca. Methylomirabilis oxyfera.” Therefore, the newly developed reverse primer designated cmo682 specifically targets “Ca. Methylomirabilis 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. Methylomirabilis 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, Buisveld, Rotterdam, and Lichtenvoorde (Table 1), biomass was collected by centrifugation. The pellets were resus-

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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-μm membranes; Millipore). The membrane filter was used for DNA extraction. DNA from Cape Cod and Zoige soil (0.25 g) was extracted using the PowerSoil 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, ppxmA, 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-

![FIG. 1. Alignment using MEGA4 of newly developed primers for the alpha subunit of particulate methane monooxygenase (pmoA) of "Candidatus Methylomirabilis oxyfera" and nucleotide sequences encoding the pmoA genes of different methanotrophic Gammaproteobacteria (GenBank accession no. AE017282, AB253367, AB275418, AB501285, DQ295901), methanotrophic Alphaproteobacteria (DQ119048, BX49604, DQ379514, EF623812), and Verrucomicrobia (EF591085, EF591086, EF591087, FJ462791); different ammonia monooxygenase (amoA) sequences of nitrifying Betaproteobacteria (EF175100, AF042171, AL954747); amoA sequences of nitrifying Gammaproteobacteria (EF175100, AL954747); and ppxmA sequences of methanotrophic Gammaproteobacteria (EU722433) and pmoA sequences of different uncultured methanotrophs of unknown affiliation (DQ367738, EU722374, EU722375) (20). Identical nucleotides are shaded in gray. cmo568 and cmo682 are shown as the reverse complementary sequence.](https://example.com/fig1.png)

### TABLE 1. Overview of sampling locations

<table>
<thead>
<tr>
<th>Site abbreviation</th>
<th>Sampling location</th>
<th>Geographic coordinate</th>
<th>Environment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ooijpolder</td>
<td>Nijmegen, Netherlands</td>
<td>51°50′N, 5°54′E</td>
<td>Ditch</td>
<td>Ettwig et al. (4)</td>
</tr>
<tr>
<td>Zoige</td>
<td>Tibetean plateau, China</td>
<td>33°56′N, 10°52′E</td>
<td>Peat bog</td>
<td>Zhang et al. (25)</td>
</tr>
<tr>
<td>Cape Cod</td>
<td>Massachusetts, United States</td>
<td>41°14′N, 70°17′W</td>
<td>Aquifer, well F168</td>
<td>Smith et al. (18); Miller and Smith (15)</td>
</tr>
<tr>
<td>Banisveld</td>
<td>Izsorsch, Netherlands</td>
<td>51°33′N, 5°17′E</td>
<td>Aquifer, 3.2-m depth</td>
<td>van Breukelen and Griffioen (22)</td>
</tr>
<tr>
<td>Lichtenvoorde</td>
<td>Lichtenvoorde, Netherlands</td>
<td>51°59′N, 6°34′E</td>
<td>WWTP</td>
<td>WWTP Lichtenvoorde&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rotterdam</td>
<td>Rotterdam, Netherlands</td>
<td>51°55′N, 4°28′E</td>
<td>WWTP</td>
<td>van der Star et al. (23)</td>
</tr>
</tbody>
</table>

<sup>a</sup> WWTP, wastewater treatment plant.

polity 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. Methylomirabilis 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.

**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.
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