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Detection of Methanotrophs in Groundwater by PCR†

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Methanotrophic bacteria have significant potential for bioremediation, which would require methods for monitoring the presence and activity of these organisms in environmental samples. In this study, PCR was used to detect methanotrophic bacteria. Primers were designed on the basis of a partial sequence of *pmoA*, which encodes one of the proteins of the particulate methane monooxygenase. Specific amplification of a portion of *pmoA* was obtained with template DNA isolated from lab strains of methanotrophs. A *pmoA* product was also obtained by using DNA from groundwater. The identity of the PCR product was confirmed by sequencing or by amplification with a nested primer. Reverse transcriptase PCR detected *pmoA* mRNA.

Methanotrophic bacteria use methane as a source of carbon and energy. Methane is oxidized to CO_2 or is incorporated into biomass. The first enzyme of the methane oxidation pathway is methane monooxygenase. All known methanotrophs produce a membrane-associated particulate methane monooxygenase (pMMO). A few strains produce a soluble methane monooxygenase (sMMO) in response to copper limitation. Because they are capable of cometabolic oxidation of trichloroethylene (TCE) and other persistent compounds, methanotrophs have potential for bioremediation (1, 4, 9, 13, 20). The initial interest in this potential has focused on sMMO because the rate of TCE oxidation with this enzyme is very high (20). However, because pMMO can also catalyze TCE oxidation (9, 30) with a low K_s for TCE (14, 25) and because methanotrophs that lack sMMO may be widely distributed, it is essential to assess the distribution and activity of pMMO when sites are evaluated prior to and during bioremediation.

The genes encoding sMMO (*mmo*) have been cloned and sequenced (6, 7, 26). The sequence information obtained has been used to design primers for PCR that can be used to detect a portion of *mmoX* in environmental samples (15, 17, 19). Primers for amplification of a portion of the gene encoding methanol dehydrogenase have been designed and used with environmental samples, but sequencing is required to distinguish products amplified from methanotrophs from products amplified from other methylotrophs (16).

Recently, the sequence of the genes encoding pMMO, *pmoCAB*, was determined and was shown to exhibit no significant homology to the *mmo* sequence (23). PCR primers that amplify a fragment of either *pmoA* or the gene for a related enzyme, *amoA*, have been designed (11). Because *amo* encodes the ammonia monooxygenase of ammonia-oxidizing bacteria, these primers cannot be used to distinguish between methanotrophs and ammonia oxidizers. This study was undertaken to develop approaches for specifically detecting *pmo* in groundwater. To do this, primers were designed on the basis of regions that are different in *pmoA* and *amoA*. These primers amplified a 330-bp sequence when DNA from a methanotroph was used as the template; no product was amplified when DNA from an ammonia oxidizer was used. Our methods should have applications for sites being assessed for bioremediation, as well as in other environmental studies on the distribution and activity of methanotrophs.

(A preliminary report of this work has been presented previously [8].)

MATERIALS AND METHODS

Bacteria and growth conditions. *Methylomicrobium album* BG8, *Methylococcus capsulatus* (Bath), and *Methylosinus trichosporium* OB3b were grown in nitrate minimal salts medium (29) supplemented with 5 μM $CuSO_4$ under an atmosphere containing 50% methane with shaking at 200 rpm at 30°C. To prepare enrichment cultures, cells from 150 ml of groundwater were collected on filters, and the filters were incubated in the same medium. When the medium became turbid, the cultures were streaked onto plates. An isolate from Yellowstone Lake was obtained from an enrichment culture inoculated with mat material collected near a fumarole in Sedge Bay. An isolate from Lake Michigan was isolated from an enrichment culture inoculated with Green Bay sediment (5). *Escherichia coli* JM109 was grown in Luria-Bertani broth (21), and *Nitrosomonas europaea* ATCC 19718 was grown in ATCC medium 221 (2).

Molecular techniques. The DNA used for PCR was prepared with a GenomicPrep Cell and Tissue DNA Isolation Kit (Amersham Pharmacia, Milwaukee, Wis.). Alternatively, DNA was prepared with an Amersham Pharmacia GFX kit with the following modifications: solution I included 2% hexadecyltrimethylammonium bromide, solution II was a 1% sodium *N*-laurylsarcosinate solution, and solution III was a 6 M guanidine thiocyanate solution. DNA was isolated from groundwater by using a protocol modified from the protocol of Fuhrman et al. (10). Cells were collected on Durapore series GV filters (Millipore Corp., Bedford, Mass.), and 0.3% hexadecyltrimethylammonium bromide and 0.9 M NaCl were added prior to phenol extraction.

RNA was prepared from cultured bacterial cells with TRIzol (Gibco BRL, Gaithersburg, Md.) by using the recommendations of the manufacturer. To prepare RNA from groundwater, cells were collected from groundwater on Durapore filters.

The PCR amplifications were performed in 100-μl (total volume) reaction mixtures in thin-wall tubes under a layer of mineral oil (Sigma Chemical Co., St. Louis, Mo.) by using a model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Each PCR was performed in a solution that contained each deoxynucleoside triphosphate at a concentration of 200 to 400 μM, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.01 mM EDTA, 0.1 mM dithiothreitol, 5 mM Tris HCl (pH 8.0), 2% dimethyl sulfoxide, 5% glycerol, 0.1% Triton X-100, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). For PCR, the concentration of each member of the primer pair was as follows: 0.15 μM for the 16S ribosomal DNA (rDNA) primers, 0.075 μM for the *mmoX* primers, and 0.10 μM for the *pmoA* primers. The PCR conditions were as follows: incubation at 95°C for 10 min; *Taq* added; 29 cycles consisting of incubation at 94°C for 1 min, at 45°C for 1 min, at 72°C for 1 min, and (for the final cycle) at 94°C for 1 min, at 45°C for 1 min, and at 72°C for 5 min. For DNA purified from cultured cells, 20 ng of DNA was used as the template. For environmental samples the level of DNA was less than the level which could be directly quantified on an agarose gel.

To detect RNA, an uncoupled reverse transcriptase PCR (RT-PCR) was used. A DNA copy (cDNA) was reverse transcribed from the RNA by using the avian myeloblastosis virus RT and the AMV/Tfl buffer components of an Access RT-PCR kit (Promega). The cDNA was isolated by using a PCR Purification Kit

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TABLE 1. Primers used for amplification of DNA from methanotrophic bacteria

Target gene	Size of product (bp)	Primer	Primer sequence (5′–3′)[a]	Reference
amoA or *pmoA*	525	amo/pmof	GGNGACTGGGACTTCTGG	11
		amo/pmor	GAASGCNGAGAAGAASGC	
mmoX	535	mmof	GGCTCCAAGTTCAAGGTCGAGC	15
		mmor	TGGCACTCGTAGCGCTCCGGCTCG	
16S rDNA	1,507	16Sf	AGAGTTTGATCATGGCTCAG	12
		16Sr	TACGGYTACCTTGTTACGACTT	
pmoA	330	pmof1	GGGGGAACTTCTGGGGITGGAC	This study
		pmor	GGGGGRCIACGTCITTACCGAA	
pmoA	178	pmof2	TTCTAYCCDRRCAACTGGCC	This study
		pmor	GGGGGRCIACGTCITTACCGAA	

[a] Y = C or T; D = G, A, or T; R = A or G; S = G or C; N = A, G, C, or T.

(Qiagen Inc., Santa Clarita, Calif.) and was used as a template for PCR. PCR and RT-PCR products were analyzed on 1.5% agarose gels.

Environmental samples. Groundwater was collected from three wells maintained by the Department of Geosciences of the University of Wisconsin-Milwaukee. Two of these wells (wells GLRF and CAP) are 3 to 5 m deep in a sand-gravel aquifer. Well LAPHAM is 83 to 105 m deep in a dolomite aquifer.

RESULTS

The sequences of the *pmoA* and *amoA* genes were aligned by Holmes et al. (11). These investigators used primers amo/pmof and amo/pmor (Table 1) to amplify a portion of either *pmoA* or *amoA*, sequenced the PCR products, and aligned the resulting sequences. On the basis of highly conserved regions of the *pmoA* sequences that are distinct from the *amoA* sequence, primers specific for *pmoA* (primers pmof1 and pmor) were designed in the present study (Table 1). Primer pmof1 binds to a site on the DNA 165 bp downstream from the primer amo/pmof binding site.

PCR was performed with three primer pairs to simultaneously detect *pmoA*, *mmoX* (primers designed by McDonald et al. [15]), and 16S rDNA. 16S rDNA, which was amplified by universal primers (12), was used as a control for the adequacy of the DNA as a template for PCR. The expected amplification products were obtained (Fig. 1). While sMMO is present in *M. capsulatus* (Bath) and *M. trichosporium* OB3b, it is not present in *M. album* BG8 (27, 28). The negative controls, *E. coli* and the ammonia oxidizer *N. europaea*, did not yield *mmoX* or *pmoA* products (Fig. 1, lanes 8 and 9). An *amoA* product was amplified from *N. europaea* with primers amo/pmof and amo/pmor (Fig. 1, lane 10).

The identities of the PCR products were confirmed by sequencing the products obtained by using *M. capsulatus* (Bath), *M. trichosporium* OB3b, and *M. album* BG8 DNAs as templates. The sequences obtained were 97 to 98% identical to the sequence described previously (11).

Another approach used to verify the identities of the PCR products was to use the *pmoA* product as a template for amplification of another product with a nested primer (primer pmof2) (Table 1). This resulted in a 178-bp product when the *pmoA* PCR products of *M. capsulatus* (Bath), *M. trichosporium* OB3b, and *M. album* BG8 were used as templates (Fig. 2). In addition, the identity of the product obtained from *M. album* BG8 with the nested primer was verified by sequencing (data not shown). Nested PCR was also used to confirm the identities of the *pmoA* products obtained with DNA from isolates from Yellowstone Lake, Green Bay, and a groundwater enrichment culture (Fig. 1); the identities of the latter two products were confirmed by sequencing (data not shown).

DNA was prepared from groundwater obtained at three sites. These DNAs were used as templates for multiplex PCR. A *pmoA* product was obtained with DNA from all three sites, while an *mmoX* product was obtained only with DNA from one site (Fig. 3). The identity of the nested product obtained with well GLRF DNA was confirmed by sequencing (data not shown).

RNA was extracted from methanotroph lab strains and

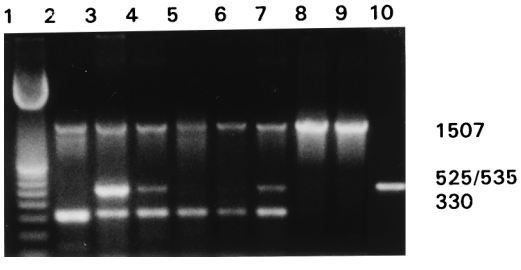

FIG. 1. Multiplex PCR. The 16Sf-16Sr, mmof-mmor, and pmof1-pmor primer pairs were used for lanes 2 through 9. The amo/pmof-amo/pmor primer pair was used for lane 10. DNA was obtained from *M. album* BG8 (lane 2), *M. capsulatus* (Bath) (lane 3), *M. trichosporium* OB3b (lane 4), a groundwater enrichment culture (lane 5), an isolate from sediment from Green Bay in Lake Michigan (lane 6), an isolate from Yellowstone Lake in Yellowstone National Park (lane 7), *E. coli* (lane 8), and *N. europaea* (lanes 9 and 10). Lane 1 contained a 100-bp ladder (Amersham Pharmacia). PCR products were identified by their sizes, as follows: 16S rDNA, 1,507 bp; *mmoX*, 535 bp; *amo-pmo*, 525 bp; and *pmoA*, 330 bp.

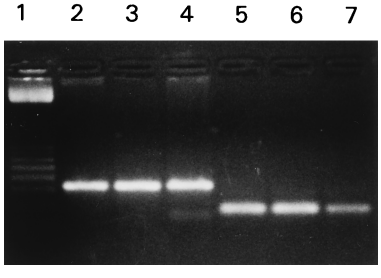

FIG. 2. Confirmation of the identify of the *pmoA* PCR product by nested PCR. The pmof1-pmor primer pair was used for lanes 2 through 4. The pmof2-pmor primer pair was used for lanes 5 through 7. The templates used for lanes 2 through 4 were DNA prepared from *M. album* BG8, *M. capsulatus* (Bath), and *M. trichosporium* OB3b, respectively. The templates used for lanes 5 through 7 were the products of the PCR in lanes 2 through 4, respectively. Lane 1 contained a 100-bp ladder (Amersham Pharmacia). PCR products of 330 bp were detected in lanes 2 through 4, and 178-bp PCR products were detected in lanes 5 through 7. No product was obtained with the pmof2-pmor primer pair when the *amo* product (Fig. 1, lane 10) was used as a template (data not shown).

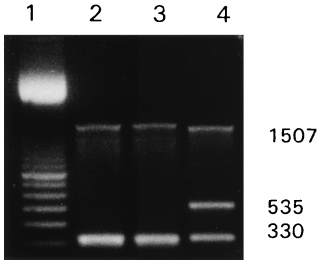

FIG. 3. Multiplex PCR analysis of groundwater. The 16Sf-16Sr, mmof-mmor, and pmof1-pmor primer pairs were used. DNA was prepared from groundwater obtained from the following sites: well CAP (lane 2), well GLRF (lane 3), and well LAPHAM (lane 4). Lane 1 contained a 100-bp ladder (Amersham Pharmacia). The 16S rDNA PCR product (1,507 bp) and the *pmoA* PCR product (330 bp) were detected in lanes 2 through 4; the *mmoX* PCR product (535 bp) was detected in lane 4. No product was obtained when distilled water was substituted for groundwater (data not shown).

groundwater. Each RNA was reverse transcribed to cDNA, which was used as a template for PCR performed with *pmoA* primers. A *pmoA* product was obtained with cDNA that had been reverse transcribed from RNA prepared from lab strains, groundwater, and a groundwater enrichment culture (Fig. 4). The identities of the PCR products were confirmed by nested PCR (data not shown). No product was obtained when the RNA preparations were used directly without an RT step (data not shown).

DISCUSSION

The pmof1-pmor primer pair is specific for pMMO, as a product was not obtained with DNA prepared from *E. coli* or the ammonia oxidizer *N. europaea*. Using a second forward primer (pmof2) provided a simple way to confirm the identity of the *pmoA* PCR product. In addition, it is possible that using nested PCR could extend the application of this approach to detection of methanotrophs in environmental samples that contain PCR inhibitors; nested PCR has been used to detect *Legionella* spp. in such samples (18).

The *pmoA* primers designed in this study could be used in multiplex PCR in conjunction with primers for 16S rDNA and *mmoX*. This allows the simultaneous detection of genes encoding pMMO and sMMO with amplification of 16S rDNA, providing a positive control.

A *pmoA* PCR product was amplified from DNA obtained from all three groundwater samples tested. In contrast to the *pmoA* product, an *mmoX* product was obtained only with DNA from one of these groundwater samples. This implies that either the levels of the methanotrophs with sMMO were below the level of detection or that the *mmoX* primers used could not detect the methanotrophs that were present. Detection of *pmoA* DNA from methanotrophs cultured from different environments (Fig. 1) and from Green Bay sediment (9a) suggested that this approach may be widely applicable.

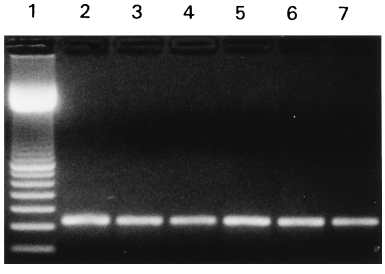

FIG. 4. RT-PCR performed with the pmof1-pmor primer pair. RNA was obtained from *M. album* BG8 (lane 2), *M. capsulatus* (Bath) (lane 3), *M. trichosporium* OB3b (lane 4), a groundwater enrichment culture (lane 5), well GLRF groundwater (lane 6), and well LAPHAM groundwater (lane 7). Lane 1 contained a 100-bp ladder (Amersham Pharmacia). A 330-bp product was detected in lanes 2 through 7.

In this study *pmoA* mRNA was detected by RT-PCR performed with RNA obtained from cultures and groundwater. This is significant because it has been suggested that detection of mRNA by RT-PCR is a better indicator of cell viability than is the detection of 16S rRNA (24). Moreover, detection of specific messages has potential applications for evaluation of activities of microbes at bioremediation sites or in bioreactors (3, 22). While in this study detection of *pmoA* DNA and RNA was qualitative, this work provides the basis for approaches that could be used to quantify methanotrophs and message encoding pMMO.

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