MICROBIAL ECOLOGY

Microb Ecol (1997) 33:21–31

Detection and Isolation of Methanotrophic Bacteria Possessing Soluble Methane Monooxygenase (sMMO) Genes Using the Polymerase Chain Reaction (PCR)*

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Received: 19 July 1995; Revised: 24 October 1995

ABSTRACT

Methanotrophic bacteria possessing sMMO activity have gained notoriety in recent years due to their ability to oxidize a wide variety of halogenated aliphatic compounds, including trichloroethylene (TCE), and are being used as the basis for developing new bioremediation processes. PCR primers were designed from DNA sequences of the alpha and beta subunits of the hydroxylase component of the sMMO from *Methylococcus capsulatus* (Bath). The *mmoY1-mmoY2* primer set was derived from the beta subunit and was specific for the *mmoY* gene from *M. capsulatus,* but failed to produce the expected 395-nucleotide (nt) fragment from *Methylosinus sporium* (ATCC 35069) or from *Methylosinus trichosporium* (ATCC 36070), even at low stringency. A second primer set, primers *mmoX1-mmoX2,* was derived from the alpha subunit and produced the expected 369-nt fragment from all three methanotrophic cultures tested at the highest stringency used (72°C). Soil and groundwater samples were tested for the presence of sMMO-containing bacteria using these two primer sets. One diesel-contaminated soil sample and one TCE-contaminated groundwater sample gave positive results after amplification of total extracted DNA using the *mmoX1-mmoX2* primers. Culture enrichment in small chemostats inoculated with the same positive samples led to the isolation of 13 cultures possessing sMMO activity and containing DNA amplifiable by the *mmoX1-mmoX2* primers. Our results indicated that attempts to directly cultivate sMMO-positive bacteria may give false negative results with some environmental samples. We recommend that primers and/or gene probes based on the sMMO be used in parallel with the naphthalene oxidation test for any environmental assessment of the methanotrophic population. RAPD-PCR analysis revealed that half of these isolates appeared to be different from each other and from *M. capsulatus, M. sporium,* or *M. trichosporium.*

*This article is NRCC Publication no. 37657
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Introduction

Methanotrophic bacteria belong to a diverse group of microorganisms known as methylotrophs and form a subgroup distinguished by their ability to obtain all of their carbon and energy from methane. Methanotrophic bacteria (methanotrophs) play an essential role in the global carbon cycle; they are responsible for most of the biological methane oxidation, thus preventing methane accumulation in the atmosphere. The oxidation of methane to methanol, the first step in the process, is catalyzed by methane monooxygenase (MMO), of which two different types have been identified: membrane-bound or "particulate" MMO (pMMO), and cytoplasmic or "soluble" MMO (sMMO) [11]. It is now recognized that methanotrophs offer high potential for applications in the bioremediation of various contaminated environments [1, 6, 8, 11–15, 19, 21, 25, 34, 36, 40, 44, 47]. This is due to the fact that the MMO enzymes, especially the sMMO, possess broad substrate specificities and can attack many pollutants such as TCE, vinyl chloride, mono- and dichlorobiphenyls, various other halogenated alkanes and alkenes, and some polycyclic aromatic hydrocarbons [1]. Unfortunately, only a limited number of species or strains produce high levels of the sMMO, and production is inhibited by concentrations of copper in the micromolar range.

Methanotrophic bacteria, particularly those with sMMO activity, offer good potential for developing new or improved bioremediation processes and products. Such products and processes could ultimately find application in the treatment of contaminated air, soil, wastewaters, and groundwater, but groundwater applications are foreseen first. Process monitoring is becoming an integral part of biological treatment schemes in the environmental area. High quality monitoring is necessary for process optimization and validation. As a consequence, a decision was made to develop biomonitoring tools that target methanotrophic bacteria.

In this study, we report the design and construction of two primer sets based on the alpha and beta subunits of the hydroxylase component of the sMMO from *Methylococcus capsulatus* (Bath). Both sets of primers were used to amplify methanotroph DNA isolated directly from hydrocarbon-contaminated soil and from TCE-contaminated groundwaters in the Montreal area, and their utilization was instrumental in the isolation of other sMMO-possessing methanotrophic cultures. Initial results using RAPD-PCR indicated that some of these isolates may differ from the most commonly encountered and well-characterized methanotrophs.

Materials and Methods

Microbial Strains and Media

Methylococcus capsulatus (Bath) (ATCC 33009), *Methylosinus trichosporium* OB3b (ATCC 35070), and *Methylosinus sporium* (ATCC 35069) were obtained from the American Type Culture Collection (Rockville, Md.). These cultures were grown using copper-free, low nitrate mineral medium (LN-NMS) [7] at 30°C in an initial atmosphere of 50% methane and 50% air. Other bacterial cultures used in the study were: *Alcaligenes denitrificans* BRI 6011 [30], *Pseudomonas aeruginosa* ATCC 9027 [29], *P. cepacia* BRI 6001 [16], *Methylobacterium extorquens* (ATCC 55366) [5], *P. putida* F1/pSMMO20 [19], *Escherichia coli* XL1-Blue/pSMMO20 [19], *Flexibacter canadensis* [49], an organonitro-degrading *Rhodococcus* sp. isolated from contaminated soil (A. Mihoc, Biotechnology Research Institute, NRC, Montreal), and a psychotrophic groundwater yeast isolate tentatively identified as *Candida* sp. (L. Whyte, Biotechnology Research Institute, NRC, Montreal).

Enrichment Culture Isolation of sMMO-Possessing Methanotrophic Bacteria

Samples were collected from different environments including forest soil (Mount Orford, Quebec), agricultural soil (St. Cesaire, Quebec), two sandy loam soils contaminated with diesel fuel, and groundwater contaminated with TCE (Ville Mercier, Quebec). Soil samples (2 g) were added to 50 ml of sterile, copper-free LN-NMS medium containing glass beads, and then vigorously shaken by hand. Immediately following agitation, the slurry, free of glass beads, was added to a 100-ml glass chemostat. Groundwater samples were concentrated 315-fold using a polysulfone hollow fiber membrane with a nominal pore size of 0.1 μm (Amicon). Concentrated groundwater (20 ml) was added to a 100-ml glass reactor (used as a semicontinuous chemostat) containing 30 ml of sterile, copper-free LN-NMS medium. The reactors were capped, placed over a magnetic stirrer, flushed continuously with a sterile mixture of 50% methane and 50% air (sterilization via a 0.22-μm filter), and incubated at room temperature for up to 3 months. Each week, one-fifth of the growth medium from each reactor was replaced with an equal volume of fresh, sterile LN-NMS medium. Prior to growth medium replacement, 1-ml aliquots were withdrawn from the reactors and added to 50-ml serum bottles containing 20 ml of copper-free LN-NMS medium; in parallel, 0.1-ml aliquots were plated directly onto LN-NMS agar plates. The serum bottles were flushed biweekly for 1 min with the methane-air mixture. Once methane consumption and CO_2 production had been determined by GC analysis of the head space gases, 0.1-ml aliquots were taken and plated onto LN-NMS agar plates. The plates were placed in modified "anaerobic jars," flushed with a 50% methane and 50% air mixture, and incubated at 30°C. Colonies expressing sMMO activity were identified using the naphthalene oxidation assay [10]. sMMO-positive colonies were purified to homogeneity. Culture purity was verified by an absence of growth on nutrient agar plates and by microscopic examination [24].

Table 1. Location and nucleotide sequence of the sMMO-based primers

Probe	Length (nt)	DNA primers	Location[a]	Sequence (30 nt)
mmoX	369	*mmoX1*	2008–2037	5′-CGGTCCGCTGTGGAAGGGCATGAAGCGCGT-3′
		mmoX2	2347–2376	5′-GGCTCGACCTTGAACTTGGAGCCATACTCG-3′
mmoY	395	*mmoY1*	3396–3425	5′-CGAGACCACGGAGCTGCGCACCGTCGACTG-3′
		mmoY2	3761–3790	5′-CGGCCTTCGGCACCGCTGTGGACTCGTCGA-3′

[a] Coding region of *M. capsulatus* (Bath).

DNA Extraction

Total genomic DNA from *M. capsulatus, M. trichosporium, M. sporium,* and from concentrated groundwater samples was extracted as described by Allen and Hanson [2] and purified by the method of Marmur [26]. The freeze-thaw method of Tsai and Olson [45] was used for the extraction of total DNA from the soil samples. Further purification, using Sephadex G-200 gel saturated with TE buffer, was conducted as follows: First, glass wool was added to a 2-ml syringe to make a coarse filter approximately 0.3-cm thick. Sephadex G-200 gel beads saturated with TE buffer were added, and the syringe centrifuged in a swinging-bucket rotor for 10 min at 1000 *g* at 4°C. Centrifugation was repeated until a gel layer roughly 1.5-cm high was obtained. A 0.1-ml volume of DNA extract was slowly loaded onto the column, followed by centrifugation; the eluate containing colorless, purified DNA was used thereafter. All these procedures were done under aseptic conditions. Occasionally, crude DNA was obtained from single colonies by boiling cell suspensions in water for 5 min, and removing cellular debris by centrifugation [27].

Oligonucleotide Primers

Primers were synthesized using an Applied Biosystems model 380A DNA synthesizer.

sMMO Primers. Two sets of primers (30 nt each) were designed from published DNA sequences of the alpha and beta subunits of the hydroxylase component of the sMMO from *Methylococcus capsulatus* (Bath) [42, 43]. Primer set *mmoX1-mmoX2* is derived from the *mmoX* gene encoding the alpha subunit. Primer set *mmoY1-mmoY2* is derived from the *mmoY* gene encoding the beta subunit. The nucleotide sequences of the primers are shown in Table 1.

RAPD-PCR Primers. Thirty sets of 9-nt random primers were tested. Only two primers amplified banding pattern genomic DNA from methanotrophic bacteria. These two primers had the following sequences: primer 0944-10, 5′-AGAAGGCCG; primer 0940-12, 5′-ACGCGCCCT.

PCR Amplification

A Perkin-Elmer Cetus model 480 thermal cycler was used for all amplifications. For amplification for the *mmoX* and *mmoY* genes, PCR reactions were prepared in a total reaction volume of 50 μl, and consisted of 5 μl [10× buffer (100 mM Tris-HCl, pH 9, 500 mM KCl, and 15 mM $MgCl_2$ (Pharmacia)], 5 μl (5–10 ng) of purified template DNA or 5 μl of the supernatant fluid from a boiled bacterial suspension, 2.5 units of *Taq* polymerase (0.5 μl) (Pharmacia), 80 pmol each of the *mmoY* primers or 27 pmol each or the *mmoX* primers, 200 μM (8 μl) of deoxynucleotide triphosphates, and water (27.5 μl). Two sets of PCR amplification conditions (temperature profiles), varying in stringency, were employed. With the less stringent conditions, template DNAs were initially denatured in a boiling water bath for 2 min, followed by 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (primer annealing), and finally 1 min at 72°C (DNA extension). With the more stringent conditions, the denatured DNAs were subjected to 30 cycles of 1 min at 94°C (denaturation) and 1 min at 72°C (primer annealing and DNA extension). All water controls and no-template controls were subjected to the same PCR conditions as the samples.

For random amplified polymorphic DNA-PCR, the reaction mixture was identical to that described above, except that 1 μl (400 pmol) of the random oligo primer and 30.5 μl of sterile water were added. Different temperature profiles were investigated in an attempt to achieve the greatest number of sharp, discreet bands in the least time possible per cycle. Optimal results were obtained using the temperature profile described by Martin-Kearley et al. [27]. A 4-min hot start (96°C) to denature the DNA was followed by 30 cycles of a 1-min ramp time from 72 to 94°C followed by 1 s at 94°C (denaturation), a 3 min ramp time from 94 to 35°C followed by a 30-s hold at 35°C (annealing), and a 75-s ramp time from 35 to 72°C followed by a 30-s hold at 72°C (extension). Following the last cycle, the sample block was kept at 4°C until electrophoresis was performed. For routine electrophoretic analyses of PCR products, 10-μl portions of the PCR reaction were used.

sMMO Gene Probe Preparation

Probes to detect bacteria possessing *sMMO* genes were prepared from *M. capsulatus* (Bath) DNA using the PCR prim-

ers listed in Table 1 and the PCR method previously described. The PCR fragments corresponding to the expected size were removed and purified from TAE-agarose gels using the Geneclean kit (BIO 101 Inc., La Jolla, Calif.). The probes were labeled with [α-^{32}P]dATP using the Rapid Multiprime DNA labeling system (Amersham), and cleaned using Bio-Spin chromatography columns as specified by the manufacturer (Bio-Rad, Richmond, Calif.). PCR fragments of the expected size range (369 bp) obtained from the amplification of DNA templates from *M. trichosporium* and *M. sporium* with *mmoX1-mmoX2* primers were also purified and labeled for Southern hybridization (slot blot) studies.

DNA Sequencing of PCR Fragments by Cycle Sequencing Reaction

PCR fragments embedded in 0.8% agarose gels were cut from the gel and purified with QIAEX gel (QIAGEN, Chatsworth, Calif.). The cycle sequencing reaction consisted of the purified PCR fragment of the expected size range (369 nt), a 17-nt sequencing oligo (*mmoX1A:* sequence = 5′TGT GGA AGG GCA TGA AG3′), and the *Taq* DyeDeoxy terminator cycle system. DNA sequencing was performed using the Applied Biosystems 373A automated fluorescent sequencer.

Southern Analysis of PCR Amplification Products

Southern blots were prepared by vacuum transfer (Hoefer, San Francisco, Calif.) onto Zeta-Probe membranes (Bio-Rad, Richmond, Calif.), as described in Sambrook et al. [35]. The DNA was fixed to the membranes by UV cross-linking (240,000 μjoules) using a UV Stratalinker 1800 (Strategene, LaJolla, Calif.) and stored at −80°C. To prepare slot blots, 10 μl of a 10^{-3} dilution of purified PCR fragments was heated in a total volume of 0.5 ml of 0.4 M NaOH and 10 mM EDTA at 100°C for 10 min. The entire alkaline DNA solution was applied to a TE-rinsed Zeta-Probe membrane attached to a slot-blot manifold (Hoefer, San Francisco, Calif.). Vacuum was applied until the wells were dry, and the membrane was rinsed with an equal volume of 0.4 M NaOH. The membrane was removed from the manifold, rinsed in 2× SSC, air-dried, and stored at −80°C. Prehybridization and hybridization of membranes were carried out as recommended in Sambrook et al. [35]. Hybridizations were carried out overnight at 65°C, after which the membranes were washed twice with solution 1 (1 mM EDTA, 40 mM $NaHPO_4$, 5% SDS, pH 7.2) at 65°C (60 min) and twice with solution 2 (1 mM EDTA, 40 mM $NaHPO_4$, 1% SDS, pH 7.2) at 65°C (60 min). Autoradiography was carried out on a Kodak X-Omat AR X-ray film at −80°C overnight. If the same membrane was to be hybridized with a different probe, the initial probe was stripped by washing twice for 20 min in 0.1× SSC/0.5% SDS at 95°C. Autoradiography was repeated to ascertain complete removal of labeled probe. Washing was repeated when necessary.

Results

Primer Specificity

The *mmoY1-mmoY2* primers were found to be very specific, giving amplification only when DNA from *M. capsulatus* (Bath) was used as template (Fig. 1). These primers failed to produce the expected fragment by PCR, even at low stringency (annealing at 55°C), from *M. trichosporium* and *M. sporium* cultures that express sMMO activity (Fig. 1). The second set of primers, *mmoX1-mmoX2,* appeared to be from a region of the gene common to all these bacteria and produced a fragment of the expected size range (369 nt) at the highest stringency used (annealing at 72°C) (Fig. 2).

In order to expand our knowledge on the specificity of the two primer sets, total genomic DNA from several methanotrophic and nonmethanotrophic bacteria were used as templates for PCR. Primers *mmoX1-mmoX2* and *mmoY1-mmoY2* failed to produce PCR amplification fragments from the following nonmethanotrophic microorganisms: a *Rhodococcus* species, *Pseudomonas cepacia* BRI 6001, *P. aeruginosa* ATCC 9027, *Alcaligenes denitrificans* BRI 6011, *Flexibacter canadensis, Candida tropicalis,* and *Methylobacterium extorquens.* As an example, the results for the first two cultures are illustrated in Fig. 3A. In addition, no amplification was observed with several natural methanotrophic isolates (naphthalene nonoxidizers) obtained during the course of this study (results not shown). Crude DNA extracts from recombinant *E. coli* XL1-Blue/pSMMO20 and from *P. putida* F1/pSMMO20 cultures possessing the entire *sMMO* gene locus from *M. trichosporium* produced the expected fragment size with *mmoX1-mmoX2* primers (results not shown). In addition, methanotrophic isolates expressing sMMO activity (for instance, mixed cultures ppc and VM 1, described below) also produced the expected fragment size with the same primers (Fig. 3A). Southern blots of the PCR fragments obtained in Fig. 3A were probed with the *mmoX* gene probe. Figure 3B shows that only the PCR products generated with the *mmoX* primers hybridized with the *mmoX* gene probe from *M. capsulatus.*

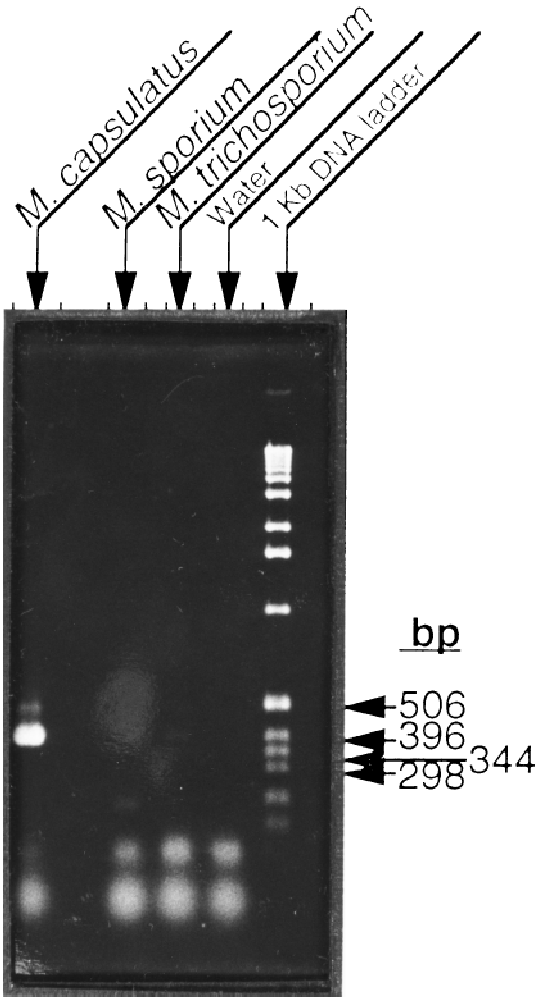

Fig. 1. Electrophoretic analysis of low stringency PCR-amplified products from various bacterial genomic DNAs using primers *mmoY1-mmoY2* for amplification of *mmoY*. Microorganisms: *M. capsulatus* (Bath) (ATCC 33009), *M. sporium* (ATCC 35069), *M. trichosporium* OB3b (ATCC 35070).

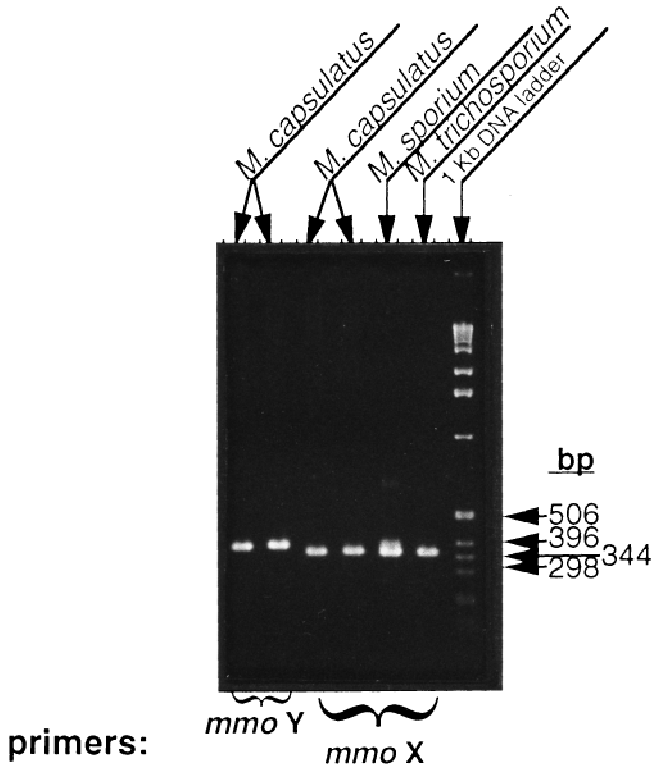

Fig. 2. Electrophoretic analysis of high stringency PCR-amplified products from various bacterial genomic DNAs using primers *mmoX1-mmoX2* and primers *mmoY1-mmoY2*. Microorganisms as in Fig. 1.

Slot-blot analysis of the PCR products (Fig. 3A) with ^{32}P-labeled *mmoX* PCR products from *M. capsulatus, M. trichosporium,* and *M. sporium,* and ^{32}P-labeled *mmoY* PCR product from *M. capsulatus* showed that the three *mmoX* PCR products cross-hybridized with each other, and did not cross-hybridize with *mmoY* (Fig. 4).

Correlation with the Naphthalene Oxidation Assay

The naphthalene oxidation assay [10] is a well-known biochemical assay for identifying and quantifying sMMO activity. The assay, however, may give false negative results due to copper contamination of the culture medium; in addition, some cultures may give variable results. In our laboratory, we have occasionally observed that the intensity of the naphthalene oxidation assay decreased upon repeated transfers of *M. capsulatus, M. trichosporium,* and *M. sporium* on solid media to the point that the enzymatic activity could not be observed. Similar observations occurred with some older cultures. The use of the *mmoX1-mmoX2* primers, however, always gave the following results: (a) cultures that were not amplified by these primers did not express sMMO activity; (b) cultures that "lost" sMMO activity were, nevertheless, always amplified by the *mmoX1-mmoX2* primers.

Direct Isolation from Soil and Water Samples

Various soil and water samples were tested for the presence of sMMO-positive bacteria by spread plating and the naphthalene oxidation assay [10]. No colony showing sMMO activity was obtained. In addition, the colonies did not hybridize with *mmoX*- and *mmoY*-specific gene probes. This confirmed an observation reported by others [8, 18, 32] that methanotrophic bacteria are difficult to culture and isolate.

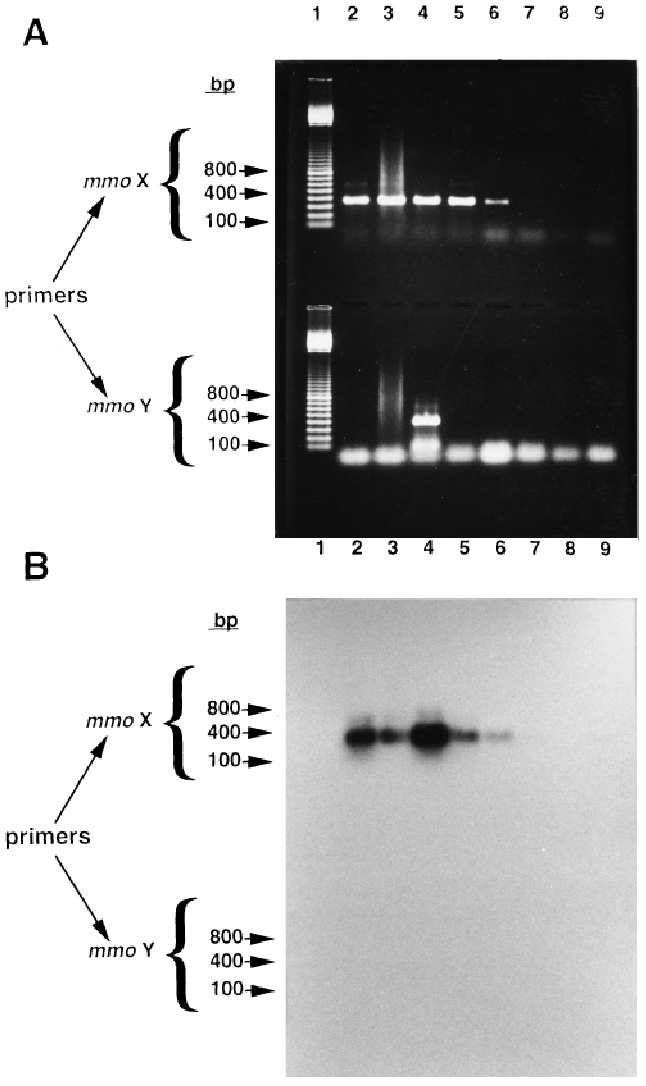

Fig. 3. (A) Electrophoretic analysis of PCR-amplified products from various genomic DNAs using *mmoX1-mmoX2* primers (*top half* of gel) and *mmoY1-mmoY2* primers (*bottom half* of gel). Lane 1, 100-bp-size ladder marker; lane 2, *M. sporium;* lane 3, *M. trichosporium;* lane 4, *M. capsulatus;* lane 5, mixed culture ppc; lane 6, mixed sample VM1; lane 7, *Rhodococcus* sp.; lane 8, *P. cepacia* BRI 6001; lane 9, negative control (water). (B) Hybridization of the products with ^{32}P-labeled *mmoX*-specific gene probe.

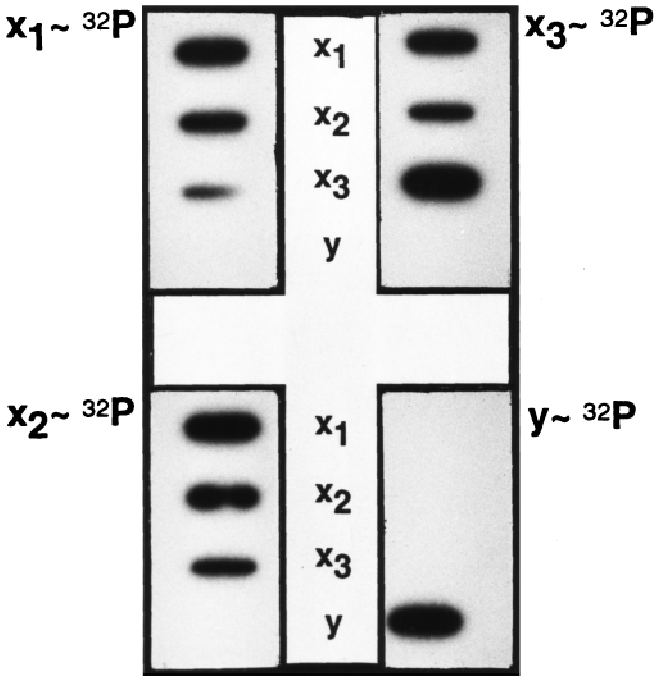

Fig. 4. Southern slot blot analysis of PCR amplification products from *M. sporium* (X_1), *M. trichosporium* (X_2), *M. capsulatus* (Bath) (X_3) using *mmoX* primers, and from *M. capsulatus* (Bath) (Y) using *mmoY* primers. The blots were hybridized with ^{32}P-labeled *mmoX* gene probe amplified from: *M. sporium* (frame X_1-^{32}P); *M. trichosporium* (frame X_2-^{32}P); and *M. capsulatus* (Bath) (frame X_3-^{32}P); and with ^{32}P-labeled *mmoY* gene probe amplified from *M. capsulatus* (Bath) (frame Y-^{32}P).

DNA Extraction from Environmental Samples

DNA extracted directly from the same environmental samples was amplified using the *mmoX1-mmoX2* and *mmoY1-mmoY2* primers. Only DNA extracted from a diesel-contaminated soil sample and from TCE-contaminated groundwater produced the expected amplification product using the *mmoX1-mmoX2* primers (Fig. 5). However, no amplification by the *mmoY1-mmoY2* primers was obtained. To validate our extraction method, the diesel-contaminated soil sample was spiked with a known quantity (10^4) of *M. tichosporium* cells; Fig. 5 shows that the expected PCR fragment was obtained with and without spiking using the *mmoX1-mmoX2* primers. With the groundwater sample, amplification of the extracted DNA also produced a fragment of the expected size using the *mmoX1-mmoX2* primers (Fig. 5). These results strongly suggested the presence of methanotrophs possessing the *sMMO* genes in some of the environmental samples tested.

Enrichment Experiments

Enrichment culture experiments using small chemostats were conducted in order to improve the probability of isolating methanotrophs possessing sMMO activity. The same environmental samples discussed above were used as sources of inoculum. This work was performed in parallel with the DNA extraction work described earlier. Approximately one month after initiation of the enrichment cultures, DNA extracted from chemostats originally inoculated with either

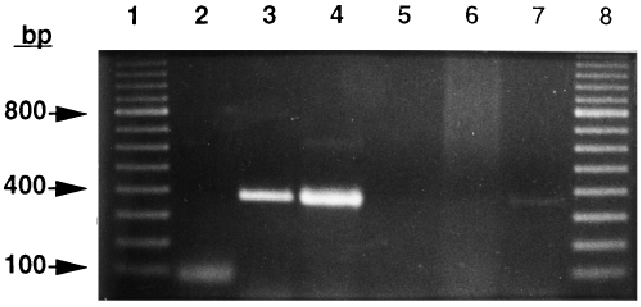

Fig. 5. Electrophoretic analysis of high stringency PCR-amplified products using *mmoX1-mmoX2* primers and genomic DNAs extracted from various environmental samples. Lanes 1 and 8 contained a 100-bp-size ladder marker; lane 2, negative control (water); lane 3, diesel-contaminated soil (sample A5); lane 4, diesel-contaminated soil (sample A5) spiked with *M. trichosporium* (10^4 cells); lane 5, diesel-contaminated soil (sample A4); lane 6, forest soil; lane 7, TCE-contaminated groundwater.

TCE-contaminated groundwater (sample VM1) or diesel-contaminated soil (sample ppc) produced the expected approximately 369-nt fragment by PCR using the *mmoX1-mmoX2* primers (Fig. 3A). The same DNA templates were not amplified with the *mmoY1-mmoY2* primers. Colonies derived from same enrichment cultures also expressed sMMO activity as measured by the naphthalene oxidation assay. Interestingly, chemostats inoculated with environmental samples from which extracted DNA was not amplifiable by the *mmoX1-mmoX2* primers resulted in the enrichment of cultures that yielded colonies showing no sMMO activity, even after three months of continuous chemostat operation. DNA obtained from these sMMO-negative colonies was not amplifiable using the *mmoX1-mmoX2* and *mmoY1-mmoY2* primers. We think it is highly probable these isolates contained only pMMO.

Following enrichment, the diesel-contaminated soil (sample ppc) yielded four pure methanotrophic cultures, whereas the TCE-contaminated groundwater sample (sample VM1) yielded nine pure methanotrophic cultures; these cultures differed in the intensity of response to the naphthalene oxidation assay. Two of the nine isolates obtained from the TCE-contaminated sample were subsequently lost. All isolates were Gram-negative short rods, and their DNA was amplified only by the *mmoX1-mmoX2* primers producing the expected PCR fragment (Fig. 6).

Initial Fingerprinting of the New Methanotrophic Cultures

To verify whether or not the new methanotrophic isolates were different from several well-characterized methanotrophic cultures (i.e., ATCC strains), DNA from these microorganisms was analyzed by random-amplified polymorphic DNA-PCR (RAPD-PCR). Two primers, among thirty primers evaluated, successfully amplified DNA from the methanotrophic cultures. The two primers had G + C contents of 66.7 and 77.8 mol %. Results shown in Fig. 7 reveal that the cultures obtained from the diesel-contaminated soils were very similar to each other and to *M. trichosporium.* The cultures obtained from the groundwater sample exhibited a higher degree of polymorphism. Of the seven isolates, two were very similar to *M. sporium* and one was similar to *M. trichosporium* (lane 20); the other cultures were not similar to either *M. trichosporium, M. sporium,* or to each other. These trends remained constant regardless of the two primers used in the amplification (data not shown). All amplifi-

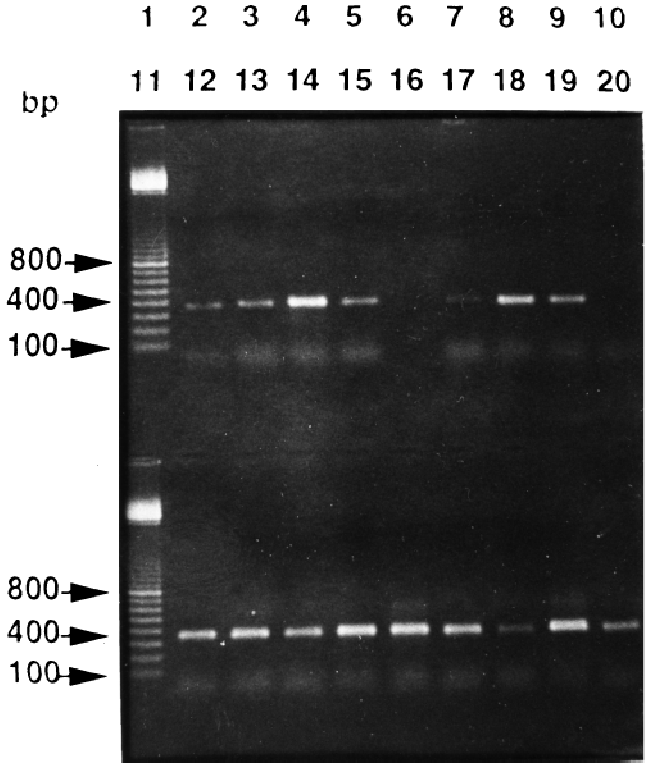

Fig. 6. Electrophoretic analysis of high stringency PCR-amplified products from various bacterial genomic DNAs using *mmoX1-mmoX2* primers. Lane 1, 100-bp-size ladder marker; lane 2, *M. sporium;* lane 3, *M. trichosporium;* lane 4, *M. capsulatus;* lane 5, methanotrophic isolate from anaerobic granular sludge (M3); lane 6, empty well; lanes 7–9, methanotrophic isolates from diesel-contaminated soil (sample A4); lane 10, negative control (water); lane 11, 100-bp-size ladder marker; lanes 12 and 13, methanotrophic isolates from anaerobic granular sludge (samples M5, M6); lanes 14–20, methanotrophic isolates from TCE-contaminated groundwater.

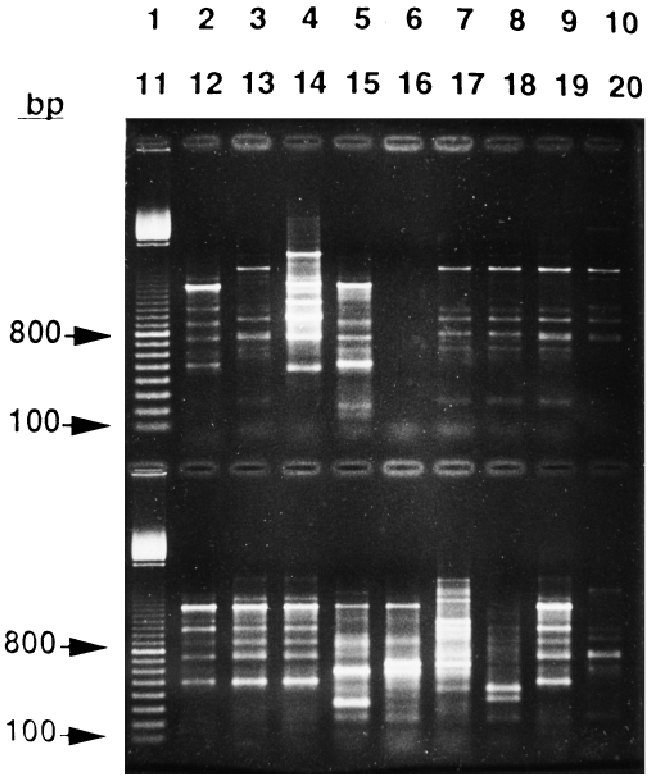

Fig. 7. RAPD-PCR profile of selected strains and isolates using primer 0940-10. Lanes 1 and 11, 100-bp-size ladder marker; lane 2, *M. sporium;* lane 3, *M. trichosporium;* lane 4, *E. coli* XL1-Blue/pSMMO20; lane 5, methanotrophic isolates from anaerobic granular sludge (sample M3); lane 6, negative control (water); lanes 7–10, methanotrophic isolates from diesel-contaminated soil (sample A5) (subsamples M12, M13, M14, M15); lanes 12 and 13, methanotrophic isolates from anaerobic granular sludge (samples M5, M6); lanes 14–20, methanotrophic isolates from TCE-contaminated groundwater (samples M1, M7, M2, M8, M9, M10, M11).

cations shown here were obtained using crude DNA templates extracted directly from boiled, single colonies. Comparisons between amplification profiles of purified and crude DNA templates revealed similar patterns (results not shown).

Discussion

In recent years, methanotrophic bacteria have attracted appreciable interest among scientists and engineers involved in many aspects of environmental microbiology and biotechnology [3, 4, 7, 13, 15, 20, 21, 34, 36, 37, 39, 41, 44, 47, 48]. Various technologies using methanotrophic bacteria are currently being developed and tested for the decontamination of air, soil, and wastewater, with special emphasis on groundwater.

Some molecular biology tools such as PCR and gene probes, are being increasingly used in the environmental field, as evidenced by some recent reports [9, 17, 23, 32, 33, 38, 46]. Some advantages of using such tools include enhanced assessment of specific microbial populations in various contaminated environments, possible isolation of microorganisms with improved degradation capabilities, and improved monitoring of the evolution of specific microbial populations during decontamination efforts. PCR and gene probes may, therefore, contribute in various ways to the development of new or improved bioprocesses and to the validation of these bioprocesses.

We have designed two sets of primers based on the nucleotide sequence of the gene encoding the hydroxylase component (protein A) of the *sMMO* from *Methylococcus capsulatus* (Bath) [42, 43]. Examination of the specificity of these two sets of primers led to an interesting observation: the *mmoY1-mmoY2* primers were specific only for *M. capsulatus* (Bath), even at low stringency (annealing at 55°C); whereas, the *mmoX1-mmoX2* primers produced the *mmoX* fragment with all three methanotrophic species examined, even at the highest stringency used (annealing at 72°C). The *mmoX1-mmoX2* primers appeared to have been designed from a region of the alpha subunit of the hydroxylase component that is common to several methanotrophs. This difference in primer specificity has obvious applications in the area of biomonitoring: the *mmoX1-mmoX2* primers could be used to follow the population of a ''group'' of methanotrophic bacteria, whereas the *mmoY1-mmoY2* primers could be used to follow the *M. capsulatus* (Bath) population and possibly those of closely related strains. Additional examination of these primers confirmed their high degree of specificity.

The naphthalene oxidation assay was regularly employed in our investigations to identify colonies or cultures expressing sMMO activity. Interestingly, sMMO activity was not always observed, although amplification was positive with the *mmoX1-mmoX2* primers. This was probably due to biochemical and physiological factors and/or to differences in the quality of the media used. On the other hand, no amplification with the *mmoX1-mmoX2* primers was always accompanied with a negative naphthalene oxidation test, indicating the absence of sMMO activity. Use of the naphthalene oxidation assay may, therefore, underestimate the quantity and quality of the methanotrophic population in natural environments, although it may be indicative of the

active proportion of the population. We strongly suggest that primers and/or gene probes based on the sMMO be used in parallel with the naphthalene oxidation test for any assessment of methanotrophic populations in various contaminated environments.

The two sets of primers and/or the corresponding gene probes were used in several preliminary microbial ecology studies. A limited number of soil and groundwater samples taken from the Greater Montreal area, some of which were known to be contaminated with diesel fuel or TCE, were investigated for the presence of sMMO-positive bacteria. As indicated earlier, direct cultivation of methanotrophic bacteria on solid media did not result in the isolation of methanotrophs expressing sMMO activity. In addition, all colonies examined were *mmoX* and *mmoY* probe-negative. These results confirmed the observation made by several authors to the fact that methanotrophic bacteria cannot be easily isolated using traditional microbiological methods. Amplification studies done with DNA extracted from the same environmental samples gave significantly different results. DNA from one soil sample contaminated with diesel and from one TCE-contaminated groundwater sample gave a detectable PCR product with the *mmoX1-mmoX2* primers. This strongly suggested the presence of sMMO-positive methanotrophic bacteria in these two samples. No amplification product with the *mmoY1-mmoY2* primers was obtained with the same DNA samples, strongly suggesting the absence of *M. capsulatus* in these samples.

In a next step, sMMO-positive cultures from the same soil and groundwater samples were obtained by employing an enrichment culture approach using small-scale chemostats. Approximately one month was required before sMMO-positive cultures and colonies could be obtained, again from the same two samples. These cultures and colonies possessed naphthalene oxidation activity and DNA extracted from these cultures and colonies was amplified by the *mmoX1-mmoX2* primers. It was interesting to note that the chemostats inoculated with environmental samples, from which extracted DNA was not amplifiable using the *mmoX1-mmoX2* primers, did not yield colonies showing sMMO activity, even after three months of operation. Nor was the DNA obtained from these colonies amplifiable using both sets of primers.

The above observations clearly demonstrate that direct cultivation of sMMO-positive bacteria may give false-negative results with some environmental samples. The use of PCR allowed us to detect the presence of sMMO-positive bacteria in some of these samples, and extended chemostat enrichments permitted the isolation and purification of at least some of these bacteria for future characterization studies.

The enrichment approaches led to the isolation of 13 pure methanotrophic isolates; four were derived from the soil samples contaminated with diesel fuel, and nine from the TCE-contaminated groundwater sample. Random amplified polymorphic DNA-PCR, using two random primers, clearly indicated that several of these isolates were different from *M. sporium, M. trichosporium,* and *M. capsulatus* (Bath). Although RAPD-PCR profiles can be variable, all DNA banding patterns of the various strains tested in this study were generally reproducible; they were always comparable, but not necessarily identical. Additional characterization of these new isolates is in progress and may yield new methanotrophic cultures with improved degradative capabilities. No reason can be advanced to explain why only 2 random primers, out of approximately 30 tested, resulted in the amplification of methanotrophic DNA. This aspect of the work is currently being investigated in more detail.

Recently, two research groups have reported the use of PCR for detecting methanotrophic bacteria in selected environmental samples [28, 31]. In the first case [31], the primers selected were specific for *sMMO* sequences of *M. trichosporium* OB3b, and these sequences were successfully amplified from DNA isolated from groundwater seeded with *M. trichosporium.* As stated by the authors, the selected primers were expected to amplify a 400-base pair (bp) fragment of the *mmoB* gene of strain OB3b. In the second study [28], the PCR primers were selected from the DNA sequence of the sMMO gene cluster of *M. trichosporium* OB3b and *M. capsulatus* (Bath). These primers were reported to be specific for four of the five structural genes in the sMMO gene clusters of several methanotrophs. The *mmoX* gene, which codes for the alpha subunit of the hydroxylase component of the sMMO, appears to be highly conserved in methanotrophs, an observation in agreement with the results obtained with our *mmoX1-mmoX2* set of primers.

We intend to use the knowledge and the molecular biology tools developed in this study to continue isolating and characterizing methanotrophs that may possess improved degradative properties, for developing new microbial products (e.g., microbial inoculants, biosurfactants) and processes for the bioremediation of various contaminated environments. Since methanotrophs play an important role in the global carbon cycle, and appear to be involved in nitrogen fixation and ammonium oxidation [18, 22], the knowledge and tools developed in the course of this study will

assist in studies of the microbial ecology of this interesting group of microorganisms.

Acknowledgments

The authors wish to thank very sincerely Mrs. Maria Pacheco-Oliver for her skilled assistance with ultrafiltration work, Mr. Marc Péloquin for his expert technical assistance in DNA probe preparation, Mrs. Sylvie Sanschagrin for providing some soil samples and relevant information, Mrs. Hélène Bergeron for her contribution to the sequencing of some PCR fragments, and finally Mr. Michael Trani for his expert review of the manuscript.

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