Detection of Bacteria in Environmental Samples by Direct PCR Without DNA Extraction

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ABSTRACT

Cultured cells and environmental samples were used directly in PCRs without the isolation of DNA. Serial dilution was used to eliminate the inhibitory effect of materials in natural samples. Primers specific for pmoA which encodes a subunit of the particulate methane monooxygenase, were used to detect and quantify methanotrophic bacteria by direct most probable number PCR. Phototrophic bacteria were detected in environmental samples by direct PCR with primers specific for pufM, and members of the bacterial domain were detected with primers for 16S rDNA. Direct PCR provides a rapid, simple, and sensitive method for detecting and quantifying bacteria in environmental samples. Detection of methanotrophic bacteria can be applied to monitoring bioremediation.

INTRODUCTION

Molecular techniques have become important tools in microbial ecology because of the limitations of culture-based analyses of microorganisms in environmental samples. It is well known that microorganisms cultured from environmental samples represent only a minor portion of the total microbial community. The use of molecular techniques allows the evaluation of the structure, dynamics, and metabolic potential of environmental samples, which is not possible to infer from data from the relatively few cultured organisms. However, the use of molecular techniques with environmental samples depends on the recovery and purity of the DNA. The approaches used in most molecular ecological studies involve the direct extraction of DNA from natural samples (19,25,27,35) or the recovery of cells from samples, followed by DNA extraction (5,11,24). However, DNA recovery and purity vary among these procedures. The contamination of DNA by substances of environmental origin that inhibit PCR has necessitated the use of extensive procedures to obtain DNA of adequate purity (11,27,35). Several investigators found that even after elaborate purification steps, it was still necessary to dilute the DNA preparations to minimize the inhibitory effect of remaining contaminants on PCR (35,41).

An alternative approach that may

avoid some of the problems associated with DNA extraction is the use of environmental samples directly in the PCR without prior isolation of DNA or pretreatment of the sample. This approach has been used with intact cultured bacterial cells (22,26,39), clinical specimens (28,31,38,40), and animals (16). Direct PCR (DPCR) on environmental samples has had only limited success (20,27), perhaps because optimal conditions were not identified. In situ PCR, in which the PCR products are detected in individual cells that have been fixed and permeabilized, is another approach used for the analysis of environmental samples (4,23).

Most probable number (MPN)-PCR has been used by investigators to quantify organisms after the extraction of DNA (7,41,46). This approach involves the serial dilution of DNA before PCR; quantitative estimates are based on the statistical analysis of replicate series in which the template is diluted to extinction. In the present study, MPN in conjunction with DPCR (MPN-DPCR) was applied to the detection of methanotrophic bacteria in environmental samples. These bacteria, which are widespread in the environment, use methane as the sole source of carbon and energy and can co-metabolize low molecular weight halogenated hydrocarbons, which are toxic pollutants (8,30,32,33,49,53). Their role in the global carbon cycle and in bioremediation applications makes the detection

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and quantification of methanotrophs in the environment important.

Methane monooxygenase (MMO) oxidizes methane to methanol and can also oxidize a variety of substrates including ground water contaminants such as trichloroethylene (TCE). There are two distinct forms of MMO—a soluble MMO (sMMO) and a particulate MMO (pMMO). The pMMO (encoded by *pmoCAB*) has been found in all known methanotrophic bacteria with one possible exception (6), while the sMMO (encoded by the *mmo* gene cluster) is restricted to a subset of methanotrophs (36). Because pMMO is widespread in methanotrophs, it serves as a useful marker for the detection of these bacteria in the environment. This can apply to studies of microbial ecology and bioremediation. Effective bioremediation requires the monitoring of relevant bacterial populations over time and in response to the augmentation of the site.

The application of DPCR to the detection of bacteria in environmental samples is not limited to methanotrophs. Primers for the amplification of *pufM* were used to detect phototrophic bacteria, and primers for 16S rDNA were used to detect members of the bacterial domain in natural samples.

MATERIALS AND METHODS

Bacteria and Growth Conditions

The methanotrophic bacteria *Methylobacterium album* BG8, *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* (Bath), *Methylocystis parvus* OBBP, and EPA (a landfill strain) (44) were grown as previously described (5). Photosynthetic bacteria were also grown as previously described (37). Cells were enumerated by direct count using a phase contrast microscope and a Petroff-Hauser counting chamber. Cultures were diluted to 2×10^8 cells/mL; 50- μ L aliquots containing 10^7 cells were stored at -20°C for DPCR analysis.

Environmental Samples

Samples YS7, YS21, and YS27 were collected with a remotely operated vehicle (Eastern Oceanics, West

Redding, CT, USA) from hydrothermal vents in Yellowstone Lake as previously described (45). The Great Lakes Research Facility (GLRF) ground water has also been previously described (5). The MW-1 ground water sample was obtained from a TCE-contaminated site that was being monitored for natural attenuation (10). Filter-sterilized ground water was obtained by recovering the filtrate using 0.22- μ m filters. Sediment (GBsed) was the upper 1–2 cm subsample from a box core obtained from a station in Green Bay, Lake Michigan, adjacent to a site that has been previously described (3). Another sediment sample was from Lake 12, a eutrophic, marl hard water lake in Washington County, WI, USA. The peat was obtained from an acid bog at the University of Wisconsin-Milwaukee Field Station in Saukville, WI, USA. The peat and sediment were initially diluted to a ratio of 1:100 with deionized, UV-irradiated water before storage. Environmental samples were stored in aliquots of 50 μ L at -20°C until they were used for DPCR analysis.

Molecular Techniques

Cultured methanotrophs were used to evaluate the DPCR method. A 50- μ L aliquot containing 10^7 cells was thawed and decimally diluted with deionized, UV-irradiated water to a theoretical concentration of 1×10^{-1} cells. Each dilution was used as a template in PCR. Serial dilution followed by PCR was performed in five replicate series for each bacterial strain. PCR amplifications were performed in 100- μ L volumes under a layer of mineral oil (Sigma, St. Louis, MO, USA) using a model 480 thermal cycler (Applied Biosystems, Foster City, CA, USA). Each PCR contained 200 μ M each dNTP, 1.5 mM MgCl_2 , 10 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 5 mM Tris-HCl, pH 8.0, 2% dimethyl sulfoxide, 5% glycerol, 0.1% Triton[®] X-100, and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA). Primers (Table 1) were used at a concentration of 0.1 μ M. The *pmoA* primers (5) have sequences that are present in 86 (64 are perfect matches and 22 have 1–3 mismatches) authentic *pmoA* sequences from cultured and uncultured methanotrophs that were de-

posited in GenBank[®] by April 2001. This includes some organisms that are quite divergent on the basis of their 16S or *pmoA* sequences (1,19,25), suggesting that these primers may be useful in identifying uncultured methanotrophs in the environment. The primers for amplification of 16S rDNA are modified from universal 16S bacterial primers (51). The primer 907GF hybridizes to the sequence complementary to 926R and has five additional G residues on the 5' end. The primer 1407GR was modified from 1407R by the addition of four G residues at the 5' end. The *pufM* primers anneal to the highly conserved regions of this gene that encode a protein of the photochemical reaction center of phototrophic bacteria.

The PCR conditions included hot start (47), touchdown (18), and prolonged annealing time in the early cycles. The touchdown involved a decrease of one degree every two cycles, followed by cycles with a constant annealing temperature. Table 1 shows the annealing times and temperatures. Following a 10-min hot start at 95°C , annealing times of 2 min were used for the first six cycles of the touchdown, followed by 1-min annealing times. Denaturation was at 95°C and extension was for 1 min at 72°C . For the final cycle, a 5-min extension time was used.

Precautions were taken throughout this work to avoid the introduction of contaminating DNA. These precautions included the use of a separate room and materials for all pre-amplification manipulations; this room was subjected to UV radiation when not in use. However, excessive high-dose UV exposure was avoided because this resulted in reduced PCR efficiency. This is probably attributable to the effect of UV on plastic, mineral oil, or other materials (9,29). Control reactions without cells or DNA were used in all reaction series to ensure that contaminating template was not present. Amplified DNA (15 μ L PCR) was analyzed on 1.5% agarose gels containing ethidium bromide. To confirm their identity, PCR products were sequenced by cycle sequencing with AmpliTaq[®] DNA Polymerase FS using a model 373 DNA Sequencer (both from Applied Biosystems).

For environmental samples, a 50- μ L aliquot was thawed and serially diluted

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Table 1. Primers and PCR Conditions Used for DPCR

Bacterial Group		Target Product				Annealing Conditions	
Detected	Gene	Size (bp)	Primer	Primer Sequence (5'→3')		Touchdown	Constant
Methanotrophs	<i>pmoA</i>	330 bp	pmof1	GGGGGAACTTCTGGGGITGGAC		53°C–42°C	45°C
			pmor	GGGGGRCIACGTCITTACCGAA		24 cycles	20 cycles
Bacteria	16S	509 bp	907GF	GGGGGAAACTYAAAKGAATTGACGG		65°C–51°C	-
			1407GR	GGGGGACGGGCGGTGTGTRC		30 cycles	
phototrophs	<i>pufM</i>	191 bp	pufmf	CGGCAACCTGTTCTACAACCCGTTCCA		65°C–51°C	55°C
			pufmr	CCCATCGTCCAGCGCCAGAA		30 cycles	10 cycles

to a ratio of 1:10 with deionized, UV-irradiated water using 50-μL volumes. Each dilution was used as a template in PCR. This was performed in five replicate reaction series. The PCR conditions were as described above.

MPN-DPCR

The MPN and confidence limits were calculated using a Microsoft® Excel® computer program developed by Briones et al. (2) to apply the algorithm of Halvorson et al. (17). To test the method, the MPN was determined by DPCR on methanotroph lab strains diluted to extinction in five replicate series. To determine the number of cells in the sample, the three critical dilutions around the endpoint were entered into the program as positive or negative reactions. A sample calculation is shown in Table 2; see Briones et al. (2) for detailed information. The MPN determined was divided by two because two copies of the *pmoA* have been identified in methanotrophs (15,48). A preliminary report of this work has been presented (14).

RESULTS AND DISCUSSION

The DPCR method was first applied to laboratory strains to optimize a protocol (Table 1) to maximize sensitivity with the *pmoA* primers. Suspensions of cells (10^7 cells) were decimally diluted to extinction, and the detection limit was determined as the lowest number of cells that, when used as a template, yielded a PCR product that could be detected on an agarose gel. For *M. albumBG8* and *M. capsulatus* (Bath), 10

cells could consistently be detected (data not shown). When purified *M. albumBG8* DNA was used as a template, the detection limit was 10 genome equivalents (data not shown). For *M. trichosporium* OB3b and *M. parvus* OBBP, 10–100 cells were required for detection and for EPA, 100–1000 cells were required (data not shown). The detection limit determined with the *pmoA* primers using the five strains of methanotrophs demonstrates that the sensitivity, to some extent, is cell specific. This differing sensitivity could reflect differences in the susceptibility of the cells to lyse under the hot-start conditions of the PCR, factors affecting the binding of the primers with the particular templates, and/or PCR efficiency, including differences in sequence, genome size, and copy number of the target gene (13,42,50).

To apply statistical analysis to the determination of bacterial numbers by

dilution to extinction, MPN-DPCR was used to quantify the number of methanotrophic cells, using the results of the five replicate dilution series of each methanotroph strain. The number of cells in the original sample was determined by MPN-DPCR and compared to the direct count (Table 3). For *M. albumBG8* and *M. capsulatus* (Bath), the direct count was eight times greater than the MPN (Table 3). This is consistent with the observation that the detection limit for these strains is approximately 10 cells. In contrast, the MPN determinations for *M. trichosporium* OB3b, *M. parvus* OBBP, and EPA are a greater underestimate of bacterial number (Table 3).

Before the application of this method to environmental samples, initial experiments were performed with seeded samples to determine if constituents in the sample would affect the sensitivity of the method. *M. album*

Figure 1. PCR with serial dilutions of environmental samples. Primers used were pmof1 and pmor. M, 100-bp ladder (Promega); +, positive control, *M. albumBG8* DNA; -, negative control, no DNA; dilutions of GLRF water and peat.

Table 2. Sample Calculation of MPN

Sample	Dilution	No. Positive	No. Negative	No. of Samples	Fraction of mL Analyzed ^a	MPN ^b
BG8	10 ⁻⁵	5	0	5	0.05	4.8 × 10 ⁷
	10 ⁻⁶	5	0	5	0.005	
	10 ⁻⁷	0	5	5	0.0005	

^aFraction of the original sample (0.05 mL) and relative quantities that were analyzed.

^bConfidence limits are determined by multiplying or dividing the MPN by 3.302 for the upper and lower limits, respectively.

Table 3. Comparison of the Number of Methanotrophs in a 1-mL Sample Determined by Direct Count and DPCR

Strain	Direct Count	MPN-DPCR ^a	95% Confidence Limits
<i>M. album</i> BG8	2 × 10 ⁸	2.40 × 10 ⁷	7.25 × 10 ⁶ to 7.90 × 10 ⁷
<i>M. capsulatus</i> (Bath)	2 × 10 ⁸	2.40 × 10 ⁷	7.25 × 10 ⁶ to 7.90 × 10 ⁷
<i>M. trichosporium</i> OB3b	2 × 10 ⁸	7.90 × 10 ⁶	2.40 × 10 ⁶ to 2.62 × 10 ⁷
<i>M. parvus</i> OBBP	2 × 10 ⁸	4.93 × 10 ⁶	1.50 × 10 ⁶ to 1.63 × 10 ⁷
EPA	2 × 10 ⁸	4.93 × 10 ⁵	1.50 × 10 ⁵ to 1.63 × 10 ⁶

^aBecause the copy number of *pmoA* in these methanotrophs is or is assumed to be two (15,48), the population estimated by MPN-PCR was divided by two.

BG8 cells serially diluted in filter-sterilized GLRF ground water or deionized water were used as templates for PCR. The endpoint (10 *M. album*BG8 cells) was not affected by the presence of materials in filter-sterilized ground water (data not shown).

The PCR protocol was then applied to a variety of environmental samples. Figure 1 shows examples of the analyses of a ground water and peat sample. Neither undiluted nor 10⁻¹ diluted GLRF ground water yielded a PCR product. A PCR product was obtained at the 10⁻² and 10⁻³ dilutions of the GLRF water. The peat sample required a 10⁻⁴ dilution to overcome the effect of inhibitors on the PCR. The identity of these PCR products was confirmed by sequencing (data not shown). In each sample, a BLAST search revealed a predominant *pmoA* sequence significantly different from laboratory strains, indicating that the product generated from the environmental sample did not

come from laboratory contamination.

For the purpose of quantifying methanotrophs in environmental samples, MPN-DPCR was determined using five replicate dilution series. Because methanotrophs may vary in the number of cells required to obtain a PCR product, quantification of methanotrophs in environmental samples cannot be made on an absolute basis but, rather, in comparison to a laboratory strain. For this purpose, the greatest dilution that was adequate as a template for the amplification of a *pmoA* product was equated to 10 *M. album*BG8 cells. This could be an underestimate of the methanotrophs present because of differences among the strains that may affect cell lysis or PCR efficiency. It is also possible that the methanotrophs in the environmental samples may differ in *pmoA* copy number. Moreover, it is necessary to consider that, as with other molecular ecology methods, dead bacteria and/or free DNA in natural

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Table 4. Quantification of Methanotrophs in Environmental Samples by MPN-DPCR

Sample	Greatest Dilution Yielding PCR Product ^a	No of <i>M. album</i> BG8 Equivalents/mL	95% Confidence Limits
Ground Water			
GLRF	10 ⁻² (3), 10 ⁻³ (2)	4.93 × 10 ⁴	1.50 × 10 ⁴ to 1.63 × 10 ⁵
MW-1	10 ⁻¹ (2), 10 ⁻² (3)	7.90 × 10 ³	2.40 × 10 ³ to 2.62 × 10 ⁴
Lake Water ^b			
YS7	10 ⁻¹ (5)	2.40 × 10 ²	7.25 × 10 ¹ to 7.90 × 10 ²
YS21	10 ⁻² (5)	2.40 × 10 ³	7.25 × 10 ² to 7.90 × 10 ³
YS27	10 ⁻² (5)	1.60 × 10 ³	4.84 × 10 ² to 5.30 × 10 ³
peat	10 ⁻⁴ (1), 10 ⁻⁵ (4)	1.30 × 10 ^{7c}	3.94 × 10 ⁶ to 4.29 × 10 ⁷
GBsed	10 ⁻⁴ (4), 10 ⁻⁵ (1)	3.29 × 10 ^{6c}	9.95 × 10 ⁵ to 1.09 × 10 ⁷

^aNo. of replicates indicated by parentheses
^bBacteria in samples were concentrated by centrifugation before analysis; calculation of the number of *M. album* BG8 equivalents reflects this concentration.
^cNo. of *M. album* BG8 equivalents/g

samples may be detected by PCR (12). Using this approach, methanotrophs were quantified as *M. album* BG8 equivalents in ground and lake waters, peat, and sediment (Table 4).

To evaluate the application of DPCR to the detection of other bacterial groups, two other primer pairs were tested. The specificity of the *pufM* primer pair was verified by PCR using *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, and *Rubrivax gelatinosus* cells as templates. Based on sequence alignment, these primers are expected to amplify a *pufM* product from other phototrophic bacteria including *Chromatium vinosum*, *Roseobacter denitrificans*, *Erythrobacter*, and *Blastochloris sulfoviridis*. PCR with the *pufM* primers amplified a single product of the expected size with the Lake 12 sediment (data not shown). A product of the expected size was obtained with the 16S primers using GLRF ground water as template (data not shown). For the 16S amplification, the number of cycles was limited to 30 (Table 1); additional cycles resulted in a product in the no-DNA control. This product is probably attributable to DNA contaminating the *Taq* DNA polymerase, as has been observed by others (21). With the appropriate primer design and PCR condi-

tions, DPCR should be applicable to many bacterial groups, including those relevant to bioremediation and pathogens.

Successful DPCR requires primers and PCR conditions that maximize sensitivity. Optimization of conditions is important for PCR and especially critical for DPCR. If the detection limit is not sufficiently low, then the amount of template will be insufficient for detection in diluted samples. This is illustrated by comparing sensitivity of the detection of *M. trichosporium* OB3b cells with primers for *pmoA* and *mmoX* (34); the latter encodes a protein of the sMMO. While 10–100 cells were sufficient to serve as a template for the former, 10⁴ cells were required for the latter under all of the conditions tested (data not shown). While the *mmoX* primers were adequate for the amplification of a DNA template extracted from ground water (5), this primer pair could not be used to obtain a product by DPCR of such samples (unpublished data). It is possible that the greater sensitivity of PCR with the *pmoA* primers compared to the *mmoX* primers is attributable to a greater tendency of the latter to form primer-dimers and hairpins. These nonproductive interactions would compete for template binding, particularly at low template concentra-

tions. Another feature incorporated in primers used in this work is a 4- to 5-G nucleotide extension at the 5' end because preliminary experiments suggested that these additional nucleotides increased yield. While these extensions will not anneal to the template in the initial cycle, they will be incorporated into the product that serves as a template in subsequent cycles, thus stabilizing the priming event. Alternatively, C nucleotides may be used to avoid the potential interaction among adjacent G nucleotides (43).

In addition to primer design, PCR conditions must be optimized for successful DPCR. Touchdown PCR uses a range of annealing temperatures. This may be particularly advantageous for environmental samples in which the sequences of templates present in the sample are unknown. Therefore, a melting temperature for primer/template binding cannot be calculated. Touchdown is suggested to compensate for suboptimal annealing temperatures and buffer conditions, including Mg²⁺ concentration (18). Extended annealing time in the early cycles is intended to compensate for differences in template density, which would be important in dilute samples. By increasing the annealing time in the first six PCR cycles, the detection limit for *M. trichosporium*

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OB3b with the *pmoA* primers was improved (data not shown). Finally, the sensitivity can be increased by a large number of cycles. However, for templates that may be present as contaminants of the *Taq* DNA polymerase, such as 16S rDNA, the sensitivity must be sufficient for DPCR, yet not so sensitive that contaminating DNA is amplified.

Because template integrity is essential to PCR, precautions were taken to minimize the impact of degradation. The samples were rapidly aliquoted in 50- μ L volumes, stored frozen, and thawed immediately before dilution and DPCR. In addition, primers were chosen that required a small (≤ 500 bp) template.

Because of its speed, simplicity, and ability to detect bacteria directly with minimal sample manipulation, DPCR is a useful approach for the detection and quantification of bacteria in environmental samples. However, this method has its limitations. The most important factor that determines the amount of DNA available is cell lysis efficiency (35). While this method may only be applicable for those cells that would lyse after being frozen once then heat-treated for 10 min (the PCR hot start), it is possible that modifications to this method may allow for the detection of other cell types. Another limitation of DPCR is that the least abundant bacteria will not be present in the most dilute samples.

The method chosen for the study of bacteria in environmental samples is dictated by experimental goals, the nature of the sample, and the bacterial group being assessed. It has been suggested that, ideally, multiple methods should be used for the detection of organisms to avoid a potentially biased view of the community of organisms that may occur when a single method is used (52). While the use of DPCR is limited to those samples that have a sufficiently high concentration of methanotrophs (or other target groups) to withstand the dilution necessary to eliminate the effect of inhibitors, a diverse array of environments tested meet this criterion, suggesting that this method should be widely applicable. We suggest that DPCR is a useful tool for some applications and may be used in conjunction with other methods for the analysis of microbes in the environment.

In conclusion, DPCR is a simple and rapid method that may be used qualitatively to detect the presence of a particular target group in natural samples. DPCR may also be used quantitatively to assess changes in the magnitude of the target group over time or in response to changes in the environment. DPCR may be used in conjunction with MPN analysis as described here or in competitive PCR (unpublished data). The detection and quantification of methanotrophs at TCE-contaminated sites being assessed for natural attenuation (Table 4, MW-1) suggest that this method will be suitable for monitoring bioremediation. DPCR may also be used preparatively to obtain PCR products for cloning, sequencing, or other analytical techniques. Such techniques could allow qualitative assessment of the microbial community diversity.

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