New approaches to the assessment of microbes in groundwater : application to bioremediation and detection of pathogens. [DNR-155] [2002]

Title: New Approaches to the Assessment of Microbes in Groundwater: Application to Bioremediation and Detection of Pathogens

Project I.D. DNR#155

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Period of Contract: 7/1/00 - 6/30/02

Background/Need: Groundwater monitoring, Bioremediation
Detection of pathogens

Objectives:

1) Development of molecular methods for the detection of a variety of bacteria in groundwater, including relevant pathogens

2) Development of molecular methods for the assessment of the population of methanotrophic bacteria in groundwater

Background:

Because traditional culture-based methods of bacterial detection are of limited usefulness for environmental applications, molecular methods for the detection and quantification of bacteria are essential. This is relevant to the monitoring of bioremediation and of particular importance for pathogens, which may be may not be detected by culture-based methods. In earlier work, we applied Direct PCR (DPCR) to the detection of methanotrophic bacteria in environmental samples. In this work, this approach is extended to other bacteria, including pathogens.

To accurately predict the process of natural attenuation and to maximize the potential for bioremediation, it is necessary to understand the microbial processes at work. Ideally, it would be useful to know the composition of the bacterial population at a site and to be able to correlate particular microbes with reductions in the target chemical and with amendments of the site. This would lead to improved methods for monitoring and improved strategies for *in situ* bioremediation in which treatments of the site could be designed to stimulate the activity of those bacteria most effective in the degradation. In this research project, an approach to bacterial population analysis coupling DPCR to Single-Stranded Conformational Polymorphism (SSCP) was developed. This was applied to lab strains and natural populations of methanotrophic bacteria.

Abbreviations:
DPCR - direct PCR PCR - polymerase chain reaction
DGGE - denaturing gradient gel electrophoreses
MPN-PCR - most probable number PCR
SSCP - single-stranded conformational polymorphism

Research Results:

Research optimizing DPCR and applying it to methanotrophs and other bacteria and using it in conjunction with SSCP for methanotroph population analysis has been completed. This work is summarized below and the research products referenced below are listed on pg. 20.

1. Use of Direct PCR (DPCR) for the detection of bacteria in groundwater

a. Optimization of Direct PCR

Traditional culture-based methods of bacterial detection are of limited use for environmental applications. Fewer than 1% of bacteria can be cultured from the environment. Even bacteria that are readily be cultured may, under some circumstances, enter a viable non-culturable state in the environment. There is a growing recognition that important pathogens that may be transmitted through water may enter a viable non-culturable state under environmental conditions.

The limitations of culture-based methods for detection and analysis of microorganisms in the environment have increased the importance of molecular methods. These methods are based on the detection of DNA (or RNA) sequences specific for the organism/s of interest. PCR methods offer the greatest sensitivity because the sample is amplified in the process.

Obstacles to the application of conventional PCR to environmental samples are related to the quality and quantity of DNA template recovered from such samples. We have developed a simplified approach that obviates the need for DNA purification. In DPCR, the sample is added directly to the PCR reaction.

Prior to the research supported by this contract, we applied DPCR to the detection of methanotrophic bacteria, a group of importance in bioremediation. In order to make DPCR widely applicable to a variety of bacteria, it is necessary to identify the key elements in primer design and PCR conditions that make this approach successful. This was evaluated by comparing the sensitivity of DPCR detection of different bacterial groups and testing different primer pairs and conditions with a particular bacterium. Using this approach, we have identified elements that should optimally be incorporated into the strategy to allow application of DPCR to a wide range of bacteria targets (Table 1, pg. 3). DPCR was extended to the detection of phototrophic bacteria and members of the Bacterial Domain. These results were reported at scientific conferences (3, 4, 5) and published in a research paper in Biotechniques (1). This work forms the basis of the extension of the DPCR technique to the detection (and quantification) of a variety of bacteria, including important pathogens.

Table 1. Parameters for optimization of DPCR

1. primer design that minimizes competing reactions caused by the formation of hairpins, primer-dimers, and self dimers

2. minimum degeneracy; it is preferable to use inosine at degenerate positions rather than multiple bases

3. 5' G or C clamp may stabilize the primer hybridization

4. primers pairs that amplify a product ≤500 bp

5. PCR conditions that include "touch-down" and prolonged annealing time in the early cycles

6. empiric optimization of annealing temperatures to achieve maximum sensitivity

b. Quantitative DPCR

DPCR was applied to the quantification of methanotrophic bacteria in environmental samples**.** For this purpose**,** samples were diluted to extinction and bacterial number calculated by most-probable number analysis. This MPN-DPCR method is described in detail in Fode-Vaughan et al. (1).

c. DPCR detection of *E. coli* O157:H7

Disease caused by Shiga toxin-producing *Escherichia coli* (STEC) has also become a major public health problem. In the U.S., the most common STEC is *E. coli* O157:H7 which may produce either or both Shiga toxins, Stx1 and Stx2. *E. coli* O157:H7 may be transmitted by food or water, the latter including both recreational and drinking water. The sources of outbreaks of *E. coli* O157:H7 infections have been identified as both groundwater and surface water. A likely source of contamination of aquatic systems is cattle manure and agricultural run-off. Dairy and beef cattle may be carriers of this organism because these animals lack receptors for specific toxins and do not exhibit disease. *E. coli* O157:H7 persists in cattle manure and manure-amended soil and experiments with models have suggested that it may leach through soil. Rapid methods to identify *E. coli* O157:H7 are important to identify the source of outbreaks and to assure public safety.

(1) Strain used and preparation of DNA

The Centers for Disease Control and Prevention type strain G5244 of *E. coli* O157:H7 was obtained for use as a control. Initial experiments and optimization was performed with DNA extracted from this organism.

(2) PCR primer design and testing for detection of *E. coli* O157:H7

Our strategy is to use primers that will amplify a portion of the DNA encoding either of the two Shiga toxins of *E. coli* O157:H7. Primer design was evaluated in the context of sequences that have been deposited in GenBank. Primers were used that meet the criteria stated above (see Table 1). Testing of two primer pairs (Table 2) *in silico* indicated that they should amplify a product from the two Shiga toxin sequences (*stx1* and *stx2)* that are available.

Table 2. Primers for amplification of *stx1* and *stx2*

Primer	Target	Sequence
*stx2*F	*stx2*	TTCTTCGGTATCCTATTCCC
s*tx2*R	*stx2*	ATGCATCTCTGGTCATTGTA
*stx1*F	*stx1*	CAGTTAATGTGGTGGCGAAG
s*tx1*R	*stx1*	CTGTCACAGTAACAAACCGT

These primers for the PCR amplification of DNA sequences encoding the Shiga-like toxins of *E. coli* O157:H7 were tested using DNA as a template. A single product of the predicted size was obtained (Fig. 1). No product was obtained with control *E. coli* that is not of the O157:H7 type (Fig. 1).

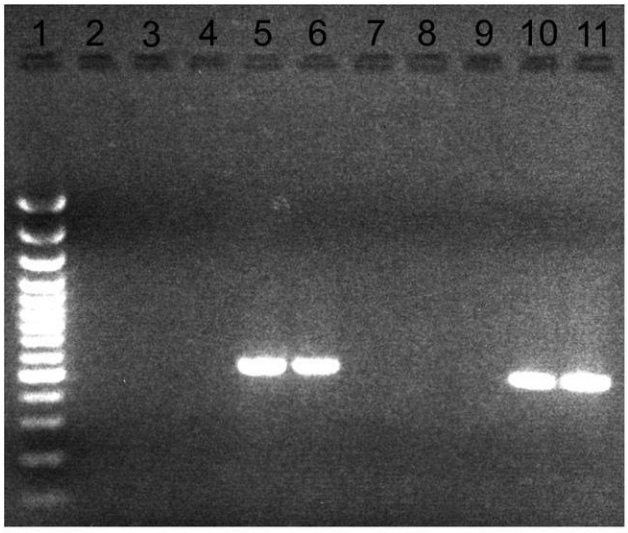

Fig. 1. Electrophoretic analysis of PCR products. PCR with primers for *stx1* toxins in lanes 3-6 and *stx2* in lanes 8 - 11.
1. size marker
2. blank
3. no DNA control
4. control *E. coli*
5. *E. coli* O157:H7
6. *E. coli* O157:H7
7. blank
8. no DNA control
9. control *E. coli*
10. *E. coli* O157:H7
11. *E. coli* O157:H7

Conditions for PCR were empirically optimized to maximize sensitivity of detection of *stx2*. The components of the PCR reaction are shown in Table 3. The temperature program is show in Table 4.

Table 3. Components of PCR reaction

component	concentration
$MgCl_2$	1.5 mM
DMSO	2%
dNTPs	200 μM
Taq polymerase	2.5 units/50 μl

Table 4. Temperature protocol for PCR

1. 95°C 10 min.
2. 95°C 1 min.
3. 53°C 2 min. –0.5°C/cycle
4. 72°C 1 min.
5. Go to Step 2 5X
6. 95°C 1 min.
7. 50°C 1 min. –0.5oC/cycle
8. 72°C 1 min.
9. Go to Step 6 17X
10. 95°C 1 min.
11. 45°C 1 min.
12. 72°C 1 min.
13. Go to Step 10 19X
14. 95°C 1 min.
15. 45°C 1 min.
16. 72°C 5 min.
17. 4°C

(3) Use of nonculturable *E. coli* as a template for DPCR

In order to maximize lab safety, conditions were empirically evaluated to identify those that render *E. coli* non-viable, but useful as a template for DPCR. Preliminary experiments were performed with a non-culturable lab strain. These chemically treated cells were shown to be nonviable and were then evaluated with suitable primers (*malF*) that were designed in conjunction with another project in my laboratory. A *malF* PCR product of the expected size was obtained.

(4) DPCR of *Escherichia coli* O157:H7

When *E. coli* O157:H7 was serially diluted in double distilled water and each dilution was used as a template for DPCR with the *stx2* primer pair, a 482 bp PCR product was formed in every tube with sufficient template. In five replicate series, one to ten cells were required to obtain a product (Fig. 2). Based on the ratio of the direct count (2.0 X 10^8 cells/ml) to the MPN-DPCR estimation of the cell number (1.58 X 10^8 cells/ml), the calculated detection limit is 1.27 cells. Similar experiments were performed to detect the *stx1* template. DPCR with the *stx1* primer pair amplified a 513 bp product; the detection limit for *E. coli* O157:H7 was determined to be 2.88 cells.

(5) DPCR of STEC in environmental samples.

DPCR of STEC suggests that this method should provide a means to detect *E. coli* O157:H7 and other STEC in environmental samples. To test this, groundwater to which *E. coli* O157:H7 was added was serially diluted and each of these dilutions was used as a template in PCR (Fig. 2). No PCR product was detected in the undiluted sample (10^3 *E. coli* O157:H7 cells in groundwater). The absence of a PCR product in the undiluted groundwater is attributable to substances in the sample inhibitory to PCR. This inhibition was relieved by dilution. These results indicate that were *E. coli* O157:H7 present in such a sample at a level of 200 cells/ml (10 cells in 50 µl diluted 1:10 to relieve inhibition), it would test positive in DPCR. Similarly, seeded river water was also tested (not shown). This work is reported in a manuscript submitted for publication (2).

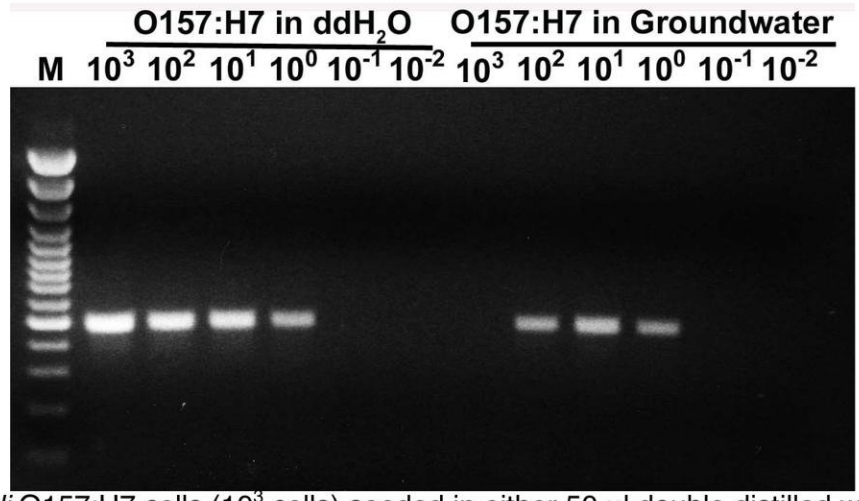

Fig 2. *E. coli* O157:H7 cells (10^3 cells) seeded in either 50 µl double distilled water or groundwater. Serial dilutions were carried out in double distilled water to a theoretical concentration of 10^{-2} cells and each tube used in PCR with the Stx2 primer pair. Note that the initial tube for the groundwater, in which the cells were seeded, does not show a positive reaction and is apparently inhibited by constituents present in the groundwater. No DNA control was negative (not shown). For quantitative analysis, 10^7 cells were added to the initial tubes of five replicate series used in DPCR-MPN analysis. One cell was sufficient for amplification of a PCR product in 3 replicates and 10 cells were required in 2 replicates; the calculated detection limit is 1.27 cells. M, DNA ladder.

(6) Analysis of surface water samples provided by the DNR

We applied DPCR detection of *E. coli* O157:H7 to water samples provided by the DNR. We did not find evidence for this organism in the samples tested. In the course of testing this material, we also evaluated the use of an enrichment culture step. Environmental samples were inoculated into EC broth with novobiocin. DPCR was conducted on these enrichment cultures. While the cultures were negative by this test, we did learn that a 1:10 dilution of the enrichment culture was sufficient to relieve inhibition. This suggests an alternative "hybrid" protocol for evaluation of environmental samples for *E. coli* O157:H7. By performing DPCR on enrichment cultures, it should be possible to detect this pathogen when it is present at a level below the DPCR detection limit and confirm the presence of *E. coli* O157:H7 more rapidly than is possible by conventional culture-based methods.

d. DPCR detection of *Helicobacter pylori*

H. pylori causes gastric diseases in humans including chronic gastritis, ulcers, and is associated with gastric adenocarcinoma and lymphoma. *H. pylori* infection is widespread, estimated to be 40-70% worldwide. The presence of *H. pylori* in water has been reported and on the basis of epidemiological evidence, water has been suggested to be a potential source of *H. pylori* infection. Prolonged survival of *H. pylori* in water has been demonstrated.

H. pylori undergoes a morphological transformation from a helical to a nonculturable coccoid form under various environmental conditions. The coccoid form is suggested to be viable and infectious. This implies that culture-based methods may not reliably detect *H. pylori* in water samples. As a result, DPCR detection of *H. pylori* would be a valuable tool.

(1) Primers, cell treatment, DPCR

Specific primers (Table 5, pg. 8) for amplification of a fragment of the *H. pylori ureA* gene were designed on the basis of work published by others. Combinations of primer pairs and PCR conditions were tested with *H. pylori* DNA. For this purpose, we obtained cultures of *H. pylori* N6 from Dr. Suhas Phadnis at the V.A. hospital in Milwaukee.

After preliminary optimization of primer design and PCR conditions with this DNA, DPCR with cells was tested. To prepare cells for DPCR, *H. pylori* cells were treated under the conditions that we applied to *E. coli* O157:H7 cells to render them non-culturable. We have successfully performed DPCR using these treated *H. pylori* cells as template (Fig. 3, pg. 8).

Table 5. Primers for amplification of *H. pylori ureA* sequence

Primer	Target	Sequence
HpF	*ureA*	ATATTATGGAAGAAGCGAGAGCTGG
HpR	*ureA*	ATGGAAGTGTGAGCCGATTTG

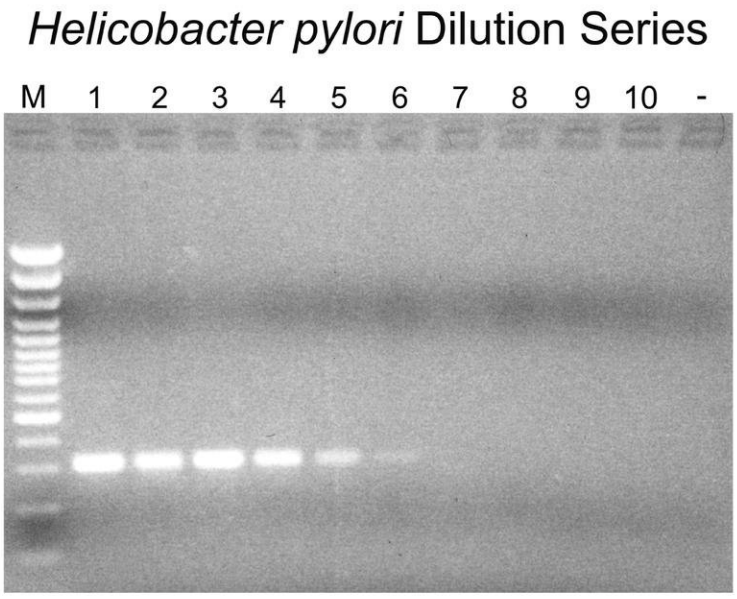

Fig 3. DPCR of *H. pylori*.

A suspension of treated *H. pylori* cells was decimally diluted and used as a template for PCR. A PCR product of 314 bp was formed in each tube with sufficient template. The template was diluted to extinction.

Lanes:
M = molecular weight markers
lanes 1 - 10 = serial 10-fold dilutions of a suspension of *H. pylori* cells
- = no DNA control (negative control)

(2) Optimization of DPCR detection of *H. pylori*; Quantification of *H. pylori* by DPCR

Conditions for DPCR for detection of *H. pylori* were empirically optimized for maximal sensitivity. *H. pylori* cells were added to distilled water, serial dilutions performed, and each dilution was used as a template for PCR. The template was diluted to extinction. In 5 replicates of decimal dilutions, between 10 and 100 cells were required to obtain a PCR product. MPN-DPCR (most probable number Direct PCR) estimated the detection limit to be 29 cells. This should provide an effective and efficient means to detect *H. pylori* in environmental water samples. This is a preferred alternative to culture-based methods. In the environment, many bacteria enter a viable, nonculturable state. As a result, culture-based methods of microbial detection would yield false negatives. This may be a particular problem with *H. pylori*, as this organism enters a nonculturable (but possibly infectious) state even under laboratory conditions.

(3) Use of DPCR for the detection of *H. pylori* in water samples

Water samples were obtained, concentrated and subjected to DPCR. One Wisconsin groundwater sample was tested and showed no evidence for *H. pylori*. A water sample from a municipal water system (supplied from a reservoir to a municipality not in Wisconsin) yielded a PCR product of the appropriate size in 3 replicate DPCR reactions to detect *H. pylori.* The identity of the PCR product amplified from this drinking water was confirmed as *H. pylori ureA* by determination of DNA sequence. This demonstrates the usefulness of this DPCR technique for the detection of *H. pylori* in water samples.

Because highly sensitive methods for the detection of *H. pylori* in water have not been available, there is little information to provide a context to assess the significance of detection of *H. pylori* in drinking water. It is possible that *H. pylori* is present in many water supplies and water could be a source of infection. Alternatively, this may be an episodic occurrence. In this regard, it should be noted that while *H. pylori* was repeatedly detected in one sample, it was not detectable by DPCR in another sample obtained from the same source 41 days later. This could reflect the impact of recent precipitation. It could also reflect inconsistency in the effectiveness of the chlorination process; *H. pylori* is sensitive to treatment with chlorine but less so than the indicator organism *E. coli*. This work has been reported in a manuscript submitted for publication (2).

4. Development of methods for analysis of populations of methanotrophic bacteria on the basis of SSCP (Single-stranded conformational polymorphism)

To accurately predict the process of natural attenuation and to maximize the potential for bioremediation, it is necessary to understand the microbial

processes at work. Prior to the research reported here, we applied DPCR to the detection of methanotrophic bacteria. This is a group of bacteria important in the degradation of halogenated aliphatics and other compounds that may be pollutants in groundwater. Because of their role in bioremediation, it is important not only to have the ability to detect and to quantify these organisms, but also to distinguish among them. Ideally, it would be useful to know the composition of the methanotroph population at a site and to be able to correlate particular populations with reductions in the target chemical and with amendments of the site. This would lead to improved methods of *in situ* bioremediation in which treatments of the site could be designed to stimulate of activity of those bacteria most effective in the degradation. It would also allow monitoring and assessment during the progress of the remediation. Moreover, the most effective degraders identified could be used for inocula for bioreactors.

PCR approaches to microbial population analysis are based on methods that distinguish PCR products amplified with the same primers from different templates. A traditional approach is denaturing gradient gel electrophoresis (DGGE). An alternative that avoids some of the problems associated with DGGE is single-stranded conformational polymorphism (SSCP). Both of these methods provide a fingerprint of the microbial population by electrophoretic resolution of PCR products amplified from environmental samples. By providing a fingerprint of the methanotroph population, SSCP would allow analysis of changes in the population over time, and correlation of changes in the population with treatments of the site and with the degradation of the target chemicals.

The SSCP method is based on the principle that single-stranded DNA has a folded structure that is determined by intramolecular interactions based on its primary sequence. When this folded structure is subjected to electrophoresis on a non-denaturing acrylamide gel, DNA molecules that differ by even a single base may migrate differently and be resolved.

a. Gel electrophoresis for detection of SSCP

Conditions for the resolution of PCR products on the basis of SSCP were tested by using lab strains of methanotrophic bacteria. The *pmoA* PCR products obtained using these strains as a template were loaded onto non-denaturing polyacrylamide gels. Gels of varying composition were cast and used for the electrophoresis of PCR products under different conditions. A wide range of variables including acrylamide concentration, acrylamide/cross-linker ratio, denaturation conditions, continuous vs. discontinuous gels, gel size, temperature, voltage and duration of electroporesis have been evaluated (see Table 6, pg. 11). An example of this analysis is shown in Fig. 4 (pg. 11). Optimized resolution was obtained with a 14% acrylamide gel with a 99:1 ratio of acrylamide to crosslinker (Fig. 5, pg. 12). Higher percentage acrylamide gels produced distinct bands, but the separation of the bands was reduced. In addition, a critical variable was the temperature at which the electrophoresis was performed. Maintaining the temperature at 4°C was optimal. As shown in Fig. 4, even

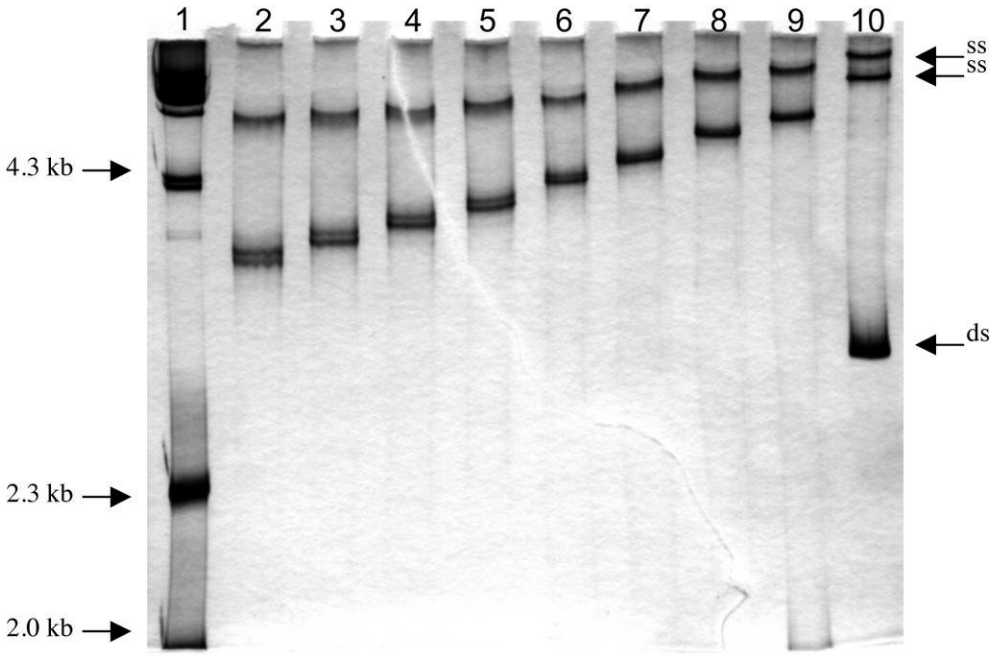

Fig. 4. Optimization of gel conditions for SSCP analysis. *M. album* BG8 PCR products (5 μl) were amplified from whole cells using our standard *pmoA* PCR conditions. Samples were loaded from left to right at approximately 45 min. intervals (except lane 10 which was loaded 75 min. after lane 9). The total electrophoresis time was 8 hours and 15 min. The first lane is λ *HindIII* / ϕX *HaeIII* marker and was loaded at the same time as the first sample. Both double-stranded (ds) and single-stranded (ss) products (arrows) are present for Lane 10.

Table 6. Various gel conditions tested for separation of PCR products for SSCP analysis.

Gel Type	%Acrylamide	Acrylamide: Bis-acrylamide ratio	Temperature
Nondenaturing-PAGE	6, 8	49:1	22°C
Nondenaturing-PAGE + 5% glycerol	8	49:1	22°C
Discontinuous Top—75% formamide Bottom—0%, 5%, 10% glycerol; 0.5XTBE	Top—8 Bottom—6	Top—19:1 Bottom—49:1	22°C
Nondenaturing-PAGE	8,10, 12, 14, 17, 20	49:1	4°C
Nondenaturing-PAGE	14, 20	99:1	4°C
Nondenaturing-PAGE	14	49:1	4°C

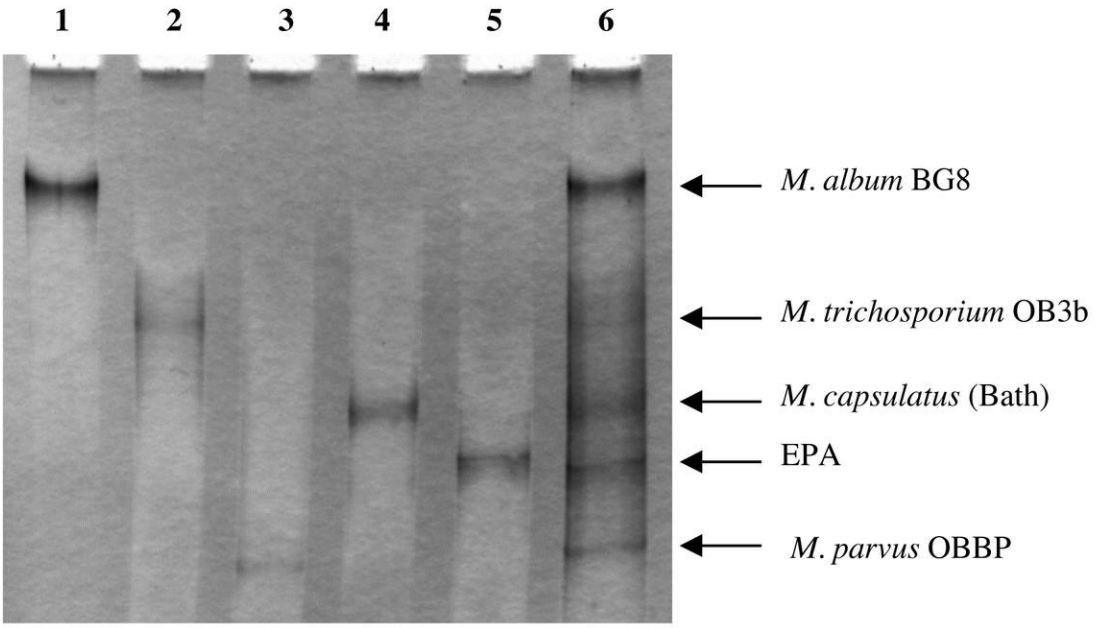

Fig. 5. Optimized conditions for SSCP analysis of five methanotroph lab strains. A 14% (99:1) non-denaturing polyacrylamide gel was used to resolve products from five methanotroph lab strains using a two-step amplification (see below). Lanes 1-5, *M. album* BG8, *M. trichosporium* OB3b, *M. parvus* OBBP, *M. capsulatus* Bath, EPA, respectively. Lanes 1-5 are products from each strain individually. Lane 6 is combined, products from all five strains.

though the double-stranded PCR products were denatured before loading onto the gel, a significant amount of the product renatured and migrated as a double-stranded product. Also, the double-stranded product from each strain produced more than the expected two bands representing the single-strands of DNA on the SSCP gels. These extra bands are assumed to be due to heteroduplex formation or the formation of more than one stable structure by a single strand of DNA.

In an attempt to prevent double-stranded DNA from renaturing and/or the formation of heteroduplexes, two different loading buffers containing higher concentrations of denaturant were evaluated. One contained 0.5 M NaOH and 10 mM EDTA and the other contained 7 M urea in 50% formamide. These buffers did assist in preventing the renaturation of single-strands representing single-stranded DNA for the individual strains, but the bands were more diffuse (not shown). These conditions did not prevent the formation of the extra bands (probably heteroduplexes) that were observed upon combining PCR products.

A discontinuous gel (Table 6, pg. 11) was also evaluated to help prevent the formation of heteroduplexes. It may have minimized the formation of

heteroduplexes, but the bands were more diffuse and the resolution of bands representing the single-stranded DNA from individual strains was diminished (not shown).

b. Generation of single stranded DNA for SSCP

SSCP requires electrophoretic resolution of single-stranded PCR products. Because it was difficult to prevent the two strands from reannealing, different procedures for obtaining predominantly single-stranded DNA were evaluated. These procedures included: 1) λ-exonuclease digestion of one strand of a PCR product which was amplified using one phosphorylated primer, 2) asymmetric PCR, 3) asymmetric PCR followed by exonuclease digestion of a phosphorylated strand (the limiting primer was the phosphorylated primer), and 4) a two-step amplification—an initial PCR using the standard protocol followed by asymmetric PCR.

Digestion of one strand of the PCR product by λ-exonuclease resulted in a predominance of single-stranded DNA; however, the digestion was not complete. Three bands representing the double-stranded product and the two single-strands were observed (not shown).

Single step asymmetric PCR (one primer limiting in the PCR) was also attempted to obtain single–stranded DNA. Double stranded product and both major and minor bands representing single strands of DNA were observed in SSCP analysis (not shown). Also, the amount of single-stranded product generated from strain to strain was not consistent. When asymmetric PCR (the phosphorylated primer at a limiting concentration) was followed by λ-exonuclease digestion, only one single strand of DNA was detected, but there was a significant loss of DNA due to purification steps.

A two-step amplification, in which a fraction of the initial PCR reaction using the standard *pmoA* PCR conditions was used as the template in an asymmetric amplification that included only one primer, resulted in the most abundant production of predominantly single-stranded DNA. This two-step amplification produced the most single-stranded product of one predominant strand for each methanotroph lab strain (Fig. 6, pg. 14). The optimized amplification and gel conditions were used on both whole cells and DNA extracted from each strain to determine if there would be a difference in banding patterns between the two sources of template DNA (Fig. 6). Approximately 6 ng of DNA from each strain and 5 X 10^5 whole cells from each strain were used in the initial PCR—enough template to ensure that the plateau phase would be reached in each reaction. The products were observed on an agarose gel to ensure that equal amounts of products were obtained for each reaction. Reamplification was performed using 1μl of each initial PCR reaction. Ten microliter aliquots of the double-stranded products from the first round of amplification and 10 μl of the asymmetric reamplification products from each

strain using both DNA (Fig. 6A) and whole cells as template (Fig. 6B) were resolved on non-denaturing polyacrylamide gels. As shown, there was essentially no difference in the banding patterns obtained between purified DNA or whole cells used as template. Banding patterns for the products from the first round of amplification show multiple bands. Two of these bands are expected to be the single-strands of the PCR products and the other bands are assumed to be heteroduplexes (or there may be more than one stable structure assumed by a single strand of DNA).

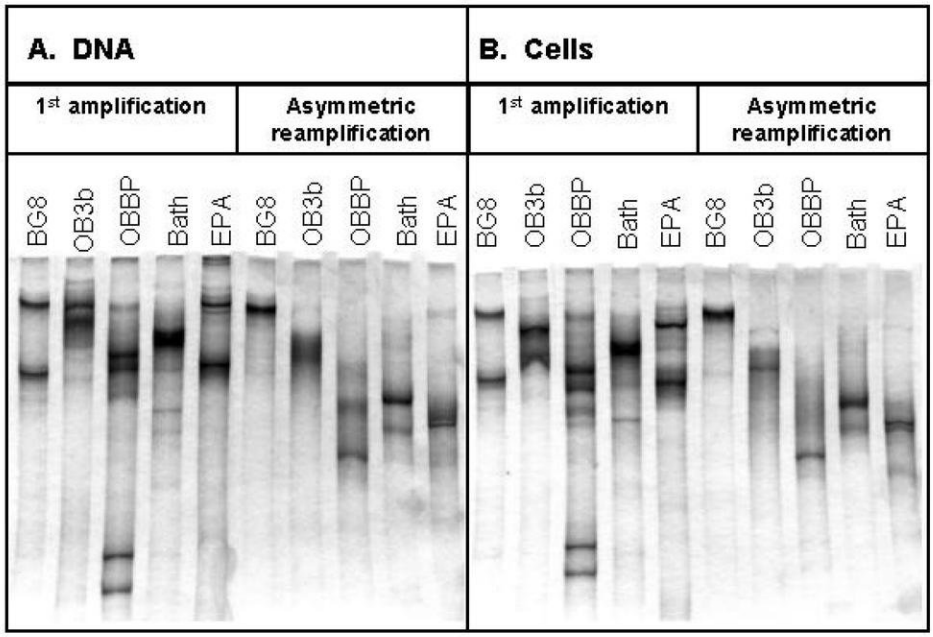

Fig. 6. SSCP analysis of PCR products amplified from purified DNA versus whole cells of five methanotroph lab strains. A. Purified DNA used as template. B. Whole cells used as template. 1st amplification, standard *pmoA* PCR protocol. Asymmetric reamplification, portion of reaction from 1st amplification (1 μl) reamplified using only pmor primer and standard *pmoA* PCR protocol. BG8 = *M. album* BG8, OB3b = *M. trichosporium* OB3b, OBBP = *M. parvus* OBBP, Bath = *M. capsulatus* (Bath), EPA.

c. Resolution of DPCR products from mixed templates

For analysis of environmental samples, it is necessary to resolve products amplified from a mixture of templates. To evaluate this, combined PCR products and PCR products amplified from combined templates were resolved on the basis of SSCP. PCR products from the asymmetric reamplification from each strain using both DNA or whole cells as template were combined (10 μl each, 50 μl total), vacuum concentrated, and resolved on an SSCP gel (Fig. 7). Also, DNA or whole cells from each strain were combined (6 ng each of DNA for 30 ng total; 10^5 cells each for 5 X 10^5 cells total) and used as template for the initial PCR and then reamplified asymmetrically. The entire PCR reaction from the reamplification was vacuum concentrated and loaded onto the gel. All five bands

representing each of the five strains were resolved (Fig. 7). Essentially the same pattern was obtained whether the samples were combined prior to or after amplification.

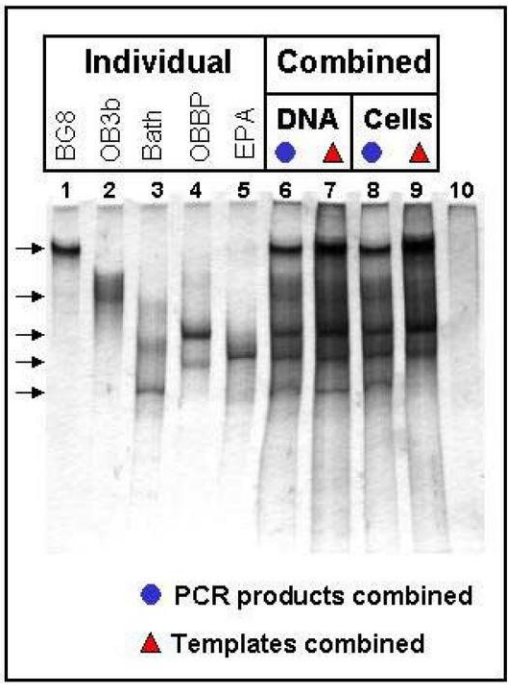

Fig. 7. SSCP analysis of combined PCR products amplified from of five methanotroph lab strains individually and PCR products from combined templates using purified DNA versus whole cells as template. Lanes 1-5, *M. album* BG8, *M. trichosporium* OB3b, *M. parvus* OBBP, *M. capsulatus* Bath, EPA, respectively. Lane 6, combined PCR products from purified DNA. Lane 7, combined purified DNA as template (100µl). Lane 8, combined PCR products from whole cells. Lane 9, combined whole cells as template (100µl). Lane 10, no DNA control.

The results of these experiments have defined conditions that can be used for DPCR two step asymmetric amplification and electrophoretic resolution for the analysis of the methanotroph community on the basis of SSCP.

d. Application of SSCP to environmental samples

The optimized amplification and gel conditions were applied to groundwater samples (Fig. 8, pg. 16). An initial dilution series of each ground water sample was used in PCR to determine the dilution(s) in which there was amplifiable *pmoA* template. One microliter of the PCR reaction from one such dilution was used in asymmetric PCR to generate predominantly single-stranded DNA. The entire reamplified PCR reaction was vacuum concentrated and resolved on a SSCP gel (Fig. 8, pg. 16). As shown, there are bands in unique positions as compared to the lab strains, suggesting that these bands represent

single strands of DNA amplified from indigenous organisms. Samples taken from well MW-6 three months apart show a predominant band that is present in only one of the samples. This suggests that this method will be useful in identifying changes in methanotrophic communities at different sites over a period of time. However, additional work was needed to improve the reproducibility of this method.

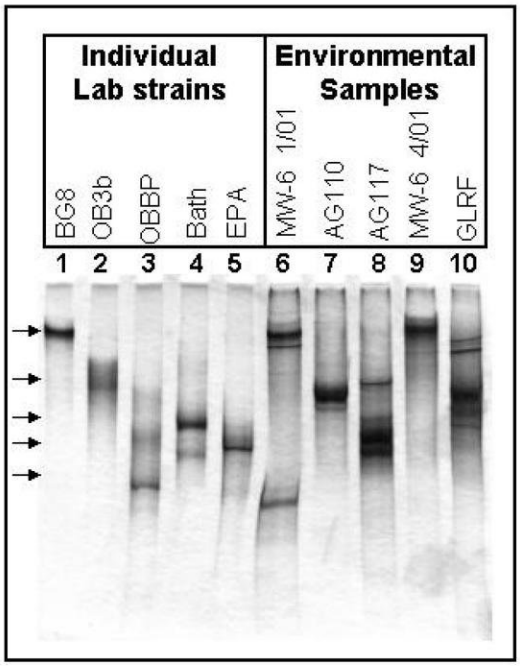

Fig 8. SSCP analysis of environmental samples. Lanes 1-5, *M. album* BG8, *M. trichosporium* OB3b, *M. parvus* OBBP, *M. capsulatus* (Bath), EPA, respectively. Lane 6, MW-6 1/01(amplified from undiluted groundwater collected in January 2001). Lane 7, AG-110 (amplified from 1:10 diluted groundwater). Lane 8, AG-117 (amplified from 1:10 diluted groundwater). Lane 9, MW-6 4/01(amplified from undiluted groundwater collected in April 2001). Lane 10, GLRF ground water (amplified from 1:10 diluted groundwater).

e. Evaluation of reproducibility

DPCR conditions were evaluated to determine those that result in the most reproducible pattern that will allow us to identify with confidence the predominant methanotroph population/s in samples. The results that we have obtained show some sample to sample variability (Fig. 9; compare lanes 1-5). The results of the secondary amplification are quite reproducible (Fig. 9; compare lane 6 to 7 and 8 to 9). Therefore the variability that we have observed is due to PCR bias in the primary DPCR reaction and/or sampling error. The former would be relieved by pooling primary PCR reactions. The latter would be relieved by

maximizing the sample used as template in the primary reaction. We examined the effect of these conditions: volume of primary PCR reaction used as a substrate for secondary asymmetric amplification, the effect of pooling primary DPCR reactions to compensate for any effect of PCR bias (see Fig. 9), and the use of different concentrations of the environmental sample in the PCR reaction to examine the effect of sampling error. Based on these analyses, a recommended protocol was developed Table 7 (pg. 18).

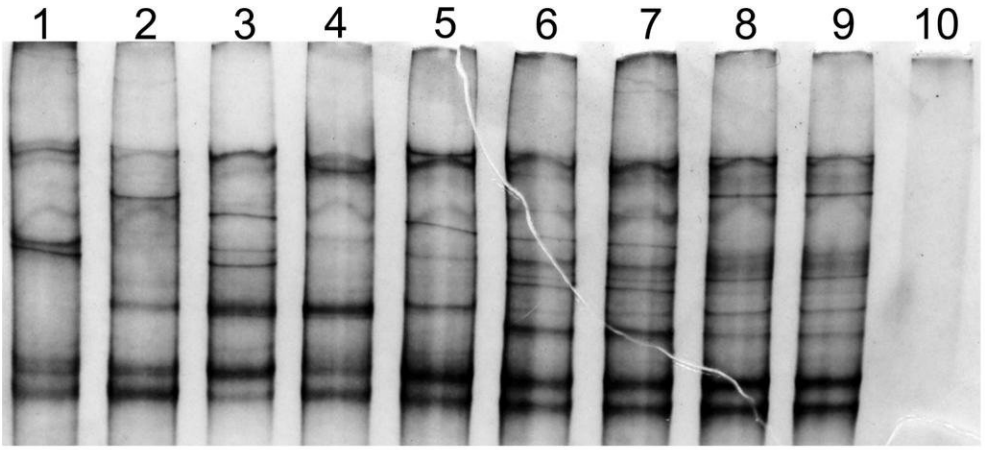

Fig. 9. Evaluation of Reproducibility of SSCP Analysis

All SSCP products were amplified from a single environmental sample taken from a monitoring well at a site in Wisconsin undergoing managed bioremediation.
Lanes 1 - 5 are independent 1^{o} and 2^{o} amplifications. Note that there is some variability.
Lanes 6 and 7 are independent 2^{o} amplifications of pooled independent 1^{o} amplifications 1-5. Comparison of 6 and 7 demonstrates reproducibility of 2^{o} amplification.
Lanes 8 and 9 are independent 2^{o} amplifications of different (6-10) pooled independent 1^{o} amplifications.
Lane 10 - No DNA control
Comparison of lanes 6 and 7 with 8 and 9 shows that these are highly similar. Note that the same major bands are present implying that these represent the predominant methanotroph species. Pooling of samples overcomes variability that may be due to PCR bias in individual PCR reactions.

Table 7. Improved Protocol for SSCP Analysis

1. Serially dilute 10-fold concentrated environmental samples and perform DPCR on dilutions. Identify the most concentrated sample in which a PCR product is formed. This would be the first sample in the series in which PCR is not inhibited by substances from the environmental sample.

2. Pool the products from 10 PCR reactions obtained using the maximally concentrated template.

3. Use 1 - 2 μl of the pooled PCR reaction for use as template for the secondary asymmetric amplification.

4. Resolve the products of the secondary asymmetric amplification on an SSCP gel.

e. Determination of DNA sequence of SSCP bands

An important potential application of this DPCR/SSCP method would be to identify the predominant organisms whose presence can then be correlated with augmentation at a site or reductions in the target chemical/s. To do this, it will be necessary to correlate the predominant bands in SSCP with particular species. This could be achieved by sequence determination of the SSCP bands and correlation with the sequences of PCR products amplified from particular strains.

We have developed methodology for this purpose. We have recovered SSCP bands, and amplified the DNA forming the band by PCR and sequenced the PCR product. The steps in this process, which we are continuing to optimize, are listed below (Table 8).

Table 8. Protocol for Sequence Analysis of SSCP bands

1. Resolve DPCR products by SSCP.

2. Excise gel bands.

3. Elute the DNA in 25μl water by incubation at 4^{o}C for 96 hr.

3. Dilute the eluted DNA 1:10 in water and use 2μl as a substrate for PCR.

4. Amplify PCR product.

5. Purify PCR product using Qiagen QIA quick PCR purification kit.

6. Sequence purified PCR product.

Summary of Results:

1. Optimization of DPCR and development of a general strategy for application of DPCR to various bacteria

2. Quantitative use of DPCR through MPN-DPCR

3. DPCR detection of *E. coli* O157:H7 applied to environmental samples

4. DPCR detection of *H. pylori* applied to environmental samples

5. Development of SSCP analysis of DPCR products for evaluation of methanotroph populations in environmental samples.

Conclusions and Implications:

Because traditional culture-based methods of bacteria detection are of limited usefulness for environmental applications, molecular methods for the detection and quantification of bacteria are essential. DPCR is an improved molecular approach that should facilitate assessment of the presence of specific bacteria. This is relevant to monitoring of bioremediation and of particular importance for pathogens, which may be may not be detected by culture-based methods.

Monitoring and management of natural attenuation and bioremediation require an understanding of the microbial processes at work. Knowledge of the composition of the bacterial population at a site and correlation of particular microbes with reductions in the target chemical and with amendments of the site would lead to improved methods for monitoring and improved strategies for *in situ* bioremediation. In regard to the latter, treatments of the site could be designed to stimulate the activity of those bacteria most effective in the degradation. In addition, this would provide a means to identify the optimal inocula for bioreactors.

This research project developed an SSCP method for the analysis of populations of methanotrophic bacteria. This should provide a useful means to assess the role of these organisms in degradative processes in the environment. It would be valuable to extend both DPCR quantification and SSCP population analysis to other bacteria groups that play a role in bioremediation. This would provide a more complete picture of the microbial processes at work and could serve as the basis for improved remediation strategies and monitoring recommendations.

Research products

Research papers

(1) Fode-Vaughan, K. A., C. F. Wimpee, C. C. Remsen and M. L. P. Collins. 2001. Detection of bacteria in environmental samples by Direct PCR without DNA extraction. BioTechniques 31: 598-607.

(2) K.A. Fode-Vaughan, J. A. Benson, J. S. Maki, and M. L. P. Collins. 2002. Detection of Pathogens in Environmental Samples by Direct PCR. (submitted)

Papers Presented at Scientific Meetings

(3) Collins, M. L. P. 2000. Detection of bacteria in environmental samples by direct PCR. Invited presentation at the Midwest Molecular Microbial Ecology Conference. DeKalb IL, July 2000.

(4) K. A. Fode-Vaughan, C. F. Wimpee, J. Maki, M. L. P. Collins. 2001. Detection of bacteria in environmental samples by Direct PCR without DNA extraction. General Meeting of the American Society for Microbiology, Orlando FL, May 2001

(5) K. A. Fode-Vaughan. 2001. Analysis of bacterial populations in natural samples using direct PCR. presentation to the Milwaukee Microbiology Society, Milwaukee WI, May 2001.

Thesis

(6) K. A. Fode-Vaughan. 2001. Development of methods for the detection, quantification, and community analysis of methanotrophic bacteria in environmental samples. M.S. thesis. University of Wisconsin-Milwaukee.

Invention Disclosures

(7) Method to detect *Escherichia coli* O157:H7 in environmental samples and food by Direct PCR, filed in the Graduate School, University of Wisconsin-Milwaukee

(8) Detection of *Helicobacter pylori* by Direct PCR, filed in the Graduate School, University of Wisconsin-Milwaukee

Key words

PCR, Direct PCR, methanotrophic bacteria, bioremediation, TCE, *Helicobacter pylori*, *E. coli* O157:H7, Shiga toxin