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Simultaneous Detection of Microorganisms in Soil Suspension Based on PCR Amplification of Bacterial 16S rRNA Fragments

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ABSTRACT

The effect of buffer composition on si-multaneous PCR amplification of 16S rRNA gene fragments of five bacterial species was examined using a number of different buffer systems. Tris-based PCR buffers at final concentrations of 10 mM proved unreliable. However, when the final concentration of Tris was increased to 75 mM, all five sam-ples were routinely detected. The use of other buffers, 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO) and 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid (CAPSO), resulted in PCR amplification of five prod-ucts even at low final concentrations (10 mM). The presence of certain proteins in the amplification reaction could overcome an inhibitory effect seen when soil suspension was present in the reaction, as might occur when testing field samples for the presence of bacteria. Bovine serum albumin was found to be the most effective additive test-ed in overcoming inhibition.

INTRODUCTION

Detection of bacterial species in en-vironmental samples using convention-al microbiological techniques can be laborious and time-consuming. Poly-merase chain reaction (PCR) (5) has the potential to allow rapid detection of any bacterial species for which specific amplification primers are available. While PCR is an attractive method for identifying bacteria, there are a number of problems associated with field sam-ples that must be addressed before suc-cessful detection can be achieved. En-vironmental samples can contain a number of different bacterial species, requiring the isolation of a large amount of sample DNA to enable indi-vidual species to be detected. Depend-ing on the source of the sample, various

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inhibitors of PCR amplification may be present, such as humic and fulvic acids (6). Finally, DNA can be difficult to isolate in an amplifiable form from field samples due to the presence of heavy metal ions or other DNA-damaging agents (7). This report attempts to address the first two problems by investigating the PCR conditions that allow the detection of several species simulta-

neously and the inhibitors of PCR amplification in environmental samples, specifically those present in soil.

It would be advantageous to detect a number of species directly from environmental samples with a minimum of sample pretreatment. To achieve this goal, as a test system, we have developed a method that allows the simultaneous amplification (multiplex) of

different-sized fragments of the 16S ribosomal RNA (rRNA) genes of *Lactobacillus delbrueckii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Yersinia pestis* (or the non-pathogenic strain *Yersinia pseudotuberculosis*) in the presence of PCR inhibitors found in soil.

To achieve successful amplification of the five target sequences in the same reaction, the effect of the amplification buffer was examined. The original buffer composition used for PCR (10× buffer: 0.1 M Tris-HCl, pH 8.3, 500 mM KCl) is suitable for most purposes; however, altered buffer composition has been found to be necessary in certain applications, such as PCR of long DNA fragments (1,4) or amplification of two different-sized, mini-satellite alleles with a single primer pair (2). Less temperature-sensitive buffers such as Tricine have been found to produce more reliable results in these cases by maintaining a higher pH at the primer annealing and polymerase extension steps in the reaction. In addition, it has been suggested that potassium chloride can reduce the product yield (4). In this report, we examine the ability of three commercially available Tris-based buffers and buffer formulations based on 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO) (pK_a 9.0) and 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid (CAPSO) (pK_a 9.6) to allow or enhance multiplex amplification of all five PCR products. The effect of substituting ammonium sulfate for potassium chloride on amplification was also examined.

While developing suitable multiplex amplification conditions, the inhibitory effect of soil suspension on PCR amplification of *E. coli* 16S rRNA gene fragments was investigated. Previous methods for removing PCR inhibitors from soil suspension have involved the use of Sephadex® G-200 spin columns to separate DNA from inhibitors (6). Other widely used methods for DNA purification such as caesium chloride gradient centrifugation and agarose gel electrophoresis can also be used. However, such methods do not allow direct PCR analysis of samples. We hypothesised that the inhibitor(s) in soil were preventing amplification by binding to

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the polymerase or to the target DNA. This might then be overcome by introducing another component to the reaction with a higher affinity for the inhibitor. The ability of various proteins to overcome the inhibition of PCR by soil suspension was assessed, and the most effective additive was used in multiplex amplification of target fragments from controlled DNA/soil samples.

MATERIALS AND METHODS

Bacterial Strains and DNA

L. delbrueckii (No. 8130), *P. aeruginosa* (No. 8297) and *E. coli* (No. 8277) bacterial strains were obtained from the National Collections of Industrial and Marine Bacteria Ltd. (Aberdeen, Scotland, UK). *Y. pseudotuberculosis* (Catalog No. 401933) was obtained from ATCC (Rockville, MD, USA). *Y. pestis* DNA (strain EV76) was prepared at CBDE (Porton, Wiltshire, UK) and *B. subtilis* (NCIMB No. 3610) was a gift from A. Glover (University of Aberdeen).

For PCR amplification, boiled cell lysates diluted 1:10 in water were prepared from stationary cultures of *L. delbrueckii*, *P. aeruginosa* and *E. coli* (without quantification of cell number or DNA content), while genomic DNA samples were prepared for the *E. coli*, *Y. pseudotuberculosis*, *Y. pestis* and *B. subtilis* strains.

Preparation of Soil Suspension

An autoclaved 10% (wt/vol) soil solution was prepared from a soil sample (clay soil) taken from a cultivated plant bed in the Cruickshank Botanical Gardens (Aberdeen, Scotland, UK) and used as a standard for all the experiments described. Before use, the sample was shaken to resuspend particulate matter and the supernatant, containing solid material, used for test amplifications.

Multiplex PCR Primer Design

After alignment of the published sequences with CLUSTAL (7), we de-

Table 1. Amplification Buffers Tested in Multiplex Reactions

No.	10× Buffer Composition
1	100 mM Tris, pH 8.8, 500 mM KCl, 1% Triton® X-100
2	100 mM Tris, pH 8.3, 500 mM KCl
3	100 mM CAPSO, pH 9.4, 200 mM (NH ₄) ₂ SO ₄ , 0.1% Tween 20
4	100 mM CAPSO, pH 9.4, 500 mM KCl, 0.1% Tween 20
5	100 mM AMPSO, pH 9.4, 200 mM (NH ₄) ₂ SO ₄ , 0.1% Tween 20
6	100 mM AMPSO, pH 9.4, 500 mM KCl, 0.1% Tween 20
7	750 mM Tris, pH 9.0, 500 mM KCl, 0.1% Tween 20
8	750 mM Tris, pH 9.0, 200 mM (NH ₄) ₂ SO ₄ , 0.1% Tween 20

Buffers 1, 2 and 8 were obtained from Advanced Biotechnologies. Buffers 3–6 were prepared using reagents from Sigma Chemical. All buffers contained 20 mM MgCl₂.

signed a single 16S rRNA gene 3'-end "universal" primer 5'-TGGACTAC-CAGGGTATCTAATCCTGT and specific primers at unique 5' sites:

Y. pestis/Y. pseudotuberculosis (GenBank® Accession No. X67464) 5'-GC-GGCAGCGGGAAGTAGTTTACTA

B. subtilis (Accession No. X60646) 5'-GGGCTAATACCGGATGGTTGT

E. coli (Accession No. J01859) 5'-

AGGGAGTAAAGTTAATACCTTTGC
P. aeruginosa (Accession No. X0-6684) 5'-GTAAAGCGCGCGTAAGT-GGTTTCAGC

L. delbrueckii (Accession No. X52654) 5'-TGGAAGTGCATCGGA-AACTGTCAT.

In conjunction with the universal primer, each specific primer should produce products of 742, 652, 356, 238

Figure 1. The effect of buffering capacity and choice of salt on multiplex PCR amplification. Lane 1, no DNA control with Table 1, buffer 8. Lanes 2–9, multiplex PCR with Table 1, buffers 1–8. Lane 10, ϕ X174 *Hae*III fragments (Life Technologies, Paisley, Scotland, UK).

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and 182 bp, respectively. Each 50- μ L multiplex amplification reaction contained 25 pmol of the universal primer and 5 pmol of each of the species-specific primers.

PCR Amplification

Amplification reactions were carried out on a Techne PHC-3 Thermal Cycler (Scotlab, Coatbridge, Scotland, UK) using the following conditions, unless otherwise stated: reactions (50 μ L), containing 0.1 mM dNTPs (Advanced Biotechnologies, London, England, UK), were overlaid with mineral oil and heated to 94°C for 5 min. *Taq* DNA Polymerase (2.5 U) (Boehringer Mannheim, East Sussex, England, UK) was added under the oil, and the PCR then carried out for 23 cycles of 94°C for 1 min; 63°C for 30 s; 72°C for 1 min; followed by a final extension step at 72°C for 5 min.

Figure 2. Multiplex amplification in the presence of soil suspension and BSA. Lanes 1–5, individual species amplified with multiplex primer set in the order: *P. aeruginosa*, *L. delbrueckii*, *E. coli*, *B. subtilis* and *Y. pestis*, respectively. Lanes 6–8, multiplex amplification from 5 μ L of concentrated soil/DNA sample in the presence of 100, 10 and 0 μ M BSA, respectively. Lane 9, 100-bp marker (Life Technologies).

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Amplification Buffer Assessment

Multiplex PCR was carried out using eight different buffer formulations (Table 1). Reactions (50 μ L) were set up containing 10, 20 and 50 ng of *E. coli*, *B. subtilis* and *Y. pseudotuberculosis* DNA, respectively, 2 μ L of *P. aeruginosa* and *L. delbrueckii* lysates and the appropriate buffer at 1 $\times$ concentration. Amplification was carried out as described. Ten microliters of each reaction were electrophoresed through a 2% agarose/TAE gel.

Soil Inhibition PCR

Nine proteins: soybean trypsin inhibitor, carbonic anhydrase, glucose 3-phosphate dehydrogenase, ovalbumin, phosphorylase B, β -galactosidase, IgG (bovine), myosin (Sigma Chemical, Poole, Dorset, England, UK) and bovine serum albumin (BSA) (Miles Diagnostics, Slough, England, UK) were tested for their ability to overcome the PCR inhibition by soil suspension. They were tested at 10 μ M and each one-tenth dilution therefrom to 1 nM in 50- μ L reactions containing 25 pmol of each of 16S rRNA primers (position 637) 5'-TGATACTGGCAAGCTTGA-GTCTCGT and (position 1020) 5'-AGGCACATTCTCATCTCTGAAAACT (primers designed from the *E. coli* gene sequence that produce a 391-bp PCR product), 2 μ L of *E. coli* lysate and 0.05 μ L of 10% soil solution. Buffer 8 (containing 25 mM $MgCl_2$; see Table 1) was used for these reactions. Amplification was carried out as described above except that extensions were for 30 s. The total reaction mixture (50 μ L) was electrophoresed through a 1% agarose/TAE gel to ensure detection of the product of a successful amplification.

Multiplex PCR in the Presence of Soil Extract

A soil suspension/DNA sample was prepared containing 200 μ L of autoclaved 10% soil suspension, 54 ng of *Y. pestis* DNA, 200 ng *B. subtilis* DNA and 20 μ L of *E. coli*, *P. aeruginosa* and *L. delbrueckii* lysates. DNA was concentrated using WizardTM DNA purification resin (Promega, Southampton,

England, UK) and eluted from the spin column in 50 μ L TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 80°C. Five microliters of the concentrated sample were used per 50 μ L reaction with buffer 8 containing 25 mM $MgCl_2$ (Table 1). A series of reactions were prepared containing 0, 10 or 100 μ M BSA. Amplification was carried out as described above. Ten microliters of each reaction were electrophoresed through a 2% agarose/TAE gel.

RESULTS

Amplification Buffer Assessment

Amplification of all five products was not observed in buffers containing a low (10 mM) final concentration of Tris (Figure 1, lanes 2 and 3). Occasionally, one or two faint products are observed with these buffers. However, amplification with these buffers was not reliable. Amplification with buffers 7 and 8 (Table 1) suggests that it is the increased buffering capacity (75 mM Tris final concentration) that results in successful amplification of all five products (Figure 1), although the effect of Tween[®] 20 cannot be formally discounted. Amplification was observed in reactions containing low final concentrations (10 mM) of AMPSO or CAPSO (Figure 1, lanes 4–7). These reactions demonstrated that potassium chloride and ammonium chloride were interchangeable at the concentrations used and did not appear to affect the yield of multiplex PCR products.

Soil Inhibition PCR

Only carbonic anhydrase, ovalbumin, BSA and myosin were found to overcome PCR inhibition by soil suspension. Myosin allowed amplification at 10 μ M concentration; ovalbumin and carbonic anhydrase were useful in the 10–1 μ M range; while BSA allowed amplification between 1 and 0.1 μ M. Further experiments demonstrated that 10 or 100 μ M BSA were optimal for overcoming PCR inhibition by soil extract (not shown). Including BSA at concentrations above 100 μ M resulted in the formation of a solid white precip-

itate in the reaction tube during the PCR.

Multiplex PCR in the Presence of Soil Suspension

PCR amplification of the five target fragments was achieved from the concentrated DNA/soil sample, by including 10 or 100 μ M BSA in the reactions (Figure 2). Prior concentration of the sample with DNA binding resin did not remove all of the inhibitory compounds (Figure 2, lane 8). When amplification was attempted from 6 μ L of the DNA/soil sample, without prior concentration, only the three smallest products were visible after 23 cycles of amplification (not shown).

DISCUSSION

In this report, we provide a method for amplifying bacterial 16S rRNA gene fragments from several species simultaneously in the presence of inhibitory compounds found in soil.

The buffers used successfully have a higher buffering capacity and a higher initial pH than conventional reaction buffers. The AMPSO and CAPSO buffers were chosen for their high pK_a values, with the aim of maintaining optimal conditions for polymerase activity at high temperatures. It is anticipated that other high pK_a buffers would also be effective for the same reason. By increasing the final concentration of Tris to 75 mM, with a subsequent increase in buffering capacity, the temperature sensitivity of the system is reduced. The use of higher pH buffers at the *Taq* DNA polymerase extension temperature of 72°C reduces the rate of polymerase dissociation from primer/template complexes, resulting in a higher level of fully extended products at the end of each cycle. The fidelity of polymerase recognition of primer/template complexes can also be increased, thereby reducing the chance of obtaining spurious reaction products through mis-priming events. In a multiplex PCR, the improved properties of the reaction buffer would increase the yield of specific products, while reducing the effect of mismatch hybridization between closely related sequences. The

same buffer characteristics have been suggested to be necessary for the amplification of long PCR products (4).

The results reported here suggest that protein additives overcome inhibition of PCR by binding to the inhibitory compounds in soil, thereby preventing its interaction with the polymerase or the target DNA. In this study, we found BSA to be the most suitable additive. However, it may be possible to identify a more effective molecule for this purpose. For example, serum albumins from other species may be more effective than BSA. In unpublished work, we found that humic acid mimics the inhibitory effect of soil, even if it is not the sole inhibitory compound present (6). By using 100 μ M BSA in the reaction, the inhibitory effect of at least 4 μ g of purified humic acid could be overcome. Therefore, an initial comparison of amplification reaction additives could be made with a humic acid standard before testing on field samples. If bacteria are present at low numbers in test samples, concentration of target DNA (as performed with the soil/DNA mixture prepared for test amplification) or an increase in PCR cycle number may be necessary to detect all species present in a sample. Indeed, the variation in the relative amplification signal observed in Figures 1 and 2 for *B. subtilis*, *Y. pestis* and *Y. pseudotuberculosis* products when the source (lysate or purified DNA) or amount of DNA is varied underlines this point.

While the work described here was aimed at detecting bacteria in environmental samples, the buffers shown to be of use in multiplex amplification may be applicable in other situations where it would be advantageous to amplify several products simultaneously. The inclusion of BSA in amplification reactions will allow environmental samples containing lower levels of humic acid than soil from sources such as sewage or water supplies to be easily analyzed by PCR.

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