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Table 2. Comparison of Number of Fragments (100–400 bp) Amplified by Double and Single Primers in Five Accessions of Sweet Potato (*I. batatas* Accessions 1–5)

Primer	No. of Fragments	No. of Polymorphic Fragments
HVH(TG) ₇ + (AG) ₈ T	73	60
BDB(CA) ₇ + (AG) ₈ T	46	38
(AG) ₈ T	28	26
BDB(CA) ₇	36	31
HVH(TG) ₇	2	2

its efficiency, the method described here is also preferable to some previously reported ISSR detection methods (1,4). The increased value in the number of bands resolved may make the present approach more appealing and thus increase its use in genetic analysis. We have successfully used the method in the phylogenetic analysis of 27 accessions of seven *Amaranthus* species, and the relationships resolved among the intra- and interspecific accessions are largely consistent with those revealed based on AFLP data (Xu and Sun, unpublished data).

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Received 22 September 1999; accepted 14 February 2000.

This work was supported in part by a grant from Hong Kong Research Grants Council to M.S. Address correspondence to Dr. Mei Sun, Department of Zoology, University of Hong Kong, Pokfulam Road, Hong Kong, PRC. Internet: meisun@hkucc.hku.hk

Junchao Huang and Mei Sun
University of Hong Kong
Hong Kong, PRC

Use of Dialysis to Prepare Bacterial DNA Suitable as PCR Template

BioTechniques 28:1072-1076 (June 2000)

The availability of reagent kits for PCR amplification of selected DNA regions has made this technique routine in research, diagnostic and educational laboratories. Nevertheless, successful amplification can be elusive in specific instances.

In a multiphasic characterization of 81 new isolates and 10 stock strains (3) of *Caulobacter* spp., a major problem was encountered in PCR amplification of putative 16S rRNA genes for analyzing similar restriction fragment length patterns. Specifically, although InstaGene™ (Bio-Rad Laboratories, Hercules, CA, USA) supernatants prepared from broth-cultivated cells were successfully amplified, preparations from cells cultivated on agar- or agarose-containing media were unsuitable as templates for *Taq*-catalyzed gene amplification. Of several remedies tested, dialysis of the DNA preparations against TE buffer was effective in making the preparations suitable as templates.

The bacterial isolates were maintained on slants of PyCM medium

[0.25% (w/v) Bacto peptone, 0.05% (w/v) Bacto yeast extract, 1 mM CaCl₂, 1 mM MgSO₄ and 1% (w/v) Bacto agar]. Routinely, cells were cultivated in PyCM broth at 30°C with gyratory agitation, collected from 1.5 mL culture by centrifugation at 15 000× g for 15 min, then lysed in 200 µL InstaGene matrix (Bio-Rad Laboratories). The absorbent beads were sedimented at 13 000× g for 3 min. The supernatant was used immediately or frozen and stored at -70°C; frozen preparations were thawed on ice and recentrifuged immediately before use. This procedure yielded supernatants containing more than 100 µg DNA/mL, as estimated from absorbance read at 260 nm.

Amplification of 16S rRNA genes employed the GeneAmp® PCR kit (PE Biosystems, Foster City, CA, USA) with AmpliTaq® or with RedTaq™ (Sigma, St. Louis, MO, USA). A buffer, 96 µL premixed with deoxyribonucleotides, 0.5 µM (final concentration) each primer and 2.5 U polymerase, was mixed into 4 µL InstaGene supernatant in a 650-µL microcentrifuge tube; the surface was then covered with approximately 65 µL mineral oil. The mixture was heated in a PTC-100™ thermal cycler (MJ Research, Waltham, MA, USA) at 94°C for

Figure 1. Agarose gel electrophoresis of PCR products amplified from DNA extracted from agar-cultivated cells and dialyzed for zero, one, two or three days, as indicated by the numbers. Lanes 1–4, *C. leidyi* strain CB37; lane 5, λ DNA-*Bst*II digestion (Sigma); lanes 6–9, *C. crescentus* strain CB2.

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4 min, then cycled 30 times (1.5 min at 94°C, 1 min at 42°C, 2 min at 72°C, with a final extension for 5 min at 72°C) (4). Five microliters of reaction mixture was mixed with 2.5 µL 3× tracking dye [0.028% (w/v) bromophenol blue in 30% (w/v) glycerol], loaded onto a 1% (w/v) agarose gel prepared in TAE buffer (pH 8.0) containing 0.1 ng ethidium bromide/mL (2) and electrophoresed at 100 V for 2 h in TAE buffer.

Primers used in this study were prepared by Operon Technologies (Alameda, CA, USA). A forward primer, 5'-AAC^c/TGAGAGTTTGAT^c/CTG-3', was designed on the basis of 16S rRNA sequences available for *Caulobacter* spp. from the GenBank® database (www.ncbi.nlm.nih.gov) (5,6) and used in combination with the reverse primer 5'-GGTTACCTTGTTACGACTT-3'. These primers overlap by 14 and 19 bases, respectively, the universal eubacterial primers designated fD1 and rP1 by Weisburg et al. (7). Reactions using these primers eventually yielded an amplification product of approximately 1400 bp from the 81 new isolates and all ten stock strains of *Caulobacter* spp. tested, as well as from a Gram-negative, non-stalked, rosette-forming rod (LWR1) isolated as an ecologic congener of the caulobacters. No other

product was detected from any *Caulobacter* strain or from LWR1.

Although the stock-culture medium was PyCM agar, 27 of the 81 recent *Caulobacter* isolates did not grow normally in PyCM broth. These strains, plus seven that did grow well in broth, were therefore streaked on PyCM agar for DNA preparation. Cells were lifted from the agar surface with a platinum loop [carefully, to avoid gouging the agar (1)], suspended in broth and subsequently treated in the same manner as broth-cultivated cells. However, such InstaGene supernatants were unsuitable as PCR templates. Substitution of electrophoresis-grade agarose for Bacto agar in the PyCM medium did not yield DNA preparations suitable for amplification, nor did washing the cells gently into broth from the agar or agarose surface.

To test the possibility that an inhibitory solute was present, each of the unsuitable InstaGene supernatants was transferred to SpectraPor dialysis tubing (Thomas Scientific, Swedesboro,

NJ, USA), dialyzed at 5°C for three days against 5000 volumes TE buffer (pH 8.0) and changed once daily. After three days of dialysis, these preparations became suitable PCR templates.

Unlike all other steps before electrophoresis, dialysis was not performed aseptically, even though it preceded the amplification step. To determine if a shorter dialysis time would be sufficient, InstaGene supernatants were prepared from agar-cultivated cells of four strains, dialyzed for zero, one, two or three days, then tested for suitability as PCR templates. The results varied with strain: dialysis for one day was sufficient for strains CB37 (Figure 1, lanes 1–4), CB-311, and (although three days appeared preferable) CB382; but a two-day period was necessary (and three days optimal) for CB2 (Figure 1, lanes 6–9).

To determine whether the product from preparations dialyzed for three days was identical to the product obtained aseptically from broth-cultivated cells, the restriction fragment patterns

Figure 2. Acrylamide gel electrophoresis of restriction endonuclease digests of PCR products. DNA prepared from broth-cultivated cells (B) or from agar gel-cultivated cells dialyzed three days (G). Amplification product digested with *AluI* (A), *DdeI* (D) or *MspI* (M). Lanes 1–6 isolate CB311; lane 7, pBR322 DNA-*MspI* digestion (Sigma); lanes 8–13, *C. leidy* strain CB37.

Figure 3. Agarose gel electrophoresis of PCR products amplified from DNA extracted from *E. coli* strain 3300 and the GeneAmp® PCR kit λ control. Lanes 1–3, respectively: *E. coli* DNA extracted from broth-grown cells, from agar-grown cells and from agar-grown cells dialyzed for three days. Lane 4: λ DNA-*BstEII* digestion (Sigma); lanes 5–7, respectively: λ control alone, plus 5 µL agar extract plus 5 µL dialyzed agar extract. Lanes 8–10, respectively: λ control plus 5 µL *E. coli* DNA from broth-grown cells, from agar-grown cells and from agar-grown cells dialyzed for three days.

Figure 4. Agarose gel electrophoresis of PCR products amplified from DNA extracted from *C. crescentus* strain CB2 and from the GeneAmp PCR kit λ control. Lanes 1–4, respectively: *C. crescentus* DNA extracted from broth-grown cells undialyzed, dialyzed for three days, undialyzed plus 5 µL agar extract and undialyzed plus 5 µL agar extract dialyzed for days. Lanes 5–6: *C. crescentus* DNA extracted from agar-grown cells undialyzed and dialyzed for three days. Lane 7: λ DNA-*BstEII* digestion (Sigma); lanes 8–10, respectively: λ control alone, plus 5 µL agar extract, plus 5 µL dialyzed agar extract. Lanes 11–13, respectively: λ control plus 5 µL *C. crescentus* DNA from broth-grown cells, and from agar-grown cells undialyzed and dialyzed for three days.

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for two strains were determined with each type of DNA preparation after digestion with *AluI*, *DdeI* or *MspI* (Sigma). Each digestion mixture contained 10 μ L reaction mixture as substrate, 1 μ L appropriate 10 $\times$ buffer and 0.3 μ L enzyme, and was incubated for 2 h at 37°C, then stored frozen at -70°C. Each mixture was thawed on ice, mixed with 5 μ L 3 $\times$ tracking dye and loaded onto an 8% acrylamide gel and electrophoresed in 0.5 $\times$ TBE buffer (2) at 150 V for 4 h. The gel was then stained for 45 min in 250 mL 0.5 $\times$ TBE buffer containing 1 ng ethidium bromide. The fragment patterns (Figure 2) for each strain with each enzyme were identical for DNA preparations from either broth- or agar-cultivated cells, and the patterns for each strain were distinct from those of the other strain, evidence that the products in the dialyzed preparations were identical to those prepared aseptically.

The phenomenon was not peculiar to *Caulobacter* preparations or to PyCM agar. InstaGene preparations were made from *E. coli* strain 3300 cultivated in tryptone media [1% (w/v) Bacto

tryptone, 0.5% (w/v) KCl, 0.5 mM CaCl₂ as broth; 1% Bacto tryptone, 0.25% NaCl, 0.25% KCl, 0.5 mM CaCl₂ and 1% Bacto agar for solidified medium] and used as templates with the same primers used for the *Caulobacter* reactions. The InstaGene preparation from broth-grown cells was amplifiable and yielded one major amplification product of 1500 bp and two smaller, faint bands (Figure 3, lane 1). However, as with *Caulobacter* preparations, amplification products (1500 bp and the two smaller, faint bands) were detectable only after dialysis of the InstaGene preparation from agar-grown cells (Figure 3, lanes 2 and 3).

When a mock cell suspension was prepared by gently scraping the surface of sterile PyCM agar and carrying it through the InstaGene procedure, the resulting supernatant did not affect amplification of DNA from broth-cultivated *C. crescentus* (Figure 4, lanes 3 and 4). Similarly, when undialyzed preparations from agar-cultivated cells were added to the " λ control" provided with the PCR kit, amplification was not inhibited. Instead, each control mixture containing 5 μ L *C. crescentus* DNA preparation yielded a second, minor product (approximately 400 bp) in addition to the λ product (450 bp) (Figure 4, lanes 8–13); each mixture containing 5 μ L *E. coli* DNA preparation yielded predominantly a 400-bp product (Figure 3, lanes 5–10), presumably a "template selectivity" effect (4). The effects of the bacterial preparations on λ DNA amplification were the same for each bacterium, whether the cells had been harvested from broth or agar medium.

We infer from these studies that the physical environment during growth of bacteria affects the suitability of the InstaGene-extracted DNA as a template for PCR amplification. It is possible that the effect of dialysis in converting unsuitable preparations to usable templates could have been achieved by storing them at 5°C rather than frozen, or that a different method of extraction would have yielded amplifiable DNA from agar-grown cells. However, because amplifiable, digestible products were eventually obtained and the comparative study of *Caulobacter* isolates was concluded, this methodological study was not pursued further. Never-

theless, the results imply that whenever PCR amplification fails to yield the expected product from DNA extracted from a pure culture of bacteria, its suitability might be improved by dialysis. The results also suggest that DNA preparations from environmental samples or other mixed microbial populations may fail to yield specific gene sequences, despite sufficient sequence similarity to the primers, because of the physical state of the extracted DNA.

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The authors acknowledge support from the National Science Foundation, award number MCB-9723157. Address correspondence to Dr. Jeanne S. Poindexter, Department of Biological Sciences, Barnard College, Columbia University, 3009 Broadway, New York, NY 10027-6598, USA. Internet: jpoindexter@barnard.edu

Received 19 May 1999; accepted 11 February 2000.

Elsa Y. Hwang, Jennifer L. Harry and Jeanne S. Poindexter
*Barnard College
Columbia University
New York, NY, USA*