the second PCR. Furthermore, the two inverse PCR primers, P1 and P2, can be used to delete the same internal region from a series of DNA constructs containing the target region.

Similarly, an internal deletion can be made by a PCR-ligation-PCR approach as described by Ali and Steinkasserer (1). According to this method, for example, the first-round PCR can be performed in two separate reactions by primers V1/P2 and P1/V2, followed by ligation of the two PCR products and reamplification of the desired PCR product from the ligation mixture by primers V1/V2. The final PCR product will be equivalent to our PCR product after the second round of PCR. Compared with the method of Ali and Steinkasserer, our method has the following advantages. First, our procedure is completed by merely two PCRs instead of three. Second, our ligation is mostly a self-ligation with only a single ligated product with one orientation, while the method of Ali and Steinkasserer generates multiple products with different orientations, which can cause difficulty with identifying the correct PCR product in the end. Thus, our two-step PCR method is a useful alternative for generating an internal deletion.

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Reduction of False Positives in Prokaryotic mRNA Differential Display

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Since their introduction in 1992, mRNA differential display (DD) (9) and the closely related RNA fingerprinting by arbitrarily primed polymerase chain reaction (RAP-PCR) (10,18) have superseded subtractive hybridization as the methods of choice for the successful identification of differentially expressed genes. The features that have made both methods attractive to researchers studying eukaryotic systems include their versatility in allowing multiple sample conditions to be compared simultaneously, degree of sensitivity to low-abundance messages and the requirement for low amounts of total RNA. Such features are also appealing to researchers studying prokaryotic systems. However, while several thousand eukaryotic DD applications have been reported, these methods have only recently been applied to bacterial systems as a means of studying differential gene expression (4,5,8,19). Furthermore, because many of the improvements to DD have been

Benchmarks

exclusively applied to eukaryotic systems, it would also be beneficial to streamline the technique for use in prokaryotic systems, thus further maximizing DD's potential.

Because most prokaryotic mRNAs do not have poly(A) tails (2,14), an arbitrary 10-bp primer was used for the reverse transcription (RT) reaction. For the subsequent PCR, a degenerate primer based on the Shine-Dalgarno sequence was used in place of an arbitrary primer (5). Degenerate primers have previously been used to clone family-specific genes using differential display methods (7,17). The arbitrary

primer can be designed to decrease the probability of ribosomal annealing and priming. However, such amplification of ribosomal RNAs (rRNAs) remains problematic due to mispriming events that occur during the RT step because of the much greater abundance of rRNA over messenger RNA (mRNA) (3,12). Consequently, this presents a difficulty in identifying authentic mRNA-derived PCR products upon DD gel analysis. Based on our experience, approximately 40% of putative differentially expressed bands identified from the DD gel are of ribosomal origin (data not shown). Because of this difficulty, a method is needed to assist with discriminating prokaryotic mRNA- vs. rRNA-derived PCR products. Use of the antibiotic rifampicin, a potent bacterial transcriptional inhibitor of RNA polymerase activity (2,11,14), is demonstrated to achieve this goal. We postulated that by inhibiting bacterial RNA polymerase activity and the subsequent initiation of mRNA synthesis, the eventual decay of differentially expressed messages, in the absence of de novo synthesis, would be readily apparent on the DD gel. In turn,

this would allow for the discrimination between authentic differentially expressed bands from the more stable rRNA bands.

Our lab is interested in monitoring microbial gene expression in the presence of radionuclides and other heavy metals in contaminated soil environments. *Pseudomonas putida* G7 was chosen as a model organism for the study of radionuclide induction in pure culture as a prelude to our work in soils. An overnight culture of *P. putida* G7 was grown at 30°C in a yeast extract polypeptone glucose (YEPG) medium. Following overnight incubation, 2 mL of culture were added to 50 mL of fresh YEPG media and allowed to incubate until cells reached the mid-log phase of growth (optical density [OD]₆₀₀ ca. 0.4). Following this incubation, 3 mL of the culture were used to inoculate each of two additional flasks, one with and one without 200 µM uranyl acetate. Cultures for DD analysis were grown to early log phase (OD₆₀₀ = 0.2), which amounted to 2 h for control cultures and 24–30 h for cultures exposed to uranyl acetate. An

Figure 1. Differential display of uranyl acetate-exposed *P. putida* G7 cells treated with rifampicin. Cell cultures were exposed to 200 µM uranyl acetate for 24–30 h. Before cell harvesting, an aliquot was taken (T = 0) followed by the addition of rifampicin as described. Following the addition of rifampicin, aliquots were taken at time intervals of 5, 10, 20, 30, 40 and 60 min, respectively. Samples were processed and subsequently used for DD. Lane 1, 100-bp ladder; lane 2, without rifampicin (T = 0); lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 40 min; lane 8, 60 min.

Figure 2. (A) Confirmation of uranyl acetate induction in *P. putida* G7 by RNA slot-blot analysis. Five micrograms each of control and uranyl acetate-exposed, rifampicin untreated total RNA were blotted onto a nylon membrane and probed using the insert from clone 60.3–950. **(B)** 16S rRNA normalization experiment confirming equal loading of total RNA onto each representative slot.

Figure 3. (A) Confirmation of differential expression of clone 60.3-850 by RNA slot blot analysis. Five micrograms each of control and uranyl acetate-exposed, rifampicin untreated total RNA were loaded onto each slot. **(B)** 16S rRNA analysis to verify equal loading of total RNA onto each slot.

aliquot of uranyl acetate-exposed culture (designated T = 0) was collected before the addition of rifampicin. An aliquot from a 20 mg/mL stock solution of rifampicin was added to each of the two cell cultures to produce a final concentration of 250 µg/mL before RNA extraction. Ten-milliliter aliquots were removed at time intervals of 5, 10, 20, 30, 40 and 60 min, respectively, and processed for total RNA isolation using a modification of the hot phenol method (5).

DD was conducted as previously described (5). The final concentrations of components in the RT reaction were: 200 µM dNTPs, 5 mM dithiothreitol, 50 U Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Life Technologies, Gaithersburg MD, USA), 0.2 µg total RNA, 0.4 µM primer and 1× M-MLV Reaction Buffer (Life Technologies) in a total reaction volume of 20 µL. The 10-bp arbitrary primers were derived from the Arbitrary 10-mer Primer Kit (Genosys Biotechnologies, The Woodlands, TX, USA). The RT reaction was carried out in a DNA Thermal Cycler 480 (PE Biosystems, Foster City, CA, USA) using the following conditions: ramp 50° to 30°C for 15 min; 37°C for 1 h; 95°C for 5 min followed by incubation at 4°C.

The PCR step was performed with a GeneAmp® PCR System 2400 (PE Biosystems) incorporating ³²P (ICN Biomedicals, Costa Mesa, CA, USA) or ³³P-labeled nucleotides (Andotek, Irvine, CA, USA) for visualization by autoradiography. The 15-µL PCRs contained: 0.3 U *Taq* DNA Polymerase (Life Technologies), 20 µM dNTPs, 6% dimethyl sulfoxide, 0.06 µM primer, 0.25 µL labeled nucleotide, 0.001% Triton® X-100 and 1× PCR Buffer (PE Biosystems). Following addition of 3 µL of the RT reaction, the PCR conditions used were 94°C for 15 s, 40°C for 30 s and 72°C for 60 s for a total of 40 cycles. This was followed by a 7-min final extension step at 72°C.

Two and one-half microliters of each RT-PCR were mixed with 2.5 µL of a denaturing loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and incubated in a 95°C water bath for 3 min. The PCR products were loaded side by side onto a 340-µm-thick 61- × 33-cm, 4.5% Denaturing Acrylamide Gel (Genomix, Foster City, CA, USA). The gel was run on an LR Sequencing Apparatus (Genomix) for 2 h at 2700 V. The gel was then dried and exposed to BioMax® MR film (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) at room temperature for 12–24 h.

Three putative differentially expressed bands of 775, 850 and 950 bp mol wt were observed to decrease in intensity with increasing time of exposure to rifampicin (Figure 1). The three candidate bands were excised from the gel, eluted in diethyl pyrocarbonate (DEPC)-treated water and reamplified using the same PCR conditions as used for DD. The number of cycles used for PCR reamplification was decreased from 40 cycles to 35 cycles to minimize any potential background effects. Subsequent PCR reamplification products were cloned into the pCR® 2.1 vector using the Original TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA). To verify differential expression, RNA slot-blot analysis was performed according to the method of Sambrook et al. (16) using rifampicin untreated control and uranyl acetate-exposed *P. putida* G7 total RNA from early to mid-log phase of growth. Each of the cDNA

Figure 4. (A) Confirmation of differential expression of clone 60.3–775 by RNA slot-blot analysis. Five micrograms each of control and uranyl acetate-exposed, rifampicin untreated total RNA were loaded onto each slot. (B) 16S rRNA analysis verifying equal loading of total RNA onto each slot.

Benchmarks

fragments was labeled using a Prime-It® II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) and subsequent hybridizations were incubated overnight at 68°C.

The three candidate bands previously identified on the DD gel, designated 60.3–775, 60.3–850 and 60.3–950, respectively, were each verified to be differentially expressed (Figures 2–4). Levels of induction between control and uranyl acetate-exposed samples were examined using the ImageQuant® program (Molecular Dynamics, Sunnyvale, CA, USA). The increased transcriptional activities observed were 7-fold, 3-fold and 2-fold higher for bands 60.3–950, 60.3–850 and 60.3–775, respectively. Subsequent sequencing of clones 60.3–775 and 60.3–850 showed less than 65% sequence similarity to any of the GenBank® sequences using the Blast-N and Blast-X algorithms (1), suggesting putative novel sequences. Sequencing results of clone 60.3–950 showed 98% sequence similarity to the *rpoB* gene, which encodes for the β -subunit of RNA polymerase (6,11,13, 15). Interestingly, there is evidence to support a transient increase in transcriptional activity of the *rpoB* gene upon initial exposure to rifampicin (13). There is also evidence to support a mutational event occurring in a discrete region of the *rpoB* gene resulting in resistance to rifampicin (6,11,15). RNA slot-blot analysis suggests that up-regulation of the three differential transcripts is due to the presence of uranyl-acetate, rather than rifampicin. However, because of the much slower growth rate of cells grown in uranyl acetate, the differentially expressed genes could be related more specifically to growth rate rather than uranyl acetate induction.

While discriminating between cloned rRNAs and mRNAs can be accomplished by using northern blots or RNA slot blots, this activity is labor-intensive. Thus, avoiding rRNA-derived PCR products before cloning and screening would save time and resources. Therefore, the bacterial transcriptional inhibitor rifampicin was used as an internal control to aid in the screening of differentially expressed mRNAs allowing discrimination from the more stable rRNA molecules on the DD gels.

This approach along with other subsequent screening procedures (20) can further reduce the number of false positives to a tolerable minimum that allows effective use of DD for the identification and isolation of differentially expressed genes in prokaryotic systems.

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