Optimization of Differential Display of Prokaryotic mRNA: Application to Pure Culture and Soil Microcosms

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The differential display (DD) technique, which is widely used almost exclusively for eukaryotic gene discovery, was optimized to detect differential mRNA transcription from both pure-culture and soil-derived bacterial RNA. A model system which included toluene induction of todC1 in Pseudomonas putida F1 was used to optimize the procedure. At 24-h tod induction was determined to be approximately 8 x 10⁷ transcripts/µg or 0.08% of the total mRNA. The primer concentration, primer length, annealing temperature, and template, deoxynucleoside triphosphate, and MgCl₂ concentrations were varied to optimize amplification of a todC1 fragment. The limit of detection of todC1 by DD was found to be 0.015 ng of total RNA template or approximately 10⁸ transcripts. Once optimized, a todC1C2 gene fragment from P. putida F1 RNA was detected by using an arbitrary primer for the reverse transcriptase step in conjunction with the same arbitrary primer and a Shine-Dalgarno primer in the PCR. To verify the results, an arbitrary primer was used to detect recovery of a new salicylate-inducible naphthalene dioxygenase in Burkholderia cepacia JS150. The method was then used to detect mRNA induction in both inoculated and uninoculated toluene-induced soil microcosms. Several putative differentially expressed partial gene sequences obtained from the uninoculated microcosms were examined, and one novel fragment was found to be differentially expressed.

The methods used for isolation and quantification of mRNA in environmental samples are designed to specifically measure in situ gene expression and activity. Direct extraction of mRNA from soils (24) and quantification of mRNA by an RNAse protection assay (11) have been performed for naphthalene dioxygenase in soils and for soluble methane monoxygenase in aquifer sediments (27). Reverse transcriptase (RT) PCR amplification of mRNA for soluble methane monoxygenase in activated sludge (25) and for lignin peroxidase in soils (6) has also been performed. These methods of mRNA analysis are a natural complement to DNA extraction and hybridization or PCR analysis for detecting sequences of catabolic genes or for determining ribosomal DNA concentrations in natural samples (12). However, the previously described mRNA analytical methods are limited by their need for a priori information on gene sequences in order to design specific probes or primers for mRNA measurement.

The differential display (DD) technique and the closely related RNA arbitrarily primed PCR (RAP-PCR) method have been used to detect and isolate differentially expressed genes under induced and uninhibited conditions in both eukaryotes and prokaryotes (10, 18, 20, 32). The DD procedure, in which a poly(T) primer is used for the RT reaction and an additional arbitrary primer is used for the PCR, has been used exclusively in eukaryotic expression studies. The RAP-PCR method differs from the DD technique in that arbitrary primers are used for both the RT and PCR steps, and the RAP-PCR method has been used for both eukaryotic and prokaryotic studies. In the procedure described here an arbitrary primer is used for the RT reaction, and the same arbitrary primer is used in conjunction with a Shine-Dalgarno (SD) primer for the PCR.

The objectives of this investigation were (i) to optimize and define reproducible conditions for using the DD technique to recover novel mRNAs from bacteria and (ii) to explore the use of DD for recovering cryptic or unknown RNA sequences transcribed under in situ conditions in soil. While DD has very recently been applied to environmentally related research, the focus has been limited to eukaryotes. This technique has been used to discover genes induced in a white rot fungus by pentachlorophenol (16) and in rat Sertoli cells by cadmium acetate and polychlorinated biphenyls (30). In this regard, the DD technique potentially could be used to identify known or cryptic microbial genes that are differentially expressed under altered field conditions, such as chemical exposure, oxidative stress, extreme pH, anaerobiosis, heat shock, and starvation.

MATERIALS AND METHODS

Cultivation of strains. A single colony of Pseudomonas putida F1 (13) was used to inoculate 100-mI portions of YEPG medium (1.0 g of dextrose per liter, 2.0 g of Polypeptone per liter, 0.2 g of yeast extract per liter, 0.2 g of NH₄NO₃ per liter; pH 7.0) in 250-mI flasks at 26°C, and the flasks were shaken at 225 rpm overnight. One milliliter of culture was collected and washed three times with phosphate-buffered saline (8.0 g of NaCl per liter, 0.2 g of KCl per liter, 1.15 g of Na₂HPO₄ per liter, 0.2 g of KH₂PO₄ per liter; pH 7.0). The cells were suspended in 100 ml of toluene-saturated minimal salts medium to induce tod gene expression under the same growth conditions. Similarly, colonies of Burkholderia cepacia JS150 (14) were used to inoculate YEPO medium for control cultures and YEPPS medium (0.2 g of yeast extract per liter, 2 g of peptone per liter, 0.5 g of Na₂H₄O₆ per liter, 2.7 g of Na₂C₃H₅O₃ per liter, 0.2 g of NH₄NO₃ per liter; pH 7.0). The latter medium was used to induce nahA transcription (11). Minimal salts buffer (MSB) contained (per liter) 4.0 g of NaNO₃, 1.5 g of KH₂PO₄, 0.005 g of FeCl₃, 0.2 g of MgSO₄, 0.01 m NaCl, and 0.5 g of Na₂HPO₄.

Reagents and chemicals. Unless otherwise noted, chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Molecular biology enzymes and reagents were obtained from Bethesda Research Laboratories (BRL), Gaithersburg, Md.

Primer synthesis. All primers were synthesized in house by using a DNA synthesizer (model Oligo 1000; Beckman, Fullerton, Calif.). Full-length primers were purified with reverse-phase cartridges (Glen Research, Sterling, Va.) by using the manufacturer’s protocol. The todC1 primers used were tod13a (GT GCTGCAACCATG; antisense) and tod13b (CAGATCGTGCAACC; sense) or tod10a (TGCTGCAAC; sense) and tod10b (CAGATCGTG; antisense). These primers amplify a 384-bp fragment from the todC1 gene of P. putida F1 (34). todC1 primers tod20a (ATGAATCAGACCGACACATC) (antisense) and
todd30s (AGACCCTCTAGGTCCGACACCAGACGATC) (sense) amplify a 940-bp fragment from *P. putida* F1 (34). The following arbitrary 10-base primers were used as described previously (11). Colony lifts and hybridizations were also performed on the plates as previously described (11), and the plates were probed with an *F1* cDNA fragment.

**RNA extraction from soil microcosms.** Ten milliliters of a soil slurry was added to an extraction solution consisting of 5 ml of extraction buffer (100 mM NaH2PO4, 1 M NaCl, 20 mM EDTA, 0.5% sodium dodecyl sulfate), 5 ml of phenol (pH 8.0; equilibrated with Tris), and 5 ml of chloroform-prepared DNA standards. The blots were processed as described above and were probed with a 32P-labeled *nahA* probe.

**Optimization of DD of prokaryotic mRNA.** To optimize the DD reaction conditions, we varied several parameters in parallel by using a set of specific *F1* primers to allow optimization of the following DD reaction conditions: (i) template concentration (15, 1.5, 0.15, and 0.015 μg); (ii) magnesium concentration (8, 4, 2, and 1 mM); (iii) primer concentration (2, 0.2, 0.02, and 0.002 μM); (iv) annealing temperature (50, 40, and 30°C); (v) deoxyribonucleotide triphosphate concentration (200, 20, and 2 μM); and (vi) primer length (10 and 13 bases). The concentrations of all of the other components were maintained at the values described above.

cDNA was synthesized by Moloney murine leukemia virus (MMLV) RT (BRL). A *F1* cDNA antisense 10- or 13-base primer was used for the initial optimization experiments, and subsequently, arbitrary 10-base primers were used. After optimization, in all subsequent experiments the final amounts and concentrations of components (in a reaction volume of 20 μl) were as follows: each dNTP, 200 μM; dithiothreitol, 5 mM; MMLV RT, 30 nM; total RNA, 200 ng; each primer, 0.4 μM; and 1× MMLV reaction buffer (BRL). The RT reaction was carried out with Perkin-Elmer (model 480 thermal cycler) for 94°C for 15 s, 40°C for 30 s, and 72°C for 60 s (for the model 2400 thermal cycler), followed by a 10-min extension step at 72°C.

The PCRs were performed with thermal cycles (model 480 and 2400; Perkin-Elmer) using 20-μl reactions. For each reaction, 2 μl of the RT reaction mixture, the solution was subjected to 40 cycles consisting of 94°C for 30 s (denaturing), 40°C for 2 min (annealing), and 72°C for 1 min (extension) (for the model 480 thermal cycler) or 94°C for 15 s, 40°C for 30 s, and 72°C for 60 s (for the model 2400 thermal cycler), followed by a 10-min extension step at 72°C.

The PCRs were electrophoresed on 4.5% denaturing acrylamide gels (340 μl by 61 cm by 33 cm; Genomyx, Foster City, Calif.) in side-by-side fashion. Four-microliter portions of the RT-PCR mixtures were loaded onto the sequencing gels along with 4 μl of denaturing loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) after the mixtures were boiled in a water bath for 2 min. Each gel was electrophoresed with a sequencing apparatus (model LR; Genomyx) for 2 h at 2,700 V. The gel was then dried directly on the glass by three cycles of sequential washing (2 min) in water and drying (15 min) to dissolve the agar. The dried gel was then exposed to X-ray film at −70°C (11) to visualize the bands. DNA bands were visualized by autoradiography. The final amounts and concentrations of components in the 27-μl reaction mixtures were as follows: Taq polymerase (BRL), 0.25 U; each dNTP, 20 μM; dimethyl sulfoxide, 0.5%; each primer, 2 μM; labeled nucleotide, 0.25 μM; 10× Triton X-100, 0.1%; and 1× PCR buffer (1.5 mM MgCl2; Perkin-Elmer). Following addition of 3 μl of the RT reaction mixture, the solution was subjected to 40 cycles consisting of 94°C for 30 s (denaturing), 40°C for 2 min (annealing), and 72°C for 1 min (extension) (for the model 480 thermal cycler) or 94°C for 15 s, 40°C for 30 s, and 72°C for 60 s (for the model 2400 thermal cycler), followed by a 10-min extension step at 72°C.

**DD analysis of toluene-induced RNA with arbitrary primers.** After optimization with specific primers, an attempt was made to detect *tod* transcription after the preparation was first enriched for *tod* cDNA by performing an RT reaction with an *F1*-specific primer. This enrichment step was followed by a PCR amplification step using the optimized DD reaction conditions, and subsequently, arbitrary 10-base primers were used. Following this intermediate experiment, an attempt was made to detect *tod* transcription by using arbitrary primers and/or primer SD14 for both the RT and PCR steps. Primers 70.3, 80.02, and 80.05 were used for a DD analysis in which primer 70.3 was used for the RT reaction and primer SD14 was used in conjunction with the same arbitrary primers for the PCR.

**DD analysis of salicylate-induced RNA with arbitrary primers.** Salicylate-induced and uninuced *B. cenocepacia* JS150 RNA were used for a series of reactions. In this analysis arbitrary primers 60.1, 60.5, 60.6, 60.8, and 80.7 were used separately for the RT reaction, and primer SD14 was used in conjunction with the same arbitrary primers for the PCR.

**Cloning of DD-derived PCR products.** Cloning was performed with a TA cloning kit (Invitrogen, San Diego, Calif.) by following the manufacturer's pro-
RESULTS

Quantitation of \textit{tod} and \textit{nah} transcription. Toluene-induced \textit{tod} gene expression in \textit{P. putida} F1 was detected after 3 h, and maximal induction occurred at 24 h (data not shown). \textit{tod} transcripts were not detected in the uninduced sample. By comparison with known amounts of \textit{tod} DNA, the amount of \textit{tod} RNA was determined to be 0.04 ng/\mu g of total RNA (Fig. 1A). The number of \textit{tod} transcripts was estimated by (i) calculating the number of moles of \textit{tod} RNA by dividing the mass (in grams) by the molecular mass of the \textit{tod} standard (940 bases; 330 Da/base) and (ii) multiplying the number of moles of \textit{tod} RNA by Avogadro’s number (6.022 \times 10^{23}). On this basis the number of \textit{tod} transcripts was estimated to be $8 \times 10^7$ transcripts/$\mu g$ of total RNA. By comparison with known amounts of \textit{nah}A DNA, the amount of \textit{nah}A-like transcripts from \textit{B. cepacia} JS150 was determined to be 0.05 ng (Fig. 1B). The number of \textit{nah}A transcripts was estimated to be $9 \times 10^7$ transcripts/$\mu g$ of total RNA by using 1,030 bases as the length of the \textit{nah}A standard.

Optimizing prokaryotic DD analysis by using specific primers. (i) Optimization conditions. In the electrophoretic gel analysis in which the Genomyx model LR apparatus was used, the average number of amplification products represented by visible bands was more than 70 products per lane, and the lengths ranged from 100 bp to 2 kbp. After the initial optimization, electrophoresis of the RT-PCR products from \textit{P. putida} F1 RNA was successful and resulted in a \textit{todC1} fragment of the expected size that was detected only in induced samples. When a series of decreasing RNA template concentrations were used, the \textit{todC1} fragment could still be detected when the amount of total RNA was 0.015 ng, and there was only a slight decrease in intensity under these conditions (Fig. 2A). The lower template concentration also decreased the total number of visible bands per lane. When a primer concentration of 0.2 $\mu M$ was used, the magnesium concentration did not appear to be critical, so a concentration of 1.5 $M$ was used (Fig. 2B).

![FIG. 1. Quantitation of induced transcripts from pure cultures. (A) \textit{P. putida} F1 cells grown in the presence or absence of toluene. Column 1, 10, 3, 1, 0.1, 0.03, and 0.01 ng of \textit{tod} DNA used as standards; columns 2 and 3, 10 $\mu g$ of total RNA from uninduced and induced \textit{P. putida} F1 applied to the membrane in duplicate. A $^{32}$P-labeled \textit{todC1} probe was hybridized with the blotted RNA. (B) \textit{B. cepacia} JS150 cells grown in the presence or absence of salicylate. Column 1, 10, 3, 1, 0.1, 0.03, and 0.01 ng of \textit{nah}A DNA used as standards; columns 2 and 3, 10 and 1 $\mu g$, respectively, of total RNA from uninduced and induced \textit{B. cepacia} JS150 applied to the membrane and probed with a $^{32}$P-labeled \textit{nah}A fragment.](http://aem.asm.org/toc)

![FIG. 2. Determination of the lower limit of the RNA template concentration and optimization of MgCl$_2$ concentrations for DD reactions. RNA from toluene-induced \textit{P. putida} F1 cells was processed for DD. (A) Different amounts of template RNA. Lane 1, 100-bp ladder; lane 2, 15 ng; lane 3, 1.5 ng; lane 4, 0.15 ng; lane 5, 0.015 ng. (B) Effects of different MgCl$_2$ concentrations on the complexity of the band pattern. Lane 1, 100-bp ladder; lane 2, 8 mM; lane 3, 4 mM; lane 4, 2 mM; lane 5, 1 mM. The position of the \textit{todC1} fragment is indicated by arrows.](http://aem.asm.org/toc)
The optimal primer concentration was found to be 0.2 mM, band intensity also dramatically decreased (data not shown). When the primer concentration was lower than 0.02 mM, the band pattern was observed when the annealing temperature was increased from 30 to 50°C (Fig. 4A). Primer length also had a great influence on the fingerprint band patterns (Fig. 4B); more bands per lane were observed with longer primers.

(ii) Verification of differential expression. In the optimization experiment performed with tod-specific primers, toluene induction of P. putida F1 yielded a differentially expressed band at 410 Da (Fig. 5, lane 2). This band was excised from the gel, reamplified, cloned, and designated clone tod410. We verified that this clone was differentially expressed band at 170 Da was excised (Fig. 5, lane 4), reamplified, and cloned, and this band was designated clone 70.3-170. We verified that this clone was differentially expressed by using the reamplified PCR product to probe RNA slot blots. Sequence analysis revealed that this differentially expressed PCR fragment was an overlapping but nonidentical toluene dioxygenase fragment 5’ to the todC1 gene extending into the todC2 region.

(ii) Salicylate induction and verification. The RNA fingerprints obtained with salicylate-induced and inoculated B. cepacia JS150 cells did not result in differential bands when primers 60.1, 60.5, 60.8, and 80.7 were used, but primers 60.3 and 60.4 yielded several differential bands (Fig. 8). Cloning and sequencing of reamplified clone 60.3-380 (Fig. 8, lane 3) revealed that it exhibited 90% homology with a Pseudomonas reductase (ntdAc) gene (32). We verified that clone 60.3-380 was differentially expressed by RNA slot blotting (Fig. 9A). Probing slot blots with clone 60.3-325 revealed that this clone was a ribosomal subunit, and this clone was used as an indicator of equal slot blot loading (Fig. 9B).

DD analysis of soil microcosm-derived RNA. (i) Inoculated microcosms. The RNA fingerprints obtained with pure-culture and inoculated soil microcosms were virtually identical (Fig. 10). However, the todC1 band was fainter in the soil sample than in the pure-culture sample (Fig. 10, lane 5). The recovery of the 32P-labeled internal standard RNA from the soil was approximately 75% when the protocol described above was used.

Compared to the RNA fingerprints generated with specific primers, more bands per lane were observed when single arbitrary primers were used. Seven differential bands were detected on the DD gel when the SD primer and arbitrary primer 70.3 were used (Fig. 7, lane 3), and we verified that one of the bands was differentially expressed by using the reamplified PCR product to probe RNA slot blots. Sequence analysis revealed that this differentially expressed PCR fragment was an overlapping but nonidentical toluene dioxygenase fragment 5’ to the todC1 gene extending into the todC2 region.

DD analysis with arbitrary primers. (i) Toluene induction and verification. When a specific tod primer was used for the RT step and arbitrary primer 70.3 was used for the PCR step, a differentially expressed band at 170 Da was excised (Fig. 5, lane 4), reamplified, and cloned, and this band was designated clone 70.3-170. We verified that this clone was differentially expressed by RNA slot blotting (Fig. 6B), and it was found to flank bases 1035 to 1203 of todC1. Clone 70.3-350 obtained in this experiment was determined to be a ribosomal subunit and was used as an indicator of equal loading on the slot blot (Fig. 6C).

FIG. 3. Optimization of nucleotide and primer concentrations for DD reactions. RNA from toluene-induced P. putida F1 cells was processed for DD. (A) Effects of different nucleotide concentrations on the complexity of the band pattern. Lane 1, 100-bp ladder; lane 2, 200 μM; lane 3, 20 μM; lane 4, 2 μM; lane 5, 0.2 μM. (B) Effects of different primer concentrations on the complexity of the band pattern. Lane 1, 100-bp ladder; lane 2, 2 μM; lane 3, 0.2 μM; lane 4, 0.02 μM; lane 5, 0.002 μM. The position of the todC1 fragment is indicated by arrows.

FIG. 4. Optimization of annealing temperature and primer length for DD reactions. (A) Effects of different annealing temperatures on the complexity of the band pattern. RNA from toluene-induced P. putida F1 cells was processed for DD. Lanes 1 and 5, 100-bp ladder; lane 2, 30°C; lane 3, 40°C; lane 4, 50°C. (B) Effects of different primer lengths on the complexity of the band pattern. Lanes 1 and 2, 10-base primer; lanes 3 and 4, 13-base primer. RNA from uninduced cells (lanes 1 and 3) or toluene-induced cells (lanes 2 and 4) was processed for DD. The position of the todC1 fragment is indicated by arrows.
(ii) Uninoculated microcosms. After 4 h, when soil slurry samples were processed for total RNA, the cultured heterotrophic plate counts were 2.23 \( \pm 0.15 \times 10^6 \) CFU/g for the microcosm without toluene and 2.07 \( \pm 0.31 \times 10^6 \) CFU/g for the toluene-induced microcosm. The sizes of the \( \text{todC1} \)-positive populations in the uninduced microcosms and the toluene-induced microcosms were 5.17 \( \pm 1.0 \times 10^5 \) and 4.00 \( \pm 2.2 \times 10^5 \) CFU/g, respectively. The results of mass determinations for soil-derived total RNA obtained by hybridization of \( ^{32} \text{P} \)-labeled universal rRNA oligonucleotides agreed with the results of determinations based on \( A_{260} \) values (data not shown). Extraction of uninoculated soil microcosms yielded 9.8 and 9.4 \( \mu \text{g} \) of total RNA from toluene-induced and uninduced soils, respectively. Because of the low RNA yield, the RNA integrity was not checked by visualization on denaturing gels. DD experiments performed with RNA derived from the uninoculated microcosm yielded several differential bands (Fig. 11). When these bands were cloned and sequenced, clone 70.3-400 was found to be both unique and differentially expressed. Clone 70.3-350 (lane 4, band a) was shown to be a ribosomal subunit.

**DISCUSSION**

Optimization of the DD technique for prokaryotic RNA. The DD technique involves several steps, including (i) isolation of intact RNA from organisms, (ii) reverse transcription of total RNA with either an arbitrary primer or an oligo(dT) primer (for eukaryotic systems) to generate cDNA, (iii) PCR amplification with an arbitrary primer or an arbitrary primer paired with an oligo(dT) primer (for eukaryotic systems) to amplify the cDNA, (iv) separation and detection of the differential PCR products on sequencing gels, (v) reamplification and cloning of the differential PCR products, (vi) verification of the differential expression of the isolated gene fragment by Northern blotting, RNA slot blotting, RNase protection assay, or RT-PCR; and (vii) comparison of sequences with known sequences in gene sequence databases.

Our initial attempts in which we used a previously described prokaryotic RAP-PCR protocol (32) yielded only rRNA fragments from the putative DD bands (data not shown). Therefore, parameters such as annealing temperature, primer size and concentration, magnesium and nucleotide concentrations, and RNA template concentration were empirically examined to optimize the procedure for application to RNA derived from both prokaryotic pure cultures and soil communities.

Even when specific primers were used to specifically amplify the \( \text{todC1} \) gene, approximately 70 other distinct bands, many

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**FIG. 5.** DD analysis of toluene-induced \( P. \ putida \) F1 cells performed with specific and arbitrary primers. RNA from uninduced cells (lanes 1 and 3) and toluene-induced cells (lanes 2 and 4) was reverse transcribed by using primer tod13a. Subsequently, primer tod13s (lanes 1 and 2) or arbitrary primer 70-3 was used in conjunction with primer tod13a for PCR. Lane 5 contained a 100-bp ladder. The arrows indicate the positions of bands that were cloned and sequenced. Clone tod410 (lane 2) and clone 70.3-170 (lane 4, band b) were verified as differentially expressed. Clone 70.3-350 (lane 4, band a) was shown to be a ribosomal subunit.

**FIG. 6.** Confirmation of differential expression in \( P. \ putida \) F1 by RNA slot blot analysis. Five micrograms of uninduced or toluene-induced pure-culture total RNA was blotted onto nylon membranes in duplicate and probed with \( ^{32} \text{P} \)-labeled inserts from clones tod410 (A), 70.3-170 (B), and 70.3-350 (C).
probably ribosomal in origin, were also amplified. Since the primers were designed in such a way that specific annealing of the *P. putida* rRNA to the 3′ end of the primer was diminished, the observed amplification of rRNA was presumably due to mispriming of mismatched primer-template duplexes. Theoretically, the annealing temperature influences the DD results; the higher the annealing temperature, the smaller the number of mispriming events. However, our data show that the annealing temperature did not have much influence on the total number of bands generated or the band patterns. When the annealing temperature was increased from 30 to 50°C, only a slight band pattern change was observed (Fig. 4A). This finding may be explained by the fact that the PCR enhancers Triton X-100 and dimethyl sulfoxide were added to the PCR mixture, which reportedly increases PCR efficiency and specificity (22). Alternatively, it may be attributed to the template or the primer used (20).

Primer length also had a great influence on the fingerprint band patterns. Compared to fingerprints generated with 10-base primers, more bands per lane were obtained when 13-base primers were used (Fig. 4B). This was most probably due to increased mispriming with the longer primers. After the initial optimization work, the best results were obtained with an arbitrary 10-base primer for the RT step and primer SD14 in conjunction with the same arbitrary primer for the PCR.

Primer concentration also had a great influence on the band pattern. The best primer concentration was determined to be in the range from 0.2 to 2 μM. At concentrations greater than 2 μM, nonspecific priming increased (data not shown); at concentrations less than 0.2 μM band intensity and complexity decreased. Nucleotide concentrations were also critical for good fingerprints. At concentrations greater than 20 μM, the rate of incorporation of the radiolabeled dNTP was low, resulting in a lower band intensity; at concentrations less than 2 μM, bands were barely visible, most probably due to depletions of dNTPs (Fig. 3A, lane 5).

Once parameters were optimized by using specific rDNA primers, the procedure was modified for application to unknown sequences by using an arbitrary primer for the RT step and a primer for the SD region in conjunction with the same arbitrary primer for the PCR step. In contrast to a recent report which described the design of 3′ and 5′ DD primers for a particular bacterial family (10), the use of an SD primer in this study was intended to prime the 5′ regions of a wider range of prokaryotes. The primer used in this study included the ATG start codon at the 3′ end along with additional 5′ bases derived from a comparison of *Pseudomonas* and *Escherichia coli* SD regions. While the use of a 5′ SD primer generally biases amplification toward mRNA sequences in a pool of prokaryotic RNA sequences, such a primer may ignore messages that do not have SD regions. For organisms without SD regions, the use of arbitrary primers may be preferable.

**Message abundance and the arbitrary primer set.** There is some debate concerning the sensitivity of the DD technique to rare mRNAs, and no data related to prokaryotic RNA has been published previously. While it is estimated that a typical mammalian cell has 360,000 mRNA molecules per cell and 20,000 to 30,000 different mRNA species whose copy numbers range from 15 to 12,000 depending on the species (2), prokaryotes are estimated to have only 1,380 mRNA molecules

![FIG. 7. DD analysis of toluene-induced *P. putida* F1 cells performed with arbitrary primers. RNAs from uninduced cells (lanes 2, 4, 7, and 9) or toluene-induced cells (lanes 3, 5, 8, and 10) were reverse transcribed by using primer 70.3. Subsequently, primer 70.3 alone (lanes 7 and 8), primer SD14 alone (lanes 9 and 10), or both primers (lanes 2 through 5) were used for PCR. Lanes 1 and 6 contained a 100-bp ladder. The position of the *todC1C2* fragment in lanes 3 and 5 is indicated by an arrow.](http://aem.asm.org/)

![FIG. 8. DD analysis of salicylate-induced and uninduced RNA from *B. cepacia* JS150 performed with arbitrary primers. Total RNA from uninduced cells (lanes 2, 4, 6, and 8) and induced cells (lanes 3, 5, 7, and 9) were reverse transcribed with primer SD14, and this was followed by PCR performed with primer SD14 and an arbitrary 10-base primer. Lane 1, 100-bp ladder; lanes 2, 3, 6, and 7, PCR performed with primers SD14 and 60.3; lanes 4, 5, 8, and 9, PCR performed with primers SD14 and 60.4. The arrows indicate the positions where clones 60.3-380 (lane 3, arrow a) and 60.3-325 (lane 3, arrow b) were obtained.](http://aem.asm.org/)
per cell, representing 400 different mRNA species (21). Using a model eukaryotic system, Bertioli et al. showed that when the standard DD protocol was used, mRNA that accounted for less than 1.2% of the total mRNA (the mean percentage for the most prominent class of mammalian RNA) was not detected by this technique (5). At levels lower than this, even a perfectly matched primer failed to detect the mRNA template in a heterologous total RNA background. In contrast to these findings, Wan et al., using cultured HeLa cells, observed levels of DD sensitivity as low as 0.0005% of the total mRNA (31). In the present work, we demonstrated that prokaryotic catabolic mRNAs, as represented by the tod and nah messages in pure culture after induction, accounted for 0.08 and 0.09%, respectively, of the total mRNA and thus might be considered intermediate-level mRNAs. The limit of detection for the DD technique when a specific tod 13-base primer was used was 0.015 ng of total RNA, which corresponds to $10^3$ transcripts. Because the complexity of prokaryotic mRNAs is much less than the complexity of eukaryotic mRNAs, it should theoretically be easier to obtain rare transcripts from prokaryotes by the DD technique.

For the DD technique to have practical value for screening environmental samples, a limited set of primers would ideally allow detection of prominent differentially expressed transcripts. The logic of the approach developed here was that, once DD conditions were optimized by using specific primers for abundant messages, the same conditions should permit detection of the same message or other abundant messages with arbitrary primers alone. Since 10-base primers appear to function like 6- or 7-base primers and there are approximately 10,000 possible 6- or 7-base oligonucleotides (18), the chance of finding any one sequence in 1,500 bp (the maximum length of fragment separable on a Genomyx LR sequencing gel) is 0.15. On this basis and considering the abundance issue, theoretically it should take approximately seven primers to cover all abundantly expressed mRNAs. This hypothesis is supported by the fact that both the tod and nah messages were detected with a small set of arbitrary primers alone. It is reasonable to assume that the catabolic mRNAs of other microbes which use organic compounds as energy sources are also abundantly expressed. Therefore, the DD procedure may be well-suited for detection of microbial genes responsible for the degradation of organic compounds.

**DD analysis of soil microcosm RNA.** The results of the inoculated-soil RNA extraction experiment demonstrated the feasibility of using the DD technique to study gene expression in soil systems (Fig. 10). The RNA extraction procedure yielded samples that were visually clean and had $A_{260}/A_{280}$ ratios on the order of $>1.8$. The soil-derived RNA apparently did not contain contaminants that prevented primer annealing or the enzymatic processes in DD; the RNA fingerprints of inoculated soil microcosms were virtually identical to those of pure cultures. However, based on the intensity of the tod fragment on DD gels (Fig. 10), the efficiency of amplification of soil-derived RNA appears to be less than the efficiency of amplification predicted on the basis of the efficiency of RNA extraction. This may be due to PCR inhibitors in the soil-derived sample. An alternative explanation is that the tod message was partially degraded during the soil isolation procedure. Nevertheless, the reproducibility of RNA fingerprints obtained with soil-derived RNA was demonstrated by the results of the uninoculated soil microcosm experiment. The RNA fingerprints obtained from triplicate reactions by using the same RNA sample obtained from uninoculated soil microcosms were nearly identical (Fig. 11).
Of the several problems encountered with the DD procedure, the occurrence of false positives is the most vexing (7, 19, 33). In agreement with results describing the application of the DD technique to eukaryotic systems, the percentage of false positives obtained in this study was high. It is the consensus of the research community that less than one-half of the putative differentially expressed PCR products excised from gels are truly differentially expressed (1, 17, 29). Subsequent to the optimization experiments, in which an arbitrary primer was used to display toluene-induced RNA from \textit{P. putida} F1, seven differential bands were eluted; after cloning, only one band showed differential expression. Similarly, in the uninoculated soil microcosm experiment, only 1 of 12 putative differentially expressed clones that were screened for differential expression was confirmed to be differentially expressed by RNA slot blotting. The high percentage of false positives obtained in this study may be due in part to the lack of sensitivity of RNA slot blots for detecting low-abundance messages. A more sensitive detection method, such as RT-PCR, may increase the number of verified differentially expressed gene fragments. The use of a lower RNA template concentration (100 ng) in the soil microcosm experiments compared to the preferable higher concentration (200 ng) used in the pure-culture experiments may also partially account for the lower percentage of verified differentially expressed gene fragments in the soil microcosm experiment.

Because of the temperature and length of storage, the soils used for microcosm studies were relatively inactive; this may, in turn, explain the low RNA yield. This problem could be overcome by increasing the scale of the isolation procedure and using fresh soil for microcosm experiments. Another solution would be to increase the population by culturing the cells for a longer period of time. In our experiments the induction period was kept to a minimum in an attempt to discern true induction effects from changes in population due to growth of the members able to utilize toluene as a carbon source. Based on the enumeration data, there was no significant difference between the uninduced and toluene-induced microcosms when either heterotrophic or cultured \textit{todC1} populations were examined.

The sequence of the clone obtained from the uninoculated microcosm exhibited little similarity to GenBank sequences. This may not be an anomaly considering that the vast majority of organisms in the environment have not been characterized (4). Attribution of a function to this sequence will require isolation of the full-length gene from a soil-derived DNA library, followed by functional studies performed in transgenic hosts. Despite the shortcomings of the DD technique, it potentially offers a powerful approach for studying gene expression in the environment without prior culturing of cells or knowledge of sequences.

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