AFRL-SR-BL-TR-98-

0343

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	3/31/98	Final Technical 5/1/94-1/31/97

4. TITLE AND SUBTITLE
Environmental Genome Analysis: Polymerase Chain Reaction Fingerprinting on In Situ Extracted Bacterial mRNA from Contaminated Soils

5. FUNDING NUMBERS
C# F49620-94-1-0222
2312/AS
61102F

6. AUTHOR(S)
James T. Fleming and Gary S. Sayler

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Tennessee
404 Andy Holt Tower
Knoxville, TN 37996-0140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR/NL
110 Duncan Ave.
Suite B115
Bolling AFB, DC 20332-0001

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

19980511 076

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
no limitations

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of this research was to develop a polymerase chain reaction method to fingerprint mRNA obtained by in situ extraction from contaminated soils to assess the differential catabolic gene expression of a soil microbial population. This approach, which is now generally referred to in the scientific community as differential display (DD), has great potential application for the study of gene expression in the environment and the acquisition of novel genetic elements for industrial application. The major outcomes of this work are: 1) Optimization of DD for prokaryotic cells. 2) Demonstration of DD from soil derived RNA obtained from both inoculated and uninoculated soil microcosms. In the process of achieving these goals several previously uncharacterized genetic elements were isolated and cloned.

14. SUBJECT TERMS
bioremediation, RNA fingerprinting, gene expression, in situ soil Nucleic acid extraction, differential display

15. NUMBER OF PAGES
71

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	UL

Environmental Genome Analysis: Polymerase Chain Reaction Fingerprinting on In Situ Extracted Bacterial Messenger RNA from Contaminated Soils

Final Technical Report (May 1, 1994 - January 31, 1998)
Contract Number F49620-94-1-0222

Submitted to:

United States Air Force Office of Scientific Research

Prepared by

James T. Fleming, Gary S. Sayler

Center of Environmental Biotechnology
University of Tennessee
Knoxville, TN 37932
(423) 974-8080

Table of contents

Figures

Abstract

The differential display (DD) technique, 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 using toluene induction of *todC1* in *Pseudomonas putida* F1 was used to optimize the procedure. Primer concentration, primer length, annealing temperature, template, dNTP and $MgCl_2$ concentration was varied to optimize the amplification of a *todC1* fragment on sequencing gels. Once optimized, an arbitrary primer for the RT step in conjunction with the same arbitrary primer and a Shine-Dalgarno (SD) primer for the PCR reaction was used to detect *tod* transcription in *P. putida* F1 and a new salicylate inducible naphthalene dioxygenase in *P. putida* JS150. To help reduce the number of false positives, rifampicin was added to pure cultures and microcosms to allow discrimination between PCR products derived from ribosomal subunits and mRNAs. The method was then applied to detect mRNA induction in both inoculated and uninoculated toluene, cadmium and uranium induced soil microcosms. Of several putative differentially expressed partial gene sequences obtained from the uninoculated microcosms, several were verified to be differentially expressed. The work described here has led to the development of a proce*ss* for discovery and acquisition of novel genes from environmental microbial communities that avoids the traditional steps and inherent bias due to the culturing of environmental isolates.

INTRODUCTION

Methods for the isolation and quantification of mRNA from environmental samples are designed to specifically measure *in situ* gene expression and activity. Direct extraction of mRNA from soils (37) and quantification of mRNA by ribonuclease protection assay (16) has been demonstrated for naphthalene dioxygenase in soils and sMMO in aquifer sediments (41). Reverse transcriptase PCR amplification of mRNA for sMMO in activated sludge (38) and lignin peroxidase in soils (7) has also been demonstrated. These methods for mRNA analysis are a natural compliment to DNA extraction and hybridization or PCR analysis to detect gene sequences for catabolic genes or rDNA gene abundance in natural samples (17). In addition, direct mRNA analysis also provides potential evidence of *in situ* gene activity and conditions permissive of gene induction in the environment. These applications can be independent of microbial cultivation and, hence, avoid many laboratory biases. However, these previous mRNA analytical methods are limited by the need for *a priori* information on gene sequences in order to design specific probes or primers for mRNA measurement. The objective of this investigation was to explore the use of differential display (DD) to quantify and recover cryptic or unknown DNA sequences transcribed under *in situ* conditions in soil. A specific goal of these studies was to optimize and define reproducible conditions to use DD to recover novel mRNAs. While DD has very recently been applied to environmentally related research, the focus has been limited to eukaryotes. The technique has been used to discover genes induced in white-rot fungus by pentachlorophenol

(22) and rat Sertoli cells by cadmium acetate and polychlorinated biphenyls (44). In this regard, DD potentially allows identification of 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.

DD and the closely related RAP-PCR have been used to detect and isolate differentially expressed genes under induced and uninduced conditions in both eukaryotes and prokaryotes (15,23,29,49). The DD procedure, which uses a poly T primer for the RT reaction and an additional arbitrary primer for PCR has been exclusively applied to eukaryotic expression studies. RAP-PCR differs from DD in that arbitrary primers are used for both the RT and PCR steps, and as such, has been used for both eukaryotic and prokaryotic studies. The current procedure described here uses an arbitrary primer for the RT reaction and the same arbitrary primer in conjunction with a Shine-Dalgarno (SD) primer for PCR.

The original objectives of the proposal were:

1. Develop an arbitrarily primed PCR method to fingerprint mRNA obtained by in situ lysis and extraction of soil bacterial mRNA using Pseudomonas putida naphthalene catabolic gene induction as a model system in pure cultures and soil microcosms.
2. Apply the AP-RNA fingerrpinting method to the induction of Pseudomonas strain JS150 in simple and complex organic mixtures in pure culture and soil microcosms.

3. Apply the AP-RNA fingerprint method to screen differential bacterial expression in soils contaminated by JP-4 jet fuel.
4. Isolate and/or sequence and clone differentially expressed RNA fingerprint products obtained from soils for nucleic acid probe development and characterization of novel enzymes.

While the overall aim of the proposal was realized, several of the original objectives and tasks were modified. Since the writing of the proposal, the more widely used term 'differential display' has become synonymous with RNA fingerprinting and for that reason it is used in this report. In this investigation a toluene degrading *Pseudomonas putida* F1 model system approach was chosen to optimize the DD procedure rather than a naphthalene induction system because toluene is more relevant to DOD's environmental concerns. Once the DD procedure was optimized and soil microcosm experiments became feasible, metals were chosen for induction experiments instead of complex organic mixtures because: 1) metal induction events are presumed to be simpler and thus less global in effect compared with organic chemical induction. 2) metals are commonly co-contaminants with organic chemicals at DOD waste sites and present a difficult remediation challenge.

Subsequent to optimization an arbitrary primer in conjunction with a primer for the SD region is used to detect toluene induced *tod* transcription in *P. putida* F1 and salicylate induction of a previously uncharacterized *nahA* positive transcript in *P. putida* JS150. This approach is then applied to isolate several toluene,

cadmium and uranium induced differentially expressed gene fragments from both inoculated and uninoculated soil microcosms.

MATERIALS AND METHODS

Cultivation of strains. A single colony of *Pseudomonas putida* F1 (18) was used to inoculate 100 ml of YEPG media (1.0 g, dextrose; 2 g, polypeptone; 0.2 g, yeast extract; 0.2 g, NH_4NO_3 /L, pH 7.0) in 250 ml flasks at 26°C and shaken at 225 rpm overnight. One ml of the culture was collected and washed three times with phosphate buffered saline (PBS), (8.0 g, NaCl; 0.2 g, KCl; 1.15 g, Na_2HPO_4; 0.2 g, KH_2PO_4 /L pH 7.0). The cells were resuspended in 100 ml of toluene saturated minimal salts media to induce *tod* gene expression under the same growth conditions. Similarly, colonies of *P. putida* JS150 (20) were used to inoculate YEPG for control cultures and YEPSS media (0.2 g, yeast extract, 2 g, peptone, 0.5 g, $NaC_7H_5O_3$, 2.7 g, $Na_2C_4H_4O_4$, 0.2 g NH_4NO_3 /L, pH 7.0). The latter medium was used to induce *nahA* transcription. Minimal salts buffer (MSB) is composed of 4.0 g, $NaNO_3$; 1.5 g, KH_2PO_4; 0.005 g, $FeCl_3$; 0.2 g, $MgSO_4$; 0.01 g, $CaCl_2$; 0.5 g, Na_2HPO_4/L . *Pseudomonas putida* G7 was grown overnight at 30°C in YEPG.

Reagents and chemicals. Unless otherwise noted chemicals and reagents were obtained form Sigma Chemical co., St. Louis, MO. Molecular biology enzymes and reagents were obtained from BRL, Gaithersburg, MD.

Primer synthesis. All primers were synthesized in-house using a DNA synthesizer (Oligo 1000, Beckman, Fullerton, CA). Purification of full-length primers was done with reverse phase (RP) cartridges (Glen Research, Sterling,

VA) according to the manufacturer's protocol. The sequences of the *todC1* primers used were: tod13a (GTGCTCGACCATG) (anti-sense) and tod13s (CACATGCTCGACC) (sense) or tod10a (GTGCTCGACC) (anti-sense) and tod10s (CACATGCTCG) (sense). These primers amplify a 384 bp fragment from the *todC1* gene of strain *P. putida* F1(38). *TodC1* primers tod20a (ATGAATCAGACCGACACATC) (antisense) and tod30s (AGACGGTCATGTGCTCGACCACTAGTTTCG) (sense) amplify a 940 bp fragment from *P. putida* F1 DNA (51). The sequences for the arbitrary 10 bp primers used were obtained from a commercial arbitrary primer set (Genosys Biotechnologies, The Woodlands, TX) and their designations are as follows: 60.1, (CGCAGTACTC); 60.2, (GTCCTACTCG); 60.3, (CGAAGCGATC); 60.4, (GTCCTTAGCG); 60.5, (GTCCTCAACG); 60.8, (GTCCTCAGTG); 70.3, (ACGGTGCCTG); 80.3, (CCATGGCGCC); 80.7, (GCACGCCGGA). The Shine-Dalgarno primer SD14 (GGGGAACGACGATG) was derived from a comparison of several bacterial mRNA start sites (32). The sequence of *nah* specific primers were: *nahA3,* (CCTTAGCGCGTAACTACCCC) and *nahA4,* (GGTCCAGACCTTGGTGGTG)(40). These two primers flank bases 2262 to 3291 of the NAH7 plasmid allowing amplification of a 1030-bp fragment.

RNA extraction from pure culture. RNA extraction was done using two methods: RNeasy columns (Qiagen, Chatsworth, CA) and a modified hot phenol procedure (16). Small-scale extraction of RNA from RNeasy columns was the preferable source for RNA fingerprinting. Large-scale extraction of RNA by the hot phenol method was the source of RNA for RNA slot blots. The procedures

for RNA extraction with RNeasy columns were followed according to manufacturer's protocol. Total mRNA from large scale-preps was done by the hot phenol method. 50 ml of mid log phase (OD^{600}=0.6) cells were collected by centrifugation at 1,935 x g (4,000 rpm, JA-20 rotor, Beckman, Fullerton, CA) for 15 minutes in 50 ml disposable centrifuge tubes. The cells were then resuspended in 20 ml of 0.05 M $NaC_2H_3O_2$, 1% $C_{12}H_{25}NaO_4S$ (SDS) buffer (pH 5.2) at 60°C and 20 ml of phenol/chloroform (1:1) was added to the solution. The tubes were shaken by hand briefly and kept at 60°C for 5 minutes after which the tubes were shaken with the wrist action shaker for 5 minutes. The tubes were cooled on ice for 2 minutes and were then centrifuged at 1,935 x g for 15 min using a Beckman table-top centrifuge. The supernatant was transferred to a fresh tube and re-extracted three times. The final supernatant was removed to a baked 150 Corex bottle (Corning, East Brunswick, NJ) and 0.1 volume 3 M $NaC_2H_3O_2$ and 2.5 volumes of ice-cold ethanol was added to the solution. The solution was allowed to sit at -80°C for 15 minutes and centrifuged at 12,096 x g (10,000 rpm, JA-20 rotor, Beckman) for 30 minutes at 4°C. The pellets were resuspended in 500 µl of $C_6H_{10}O_5$ (DEPC) treated water, 2µl of RNase free/DNase I (30 U/µl, Boehringer Mannheim, Indianapolis) and 50 µl of Mg/DTT (100 mM) was added and allowed to incubate at 37°C for 30 minutes. The RNA solution was then extracted with the same phenol/chloroform (1:1) solution three times and again precipitated by the addition of 0.1 volume of 3M $NaC_2H_3O_2$ and 2.5 volumes of ice-cold ethanol. The RNA pellet was then washed twice with ice

cold 70% ethanol, dissolved in 100 µl DEPC water and quantified by using UV spectroscopy (Beckman, Fullerton, CA model DU-70).

RNA integrity determination. RNA integrity was verified with Northern gels (36). 10 µg of total RNA was incubated with 10 µl of formamide, 3.5 µl of formaldehyde and 2 µl of 5X MOPS buffer (0.1 M $C_7H_{15}NO_4S$, pH 7.0, 40 mM $NaC_2H_3O_2$ and 5 mM $C_{10}H_{16}N_2O_8$ (EDTA), pH 8.0) in a volume of 20 µl at 65 °C for 15 minutes. The RNA was then electrophoresed on 1.2% denaturing gels containing 18% formaldehyde along with 2 µl of loading dye and 1 µl of ethidium bromide (10 mg/ml). RNA was judged to be intact if the 16S and 23S rRNA subunits were visually well-defined bands after electrophoresis.

Uranium Induction in pure culture. Following overnight incubation, a 20ml aliquot of pure culture PpG7 was transferred to 80ml of fresh YEPG media in the presence of 200 µM uranyl acetate. Since previous literature suggests that uranium uptake occurs at a fairly rapid rate, cells were harvested following a 30 min incubation.

Cadmium Induction in pure culture. After overnight incubation a 5 ml aliquot of pure culture derived PpG7 was transferred to 95 ml of fresh YEPG media in the presence of 100 µM cadmium chloride. Cells were harvested in early log phase.

Soil Microcosms.

Toluene Induction. The soil aquifer samples were obtained from bore-holes at Columbus Air Force Base (CAFB), Columbus, MS that had been stored at 4°C for 1 year. These soils were well characterized chemically and microbiologically

as part of a natural attenuation study at the CAFB groundwater test facility (42). In initial experiments, flasks containing 10 g of soil in 20 ml water were inoculated with *P. putida* F1 at 10^8 cells/g soil and incubated at 26°C with shaking at 225 rpm overnight. To induce cells, toluene was added in the saturated vapor phase. Subsequently uninoculated microcosms were prepared using the same aquifer soil. 10 g soil was incubated in 25% YEPG for 4 h in shake flasks with and without toluene. After 4 h slurry samples were simultaneously processed for total RNA and enumeration of culturable organisms as described previously (16). Colony hybridizations were also performed using the enumerated plates for colony lifts as previously described (16) and probed with a PCR generated *todC*1 fragment.

Uranium Induction. Soil microcosms consisted of 20 grams of an aquifer soil (supplied by Columbus Air Force Base) in the presence of 80ml of quarter-strength YEPG media. Microcosms were incubated for 24 h at 30°C prior to uranium exposure. Following the 24 h incubation period, 200 µM uranyl nitrate was added to the appropriate microcosm. Following a 30 min exposure to uranium, a 10ml aliquot of the soil slurry was taken from each microcosm (control and induced) for RNA extraction. To ensure that induction was due to uranium and not nitrate two additional controls were used: a soil microcosm containing 200µM calcium nitrate and a soil microcosm containing 200µM magnesium nitrate. These two compounds were chosen on the basis that each had an appropriate molecular weight in comparison to uranyl nitrate.

RNA extraction from Soil Microcosms. 10 ml of soil slurry was added to a extraction solution consisting of 5 ml of extraction buffer (100 mM $C_4H_{11}NO_3$ •HCl (Tris) 1.4 M NaCl, 20 mM EDTA, 1% SDS), 5 ml phenol, pH 8.0 (equilibrated with Tris) and 5 ml chloroform pre-warmed to 60°C. The soil solution, in baked 25 ml Corex centrifuge tubes, was incubated at 60°C for 5 min then shaken by mechanical action on a wrist action shaker for 5 min. The tubes were then centrifuged for 15 min at 12,096 x g at 4°C and the supernatants were extracted again with 10 ml chloroform. 5 µl of linear acrylamide (Ambion, Austin, TX) was added as a co-precipitant, 15 ml of isopropanol was added and the tubes were stored overnight at -20°C. The following day the tubes were centrifuged at 12,096 x g for 15 min and the pellet was resuspended in 200 µl DEPC treated water. The soil derived RNA solution was then DNase treated, phenol/chloroform extracted and ethanol precipitated. The concentration of soil extracted total RNA was estimated on the basis of absorbance at 260 and 280 nm.

A PCR constructed *NahA* template linked to a T7 promoter was used to transcribe a labeled *NahA* RNA probe (16) according to the manufacturer's protocols (MEGAscript kit, Ambion). After precipitation, the RNA probe was dissolved in 100 µL of DEPC treated water and 1 µL of the RNA was counted by scintillation countng. 160,000 cpm of the labeled RNA solution was added to the soil sample as an internal standard to estimate the RNA recovery rate.

Preparation of radiolabled gene probes. Gene probes were prepared by random primer extension (14) or by PCR (34). For the random primer method,

the template DNA was restricted, electrophoresed in agarose gels, excised, and placed into a pre-weighed tube. 3 ml of distilled water/g of gel was added to the tube and heated at 65 °C for 5 minutes to melt the gel. 21 µl of the resulting DNA solution was used to prepare for the probe according to the manufacturer's protocols (Random Primer labeling Kit, Stratagene, LaJolla, CA). PCR generated double stranded probes were produced by substituting α-^{32}P-dCTP(600 Ci/mmol) for cold dCTP in the standard PCR reaction (41). After PCR amplification, probes were denatured by boiling for 10 min prior to being added to prehybridized filters.

Time-course analysis of *tod* gene induction. A single colony of *P. putida* F1 was used to inoculate 50 ml of YEPG broth in a 250 flask which was incubated overnight at 26°C on a rotary shaker at 225 rpm. The culture was harvested by centrifugation, washed three times with PBS buffer and resuspended in 500 ml of MSB medium saturated with toluene vapor and incubated at 26°C on a rotary shaker at 225 rpm. 50 ml of the resuspended culture was taken at 0, 1, 3, 6, 9 and 24 hr intervals. The cells were collected by centrifugation at 1,935 x g. RNA was isolated using RNeasy columns. 5 µg of each RNA sample was dissolved in a solution of 50% deionized formamide, 7% formaldehyde, 1 x SSC (0.15M NaCl, 0.015M $Na_3C_6H_5O_7$) in a total volume of 40 µl, incubated at 68°C for 15 min and cooled on ice (36). 2 volumes of 20 x SSC (3M NaCl, 0.3 M $Na_3C_6H_5O_7$) was then added to each sample and the resulting solution was slot blotted to nylon membranes (36). The membranes were dried

at 25ºC, baked at 80°C for 2 h and hybridized with a PCR generated *todC1* probe.

RNA-DNA Hybridization. Hybridization was done according to the method of Church and Gilbert (12). Membranes were incubated in prehybridization solution (0.5 M Na_2HPO_4, 1mM EDTA, 7% SDS, pH 7.3) for 4 h at 65°C in a shaking water bath. ^{32}P-labeled probe was added to the prehybridization solution and incubated overnight at the same temperature. The membranes were washed four times with high stringency wash buffer (1.17 g, NaCl; 6.3 g, Tris-HCl; 0.74 g, EDTA; 10 g, SDS/2 litter, pH 7.3). Blots were placed in plastic bags, laid on x-ray film (Kodak Biomax MR, Eastman-Kodak, Rochester, NY) at -80°C with intensifying screens and developed after 18-48 hrs.

Quantitative assays for *tod* and *nah* mRNA induction. *P. putida* F1 cultures were grown overnight with shaking at 26°C; uninduced cells were grown in 50 ml of YEPG medium and induced cells were grown in 50 ml of MSB medium in 250 flasks with toluene vapor. Cells were harvested at mid-log phase (OD^{600}~0.6), RNA was isolated with RNeasy columns and slot-blotted to nylon membranes as described above. 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ng of *todC1* DNA was also blotted on the same membrane as standards. The membrane was hybridized with a *todC1* ^{32}P-labeled probe, washed, applied to film and quantitation of transcripts was accomplished using a photo-imager (Visage 110, Kodak). Similarly *P. putida* JS150 was grown in 50 ml of YEPSS medium, the total RNA was extracted and blotted to nylon membranes along with *nahA* DNA

standards. The blots were processed as described above and probed with a ^{32}P-labeled *nahA* probe.

Optimization of differential display using *tod* specific primers. Using toluene induced *P. putida* F1 RNA as a model system, several parameters were varied in parallel using a set of specific *tod* primers to allow optimization of the differential display reaction conditions: I) template concentration: 15, 1.5 , 0.15 and 0.015 ng. 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) dNTP concentrations: 200, 20, 2, and 0.2 μM. VI) primer lengths: 10 and 13 bp. The invariant parameters for all other components were maintained at the concentrations described in the next two paragraphs.

Complimentary DNA was synthesized by Moloney murine leukemia virus (MMLV) reverse transcriptase (RT)(BRL). A *todC1* antisense 10 bp or 13 bp primer was first used for initial optimization and, subsequently, arbitrary 10 bp primers were used. The final concentration of components was: dNTPs, 200 μM; dithiothreitol, 5 mM; MMLV enzyme, 50 U; total RNA, 200 ng; primer 0.4 μM; 1 X MMLV reaction buffer (BRL) in a total reaction volume of 20 μl. The RT reaction was carried out in a thermocycler (Perkin Elmer; Norwalk, CT; model 480) using the following program: ramp 50°C to 30°C for 15 min; 37°C for 1 h; 95°C for 5 min; followed by an incubation at 4°C.

The PCR step was performed with a thermal cycler (Perkin Elmer, models 480 and 2400,) incorporating ^{32}P (ICN, Costa Mesa, CA) or ^{33}P (Andotek, Irvine, CA) labeled nucleotides for visualization by autoradiography. The final

concentrations of components in the 27 µl reaction were: *Taq* polymerase (BRL), 0.3 U; dNTPs 20 µM; dimethylsulfoxide, 6%; primer, 2 µM; labeled nucleotide 0.25 µL; 10% triton, 0.1%; 1 X PCR reaction buffer (Perkin Elmer) in 27µl. Following addition of 3 µl of the RT reaction, the solution was cycled at 94°C for 30 s (denaturing), 40°C for 2 min (annealing), and 72°C for 1 min (extension)(for the model 480) or 94°C for 15 sec; 40°C for 30 s; and 72°C for 60 s (for the model 2400). 40 amplification cycles were used for both machines followed by a 10 min final extension at 72°C.

The PCR products were run on 340 µm x 61cm x 33 cm, 4.5% denaturing acrylamide gels (Genomyx, Foster City, CA) in side-by-side fashion. 4 µl of the RT-PCR reactions were loaded onto the sequencing gel along with 4 µl of denaturing loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) after boiling at a water bath for two min. The gel was run on a sequencing apparatus (LR, Genomyx) for 2 hours at 2,700V. 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 urea. The dried gel was then exposed to to X-ray film (Biomax MR, Kodak) at room temperature for 18-48 hrs.

Differential display from pure cultures using arbitrary primers

Toluene induction. After optimization with specific primers, an attempt was made to detect *tod* transcription using arbitrary primers alone or in conjunction with the SD14 primer. Primers 70.3, 80.2 and 80.5 were randomly selected and DD was performed using the same *P. putida* toluene induced RNA under the same conditions as used with the *tod* specific primers.

Salicylate induction

Salicylate induced and uninduced *P. putida* JS150 RNA was used for a series of reactions using arbitrary primers 60.1, 60.5, 60.3, 60.4, 60.8 and 80.7 separately for the RT reaction and primer SD14 in conjunction with the same arbitrary primer for PCR.

Uranyl acetate induction. Uranyl acetate induced *P. putida* G7 was used for DD using arbitrary primers 60.3 for the RT reaction and primer SD 14 in conjunction with the same arbitary primer for PCR.

Cadmium chloride induction. Cadmium chloride induced P. putida G7 was used for DD using arbitrary primers 70.3 for the RT reaction and primer SD14 in conjunction with the same arbitrary primer for PCR.

Differential display of uninoculated soil microcosm RNA.

Toluene induction. Toluene induced and uninduced RNA extracted from uninoculated soil microcosms was used for differential display using primer 70.3 for the RT reaction and primer SD14 in conjunction with primer 70.3 for PCR. The conditions used for both the RT and PCR steps were identical to those described above with the exception that the amount of microcosm total RNA used for the RT reaction was 100 ng.

Uranium nitrate induction. . Uranyl nitrate induced soil microcosm RNA was used for DD using arbitrary primers 60.3, 60.5 and 60.1 for the RT reaction and primer SD 14 in conjunction with the same arbitary primers for PCR.

Application of rifampicin to reduce false positives

Prior to harvesting, cells were treated with the transcriptional inhibitor, rifampicin to help distinguish between those messages related to induction and other artifacts (rRNAs, tRNAs etc.). 1 ml of rifampicin (at a stock concentration of 20 mg/ml) was added to the flask containing the induced cells to give a final concentration of 200µg/ml. Following the addition of rifampicin, 15 ml aliquots were taken at the following time intervals: 5, 10, 20, 30, 40, and 60 min. Cells were harvested by centrifugation at 8,000 RPM for 5 min at 4°C and immediately stored at -80°C until ready for use.

Elution of differential display bands and PCR reamplification. Differential cDNA bands were detected by side-by-side comparison of induced versus uninduced PCR products on the autoradiogram. Gel bands were localized by overlaying the dried gel with the film. Bands were first softened by the addition of 5 µL of distilled water and then excised with a sterile razor blade. 500 µL of TE buffer was added to the gel slice and heated at 68°C for at least 2 h. The excised fragments were re-amplified by using the same conditions as the first PCR run, except that the final concentration of dNTPs was changed to 200 µM. The re-amplification products were electrophoresed into 1% low melting agarose and products with the same molecular weight as the original DD bands were excised with a razor blade. TBE buffer (250 µl) was added to the gel slice and incubated at 65°C for 10 min. Agarase (4 µl) was then added to the solution and incubated at 42°C for 2 hrs after which the mix was then filtered using a Z-spin

column (Gelman, AnnArbor, MI). The solution was extracted with phenol/chloroform (1:1) and precipitated by the addition of ethanol.

Isolation of differential cDNAs via Single Strand Conformation Polymorphism (SSCP) Gels. Differential bands were inscribed and cut out using a razor blade and eluted in 5 µl of DEPC-treated water. The bands of interest were then pulled from the gel and placed in a RNase-free microfuge tube and eluted in 50 µl DEPC-treated water. This was followed by an incubation period for two hours at 68°C. Following incubation, 5 µl of eluent was reamplified in 25 µl PCR stock mixture using the same PCR conditions which generated the differential band. The reamplified products were electrophoresed on a 5% native acrylamide (49:1 acrylamide to N, N'-methylene-bis-acrylamide); 100 mM Tris-borate (pH 8.0), 2.5 mM EDTA (TBE) gel to separate any contaminating cDNAs from the cDNA representing the true mRNA transcript observed on the differential display gel. To differentiate from contaminating cDNAs, an adjacent lane from the control (in relation to the differentially expressed band) was run as a comparison. Bands unique to the control were then inscribed and eluted in 5 µl of DEPC-treated water. Samples were placed in a RNase-free tube and 50 µl DEPC-treated water was added. 5 µl of eluent was added to 25 µl of PCR stock and run again at the same reaction conditions which generated the initial differential band. Following PCR, 25 µl of the sample was resolved on a 1.0% low-melting agarose gel for purposes of cutting out the reamplified band of interest (if necessary). Reamplification products corresponding to the molecular weight of the differential bands on the acrylamide gel were cut out with a razor

blade and resuspended in 200 µl TE buffer (pH 8.0) and incubated for two hours at 68°C.

Cloning of differential display derived PCR products. Cloning was done using a TA cloning kit (Invitrogen, San Diego, CA) following the manufacturer's protocols. 1 µL of the purified PCR product was used for the transformation. The white colonies were transferred to another plate containing 100 mg/ml of ampicillin and 40 µL of X-gal (0.1 g/5ml). Plasmid DNA was prepared by either the boiling method (21) or alkali lysis method (36). Following isolation the crude DNA was resuspended in 20 µl of TE buffer, RNase treated at 37°C for 30 minutes, extracted twice with 1:1 phenol/chloroform, precipitated and washed with 70% ethanol. The presence of inserts was checked by electrophoresis after restriction with *EcoR*I. The inserted cDNA was then eluted to 1% low melting agarose, excised and used as a template for probing.

Verification of differential gene expression. Initially gel-purified re-amplification products were labeled by the random primer extension method using the manufacturer's protocol (Stratagene) and used to probe RNA slot blots from induced and uninduced samples. This initial screening process was later eliminated. Subsequently, re-amplification products were cloned into the pCRII vector (Invitrogen), *EcoR*I restricted plasmid DNA was labeled by the random primer method and used as a probe to hybridize with slot blots from induced and uninduced samples obtained from pure culture and inoculated microcosm derived RNA .

Isolation of the Bacterial Strain(s) responsible for Differential Expression in Soil Microcosms. In order to isolate the organism(s) responsible for differential expression, sample dilutions were initially taken from soil microcosms grown overnight at 30°C. 10 ml of soil slurry was added to a dilution tube followed by 1ml transfers into 9 ml of phosphate buffer saline (pH 7.4) to give an initial dilution range of 10^0 to 10^{-8}. 100µl from each dilution tube was then spread plated on quarter-strength YEPG plates in triplicate sets to give a final dilution range of 10^{-1} to 10^{-9}. Those plates that contained 50-100 colonies were kept for colony transfer.

Colony Hybridization Experiments. Colonies were transferred onto a circular piece of nitrocellulose membrane followed by bacterial lysis using a 0.5 N solution of NaOH. Membranes were then placed on a piece of Whatman paper and allowed to air dry for 30 min. Membranes were baked at 80°C for two hours followed by prehybridization for 6 hours at 65°C. Following the prehybridization step, $1x10^7$ cpm of the labeled probe was added and hybridized overnight at 65°C. Membranes were washed as described above, air-dried for 15 min, followed by exposure to X-ray film and developed accordingly. Those colonies which were positive were patch plated onto another YEPG plate. Colony hybridizations were performed again to confirm that the correct colony which yielded the positive signal had been isolated.

Sequencing and Data search. Sequencing was done by an automated unit (Applied Biosystems 373A) at our university's Molecular Biology Resource Facility, and the obtained sequences were compared with Genbank archives

using the BLAST algorithm (2). Prior to computer analysis the automated readout was visually inspected for deletions. Both the BLASTN and BLASTX functions were used.

RESULTS

Quantitation of *tod* and *nah* transcription. Toluene induced *tod* gene expression in *P. putida* F1 was induced after 3 h and reached maximal induction at 24 h (data not shown). *Tod* transcripts were undetectable in the uninduced sample. In a comparison with known amounts of *tod* DNA, the amount of *tod* transcripts was determined to be 0.04 ng or 7.8×10^7 transcripts /μg of total RNA (Fig. 1A). In a comparison with known amounts of *nahA* DNA, the amount of *nahA*-like transcripts was determined to be 0.05 ng or 9.7×10^7 transcripts /μg of total RNA (Fig. 1B).

Optimizing prokaryotic differential display using specific primers

Optimization conditions. For the electrophoretic gel protocol described using the Genomyx LR, the average number of amplification products represented by visible bands were > 70 per lane with lengths ranging from 100 bp to 2 Kbp. After initial optimization, electrophoresis of the DD-PCR products from *P. putida* F1 RNA was successful, resulting in a *todC1* fragment of the expected size only detected in induced samples. When a series of decreasing RNA template concentrations were used, the *todC1* fragment could still be detected when the total RNA was lowered to 0.015 ng with only a slight decrease in intensity (Fig. 2A). The lower template concentration also decreased the background. When a primer concentration of 0.2 μM was used, the optimal

magnesium concentration was between 1 to 1.5 mM (Fig. 2B). The effect of changing dNTP concentration was considerable (Fig 3A). Above 1 mM, the band number and intensity dramatically decreased. The optimal primer concentration was found to range from 0.2 μM to 2 μM. Above 20 μM, mis-priming was greatly increased. When the primer concentration was lower than 0.02 μM, band intensity also dramatically decreased (Fig. 3B). Only a slight change in band pattern was observed when the annealing temperature was increased from 30 to 50°C (Fig. 4A). The primer length also had a great influence on fingerprinting band patterns (Fig. 4B); more bands per lane were obtained by using longer primers.

Verification of differential expression. For the optimization experiment using *tod* specific primers, toluene induction of *P. putida* F1 derived clone 410a (Fig. 8A-1) was verified to be differentially expressed by RNA slot blots (Fig. 9A). After sequencing and a GenBank search, both clones were found to be 100% homologous to *todC1*.

Differential display of pure culture derived RNA using arbitrary primers

Toluene induction and verification. Compared to the RNA fingerprints generated using specific primers, more bands per lane were observed when single arbitrary primers were used (data not shown). From several differential bands detected on the DD gel (Fig. 5), one of the bands from the 70.3 lane was verified to be differentially expressed using the reamplified PCR product to probe RNA slot blots. Sequence analysis revealed both of these two differentially expressed PCR fragments to be overlapping but non-identical toluene

dioxygenase fragments 5' to the 380 bp fragment amplified using the *tod* specific primers.

Salicylate induction and verification. The RNA fingerprints from salicylate induced and uninduced *P. putida* 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. 6). Cloning and sequencing of the reamplified clone 60.3-380 revealed it to have 90% homology with a *Psuedomonas* reductase (*ntdAc*) gene and 81% homology with the naphthalene dioxygenase (*nahAc*) gene. Clone 60.3-380 was verified to be differentially expressed on the basis of RNA slot blots (Fig. 9B-1). Probing slot blots with clone 60.3-325 revealed it to be a ribosomal subunit and allowed it to serve as an indicator of equal slot blot loading (Fig. 9B-2). The sequence of clone 60.3-380 was submitted to GenBank and was given the accession number AF001828.

Uranyl acetate induction and verification. Because it is difficult at times to distinguish authentic hits from the differential display, the use of the bacterial transcriptional inhibitor, rifampicin, allowed us to further delineate between 'true' mRNAs responsible for induction and other artifacts (such as rRNAs and tRNAs) which are a commonplace to the differential display. Since rifampicin specifically binds to the ß subunit of the bacterial RNA polymerase preventing further transcription initiation, one should see on the differential display gel a gradual decrease of the message which had been induced in the presence of uranyl acetate. RNA fingerprints generated by differential display resulted in 3 putative differentially expressed bands(Fig. 10). Following reamplification by PCR, PCR

products were resolved on a 1% low-melting agarose gel to confirm the correct fragment had been isolated (data not shown). Each of the three cDNAs were cloned, sequenced, and compared to other sequences on the GenBank Blast algorithm. Results from sequence analysis revealed that two out of the three sequences had the potential to be novel. Clone 60.3-850 had the closest similarity (67%) to the *Escherichia coli* RNA polymerase alpha subunit. Clone 60.3-750 had the closest similarity (67%) to the *Psedomonas putida trp*A and *trp*B genes for tryptophan synthase. Clone 60.3-1000 showed the most homology of any of the 3 sequences presented to the sequence database. Over a 370 base sequence from clone 60.3-1000 matched almost perfectly (98%) to the *rpo*B gene of *Pseudomonas putida* which encodes for the RNA polymerase ß subunit (2, 26). Based on this result it was assumed that the sequence obtained for clone 60.3– 1000) was not novel. Verification of differential expression was done using total RNA obtained from control versus induced cultures and slot blotted onto a nitrocellulose membrane. Results from slot blot analysis revealed that each of the three bands obtained from the differential display gel were confirmed to be differentially expressed (Fig. 11). This further establishes the use of rifampicin to discriminate between false positives and authentic differentially expressed transcripts from the gel.

Cadmium chloride induction. The results for the cadmium induction experiment were similar to those obtained with uranyl acetate. One putative differentially expressed band of 500 bp was obtained using the 70.3 primer.

Differential display of toluene induced soil microcosm derived RNA

Inoculated microcosms. The RNA fingerprints from pure culture and inoculated soil microcosms were virtually identical (Fig. 7). However, the *todC1* band was fainter in the soil sample versus the pure culture sample (Fig. 7). Recovery rates of ^{32}P-labeled RNA were approximately 75% from this soil using the protocol as described.

Uninoculated microcsoms. At the 4 h period, when soil slurry samples were processed for total RNA, the heterotrophic cell count was determined to be 2.23 $\pm$ 0.15 x 10^6 cells/g for the microcosm without toluene and 2.07 $\pm$ 0.31 x 10^6 cells/g for the toluene induced microcosm. *TodC*1 positive populations for the uninduced microcosms were 5.17$\pm$ 1.0 x 10^5 cells/g and 4.00 $\pm$ 2.2 x10^5 cells/g for the toluene-induced microcosms. Mass determinations of soil derived total RNA made by hybridization of ^{32}P-labeled universal rRNA oligonucleotides agreed with determinations based on absorbance (260 nm) readings (data not shown). RNA extraction of uninoculated soil microcosms yielded 9.8 ng from toluene induced and 9.4 ng from uninduced soils. Because of the low RNA yield, a check of RNA integrety by visualization was not made. DD experiments with uninoculated microcosm derived RNA yielded several differential bands (Fig. 8). Upon cloning and sequencing these bands, clone 70.3-400 was found to be both unique and differentially expressed as determined by RNA slot blots (Fig. 9C-1). Probing slot blots with clone 70.3-325 revealed it to be a ribosomal subunit and allowed it to serve as an indicator of equal slot blot loading (Fig. 9C-2).

Differential display of uranium induced soil microcosms.

Isolation of differentially expressed PCR products

Further investigation into the effects of uranium was also studied in a more complex environment using an aquifer soil obtained from Columbus Air Force Base. RNA fingerprints from the differential display gel revealed a number of putative differentially expressed bands(Fig. 12). Rifampicin was also added to the soil, excised from the gel, reamplified, and resolved on a 1% low-melting agarose gel to verify that the bands had been isolated from the differential display gel (data not shown). However, no decaying band patterns indicative of rifampicin transcriptional inhibition were observed.

Verification of differential expression and sequence analysis. To verify differential expression, RNA slot blot analysis was accomplished using total RNA obtained from the control and uranium-induced soil microcosms. Of the four putative differentially expressed fragments isolated from the differential display, one band was confirmed to be differentially expressed (Fig. 14). The differential transcript corresponding to the original differential display gel was approximately 1200bp in length. Subsequently, clone 60.3-1200 was sequenced and compared to those sequences available on the Genbank Blast algorithm. Sequence analysis results showed no similarity to any of the sequences in the GenBank database. This was expected to some extent because the soil is highly uncharacterized.

Verification that differential expression is due to the uranyl ion. Control and uranyl nitrate induced microcosms were set up along with two additional

negative control microcosms: 200 µM calcium nitrate and 200 µM magnesium nitrate. Conditions for this experiment were analogous to those conditions which had generated the differential band (clone 60.3-1200). Results from RNA slot blot analysis revealed negative hybridization to the three controls and positive hybridization in the slot which contained uranyl nitrate induced total RNA (Fig. 14).

Isolation of gene (and organism) responsible for uranium-induced differential expression. Current investigations are ongoing to isolate the gene(s) involved in uranium induction. Colony hybridization experiments have been performed (data not shown) and proven to be successful. The colony isolate(s) showing positive hybridization (data not shown) will be used for southern hybridization experiments to further characterize the gene and organism responsible for uranium induction. If results prove to be fruitful, this would demonstrate for the first time that an organism can be successfully isolated using the differential display technique further adding to this techniques versatility.

Application of anchored oligo- dT primers to assess bacterial/eukaryotic activity in soil microcosms. Three different anchored oligo-dT primers were used, 12 nucleotides in length ($T_{11}N$), where N can be either an A, C or G. Three separate RT reactions were made for each of the anchored primers using the analogous conditions as stated above. The cDNAs which are generated would then represent a subpopulation of the poly(A) tailed mRNAs. In order to investigate this, total RNA from uranium induced and uninduced soil microcosm

experiments was used (see above). Results from the RNA fingerprinting gel revealed 3 potential differentially expressed bands in those lanes which corresponded to the anchored $T_{11}C$ primer (Fig. 15). An interesting facet while observing the RNA fingerprint was the degree of similarity with respect to banding patterns between each of the three anchored primers being used. Only one of the fragments has been successfully cloned to date. Results from sequence analysis showed the closest similarity to the strain *Bacillus subtilis*.

DISCUSSION

Optimization of Differential Display for Prokaryotic RNA. Until recently the method of choice for the detection of differentially expressed genes has been subtractive hybridization (28). However, this technique requires a large amount of RNA, is very tedious, and, generally, is not reproducible (28). Two groups developed an alternative approach referred to as either DD (24) or RAP-PCR (29) that is better suited for screening multiple samples with lower quantities of RNA (for reviews see 25, 48). While most attention has been paid to the application of differential display to eukaryotic systems, the technique may actually be better suited to prokaryotic RNA. On the basis of reassociation kinetics it is estimated that a typical mammalian cell has 360,000 mRNA molecules per cell with 20,000 to 30,000 different mRNAs species that range from 15 to 12,000 copies per species (1). In contrast, prokaryotes are estimated to have only 1,380 mRNA molecules per cell represented by 400 different mRNA species (32). Because the complexity of prokaryotic mRNAs is much less that

that of eukaryotic mRNAs, it should theoretically be easier to obtain rare transcripts from prokaryotes by DD.

The differential display technique involves several steps: 1) Isolation of intact RNA from organisms; 2) Reverse transcription of total RNA using either an arbitrary primer or oligo dT primer (for eukaryotic systems) to generate cDNA; 3) PCR amplification using an arbitrary primer or an arbitrary primer paired with an oligo dT primer (for eukaryotic systems) to amplify the cDNA; 4) Separation and detection of the differential PCR products on sequencing gels; 5) re-amplification and cloning of the isolated PCR products; 6) Verification of the differential expression of the isolated gene fragment by northern blots, RNA slots blots, ribonuclease protection assay or RT-PCR; 7) Comparison of sequence similarity with known gene sequence databases permits the possible function of the gene to be inferred. The isolated gene fragment can be further used as a probe to locate and isolate the entire gene.

Initial attempts using a previously published prokaryotic RAP-PCR protocol (49) yielded only rRNA fragments from putative differentially displayed bands (data not shown). The half-lives of several catabolic mRNAs including *nah, tod* and *phe* to be greater than 10 min (unpublished data), our difficulties could not be attributed to physiological degradation of messages alone. Therefore, parameters such as annealing temperature, primer size and concentration, magnesium and nucleotide concentration, and RNA template concentration were empirically examined to optimize the procedure for application to RNA derived from both prokaryotic pure cultures and soil communities.

Once parameters were optimized using specific *tod* primers, the procedure was modified for application to unknown sequences by using an arbitrary primer for the RT step and a primer to the Shine-Dalgarno (SD) region in conjunction with the same arbitrary primer for the PCR step. In contrast to a recent report describing the design of 3' and 5' DD primers for a particular bacterial family (15), the use of a SD primer in this report is intended to prime the 5' region of a wider range of prokaryotes. An ideal SD primer would be advantageous in that it would allow preferential amplification at the 5' end of all cistrons within a polycistronic prokaryotic message. The SD region is characterized by a purine-rich region 5 to 6 bases from the start site (32). The primer used in this study includes the an ATG start codon at the 3' end with additional 5' bases based on a comparison of *Pseudomonas* and *E. coli* SD regions. Because of substitutions, albeit conservative, a simple primer based entirely on conserved sequences is infeasible. A degenerate SD primer incorporating equal molar amounts of G and A or inclusion of the altered 'P' base (6H,8H-3,4-dihydro-pyrimido[4,5-c][1,2]oxazin-7-one,8-[(5'-dimethoxytrityl-§-D-deoxyribofuranosyl),3'-[(2-cyanoethyl)-(N,N-diisopropyl)] may result in a primer that would anneal to a greater number of SD regions in a greater number of organisms. The choice of a 5' SD primer, while generally biasing amplification toward mRNA sequences in a pool of prokaryotic RNA sequences may, however, ignore those messages that do not have SD regions. The use of two arbitrary primers for prokaryotic DD may thus be potentially advantageous.

Even when specific primers were used to specifically amplify the *todC1* gene approximately 70 other distinct bands, presumably 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 is due to mismatch amplification. Theoretically, the annealing temperature will largely influence the differential display result; the higher the annealing temperature, the fewer the number of mispriming events. However, the current data shows that the annealing temperature did not have much influence on the total number of bands generated or the banding patterns. When the annealing temperature was increased from 30 to 50°C, only a slight band pattern change was observed (Fig. 4A). This may be explained by the fact that the PCR enhancers Triton-X 100 and DMSO were added to the PCR reaction mixture, which reportedly increases PCR efficiency and specificity (34). Alternatively it may be attributed to the template or the particular primer used (30).

The primer length also had a great influence on fingerprinting band patterns. Compared to fingerprints generated with 10 bp primers, more bands per lane were obtained by using 13 bp primers (Fig. 4B). This is most probably due to increased mis-priming with the longer primer. Based on experience, after the initial optimization work, the best results were obtained using an arbitrary 10 bp primer for the RT step and the SD14 primer in conjunction with the same arbitrary primer for PCR.

Primer concentration also has great influence on the banding pattern. The best primer concentration was determined to be in the range of 0.2 to 2 μM. Above 20 μM, the non-specific priming increases, therefore, increasing the background (Fig. 3B). Too low a primer concentration decreases the band intensity and complexity. Nucleotide concentrations also play a very important role in the DD technique; above 200 μM, the incorporation rate of the radiolabeled dNTP is very low and the differential band intensity is also lowered. Lower than 2 μM, all bands are not amplified most probably due to depletion of dNTPs (Fig. 3A, lane 5).

Detection of rare vs abundant messages. There is some debate as to the sensitivity of differential display to rare mRNAs and, to date, no data has been published related to prokaryotic RNA. Using a model eukaryotic system Bertioli *et al.* have shown that the standard differential display protocol as described by Liang and Pardee (24) shows a strong bias towards high copy number mRNAs (6). Messenger RNA present at less than 1.2% of total mRNA, the mean percentage of the most prominent class of mammalian RNA, was not detected by this technique. 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 have demonstrated differential display sensitivities of down to 0.0005% of the total mRNA (46). In this current work it is demonstrated that prokaryotic catabolic mRNAs as represented here by the *tod* and *nah* messages are present at 0.08% and 0.09%, respectively, of the total mRNA and are thus might be considered an

intermediate level mRNA. The limit of detection for differential display using a specific tod 13 bp primer was 0.015 ng of total RNA which corresponds to 4 X 10^3 transcripts.

Message sequence vs abundance. Another theoretical issue is whether the differential transcripts amplified DD are sequence dependent or abundance dependent. The number of PCR products generated under DD PCR conditions are far more than regular PCR because mis-matches, especially in the 5' end of the primer, are tolerated due to short primer length and low annealing temperature. This is complicated by the low dNTP concentrations that are used in order to increase the incorporation of radiolabled NTPs. Bertioli *et al.* suggest that abundant mRNAs out-compete the rare RNA for annealing and that even though amplification efficiency is proportional to the primer-template match, abundant RNAs may be detected on the DD gel, while rare perfectly matched mRNA may not be detected (6). Contrary to this position, Wan *et al.* in a comparison of subtractive hybridization and differential display, found that the latter method was not able to detect abundant messages detected by subtractive hybridization (46). On the basis of these findings, they concluded that DD is sequence dependent.

For DD to be of practical value for the screening of environmental samples a limited set of primers would ideally allow detection of the prominent differentially expressed transcripts. The logic of the approach developed was that, once DD conditions were optimized using specific primers to abundant messages, those same conditions would permit detection of the same messages using arbitrary

primers alone. This was demonstrated first by detection of the *tod* message using arbitrary primers. As proof of concept, arbitrary primers were then used to detect salicylate induction in *P. putida* JS150. By obtaining both the *nah* and *tod* transcripts with a small set of arbitrary primers, the current findings support Bertioli's position. These findings are of significance because they suggest that only a few primers are needed to display all the abundant mRNAs. It is conceivable, for other microbes which use organic compounds as a energy sources, that their catabolic mRNAs should also abundantly expressed. Therefore, the differential display procedure may be well suited for the detection of microbial genes responsible for the degradation of the organic compounds.

False positives. Of the several problems encountered with the DD procedure, the occurrence of false positives is the most vexing (9,25,48). A significant percentage of clearly differentially expressed PCR products excised from gels showed no differential induction when used as probes to hybridize RNA slot blots. During the first optimization experiment, 7 differential bands were eluted and used as probes to screen induced and uninduced RNA slot blots. Initially three differential bands were verified as differentially expressed. However, after cloning, only one (clone 410) showed differential expression; the other two showed no difference between induced and uninduced samples. Additionally, another toluene induced clone isolated with arbitrary primer 70.3 showed no differential expression on slot blots but was proved to be the *tod* gene after sequencing. Two explanations for false positives are possible: 1) Slot blots are not sensitive enough to detect a low abundance mRNA message. 2)

Because a single DD band may contain multiple cDNA species, a clone obtained from such a band may not be derived from the visually different species, representing instead a species of a similar molecular weight. As demonstrated with discovery and cloning of the *todC2* fragment; dependence on pre-screening as a necessary and sufficient condition for differential expression may result in the omission of differentially expressed clones. Even with the use of more sensitive RNA detection methods such as RT-PCR or ribonuclease protection assay, false positives cannot be completely eliminated.

In an effort to further reduce the number of false positives an attempt was made to discriminate between amplification of mRNAs and rRNAs by the addition of the prokaryotic transcriptional inhibitor. In this procedure the prokaryotic transcriptional inhibitor rifampicin was added to cultures and microcosms prior to total RNA isolation. At defined time intervals samples were taken, RNA was prepared and DD was conducted. With rifampicin treated cells a mRNA derived PCR product is visible as a band that progressively decays over time. (Fig). This approach is now generally used for all our DD studies because it significantly reduces the number of false positives.

Poly T primers. As originally devised the differential display procedure uses poly T primers that specifically allow ampification of eukaryotic mRNAs by annealling to the 3' poly A region of the transcript. Since uranium uptake experiments have also been reported with the organism *Saccharomyces cerevisiae* (43,45) it was our hypothesis that some type of regulatory response could also be obtained when a eukaryotic cell population, if any, was exposed to

the uranyl nitrate compound.. Preliminary sequencing results from an isolated clone indicated a stretch of 20 adenosine(A) nucleotides. It was surprising at first that we actually picked up a bacterial strain. However, the literature has suggested and confirmed that poly(A) tails are also found in bacterial strains (5, 33). Interestingly the two primary organisms studied with respect to polyadenalation have been *Escherichia coli* and *Bacillus subtilis*. It is noteworthy that the role of polyadenylation in bacteria is not well understood and the amount of polyadenylation that occurs between organisms does vary (5). However, Sarker et al. have done research which supports the idea that polyadenylation plays a major role in bacterial RNA metabolism (10). The poly(A) clone isolated will be used to confirm differential expression via RNA slot blot analysis. If differential expression is verified further characterization of the gene involved in uranium induction will be taken.

Differential display of soil microcosm RNA.

Toluene induction. Results from the inoculated soil RNA extraction experiment, demonstrated the feasibility of using DD to study gene expression in soil systems (Fig.7). The RNA extraction procedure yielded samples that were visually clean with 260/280 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 culture. However, based on the intensity of the tod fragment on DD gels (Fig.7), the efficiency of amplification from soil derived RNA appears to be less than would be predicted on the basis of the

efficiency of RNA extraction. This may be due to PCR inhibitors remaining in the soil-derived sample. An alternative explanation may be that the tod message was partially degraded during the soil isolation procedure.

The RNA fingerprints obtained from the uninoculated soil microcosms were as reproducible compared to the gels obtained from inoculated soils (Fig 8). A significant problem that arose with the uninoculated microcosms was the number of false positives; though 12 putative differentially expressed clones were screened for differential expression, only one was confirmed to be differentially expressed by RNA slot blots. The use of lower RNA concentrations (100 ng) for the RT compared to the preferable higher concentration (200 ng) may partially account for this problem. Alternatively, as stated above in reference to the inoculated microcosm experiment, soil-derived contaminants may result less efficient amplification of templates. In addition, because of the temperature and length of time of storage, the soils used for microcosm studies were relatively inactive which may, in turn, explain the low yield. This problem may be obviated both by increasing the scale of the isolation procedure and using fresh soil for microcosm experiments. Another solution would be to increase the population number by culturing the cells for a longer period. For these experiments the induction period was kept to a minimum in an attempt to discern true induction effects from changes in population due to growth in those members able to utilize toluene as a carbon source. Based on the enumeration data there was no significant difference between the uninduced or toluene induced microcoms on the basis of either heterotrophic or *todC*1 populations.

Uranium induction . Because of rifampicin's inability to work effectively in the soil microcosm experiments a couple of dilemmas had been presented. First, since arbitrary primers were being used, the ability of RT-PCR to also identify other RNA species (rRNAs, tRNAs..) existed even more so than in pure culture experiments. Thus, we had no other alternative but to arbitrarily choose those sequences which seemed to be differentially expressed. Furthermore, since there is a competition for the arbitrary primers being used by other sequences within the mRNA pool it would be difficult to distinguish between the correct differential cDNA product from other cDNA products of similar size on the differential display gel (11,13,39). In order to alleviate this problem of other contaminating cDNA sequences, single-strand conformation polymorphism (SSCP) analysis was accomplished. The idea of this technique relies on the basis that single-stranded cDNAs of similar size but of different sequence have a different electrophoretic mobilty in a non-denaturing gel (13). Due to this nucleotide variation between sequences one should be able to observe a separation of cDNAs on the gel. On this premise, reamplification products from both the control and induced lanes from the differential display were first denatured to separate the double-stranded cDNA followed by loading onto a native acrylamide gel. Observations from the SSCP gel revealed adequate separation and sequences unique to the control lane(s) ( ).Bands were excised from the gel and reamplified for cloning, confirmation of differential expression, and sequence analysis.

Because recent literature suggests that uranium accumulation is not dependent on any type of metabolic process i.e. transport pathway (27,35), the question was raised on the basis that the differentially expressed band isolated was due to the nitrate group and not the uranyl ion. Thus, it was imperative to devise an experiment which would either prove or disprove this claim. Magnesium nitrate and calcium nitrate were chosen as substitutes for uranyl nitrate because each had a comparable molecular weight to uranyl nitrate. The results (Fig. 14) would suggest two things: (1) induction is due to the presence of uranium in the medium and (2) some type of regulatory response exists when the cell population is exposed to uranium.

Sequence Analysis

The sequences obtained from the uninoculated microcosms bore little similarity to archived GenBank sequences. This may not be an anamoly considering that the vast majority of organisms in the environment are uncharacterized (3), Attribution of function to these sequences will require isolation of the full length gene from a soil derived DNA library followed by functional studies in transgenic hosts.

Conclusions

While we have successfully demonstrated the detection and acquisition of differentially expressed genes from soil microcosms, several present limitations of this approach must be overcome for the procedure to see widespread use. The difficulty is not in the DD process itself, but the subsequent steps by which putative differentially expressed gene fragments are verified as actually being

uncharacterized (3), Attribution of function to these sequences will require isolation of the full length gene from a soil derived DNA library followed by functional studies in transgenic hosts.

Conclusions

While we have successfully demonstrated the detection and acquisition of differentially expressed genes from soil microcosms, several present limitations of this approach must be overcome for the procedure to see widespread use. The difficulty is not in the DD process itself, but the subsequent steps by which putative differentially expressed gene fragments are verified as actually being differentially expressed. This verification process must be streamlined to permit screening of multiple samples in parallel. This is probably achievable with presently available robotic manipulators but would require a large capital investment. Until this has been achieved the method is too labor intensive for non-research applications. Despite the shortcomings of DD, it potentially offers a powerful approach to study gene expression in the environment without the prior culturing cells or sequence bias.

Acknowledgements

This work was supported by grant

AF F49620-94-1-0222 from the

Air Force Office of Scientific Research

References

1. **Alberts, B., D. Bray, Lewis, J., R. Martin, K. Roberts, and J.D. Watson.** 1994. Molecular Biology of the Cell, 3rd ed. Garland publishing, New York. p. 369.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **15**:403-10.
3. **Amann, R.I., W. Ludwig, and K.-H. Schleifer**. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial Rev. **59:**143-169.
4. **Bauer, D., H. Muller, J. Reich, H. Riedil, V. AhrenKiel, P. Warthoe, and M. Strauss**. 1993. Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucleic Acids Res. **21:**4272-4280.
5. **Belasco, J.G.** 1993. Experimental approaches to the study of mRNA decay.p. 475-493. *In* J.G. Belasco and G. Brawerman (eds.),Control of messenger RNA stability, Academic Press, San Diego.
6. **Bertioli, D. J., U. H. A., Schlichter, M. J., Adams, P. R., Burrows, H. H. Steinbib, and J. F. Antoniw**. 1995. An analysis of differential display shows a strong bias towards high copy number mRNAs. Nucleic Acid. Res. **23:** 4520-4523.
7. **Bogan, B.W., B. Schoenike, R. T. Lamar, and D. Cullen.** 1996. Manganese Peroxidase mRNA and enzyme activity levels during bioremediation of

polycyclic aromatic hydrocarbon contaminated soil with *Panerochaete chrysosporium.* Appl. Environ. Microbiol. **62**:2381-2386.

8. **Borodin, A.M., A.V. Danilkovich, R.L. Allikmets, V.M. and I.P. Chernov**. 1988. Nucleotide sequence of rpoB gene encoding the RNA-polymerase beta subunit in *Pseudomonas putida.* Dokl. Akad. Nauk. SSSR. **302**:1262-1265.
9. **Callard, D., B. Lescure, and L., Mazzolini**. 1994. A method for the elimination of false positives generated by the mRNA differential display technique. BioTechniques **16:**1096-1103.
10. **Cao, G., N. Sarkar**. 1992. Identification if the gene for an Escherichia coli poly(A) polymerase. Proc. Natl. Acad. Sci. USA. **89**:10380-10384.
11. **Chunming, L., and K.G. Raghothama**. 1995. Practical method for cloning cDNAs generated in an mRNA differential display. BioTechniques. **20**:577-579.
12. **Church G.M., and W. Gilbert.** 1984. Genomic Sequencing. Proc. Natl. Acad. Sci. USA. **81:**1991-1995.
13. **Daude-Mathieu, F., R. Cheng, J. Welsh, and M. McClelland.** 1996. Screening of differentially amplified cDNA products from arbitrarily primed PCR fingerprints using singl strand conformation polmorphism (SSCP) gels. Nucleic Acids Research. **24:**1504-1507.
14. **Feinberg, A.P., and B. Vogelstein**. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, addendum. Anal. Biochem. **137:**266-267.

15. **Fislage, R., M. Berceanu, Y. Humboldt, M. Wendt, and H. Oberender.** 1997. Primer design for a prokaryotic differential display RT-PCR Nucleic Acids Res. **25**:1830-1835.

16. **Fleming, J. T., J. Sanseverino, and G. S. Sayler**. 1993. Quantitative Relationship between naphthalene catabolic gene frequency and expression in prediction PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. **27:**1068-1074.

17. **Fleming, J. T., and G. S. Sayler.** 1995. Assessment of Gene Expression in the Environment: Quantitiative mRNA Analysis in Contaminated Soils, p. 45-58. *In* R. A. Minear, A.M. Ford, L.L. Needham, N.J. Karch (eds.), Applications of Molecular Biology in Environmental Chemistry. Lewis Publishers, Boca Raton.

18. **Gibson, D.T., J.R. Koch, and R. E. Kallio**. 1968. Oxidative degradation of aromatic hydrocarbons by microorganisms I. Enzymatic formation of catechol from benzene. Biochemistry **7**:2653-2662.

19. **Haag, E. and R. Vennu**. 1994. Effects of primer choice and source of Taq DNA polymerase on the banding patterns of differential display RT-PCR. BioTechniques. **17:**226-228.

20. **Haigler, B.E., C.A. Pettigrew and J.C. Spain**. 1992. Biodegradation of Mixtures of substituted benzenes by Pseudomonas sp. Stain JS150. Appl. Environ. Microbiol. **58**:2237-2244.

21. **Holmes, D. S. and M. Quigley**. 1981. A Rapid boiling method for the preparation of Bacterial plasmids. Anal. Biochem. **114:**193-197.

22. **Iimura, Y. and Tatsumi, K.** 1997. Isolation of mRNAs induced by a hazardous chemical in white-rot fungus, Coriolus veriscolor, by differential display. FEBS-Lett. **412**:370-4.

23. **Liang, P., L. Averboukh, and A. B. Pardee**. 1993. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. 1993. Nucleic Acids Res. **21:**3269-3275.

24. **Liang, P., and A. B. Pardee**. 1992. Differential display of eucaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967-971.

25. **Liang, P. and A. B. Pardee**. 1995. Recent advances in differential display. Current Opin. Immunol. **7**:174-280.

26. **Lisitsyn, N.A., G.S. Monastyrskaya, and E.D. Sverdlov**. 1988. Genes coding for RNA polymerase beta-subunit in bacteria. Eur. J. Biochem. **177**: 363-396.

27. **Marques, A.M., X. Roca, M.D. Simon-Pujol, M.C. Fuste, and F. Congregado**. 1991. Uranium accumulation by Pseudomonas sp. EPS-5028. Appl. Microbiol. Biotechnol. **35**:406-410.

28. **Maser, R. L., and J. P. Calvet**. 1995. Analysis of differential gene expression in the kidney by differential cDNA screening, subtractive cloning, and mRNA differential display. Seminars in Nephrology. **15:**29-42.

29. **McClelland, M. and J. W. Welsh**. 1994. RNA fingerprinting by arbitrarily primed PCR. PCR Methods Appl. **4:**S66-S81.

30. **McClelland, M., A. Hartwig, R. Cheng, and J. Welsh**. 1994. Arbitrarily primed PCR fingerprints resolved on SSCP gels. Nucleic Acids Research. **22:** 1770-1771.

31. **McClelland, M., D. Ralph, R. Cheng and J. Welsh**. 1994. Interactions among regulators of RNA abundance characterized using RNA fingerprinting by arbitrarily primed PCR. Nucleic Acid. Res. **22:**4419-4431.

32. **Neidhardt, C. F., J. C. Ingraham and M. Schaechter.** 1990. Physiology of the bacterial cell, a molecular approach, p. 1-29. Sinauer Associates, Inc, Sunderland, MA.

33. **Nierlich, D.P., anx G.J. Murakawa**. 1996. The decay of bacterial messenger RNA. Prog. Nucleic Acids Res. Mol. Biol. **52**:153-215.

34. **Newton, C.R. and A. Graham**. 1994. PCR, introduction to biotechniques, p. 19-20. BIOS Scientific Publishers Limited, Oxford, UK.

35. **Pons, M.P. and M.C. Fuste**. 1993. Uranium uptake by immobilized cells of *Pseudomonas* strain EPS 5028. Appl. Microbiol. Biotechnol. **39**:661-665.

36. **Sambrook, J., E. F. Fritsch, T. Maniatis**. 1989. Molecular cloning, a laboratory manual 2nd ed., p. 7.43-7.45. Cold Spring Harbor Press, New York.

37. **Sayler, G.S., J. Fleming, B. Applegate, C. Werner, and K. Nikbakht**. 1989. Microbial Community Analysis Using Environmental Nucleic Acid Extracts, p.658-660. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Morita, and A. Uchida, (eds.), Recent Advances in Microbial Ecology. Japan Scientific Societies Press, Tokyo.

38. **Selvaratnam, S., B.A. Schoedel, B.L. McFarland and C.F. Kulpa** 1995. Application of reverse transcriptase PCR for the monitoring expression of the catabolic dmpN gene in a phenol-degrading sequencing batch reactor. Appl. Environ. Microbiol. **61**:3981-3985.

39. **Shanchuan, Z., S. L. Ooi, F.C. Yang and A. Pardee.** 1996. Three methods for identification of true positive cloned cDNA fragments in differential display. BioTechniques. **20**: 400-403.

40. **Simon, M.J., T.D. Osslund, R. Saunders, B.D. Ensley, S. Suggs, A. Harcourt, W. Suen, D.L. Cruden, D.T. Gibson and G.J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene **127**:31-37.

41. **Stapleton, R.D, S. Ripp. L. Jimenez, S.C-Koh, J.T. Flemingand G.S. Sayler.** Nucleic acid analytical approaches in bioremediation: Site assessment and characterization J. Microbial Meth., in press.

42. **Stapleton, R.D and G.S. Sayler.** Molecular site assessment of natural attenuation potential for petroleum hydrocarbons. (submitted for publication: Microb. Ecol.)

43. **Strandberg, G.W., S.E. Shumate II., and J. Parrott Jr. 1982.** Microbial cells as biosorbents for heavy metals: Accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. **41:** 237-245.

44. **Syed, V., W. Gu, and N.B. Hecht.** 1997. Sertoli cells in culture and mRNA differential display provide a sensitive early warning assay system to detect changes induced by xenobiotics. J. Androl. **18**:264-273.

45. **Volesky, B., and H.A. May-Phillips**. 1995. Biosorption of heavy metals by *Saccharomyces cerevisiae.* Appl Microbiol Biotechnol. **42:** 797-806.

46. **Wan, J.S., S.J. Sharp, G.M.C. Poirier, P.C. Wagaman, J. Chambers, J. Pyati, Y.L. Hom, J.E. Galindo, A. Huvar, P.A. Peterson, M.R. Jackson, M.G. Erlander.** 1996. Cloning differentially expressed mRNAs. Nature Biotech. **14:**1685-1691.

47. **Welsh, J., K. Chada, S. S. Dalal, R. C. D. Ralph and M. McClelland**. 1992. Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res. **20:**4965-4970.

48. **Welsh, J., D. Ralph, and M. McClelland**. 1994. DNA and RNA fingerprinting using arbitrarily primed PCR. *In* J.J. Sninski, M.A.Innis, D.H. Gelfand, T.J. White, eds., PCR Protocols, 2nd ed., Academic Press, San Diego.

49. **Wong, K. K. and M. McClelland.** 1994. Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA. Proc. Natl. Acad. Sci. **91:**639-643.

50. **Zhao, S., S. L., Ooi, F. C. Yang and A. B., Pardee**. 1996. Three methods for identification of true positive cloned cDNA fragments in differential display. BioTechniques. **20:**400-403.

51. **Zylstra, G. J., R. W. McCombie, D. T. Gibson, and B.A. Finette**. 1988. Toluene degradation by Pseudomonas putida F1: genetic organization of the tod operon. Appl. Environ. Microbiol. **54:**1498-1503.

52. **Zylstra, G. J. and D. T. Gibson.** 1989. Toluene degradation by Pseudomonas putida F1. J. Biol. Chem. **264:**14940-14946.

A

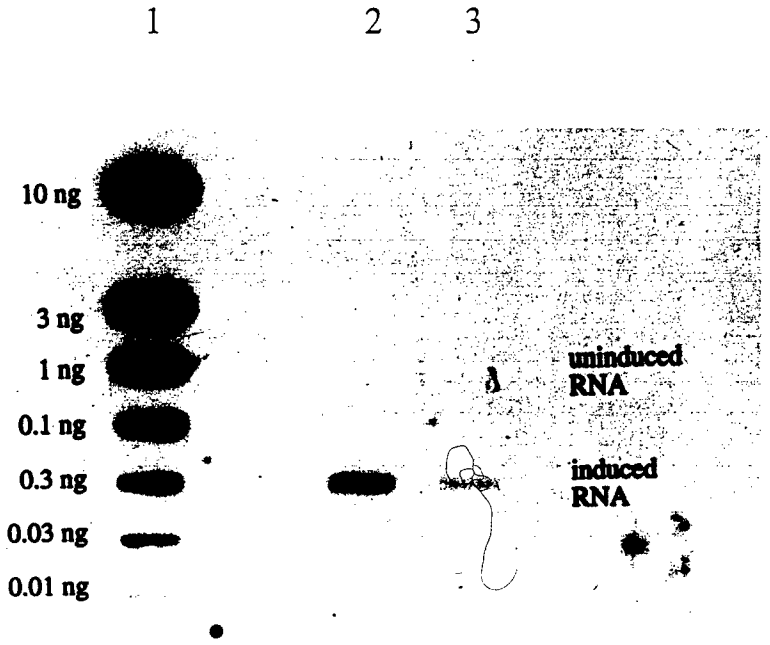
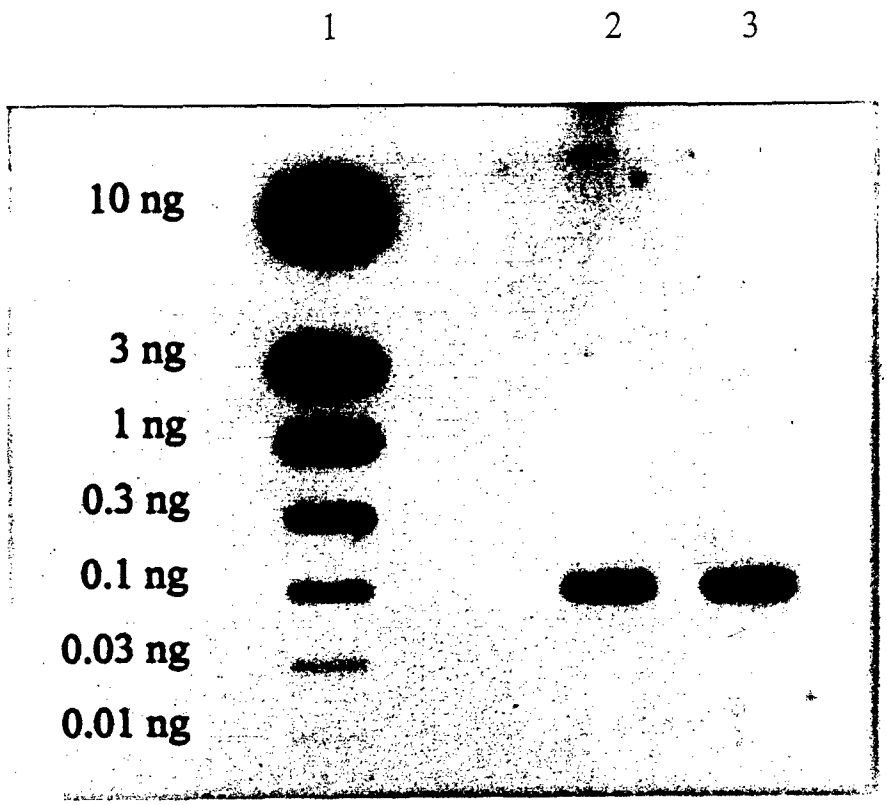


Figure 1. Quantitation of induced transcipts from pure cultures. (A) *P. putida* F1 cells grown in the presence or absence of toluene. Column 1; 10, 3, 1, 0.1, 0.03, 0.01 ng of *tod* DNA used as standards. Columns 2, 3; 10 μg of total RNA from induced total RNA from *P. putida* F1 was applied to the membrane in duplicate. A ^{32}P- labeled *todC1* probe was used to hybridize with the blotted RNA. (B) *P. putida* JS150 cells grown in the presence or absence of salicylate. Column 1; 10, 3, 1, 0.1, 0.03, 0.01 ng of *nah* DNA used as standards. Columns 2, 3; 10 μg and 1μg, respectively, of total RNA from induced total RNA from *P. putida* JS 150 was applied to the membrane and probed with a ^{32}P-labeled *nahA* fragment.

B


1
2
3
10 ng
3 ng
1 ng
0.3 ng
0.1 ng
0.03 ng
0.01 ng
uninduced RNA
induced RNA

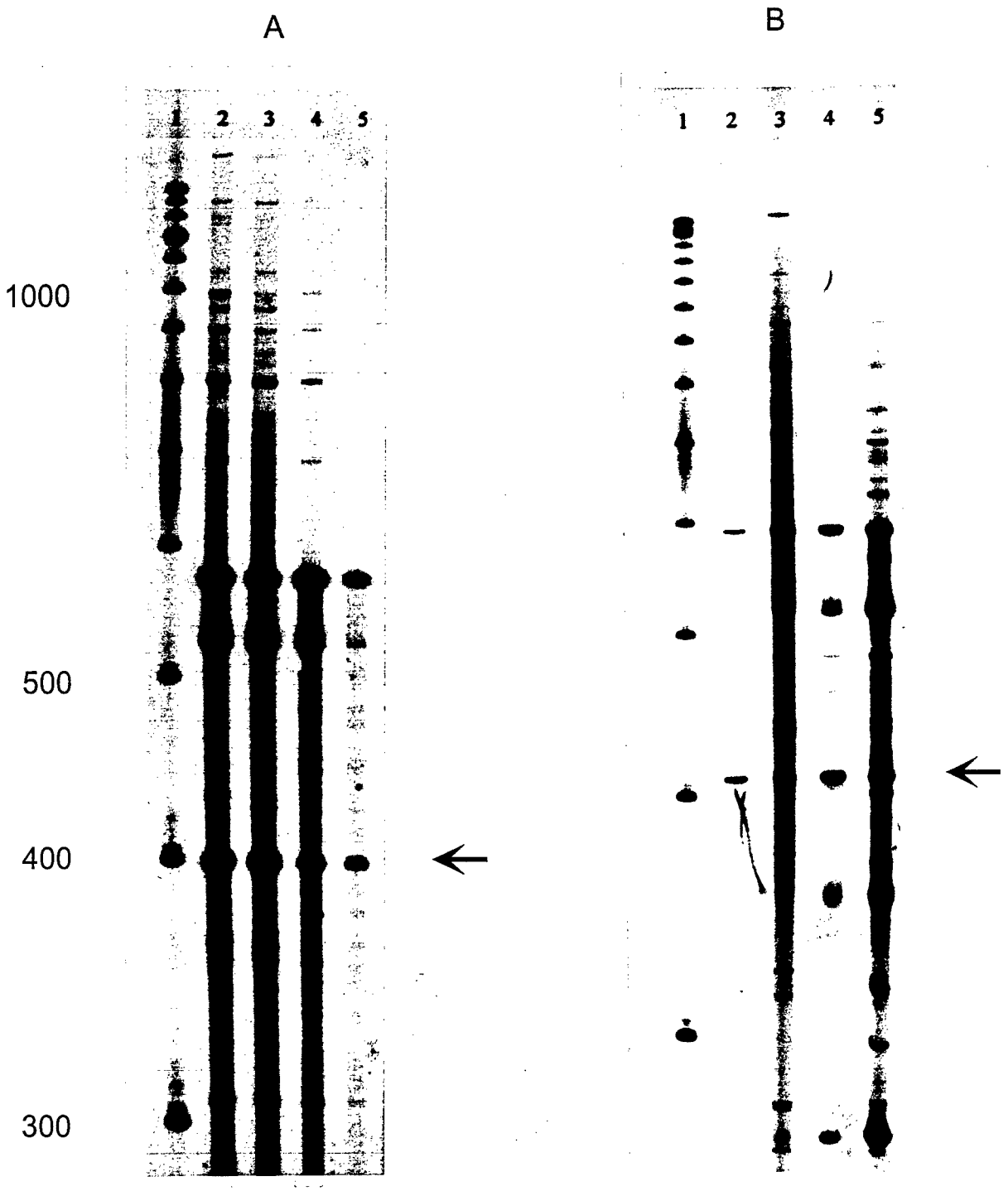


Figure 2. Optimization of RNA and $MgCl_2$ concentrations for differential display reactions. (A) Differing amounts of toluene induced RNA were used for differential display. Lane 1, 100 bp ladder; lane 2, 15 ng; lane 3, 1.5 ng; lane 4, 0.015 ng. (B) Effect of differing $MgCl_2$ concentrations on the complexity of banding pattern. Lane 1; 100 bp ladder; lane 2, 8 mM; lane 3, 4 mM; lane 4, 2 mM; lane 5, 1 mM. The *todC1* fragment is indicated by an arrow.

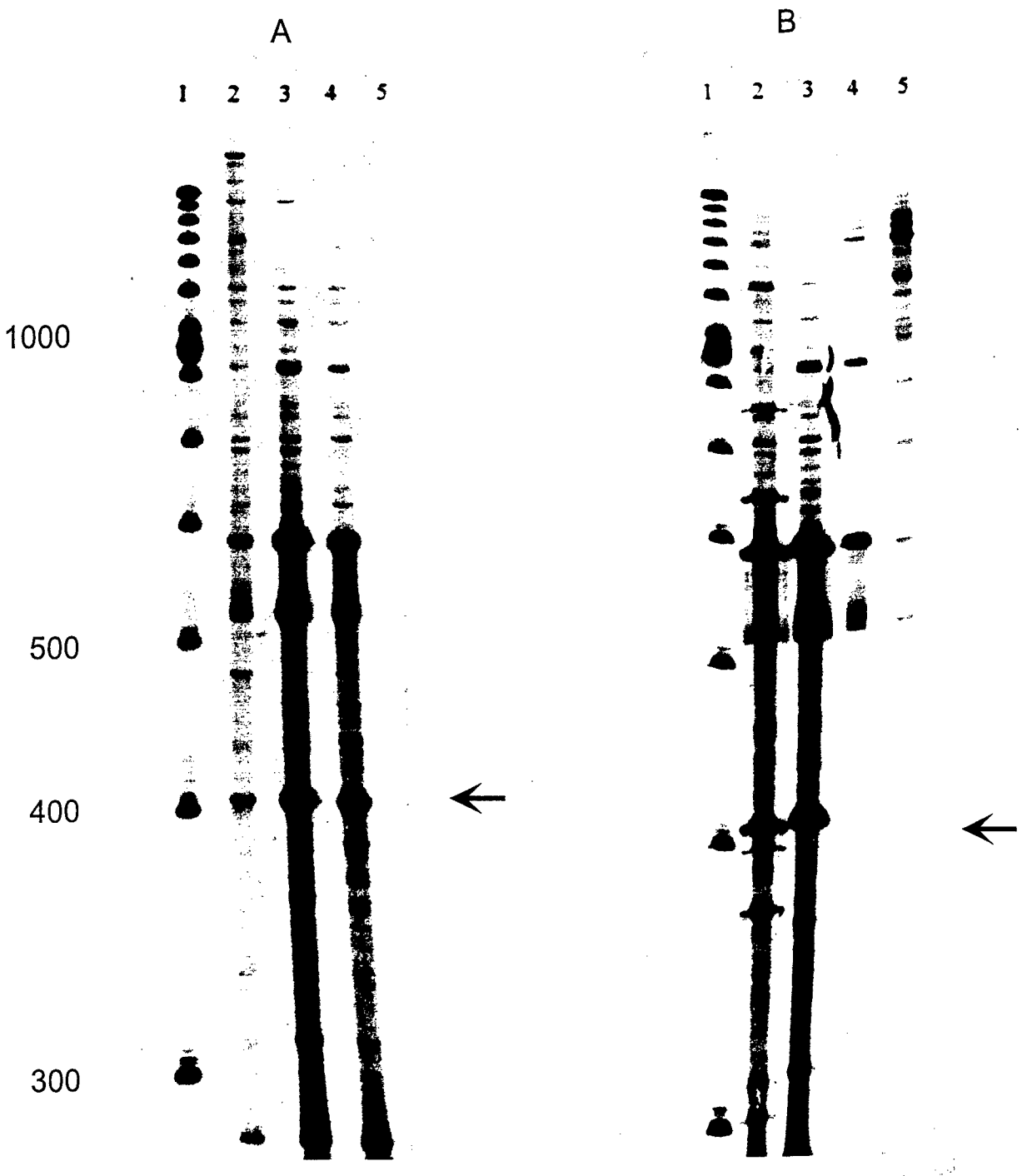


Figure 3. Optimization of nucleotide and primer concentration for differential display reactions. (A) Effect of varying nucleotide concentration on the complexity of banding patterns. Lane 1, 100 bp ladder; lane 2, 200 μM; lane 3, 20 μM, lane 4, 2 μM; lane 5, 0.2 μM. (B) Effect of varying primer concentration on the complexity of banding patterns. Lane 1, 100 bp ladder; lane 2, 2μM; lane 3, 0.2 μM; lane 4, 0.02 μM, lane 5, 0.002 μM. The *todC1* fragment is indicated by an arrow.

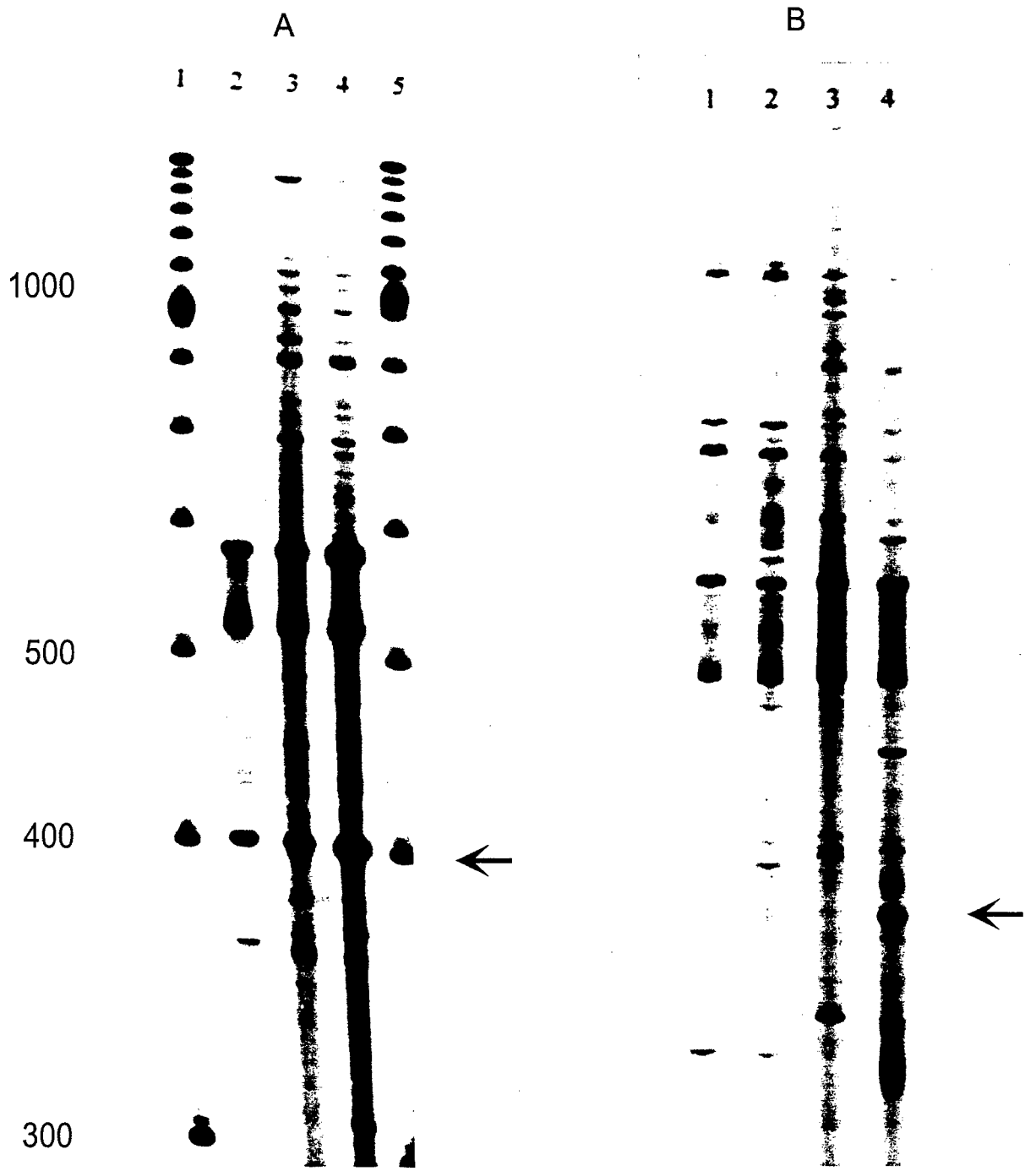


Figure 4. Optimization of annealing temperature and primer length for differential display reactions. (A) Effect of varying annealing temperature on complexity of banding patterns. Lane 1, 100 bp ladder; lane 2, 30°C; lane 3, 40°C; lane 4, 50°C. (B) Effect of varying primer length between 10 and 13 bp on complexity of banding pattern. Lanes 1, 2, 10 bp; lane 3, 4, 13 bp. The *todC1* fragment is indicated by an arrow.

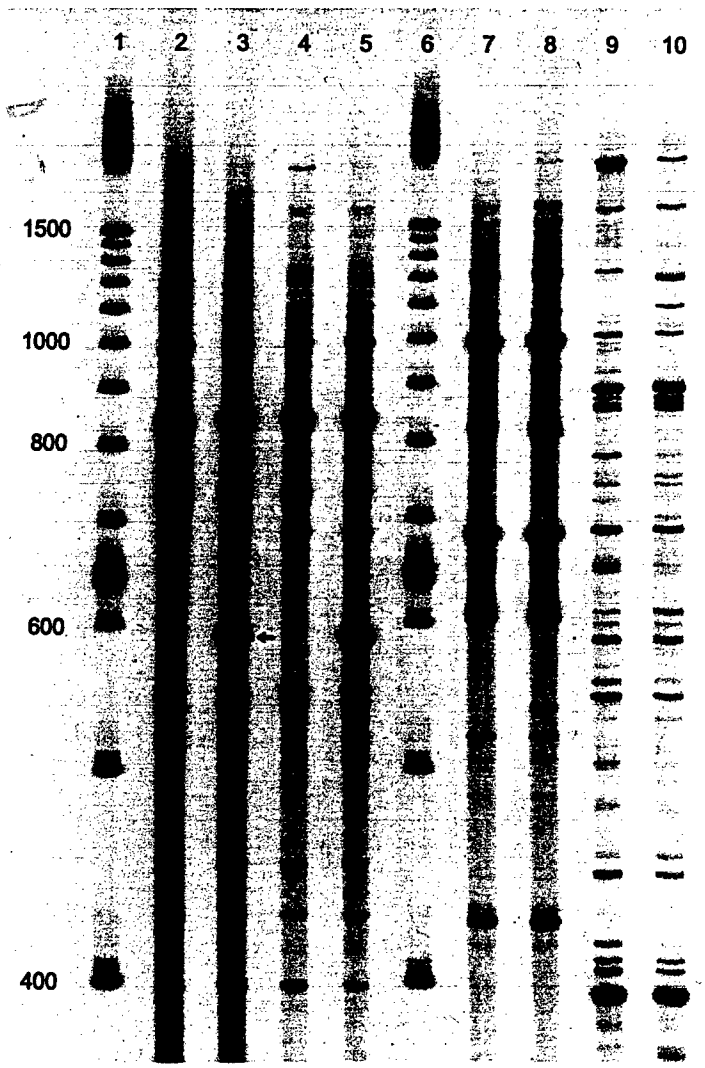


Figure 5. Differential display from uninduced and toluene induced *P.putida* F1 cells using arbitrary primers. Lanes 1, 6; 100 bp ladder. Lanes 2,4 ,7,9; uninduced. Lanes 3,5, 8,10; toluene induced. Primers used: 70.3 alone (lane7, 8) SD alone (lanes 9, 10) or both primers (lanes 1,2,3,4). The *todC1* fragment is indicated by an arrow.

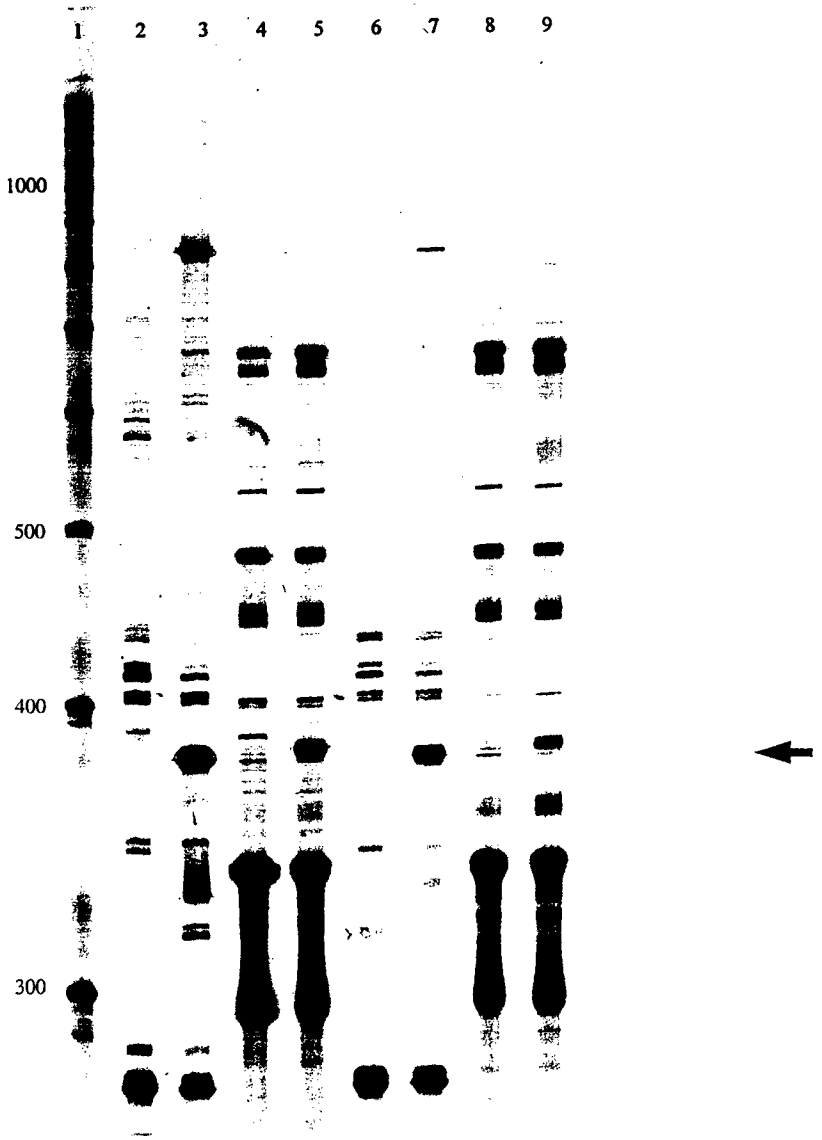


Figure 6. Differential display of salicylate induced and uninduced RNA from *P. putida* JS150 using arbitrary primers. Total RNA from uninduced and induced cells were reverse transcribed using a Shine-Dalgarno (SD13) primer followed by PCR using the SD13 primer and an arbitrary 10 bp primer. Lane 1, 100 bp ladder. Lanes 2, 3, 6, 7; PCR with primers SD13 and 60.3; lanes 3 and 7, induced; lanes 2 and 6 uninduced. Lanes 4, 5, 8, 9; PCR with primers SD13 and 60.4; lanes 5 and 9, induced; lanes 4 and 8, uninduced. The arrow points to the *nahA*-like fragment.

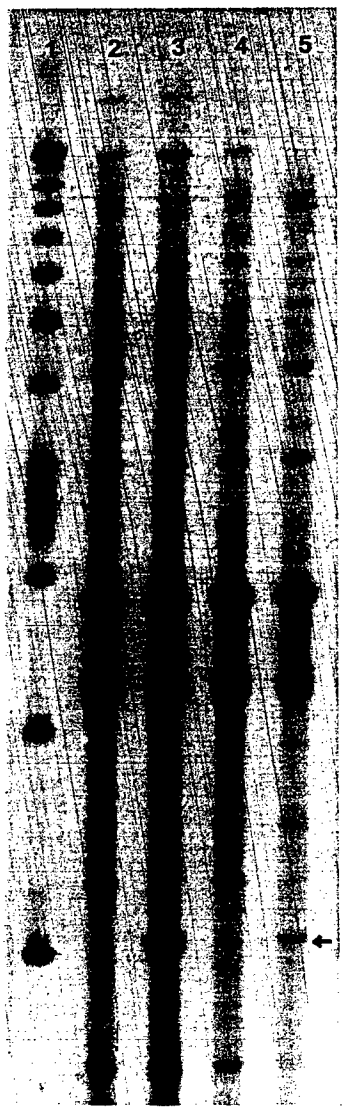


Figure 7. Differential display of toluene induced and uninduced RNA from *P. putida* F1 isolated from pure culture and soil samples. Lane 1, 100 bp ladder; lane 2, pure culture uninduced ; lane 3, pure culture toluene induced; lane 4, soil extracted uninduced; lane 5, soil extracted toluene induced. The arrow points to a band that, after cloning and sequencing, was revealed to be a *TodC1* fragment.

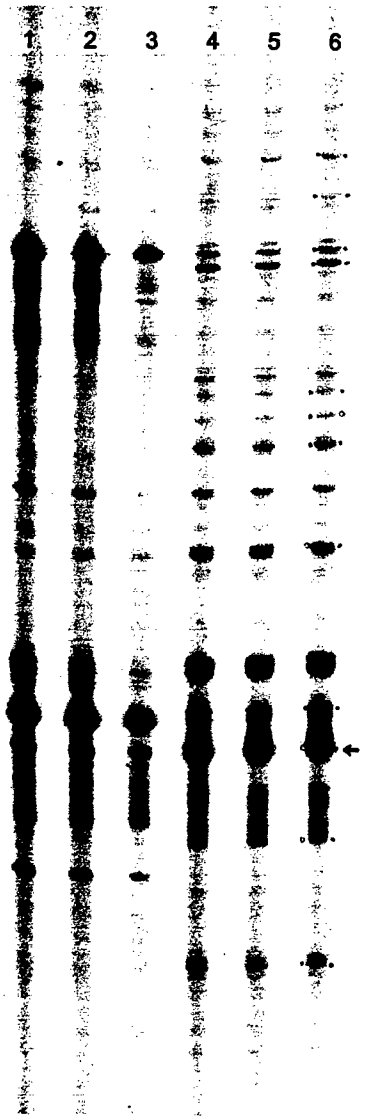


Figure 8. Differential display of toluene induced and uninduced uninoculated soil microcosms. Total RNA from toluene induced and uninduced microcosms was reverse transcribed using the SD primer and amplified using the SD primer in conjunction with primer 70.3. Lanes 1,2,3; triplicate reactions from uninduced microcosms. Lanes 4, 5, 6; triplicate reactions from toluene induced microcoms. The arrow points to a band that was reamplifed, cloned, sequenced and verified as differentially expressed.

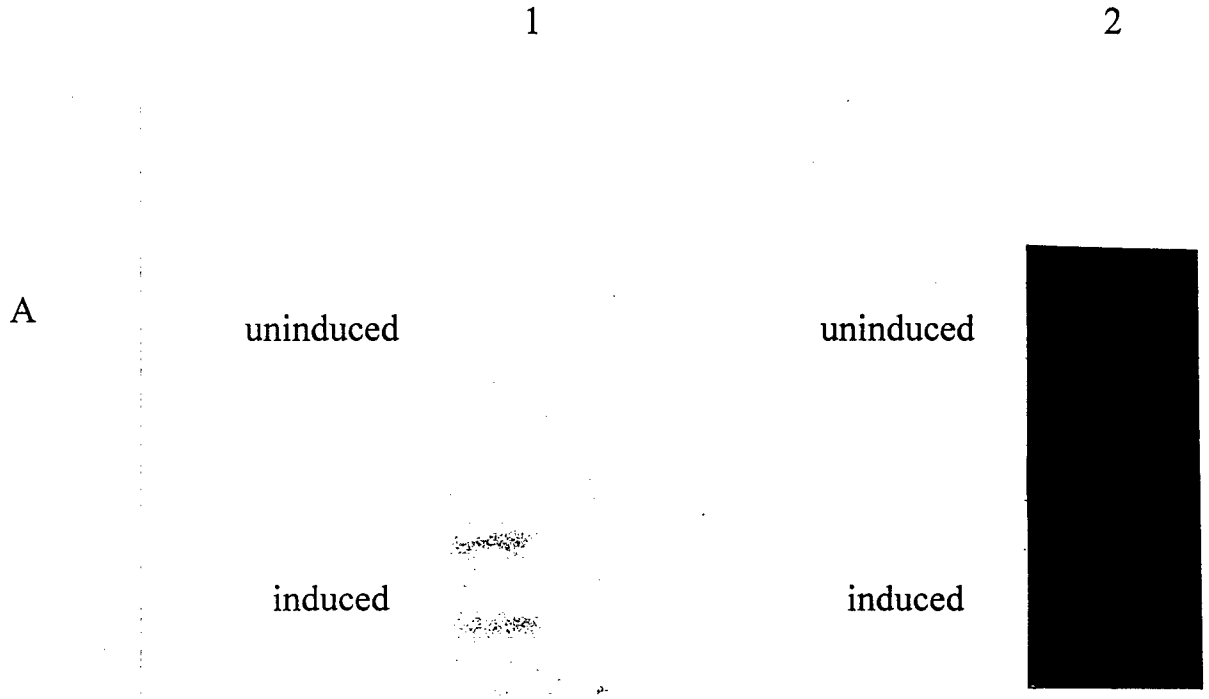


Figure 9. Confirmation of clonal differential expression by RNA slot blot analysis. Total RNA was isolated from pure cultures or soil microcosms, blotted to nylon membranes and probed with ^{32}P-labeled cloned fragments. (A) toluene induced and uninduced *P. putida* F1 RNA probed with inserts from clones 410 (1) and 170 (2). (B) Salicylate inuduced or uninduced *P. putida* JS150 RNA hybridized with clones 380 (1) and 325 (2). (C) Toluene induced or uninduced uninoculated soil microcosm RNA probed with labeled clones 400 (1) and 325 (2) .

1

2

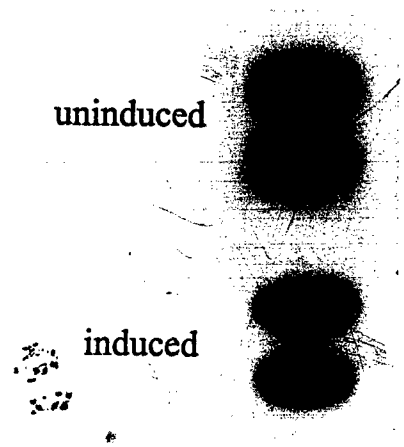
B

uninduced

induced

uninduced

induced



C

uninduced

induced

uninduced

induced

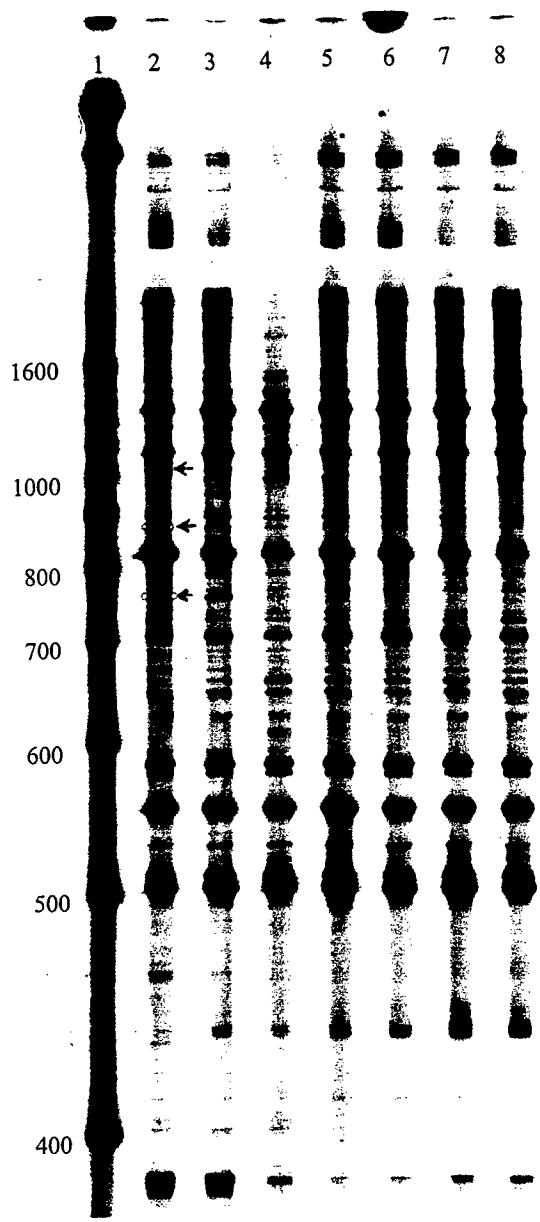


Figure 10. Differential Display of uranyl acetate induced *P. putida* G7 cells treated with rifampicin. Lane 1, 100 bp ladder; lane 2, T=0 min (without rifampicin); lane 3, T=5 min; lane 4, T=10 min; lane 5, T=20 min; lane 6, T=30 min; lane 7, T=40 min; lane 8, T=60 min.

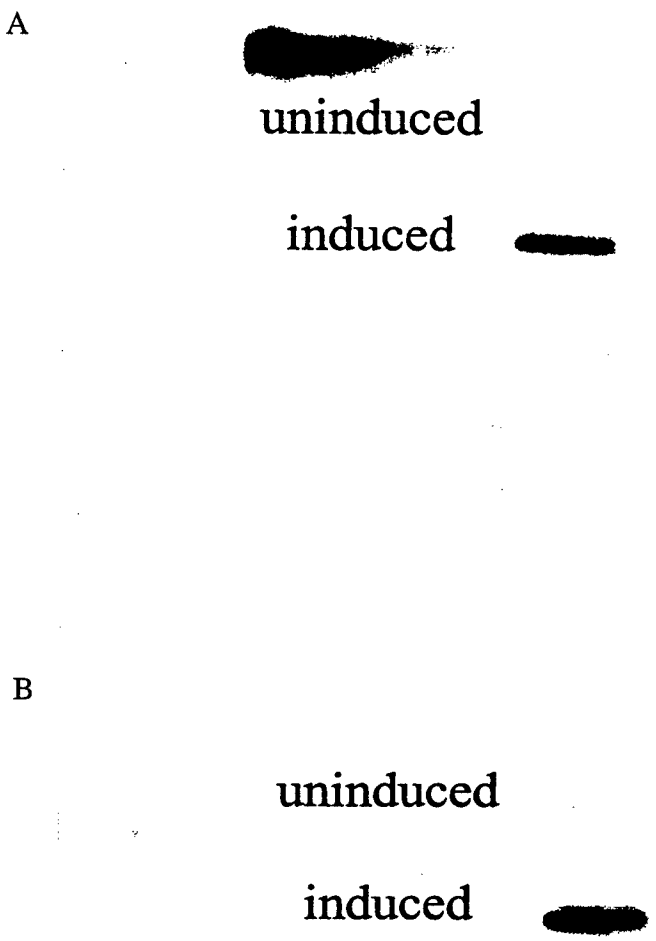


Figure 11. Confirmation of uranyl acetate induction in *P.putida*G7. A; Uninduced and uranyl acetate induced total RNA from *P. putida* G7 probed with clone 60.3-850. B; Uninduced and uranyl acetate induced total RNA from *P.putida*G7 probed with clone 60.3-775.

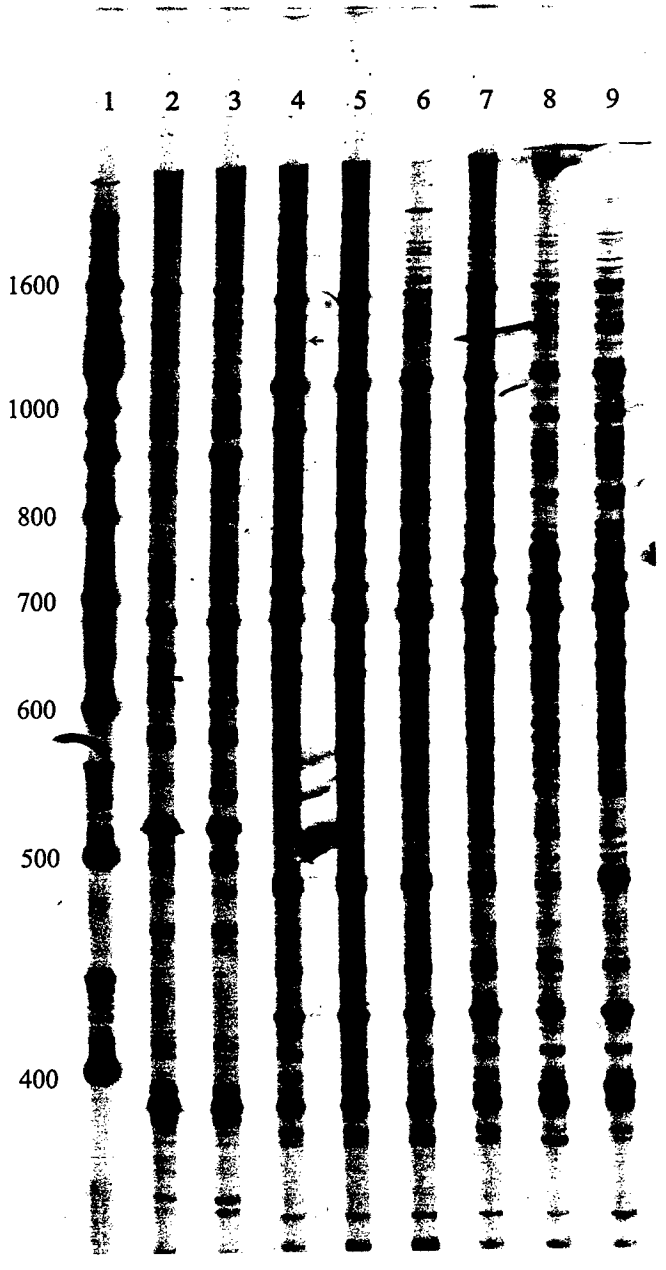


Figure 12. Differential display of uranium induced and uninduced uninoculated soil microcosms treated with rifampicin. Total RNA was reverse transcribed using a 10 bp arbitrary primer followed by PCR with the same arbitrary primer and SD14 primer. Lane 1, 100 bp ladder; lanes 2, 3, duplicate control samples; lane4, T=0 min (without rifampicin); lane 5, T=5 min; lane 6, T=10 min; lane 7, T=20 min; lane 8, T=40 min; lane 9, T=60 min. The arrows point to the bands that were isolated and reamplified.

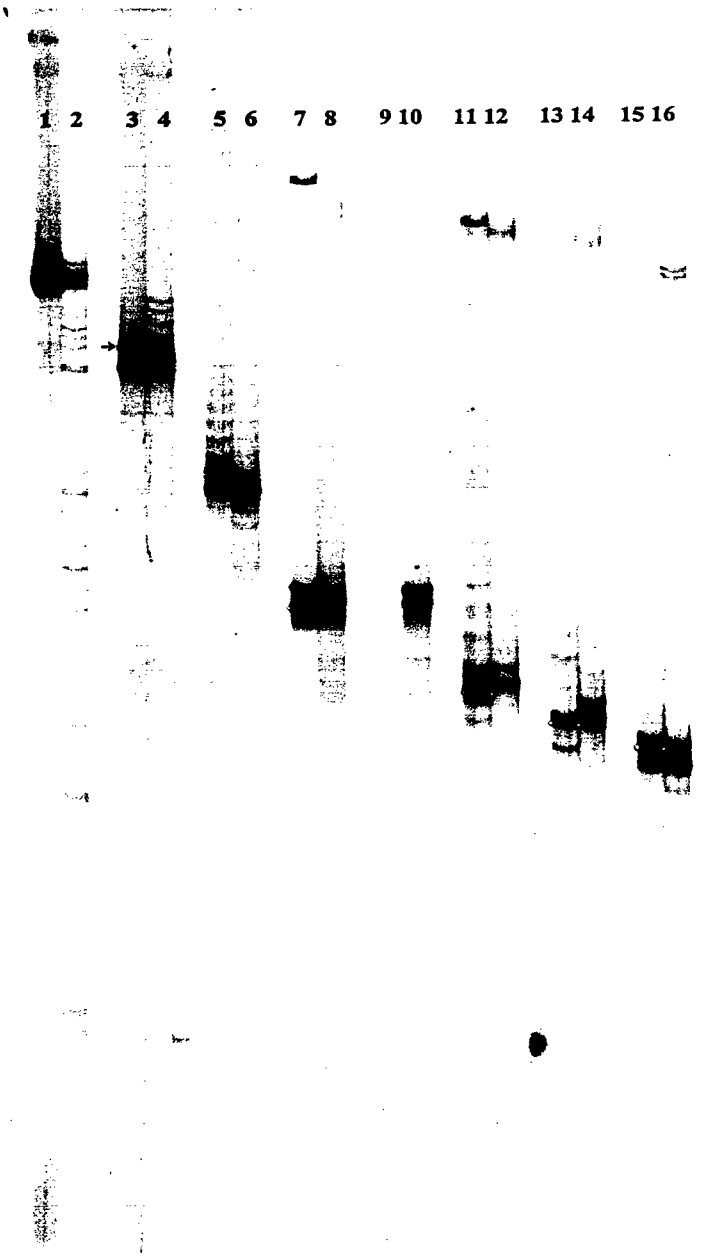


Figure 13. Single strand conformation polymorphism (SSCP) analysis of reamplified DD-PCR products from uranium induced soil microcosms. Side by side comparisons were made between putative differentially expressed and control DD-PCR products both of which were excised from the same molecular weight region of the gel. Lanes 1, 3, 5, 7, 9, 11, 13, 15, induced bands; Lanes 2 ,4, 6, 8, 10, 12, 14, 16, control bands. The arrows point to the bands isolated from the SSCP gel. Bands were reamplified and cloned to verify differential expression.

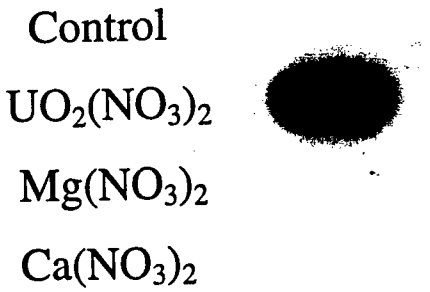


Figure 14. Confirmation of uranyl nitrate induction of uninoculated soil microcosms. Lane 1, control microcosm total RNA; lane 2, $UO_2(NO_3)_2$ microcosm total RNA; lane 3, $Mg(NO_3)_2$ microcosm total RNA; lane 4, $Ca(NO_3)_2$ microcosm total RNA. Microcosm RNA was blotted to nylon membranes and probed with uranyl nitrate induced clone 60.3-1200.

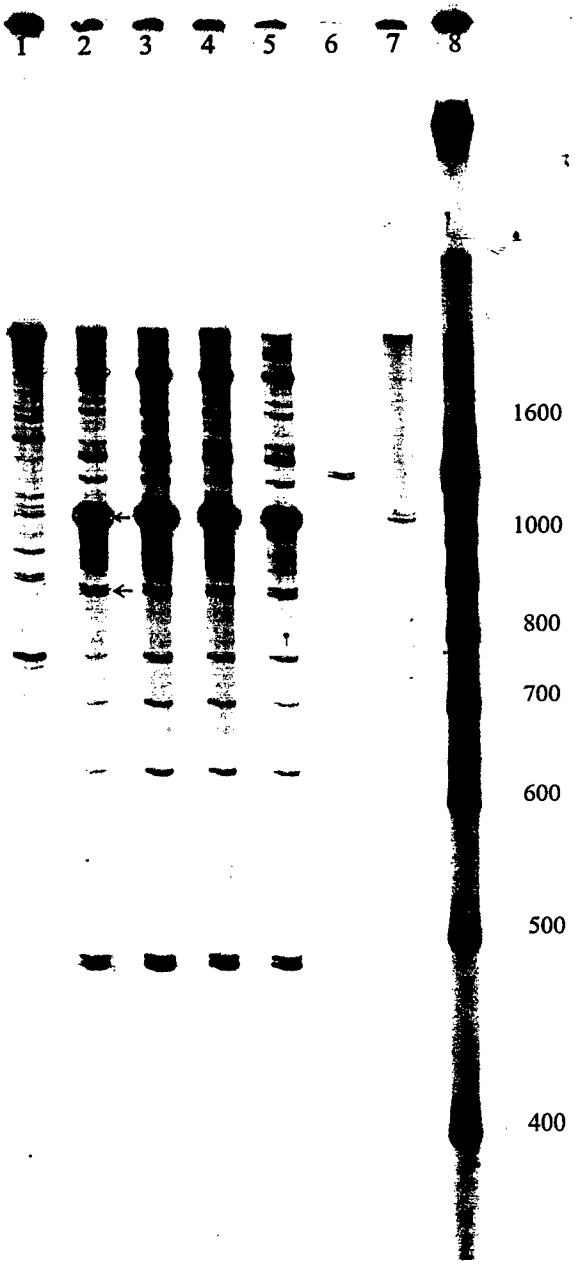


Figure 15. Differential display from induced and uninduced uninoculated soil microcosms using an anchored oligo-dT primer ($T_{11}C$). Total RNA was reverse transcribed using the anchored primer followed by PCR using the same anchored primer and a 10 bp arbitrary primer. Lane 1, control microcosm RNA; lane 2, induced microcosm RNA (T=0); lanes 3, 4, 5, 6, 7, induced microcosm RNA treated with rifampicin; lane3, T=5 min;, lane 4, T=8 min; lane 5, T= 16 min; lane 6, T=32 min; lane 7, T=60 min; lane 8, 100 bp ladder.

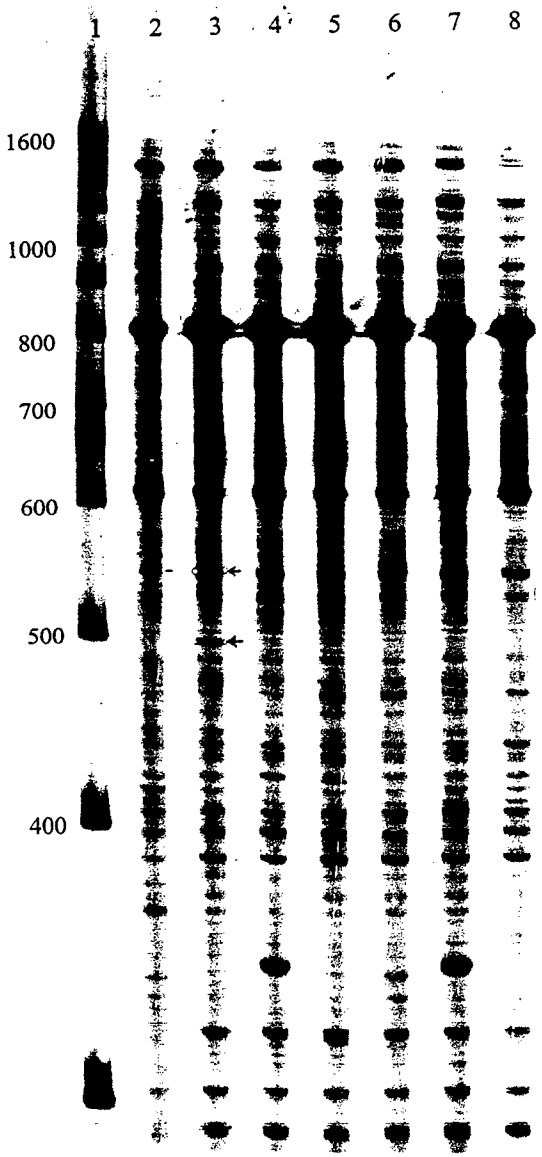


Figure 16. Differential display of cadmium induced *P. putida* G7. Lane 1, 100 bp ladder; lane 2, control total RNA; lane3, cadmium induced total RNA (T=0); total RNA from cadmium induced culture treated with rifampicin; lane 4, T=5 min; lane 5, T=10 min; lane 6, T=20 min; lane 7, T=40 min; lane 8, T=60 min. The arrows point to 2 putative differentially expressed bands.