

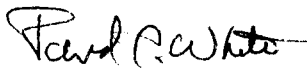
To the Graduate Councils:

I am submitting herewith a thesis written by Wen-Hsiang Yao entitled "RNA Fingerprinting of total bacterial RNA obtained by *in situ* extraction of soil microcosms inoculated with strain *Pseudomonas putida* F1." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.



Dr. Gary S. Saylor, Major Professor

We have read his thesis
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

RNA Fingerprinting of total bacterial RNA obtained by *in situ* extraction of soil
microcosms inoculated with strain *Pseudomonas putida* F1

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for the
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ABSTRACT

The differential display RT-PCR (DDRT-PCR) technique has been successfully applied to obtain catabolic gene information from environmental bacterial strains. *Pseudomonas putida* F1 RNA was used as a model system to allow optimization of this technique. A 382-bp *todC1* gene fragment was detected on a sequencing gel by using *tod* specific 13 oligonucleotides. The time course for *tod* transcripts induction and quantitation of the *tod* transcripts have also been achieved. The level of *tod* transcription was determined to be 10^7 transcripts/ 10 μ g of total RNA, which is approximately 0.004% of the total RNA. Primer concentration, primer length, annealing temperature, template, dNTP, and $MgCl_2$ concentrations have also been optimized. The detection limit for differential display of the *tod* fragment was shown to be 0.015 ng of total RNA template.

Optimization of the RNA extraction buffer and fingerprinting of RNA obtained from soil samples inoculated with *P. putida* F1 have also been successful. The banding patterns of RNA from pure culture and soil samples are almost identical. Non-specific arbitrary primers have also been used to fingerprint *P. putida* F1 RNA to assess the effect of cDNA amplification. Subsequently a 543-bp *todC2* gene fragment has been detected. Therefore, this study provides support that DDRT-PCR can be used to isolate the active genes from pure bacterial cultures and environmental soil bacteria.

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1. INTRODUCTION

Environmental microbial communities are typically heterogeneous in that they contain organisms which do and do not degrade organic pollutants. Although numerous studies have demonstrated the biodegradation of organic compounds by certain microbial communities (31,44,45,67,76), our understanding of these communities is still limited. This is especially true with the microorganisms that are poorly culturable. Therefore many new techniques have been developed to enhance the detection of the degradative potential of the microbial communities. Direct extraction of DNA from soil samples allows detection of the overall microbial populations (75). Hybridization of gene probes with DNA obtained from environmental samples has been used to identify microorganism populations with degradative genes (74,76,78). For example, the biphenyl operon has been used as a gene probe to monitor the presence of polychlorinated biphenyl (PCB) degrading microorganisms in soil (44,45). Thus gene probes can be used for molecular diagnostics in environmental bioremediation. Although the frequency and distribution of the genes responsible for degradation of hazardous compounds may be determined by nucleic acid hybridization to DNA isolated from environmental samples such as soils and sediments (9,77,86), DNA analysis provides no information about the viability, catabolic activity or gene expression of the microbial populations. A

complementary method to DNA analysis is the use of mRNA obtained from the environmental samples to assess the activity of the microbial populations.

Because all studied genes of environmental importance are regulated at the transcriptional level, this procedure may allow the study of gene expression of the environmental microbial populations.

The polymerase chain reaction (PCR) is a powerful tool for detecting low concentrations of specific DNA especially in environmental samples. The polymerase chain reaction can amplify *in vitro* a defined gene segment of interest by using two primers (64,65). By exponential amplification of a target sequence, PCR greatly enhances the detection of that specific sequence. PCR involves three steps: denaturation, annealing, and extension. The extension step is catalyzed by thermostable *Thermus aquaticus* (*Taq*) DNA polymerase. PCR products can also be generated from mRNA (5). In a preliminary step, called reverse transcription, the complementary DNA (cDNA) strand is synthesized by a reverse transcriptase by using one 3' primer followed by the three PCR steps. This method, referred to as RT-PCR, may, because of its great sensitivity, provide us with more information about active gene regulation.

In contrast to conventional PCR, arbitrarily primed PCR uses random primers to generate randomly amplified PCR products (37,96); most of which are polymorphic. This polymorphism or fingerprint can be used similarly to obtain restriction fragment length polymorphisms (RFLPs) for genetic mapping. DNA polymorphisms obtained by arbitrarily primed PCR can be used as genetic

markers (97) and to identify bacterial pathogenic strains in clinical settings (39). The difference in fingerprints between closely related organisms can also be used to subtype strains (37). Sequence polymorphisms in RNA may also be genetically mapped by arbitrarily primed RT-PCR. Fingerprinting of RNA by arbitrarily primed RT-PCR, also referred to as differential display, has also been used to detect differential gene expression in pure culture (10,95,99). This novel technique uses one arbitrary primer or a set of arbitrary primers for PCR amplification of cDNA generated by reverse transcription from mRNA. These primers will anneal to unknown sequences in the mRNA, many of which are polymorphic. The polymorphism may represent an unknown gene that is expressed in the organism. Under inducing conditions, arbitrarily primed RT-PCR potentially allows identification of those genes that are differentially expressed under altered conditions, such as oxidative stress, extreme pH, anaerobiosis, heat shock, and starvation (99). This approach does not require prior knowledge of sequence information and can generate polymorphisms from mRNA isolated from different organisms such as bacteria, fungi, plants or animals. Thus under induced and uninduced conditions, a side-by-side electrophoresis of the PCR products allows detection and isolation of the differentially expressed genes from the gel. Subsequently, the isolated gene fragment can be cloned and sequenced. Such a method may facilitate the study and isolation of transcriptionally active genes from soil microbial environments.

Successful application of the differential display technique to RNA directly extracted from soil microorganisms is difficult. RNA extracted from soils high in organic material samples is always contaminated with humic acids. These humic acids have properties similar to nucleic acids, such as charge and molecular weight, which will interfere with the RT and PCR procedures (63). Recently a new approach, direct extraction of mRNA from soil microcosms, has been developed to measure catabolic gene expression in PAH-contaminated soil by Fleming et al. (25). *In situ* isolation and quantitation of mRNA was demonstrated for the NAH7 naphthalene dioxygenase gene (*nahA*), proving that extraction of intact mRNA directly from soil microcosms is possible.

Before the arbitrary RT-PCR method was used in this work to study the gene expression of microbial populations in contaminated field samples, a model system approach was used to optimize the procedure for prokaryotic RNA. The strain, *Pseudomonas putida* F1 was used for transcription induction studies in pure culture and in soil microcosms. Toluene was added in the vapor phase to induce the degradative gene expression. Total RNA from induced and uninduced samples were extracted and DNase treated. The induced gene expression was detected by electrophoresis after arbitrarily primed RT-PCR. The isolated genes were then reamplified, cloned, and sequenced. The sequences of the obtained cDNA fragments were compared with GenBank sequences to see if they were unique or specific. Differential induction was confirmed by Northern hybridization with induced and uninduced mRNA.

After initial optimization of the differential display conditions for RNA from pure culture, toluene induced and uninduced *P. putida* F1 cells were added to soil microcosms. RNA was then extracted from each sample and the differential display procedure was performed with the soil extracted RNA. Any difference in a comparison of the fingerprint patterns obtained from uninduced and induced inoculated soils with those from pure culture permits optimization of the procedures to assess degradative gene expression of the microbial populations in the contaminated environment.

Using short *todC1* specific primers, differential expression of the *todC1* gene are hypothesized to be detectable. Confirmation of the *todC1* fragment was done by sequence analysis. The specific *todC1* fragment, was then an index for fine-tuning the RNA fingerprinting procedure for prokaryotic cells. Comparison of the fingerprinting pattern obtained from pure culture and soil RNA is hypothesized to allow the technique to be directly applied to the soil samples. The overall procedures taken to accomplish this work are illustrated in Figure 1.

The goal of the research described in this thesis was to apply the differential display technique to *Pseudomonas putida* F1 cells to measure prokaryotic gene expression under toluene inducing conditions. Specifically, the objectives were:

1. To optimize reproducible RNA fingerprints from toluene induced and uninduced *P. putida* F1 total RNA from pure culture.

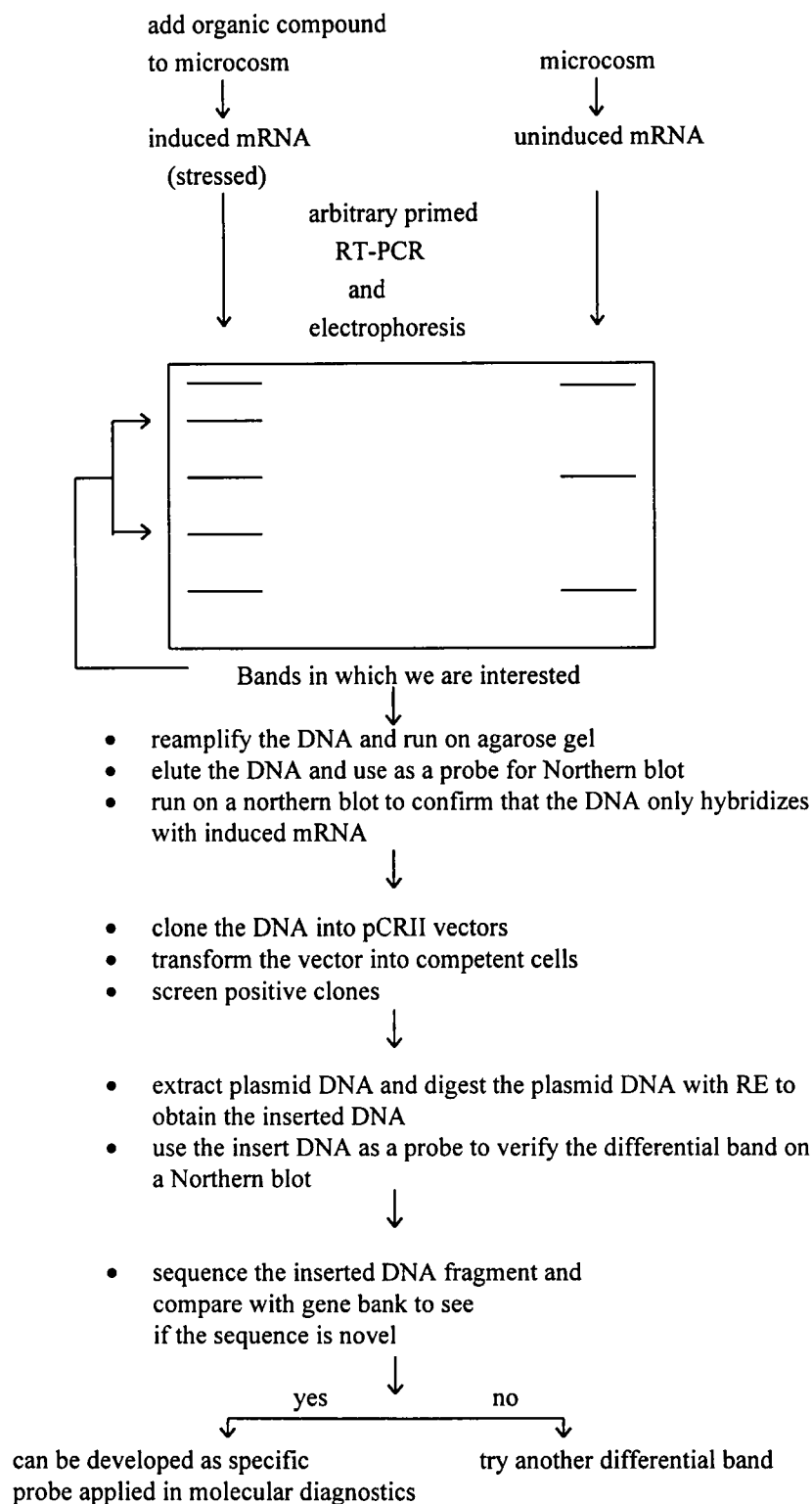


Figure 1. Outline of a procedure to detect differential genes by using arbitrarily primed PCR in microcosm system.

2. To examine the use of short arbitrary primers to screen prokaryotic transcriptional events by the differential display procedure.
3. To demonstrate RNA fingerprinting of *P. putida* F1 inoculated in soil microcosm.

2. LITERATURE REVIEW

Differential Display

Conventional methods to detect differentially expressed genes largely depend on subtractive hybridization (46). However, this technique requires a large amount of RNA and usually is not reproducible and is time-consuming (49). Recently, two groups have developed an alternative approach referred to as differential display (Liang and Pardee) or RNA fingerprinting (McClelland), which in many respects is an improvement over subtractive hybridization (Table 1). Differential display has been applied to detect differentially expressed eucaryotic mRNA. It has been successfully applied to identify and isolate differentially expressed genes in cancers (27,55,98,100), cell proliferation (17), brain (23,36,101), muscle (42), kidneys (56), embryogenesis (29,32,62), tumors (12,72,81), plants (1,32,38,40,62,71,79,90), insects (41), immune response (33), cell differentiation (35,80,87), growth factors (2,22,58), transcriptional regulators (88) and signal transduction (60) (For recent reviews see 52,56,94).

The differential display technique involves several steps: 1) Isolation of intact RNA from organisms. 2) Reverse transcription of total RNA or mRNA using an arbitrary primer or oligo dT primer (for eucaryotic systems) to generate cDNA. 3) PCR amplification using the same primer or another set of arbitrary primers to amplify the cDNA corresponding to a smaller subset of RNA. 4) Separation and detection of the differential PCR products on sequencing gels.

Table 1. Comparison of subtractive hybridization and differential display to study gene expression

<u>Subtractive hybridization</u>	<u>Differential display</u>
1. One way detection	1. Detection of both groups of differentially expressed gene
2. low sensitivity	2. high sensitivity (PCR based)
3. require large quantities of mRNA	3. 10ng - 400ng of total RNA
4. low reproducibility	4. high reproducibility
5. tedious	5. simple

Bands of interest can be excised from the gel and eluted. The eluted bands are then reamplified, cloned and sequenced. Comparison of sequence similarity with known gene sequence databases permits analysis of the possible function of the gene to be inferred. The isolated gene can be further used as a probe to isolate and locate the gene in the chromosome or plasmid.

Differential display band patterns

The factors which affect reproducibility and intensity of bands in a fingerprint are a function of a number of parameters, such as annealing temperature, primers, salt concentration, template concentration, and RNA integrity (59). Some artifacts like the production of faint bands or non-specific background smears on gels, are commonly observed by researchers (24,82). These faint bands often make interpretation of the data difficult. Eric et al. successfully replaced the oligo-dT primer with a second arbitrary primer in the PCR step to cut down the band pattern complexity; thus, the arbitrary primer enables more specific amplification compared to the oligo-dT primer. Therefore, enhanced specificity by using arbitrary primers may reduce such background signals (24).

Other laboratories have observed that primer and the specific *Taq* polymerase greatly affect the quality and consistency of the banding patterns. In most situations, mRNA is reverse transcribed to cDNA by reverse transcriptase

using an oligo-dT primer anchored 2 or 3 bases at 3' end, such as 5'-dT₁₀AC. The anchored 2 or 3 bases oligo-dT primer can reduce the number of annealing events, however, a common problem observed by other labs is that faint bands have been consistently produced by the primer choice (24). Moreover the poly-dT primer will always anneal to the 3'-untranslated region of mRNA, thus not giving useful information (24). To solve this problem, Eric et al. (24) used the arbitrary primer to amplify the cDNA sequence during the PCR step to get the information of the 5' translated region. Furthermore, Eric et al. (24) also suggested that different sources of *Taq* polymerase also will influence the band patterns. Thus, using the same source of *Taq* polymerase is necessary to keep the results consistent. Some reports also showed that different batches of *Taq* polymerase or different PCR tubes also influence the band patterns (19,24). Since the differential display technique is a very sensitive technique, minor parameter changes may influence the band patterns. Therefore, suitable conditions for a specific template may need to be tested.

RNA abundance

Bertioli et al. (14) showed that the standard differential display protocol (DDRT-PCR), published by Liang and Pardee (50) shows a strong bias towards high copy number eucaryotic mRNAs. Only mRNA present at more than 1.2% of total mRNA can be detected by this technique. At levels lower than this, even a

perfectly matched primer failed to detect the mRNA template. This may be due to the competition for the substrate by the many PCR products in the differential display reaction. The number of PCR products generated under differential display PCR conditions outnumber conventional PCR because mismatches are tolerated due to short primer length and low annealing temperature. Also low dNTP concentrations are used in order to increase the incorporation of the radiolabeled ^{32}P -dCTP. Therefore, the abundant RNA may out-compete the rare RNA in a severely competitive situation (14). Thus, the standard DDRT-PCR technique is not able to efficiently display most of the mRNA in the organism.

Liang et al. (49) showed that a perfect match of 10mer primer at both ends for a rare message did not dominate the fingerprint and other products can still effectively compete to produce fingerprints. This indicates that arbitrarily primed RT-PCR lacks abundance normalization; mismatch amplification of abundant RNA may out-compete with the low abundance RNA. Although the intensities of different bands in the same lane vary independently from each other, the intensity of certain bands between fingerprints is proportional to the concentration of its template (49). Thus, the semi-quantitative nature of arbitrary priming PCR (AP-PCR) theoretically allows detection of the degree of induction by comparing the intensity of matched bands between the samples. By decreasing the number of base mismatches in the arbitrary priming steps, one can increase the abundance normalization. However, in order to get the maximal expression information from the mRNA pool, low stringency priming

must be performed (59). Abundance normalization of RNA may be partially achieved by using additional rounds of amplification with nested primers (59,70). Nested primers are designed to improve normalization of the fingerprint with respect to mRNA abundance (70). The nested primer is the same with the first primer except that it has one or more additional arbitrary nucleotides at its 3' end. The additional 3' nucleotide, almost eliminates all the spurious nonspecific amplification products. This happens because after the first round of PCR, any nonspecific products will not be sufficiently complementary to the nested primer to serve as a template for further amplification. Thus, the sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using a nested primer strategy. RNA fingerprinting produces PCR products that are not visible on the gel (low abundance messages). A secondary round amplification by the high-stringency nesting step may amplify the low abundance mRNA (59). However, abundance normalization may not be of critical importance if the targeted mRNAs are highly expressed.

There are many more low abundance mRNAs than high abundance mRNAs in the eucaryotic RNA pool. Liang et al. (49) showed that minor differences were observed in the amplified patterns when the RNA template concentrations were decreased from 2 to 0.002 μg . Some of the difference may be due to the loss of detection of rarer mRNAs when RNA concentration is lowered.

Because the procedure in the differential display technique is always performed in the low stringency cycle, mismatch amplification is tolerated especially in the 5' end of the primer. Liang et al. (49) showed that up to 4 bp mismatch still can be amplified. The amplification efficiency is proportional to the match. However, the abundance of RNA may overcome the mismatch problem. In other words, abundant RNA which is not a perfect match may be detected in the differential gel, however, the rare mRNA may not be detected although it is perfect match (49). This suggests that only a few primers will be needed to display all the abundant mRNAs. To improve this bias, one can increase the dNTP concentration or increase the annealing temperature of the PCR to allow more specific amplification. However, this change may also influence other factors.

Primer choice

In the differential display technique, the RNA must first be reverse transcribed into cDNA. This can be accomplished by using either arbitrary primers or oligo dT primers for eucaryotic poly-A tailed mRNA. By using arbitrary primers, synthesis of first strand cDNA is initiated by reverse transcriptase at those sites in the RNA that best match the primer. The 3' nucleotides in the primer usually are more important than those in 5' end because its -OH group is required by the DNA polymerase (51). PCR amplification is achieved by using

the same arbitrary primer or another primer. First strand cDNA synthesis can also be achieved using oligo dT. The oligo dT will anneal to the 3' end of the target eucaryotic mRNA. PCR amplification is accomplished by using an arbitrary primer in conjunction with the poly-dT primer.

Anchored 3' primers such as oligo dT₁₁C, oligo dT₁₁CA and oligo dT₁₁CAG, have been used for reverse transcription and an arbitrary primer for priming the first-strand cDNA (51). The base-anchored oligo-dT primer can reduce the annealing frequency on the poly-A tailed RNA template, providing better specificity. This minimizes the under-representation of certain RNA species (8,49,51). Linskens et al. (53) observed that fingerprints amplified by a GC rich primer at the 3'-end seemed to obtain more bands on a sequencing gel.

Several modifications of primer design for differential display have been reported. In order to get more gene information from reverse transcription, the primer length used in differential display is 10 to 13 bases, which theoretically will have more annealing sites on the mRNA template than larger primers. However, these primers are too small to be successfully used as sequencing primers by using the current sequencing method. Therefore, Wang et al. (92) used a longer modified primer which contained the original short primer at 3' end in the subsequent PCR reamplification step. Therefore, the reamplified PCR product now is suitable for direct sequencing by using the same primer without further subcloning steps. To increase the reproducibility and reduce false positive results, Ayala et al. (8) directly used modified longer primers as the reverse

transcription primer and then the same primer was used for PCR amplification at a higher annealing temperature to get more specific desired products. Thus, using the shorter primer to get more annealing sites can also be achieved by using longer primers with lower annealing temperatures in the PCR reaction.

The use of oligo-dT primers ensures that only the poly A mRNA will be amplified rather than rRNA, tRNA or other structure RNAs. However, such products represent the 3'untranslated portion of the mRNA, because the 3' end of eucaryotic mRNA tends to be the non-coding region. Thus, it will provide less useful sequence information. When arbitrary primers are used, the product of the cDNA is not biased toward the 3' end region of mRNA. However, all the total RNA may be potentially amplified rather than mRNA only. This will make the identification of true differentially expressed mRNA more difficult, because the rRNA is the dominant species in the RNA pool. To avoid the 3' bias with oligo-dT primers, one can use poly-dT column to purify the mRNA and then use arbitrary primers to fingerprint the RNA. Therefore, the better primer seems to be dependent on the template and reaction condition. Best choice of primer seems to be by experimental testing.

McClelland et al. (60) and Donohue et al. (22) used particular primers, such as a nuclear localization amino acid motif or protein kinase zinc finger motif to amplify a particular gene classes. However, primers containing sequence motifs for particular classes of mRNA or sequence from degenerate primers may bias the fingerprint to a particular class of mRNA (95).

In eukaryotic organisms, poly-dT or arbitrary primers can be used as the first RT primer. However, in prokaryotic organisms, because poly-A tails at the 3' end of mRNA are rare, arbitrary primers must be used in the RT step to amplify RNA (99). Although abundant ribosomal RNA increases the false positive rate by using arbitrary primers, Kwong et al. (99) successfully used arbitrary primers to detect inducible genes from *Salmonella typhimurium* LT2 in response to peroxide stress. This demonstrates that arbitrary primers can be used to amplify inducible genes of prokaryotic cells in response to stress when suitable primers were chosen.

Screening PCR products and clones

A common problem observed by other laboratories is that a good percentage of clearly differentially expressed PCR products excised from gels show no differential induction when used as probes to hybridize against total RNA. Also experienced by others is the failure to clone the cDNAs which would reconfirm differences between induced and uninduced samples (52). Therefore, the screening procedure plays a key step in the differential display method.

There are two kinds of false positives. The first one is found when there are no truly differentially expressed PCR products inside the bands isolated from the differential display gel. The second is when multiple PCR products of the same molecular weight are found within one band, only one of which is

differentially expressed. These two kinds of false positive results will always influence the downstream work.

For the first situation, several steps can be taken to decrease the false positive. 1) When RNA is contaminated with DNA, the fingerprint will be independent of reverse transcription (49). Thus, the sample should be treated with DNase I before performing the reverse transcription. 2) The existence of abundant ribosomal RNA (rRNA) may always influence abundance normalization and cause false positive results. It may be an advantage to eliminate the rRNA before performing the reverse transcription. For eucaryotic cells, poly(T) columns can be used to purify poly(A) mRNA. However, in prokaryotic cells, which have little poly(A) mRNA, it is more difficult to purify the mRNA from the RNA pool. One possible approach to purify prokaryotic mRNA may be to use an rDNA affinity column which contains the conserved sequences of rDNA on the resin. By eluting the total RNA solution through the rRNA affinity column, one could conceivably eliminate most of the rRNA. This procedure could be performed before or after the reverse transcription. However, the latter seems better because any additional procedure to manipulate the RNA before reverse transcription will cause the change of fingerprint pattern. 3) Another way to minimize the false positive results is to run duplicate samples or repeat experiments for those lanes that show differential expression, or run RNA fingerprinting from uninduced and induced cells over a time course of induction

(84). These procedures will dramatically decrease the first type of false positive results.

For the second situation, one can screen differential bands in the cloning step or capture the real differential bands using northern blots, then directly clone the reamplified bands. There are five methods to screen clones. Each method has its own advantage and weakness. The first one uses the Northern blot to affinity capture the specific cDNA. The differential bands from the sequencing gel are eluted and used as probes to hybridize with northern blots. Fragments of cDNAs which show differences in the northern blot between induced and uninduced samples are then recovered from the membrane after hybridization. The probes are subsequently reamplified, cloned, sequenced (48). Although this technique provides a rapid and effective method for screening, selecting and cloning cDNAs, this method requires a large amount of RNA to prepare the blots and also sometimes the retrieval of cDNA is not effective.

The second approach uses plasmid dot-blotting to screen cloned cDNA fragments, using cDNA PCR products as probes (16,54). The differential band is eluted and precipitated and resuspended in TE buffer. Half of the eluted band solution is used for PCR reamplification and cloning. The other half is used to probe back to plasmid DNA obtained from the clones. Although this method can screen many cDNA clones, it cannot assure that the clone will be the true differential gene because the cloned cDNA fragments may originate from false

positives. Also because the probes are eluted from the differential bands, the radioactivity may be too low to be detected.

A similar method has also been developed to screen the clones by Vögeli-Lange et al. (91) Inserts from plasmids of different clones are blotted to the membrane. The membrane is hybridized to the uninduced DDRT-PCR products as a control. One duplicate membrane is then hybridized with induced DDRT-PCR products as treated samples. Differences between the two membranes reveal the presence of differential genes in the clones.

The third method uses restriction enzyme digestion to identify different cDNA inserts. Different cDNA inserts are used as probes to identify the true positive clones. Using multiple restriction enzymes to cut the inserted cDNA gives different banding patterns because of different restriction sites on the sequence (82). Theoretically, by using two 4-cutter restriction enzymes, a cutting frequency of one in 128 will be expected. Thus, it gives a distinguishable RFLP for cDNA fragments longer than 128 bp. However, the enzyme digestion method may not give a good classification of cDNA clones because of the unpredictable cutting sites (102).

The fourth method uses the partial DNA sequencing approach to subtype the clone (102). The partial DNA sequencing approach uses only one sequencing reactions (i.e. using either A, T, C, or G). Each insert of the plasmid is then used as a probe to identify the true positive clones.

The fifth method uses the single-strand conformation polymorphism (SSCP) to identify different clones. Different inserts from the clones are then used as probes to identify the positive clones. The SSCP procedure involves denaturation of the double strand DNA to single stranded DNA; the denatured DNA then, is run on a non-denaturing polyacrylamide gel. Because of sequence and conformation differences, different ssDNAs will have different mobility although their length may be the same (57,69).

The development of gene probes in environmental microbiology

Gene probes can be used to identify or monitor the microbial population in complex environmental samples (9,67,68,77,86). Also, functional gene probes can be used to identify related functional genes because microorganisms which have the same function tend to contain high gene similarity. By using functional gene probes such as *todC1* or *nahA*, one can monitor specific microbial populations in complex microbial communities.

Two commonly used gene probing techniques in molecular microbial studies are: DNA:DNA colony hybridization and DNA:DNA hybridization of direct DNA extraction. Colony hybridization requires that microorganisms must be culturable. Because many of the microbial species are non or poorly culturable, direct extraction and purification of DNA from environmental samples may be necessary, although direct extraction of DNA from environmental samples may

be more tedious than colony hybridization. However, it represents the true microbial population in the environmental samples.

Gene probes have been used extensively for identification of bacterial strains in environmental microbiology and biodegradation research (9,67,77,86). 16S conserved ribosomal probes can be used to study phylogenetic relationships among bacterial strains. More specific 16S ribosomal RNA probes can be used to identify or subtype strains (4,21). Also functional gene probes can be used to identify strains which have similar function(74). Thus, it is important to obtain different functional probes. The recently developed differential display technique may provide a method to hasten the development of gene probes for catabolic genes of environmental significance. A comparison of development of gene probe procedures by using differential display method with those in conventional methods are shown in Table 2.

Toluene degradation by *Pseudomonas putida* F1

Pseudomonas putida F1 possesses one of the best characterized toluene degradation systems (see Figure 2). In the first step, *P. putida* F1 oxidizes toluene by incorporation of two atoms of oxygen into the aromatic ring of toluene to form a (+)-cis-1(S), 2 (R)-dihydro-3-methylcyclohexa-3,5-diene (cis-toluene dihydrodiol) (103). NADH serves as an electron donor in the oxidation reaction. This reaction is catalyzed by a three component enzyme system called toluene

Table 2. Comparison of conventional approach with differential display to develop gene probe

Conventional		Differential display	
<u>Procedure</u>	<u>Time</u>	<u>Procedure</u>	<u>Time</u>
1. enrichment	2-4 weeks	1. cultivation or direct extraction of RNA from soil samples	1-2 days
2. isolation and cultivation	1-2 weeks	2. RT-PCR	1-2 days
3. construction of gene library	4-8 weeks	3. electrophoresis and autoradiography	2-4 days
4. screen gene library	4-24 weeks	4. confirmation of differentially expressed gene	2-4 days
5. gene characterization	4-24 weeks	5. cloning and sequencing	1 week
6. data analysis		6. data analysis	

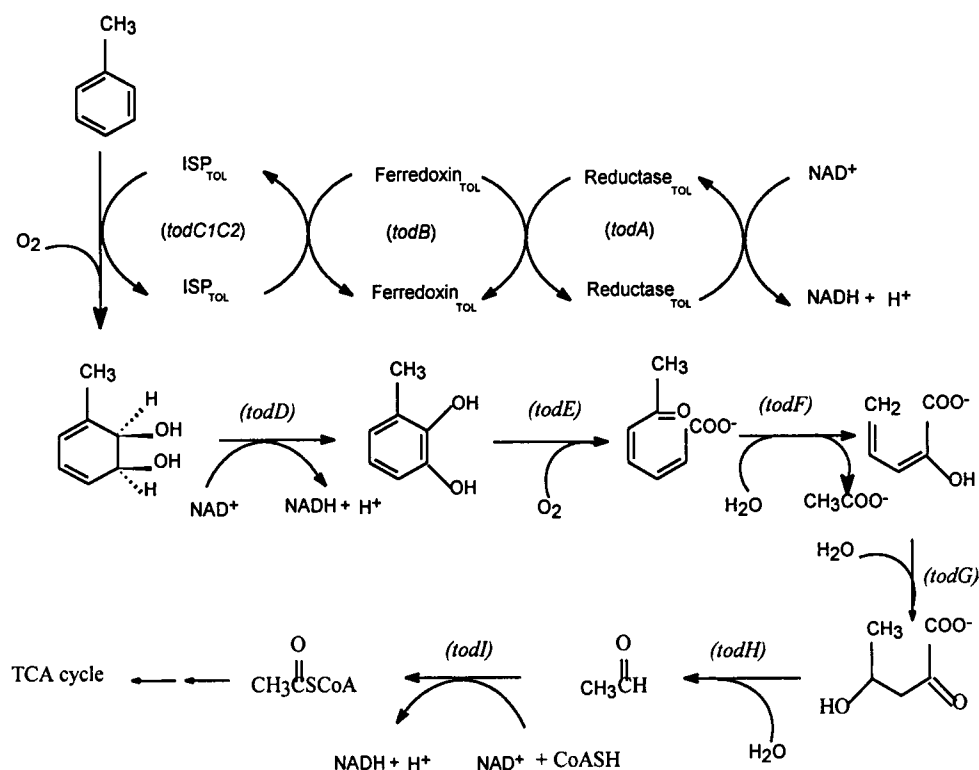
(adapted from Sayler, 1988)

dioxygenase. The components of toluene dioxygenase have been purified and characterized (103,104). A flavoprotein reductase encoded by *todA* accepts electrons from NADH and transfers them to a iron-sulfur protein, ferredoxin_{TOL} encoded by *todB*. Then, ferredoxin_{TOL} reduces another oxygenase named ISP_{TOL} (iron-sulfur protein), which contains two subunits encoded by *todC1* and *todC2*. Further metabolism of cis-toluene dihydrodiol to 3-methylcatechol involves an NAD⁺-dependent dehydrogenation catalyzed by cis-toluene dihydrodiol dehydrogenase encoded by *todD*. 3-methylcatechol is then oxidized by 3-methylcatechol 2,3-dioxygenase to 2-hydroxy-6-oxo-2,4-heptadienoate. The 3-methylcatechol 2,3-dioxygenase is encoded by *todE*. 2-hydroxy-6-oxo-2,4-heptadienoate (HOHD) is further metabolized to 2-hydroxypenta-2,4-dienoate (HPD) and acetate. This reaction is catalyzed by 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase encoded by *todF* (61).

The last three steps of toluene degradation by *P. putida* F1 are encoded by three genes named *todGHI* (43). The HPD is metabolized to 4-hydroxy-2-oxovalerate (HO) by HPD hydratase encoded by *todG*. HO is then converted to acyl-aldehyde (AA) by HO aldolase encoded by *todH*. AA is then metabolized to acetyl-CoA by AA dehydrogenase encoded by *todI*. The order of gene organization is *todFC1C2BADEGIH*. The overall toluene degradation pathway is shown in Figure 2.

The regulation of the toluene degradation reaction is still not known very well. However, previous work showed that the genes involved in toluene

A.



B.

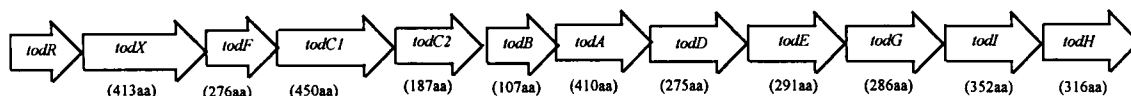


Figure 2. Toluene degradation pathway and gene organization of *tod* operon. (A) Toluene degradation pathway by *P. putida* F1. *TodC1C2AB* consist of toluene dioxygenase. *TodD*: cis-toluene dihydrodiol dehydrogenase. *TodE*: 3-methylcatechol-2,3-dioxygenase. *TodF*: 2-hydroxy-6-oxohepta-2,4-dienoate (HOHD) hydrolase. *TodG*: 2-hydroxypenta-2,4-dienoate (HPD) hydratase. *TodH*: 4-hydroxy-2-oxovalerate (HO) aldolase, and *TodI*: acylating aldehyde (AA) dehydrogenase. (B) Gene organization of *tod* operon (adapted from Zylstra, 1989 and Lau, 1994).

metabolism are coordinately induced (103). Recently a toluene-inducible promoter was discovered (93). It may be responsible for the expression of all *tod* structural genes. Another protein, TodX, was found to be a membrane protein. It exhibits high homology with the FadL protein of *E. coli*, which is an outer membrane protein required for binding and transportation of long chain fatty acids. Thus, it seems that the function of the TodX protein is likely to be involved in facilitating delivery of toluene inside of the *P. putida* F1 cell. TodR exhibits high homology to the DNA-binding domain of the transcription activation factor of the LysR family, but it was found to play no significant role in *tod* gene regulation (93). The two genes, *todRX* are located upstream of the toluene degradation genes, *todFC1C2BADEGIH*. The toluene-inducible promoter was located in front of *todX* (93).

3. MATERIALS AND METHODS

Cultivation of strains

A single colony of *Pseudomonas putida* F1 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 per liter, pH 7.0) in a 250 ml of flask 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 , pH 7.0). The cells were resuspended in 100 ml of toluene saturated minimal salts base medium (MSB) to induce *tod* gene expression in the same growth conditions. MSB was prepared as described previously (85). Total bacterial RNA was isolated from the induced and uninduced samples when the cells were harvested to mid-log phase ($\text{OD}_{600} \approx 0.6$).

RNA extraction from pure culture

RNA extraction was done using two methods, RNeasy columns (QIAGEN, Germany) and a modified hot phenol procedure (25). Small scale extraction of RNA from RNeasy columns was the 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 the manufacturer's protocol. Total mRNA from large scale-preps

was recovered by the modified hot phenol method (25). Fifty ml of mid log phase ($OD_{600} \approx 0.6$) cells was collected by centrifugation at 4,000 rpm for 15 minutes in 50 ml disposable centrifuge tubes. The cells were then resuspended in 20 ml 0.05 M NaOAc, 1% SDS at 60°C and 20 ml of 1:1 phenol/chloroform 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 4,000 rpm on the table top centrifuge for 15 minutes. The supernatant was transferred to a fresh tube and re-extracted three times. The final supernatant was removed to a baked 150 Corex bottle and 0.1 volume 3 M NaOAc 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 10,000 rpm for 30 minutes at 4°C. The pellets were resuspended in 500 μ l of diethyl pyrocarbonate (DEPC)-treated water and treated with DNase. Two μ l of RNase free/DNase I and 50 μ l of magnesium/dithiothreitol (Mg/DTT) solution was added to the solution and incubated at 37°C for 30 minutes. The RNA solution was then purified with phenol/chloroform solution for three times and then precipitated by adding 0.1 volume of 3M NaOAc and 2.5 volumes of ice-cold ethanol. The pellet was then washed twice with ice cold 70% ethanol. The RNA was then dissolved in 100 μ l DEPC water and quantified by using spectrophotometer.

RNA integrity

RNA integrity was determined according to the sharpness of the two major RNA bands, 16S and 23S rRNA (73). Ten μg of total RNA was incubated along with 10 μl of 100% deionized formamide, 3.5 μl of 37% formaldehyde and 2 μl of 5X 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer (0.1M MOPS, pH 7.0, 40 sodium acetate and 5 mM EDTA, pH 8.0) in a volume of 20 μl at 65 °C for 15 minutes. The RNA was then loaded to 1.2 % denatured gel containing 6.6% of formaldehyde along with 2 μl of loading dye (50% glycerol, 1 mM EDTA (pH 8.0)), 0.25% bromophenol blue and 0.25% xylene cyanol ff) and 1 μl of 10 mg/ml ethidium bromide (EtBr).

Primer synthesis

Primers were synthesized with a DNA Synthesizer Oligo 1000 (Beckman, Fullerton, CA). Further purification of full length primer was done with reverse phase (RP) cartridges and columns (Glen Research, Sterling, VA). The procedures were followed according to the manufacturer's protocol. *todC2* primer (ATGAATCAGACCGACACATC) and *SPEII* (AGACGGTCATGTGCTCGACCACTAGTTTCG) amplified a 940-bp *todC1* fragment. The sequence of the *todC1* primers were: GTGCTCGACCATG (anti-sense) and CACATGCTCGACC (sense) (13 mer) or GTGCTCGACC (anti-

sense) and CACATGCTCG (sense) (10 mer). These primers amplify a 382-bp fragment from the *todC1* gene of strain *P. putida* F1.

The sequence of arbitrary primers were: 70.3: ACGGTGCCTG, 80-02: CGCCCAAGCC, 80-05: ACCCAGCCG (10 mer) and Shine-Dalgarno (SD) primer: GGGGAACGACGATG (14 mer). These primers arbitrarily amplified cDNAs.

Time-course analysis of *tod* gene induction

A single colony from a YEPG plate was used to inoculate 50 ml of YEPG broth in a 250 flask and 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 solution was taken at 0, 1, 3, 6, 9 and 24 hr intervals. The cells were collected by centrifugation at 4,000 rpm. RNA was extracted with RNeasy columns (Qiagen, Germany). Five µg of each RNA was slot-blotted to a nylon membrane (ICN, Irvine, CA). The RNA slot blot was prehybridized and then was hybridized with a *todC1* probe using the same procedure as described in DNA-RNA hybridization section. The *todC1* probe was prepared by PCR using specific primer *todC2* and *SPEII* to amplify a 940 *todC1* fragment (103).

Quantitative assays for *tod* mRNA induction

Uninduced *P. putida* F1 cells were grown in 50 ml of YEPG medium overnight. Cells were harvested at $OD_{600} \approx 0.6$. Induced *P. putida* F1 cells were grown in 50 ml of MSB medium in 250 ml flask with toluene vapor. RNA was extracted with RNeasy columns and slot blotted to nylon membranes. Standard DNA was generated by using *todC2* and *SPEII* primers with the same PCR conditions as above. The PCR product was electrophoresed on a 1.8% low melting agarose and subsequently the DNA band was cut out with a razor blade. The DNA was then treated with agarase (Biolabs, Beverly, MA) and extracted three times with 1:1 phenol/chloroform. The DNA was precipitated by adding 2.5 volume of ethanol and one tenth of 3M sodium acetate at -80°C for 1 hr and then centrifuged at 12,000 rpm for 15 min at 4°C . The DNA pellet was washed with ice-cold 70% ethanol and resuspended in TE buffer and quantitated by using a spectrophotometer model DU-70 (Beckman, Fullerton, CA). 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ng/100 μl of *todC1* DNA were blotted on the same membrane with RNA as a relative standard. 100 μl of each were added to 400 μl of 0.5 N NaOH and boiled for 5 minutes before loading to the slot blot. The membrane was prehybridized and hybridized as above with a *todC1* probe. Quantitation of *tod* transcript was done by comparing with the standard using Bio Image (Visage 60, Kodak, Ann Arbor, Michigan). The procedures were followed according to manufacturer's protocol.

Reverse transcription

The first strand of cDNA was synthesized by Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Grand island, NY) by using the synthesized specific *tod* antisense primers at 37°C for 1 hr. 1 µg of total RNA was added to the reaction with 4 µl of 5X MMLV reverse transcription buffer (Gibco BRL, Grand island, NY), 1 µl of MMLV reverse transcriptase, 2 µl of 2 µM primer, 2 µl of 100 mM DDT (Gibco BRL, Grand island, NY), and 0.4 µl of 10 mM dNTP (Gibco BRL, Grand island, NY) to total reaction volume of 20 µl. The reaction was followed by incubation at 95°C for 5 minutes to denature the reverse transcriptase and RNA. 3 µl of the reaction mixture was used in the PCR reaction.

Polymerase chain reaction (PCR)

Amplification of cDNA was catalyzed by *Taq* polymerase. PCR was performed with the GeneAmp PCR kit (Perkin Elmer Cetus, Norwalk, CT) and a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). $\alpha^{32}\text{P}$ -dCTP (800 Ci/mmol, ICN, Costa Mesa, CA) was used to label DNA for autoradiography. For differential display, the final concentrations of components were 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 0.001% w/v gelatin, 0.2 µM primer; 20 mM dNTP, 0.1% triton, 6% dimethyl sulfoxide (DMSO) (SIGMA, St. Louis, MO), 1.5 U *Taq* polymerase, and 0.1 µCi/µl $\alpha^{32}\text{p}$ -dCTP to total reaction volume of 30

μl. Each amplification cycle consisted of 30 seconds at 94 °C (denaturing), 2 minutes at 50 °C (annealing), and 1 minute at 72 °C (extension). 40 amplification cycles were used for each program. The amplification cycles were followed by a 10 minutes final extension at 72°C.

For regular PCR, the reaction conditions were the same as above except 200 μM dNTP was used. For the preparation of probes, the reaction conditions were the same as regular PCR except for the addition of 1 μCi/μl α-³²p-dCTP.

Electrophoresis

The PCR products were run on a sequencing gel in side-by-side fashion. Four μl of the RT-PCR reactions were loaded onto the sequencing gel along with 4 μl of loading dye after boiling in a water bath for two minutes. The gel was run on a sequencing apparatus (Genomylx, Foster city, CA) for 2 hours at 2,700V. The gel was then dried directly on the glass plate in the machine and followed by washing three times with tap water for 3 minutes. The gel then was dried on the plate inside the machine for 30 minutes and exposed to X-ray film for 18-48 hrs.

Elution of differential cDNA and PCR reamplification

Differential cDNA bands were detected by side-by side comparison fashion. The differential bands then were softened by adding 5 μl of distilled water and cut out of the gel with a razor blade. 500 μl of TE buffer was added to

the gel slice and heated at 68°C for at least two hours. The differential bands were reamplified by using the same condition as the first PCR run. The reamplified PCR products were electrophoresed on a 2% agarose gel. The PCR reamplification products with the same molecular weight as the differential display bands were eluted to 1% low melting agarose. The DNA was used as a probe by random primer extension method (Stratagene, La Jolla, CA) or was purified as a source for cloning.

Cloning

Cloning work was done by using TA cloning kit (Invitrogen, San Diego, CA). All the procedures were followed according to manufacturer's protocols. The reamplified PCR products were electrophoresed on a 2% agarose gel. If multiple bands were detected on the agarose gel, the band which has the same molecular weight as the differential band was eluted to a low melting agarose gel. The band from the low melting agarose gel was then cut out with a razor blade. 250 µl of TBE buffer was added to the gel slice. The gel solution was then incubated at 65°C for 10 min. Four µl of agarase (Promega, Masison, WI) was then added to the solution and incubated at 42°C for 2 hrs. The mix was then filtered using a Z-spin column (GelmanScience, Ann Arbor, Michigan). The solution was subsequently purified with 1:1 phenol/chloroform. The DNA was then precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes

of ice-cold 100% ethanol and kept at -80°C for 20 minutes and centrifuged at maximal speed for 15 minutes. The pellet was washed with 70% ethanol twice and air dried for 10 minutes. Ten µl of TE buffer was added to the solution to dissolve the pellet. One µl of the purified PCR product was used for cloning. The white colonies were transferred to another plate containing 100mg/ml of ampicillin and 40 µl of 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) (0.1g/5ml). The clones were further characterized by restriction enzyme digestion and probing.

Preparation of plasmid DNA

Plasmid DNA was prepared by either the boiling method (34) or alkaline lysis method (73). Two ml of culture was grown overnight in LB broth (10 g, tryptone; 5 g, yeast extract; 10 g, NaCl/liter, pH 7.5) with 100 µg/ml of ampicillin. The cells then were harvested by centrifugation at 14,000 rpm and resuspended in 150 µl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0). 200 µl of freshly prepared solution II (0.2N NaOH and 0.1% SDS), was added and mixed by inversion. 150 µl of solution III (3M potassium acetate, 11.5% (v/v) glacial acetic acid) was then added to the lysate. The mix was then centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was then extracted once with 1:1 phenol/chloroform. Plasmid DNA was precipitated by adding 2.5 volume of ethanol at room temperature and mixed by vortexing and

allowing it to stand for 2 minutes and then centrifuged at 12,000 rpm for 5 minutes at 4°C. The crude DNA was resuspended in 20 µl of TE buffer and RNase treated at 37°C for 30 minutes. 400 µl of distilled water was added to the solution and then was extracted two times with 1:1 phenol/chloroform. Plasmid DNA was precipitated by addition of 2.5 volumes of ice-cold 100% ethanol and 1/10 of 3M acetic acid and kept at -80°C for 20 minutes and centrifuged at maximal speed for 15 minutes. The pellet was washed with 70% ethanol twice and air dried for 10 minutes.

Restriction enzyme digestion

5 µg of plasmid was added to the solution containing 2 µl of EcoRI enzyme (Gibco BRL, Grand Island, NY) and 2 µl of 10X reaction buffer 3 (Gibco BRL, Grand island, NY) to form a total reaction volume of 20 µl. The mix was incubated at 37°C for 30 minutes in a water bath. It was then run on a 1.5% agarose gel. The inserted cDNA was then eluted to 1% low melting agarose, cut out with razor blades, and directly used as a template for the probe.

Preparation of radiolabeled gene probes

Gene probes were prepared by random primer extension or by PCR. Templates were produced by reamplifying the differential bands and eluted into 1.2% low melting agarose. The desired DNA fragment was cut from the gel and

put into a preweighed tube to obtain the weight of the gel. Three ml of distilled H₂O per gram of gel was added to the tube and heated at 65°C for 5 minutes to melt the gel. 21 µl of the DNA solution and 5 µl of $\alpha^{32}\text{P}$ -dCTP were used to prepare the probe using random primer method. Further procedures were followed according to the manufacturer's protocols (Random Primer labeling Kit, Stratagene, LA Jolla, CA). PCR-generated double stranded probes were produced by standard PCR as described above. The probes were heated for 10 minutes before adding to prehybridized filters.

Preparation of slot blots

Total RNA was denatured by heating 10 ug of the RNA along with 200 µl of formamide, 70 µl of formaldehyde, and 20 µl of 20X SSC at 68°C for 15 minutes. A biodot SF slot blot apparatus (Bio-RAD, Melville, NY) was used for filtering the solution through a BiotransTM nylon membrane (ICN biochemicals, Cleveland, Ohio) that had been pre-moistened with 20X SSC. RNA solution was drawn through the filter under a vacuum of 25 cm Hg. The RNA solution was followed with two washes of 10X SSC solution. The filters were then put under UV light for 5 minutes and baked for 2 hours at 80°C.

RNA-DNA Hybridization

Hybridization was done according to the method by Church and Gilbert (20). Membranes were incubated in prehybridization solution (0.5 M Na_2HPO_4 , 1mM EDTA, 7% SDS, pH 7.3) for 4 hours at 65 °C in a shaking water bath. Radiolabeled probe was added to the prehybridization solution and incubated for 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 exposed to x-ray film (Kodak BIOMAXTM MR, Eastman Kodak, Rochester, NY) at -80°C with intensifying screens. Film was developed after 18-48 hrs.

Screening of positive clones

The reamplified PCR products were electrophoresed in 1 % low melting point agarose and the bands of similar MW to the bands on the sequencing gel were excised and eluted. These PCR products were used to probe RNA slot blots from induced and uninduced samples. Only the differential bands which were verified to be differentially expressed were used for further cloning. After the differential bands were cloned to the pCRII vector (Invitrogen, San Diego, CA), plasmid DNA was purified by the alkali lysis method and digested with restriction enzymes to obtain the insert. The inserted cDNA was used as a

probe to hybridize with slot blots from induced and uninduced samples. Only the inserts which showed differential signal were sequenced.

Sequencing and Data search

Sequencing was done by an automated unit (Applied Biosystems 373A, Foster city, CA) (University of Tennessee, Department of Microbiology Molecular Biology Resource Facility). Double-stranded plasmid DNA was used for sequencing reactions. M13 forward primer (17 mer: GTAAAACGACGGCCAGT) and reverse primer (17 mer: CAGGAAACAGCTATGAC) were used as sequencing primers.

The DNA sequence information was used to search the nucleic acid databases (GenBank/National Center for Biotechnology Information [NCBI]) for similar sequence (3). This was performed by sending the sequence by e-mail to the GenBank BLAST server.

Soil microcosm simulation

RNA was obtained from soil microcosms inoculated with *Pseudomonas putida* F1 cells, which were intended to mimic a contaminated environment. The soil samples were obtained from bore holes at Columbus air force base (CAFB), Columbus, MS. They were well characterized chemically and microbiologically as part of a natural attenuation (NAT) study at the CAFB groundwater test

facility. 16S universal oligo probing revealed there were about 8.67×10^7 cells/g soil. Slot blot hybridization of the soil with specific probes indicated there were alkane, naphthalene, toluene, and xylene degraders. Microcosms were inoculated with *Pseudomonas putida* F1 in 10^8 cells/g soil and incubated at 26 °C and shaken at 225 rpm overnight. To induce cells, toluene was added in the saturated vapor phase.

Optimization of extraction buffer for soil samples

Different concentrations of hexadecyltrimethyl ammonium bromide (CTAB), sodium dodecyl sulfate (SDS), Na_2PO_4 , polyvinylpyrrolidone (PVP), pyrophosphate glass and sodium pyrophosphate (PYRO) were tested to determine the best buffer system for soil RNA extraction. A PCR constructed *nahA* template linked with T7 promoter was used for *in vitro* transcription reaction (15). Single stranded *nahA* mRNA probe synthesis was carried out with T7 RNA polymerase in the presence of α - ^{32}P -UTP as a radioactive substrate. The procedures were recommended by the manufacturer's protocols (MEGAscript kit, Ambion, Austin, Texas). After precipitation of the RNA probe, the RNA was dissolved in 100 μl of DEPC and 1 μl of the RNA was taken to count the radioactivity by using a model LS3801 liquid scintillation counters (Beckman, Fullerton, CA). 1 μl of the solution was added to the soil sample as an internal standard to trace the RNA recovery rate.

A small scale extraction buffer system (1.5 ml centrifuge tube) was used to screen the different buffers. 500 μ l of the above buffer was added to the centrifuge tube with 100 μ l of soil slurry and probes. 600 μ l of 1:1 phenol/chloroform were then added to extract the RNA probe. The solution was vortexed for 30 seconds and kept at 60°C for 5 minutes. The tubes were then centrifuged at maximal speed at 4°C for 5 minutes. The supernatant was transferred to another tube and 5 μ l of the solution was taken to count the radioactivity.

Buffer systems tested for small scale prep of RNA from soil samples included:

1. 2% CTAB (w/v)
2. 2% CTAB + 1% PVP
3. 2% SDS
4. 2% SDS + 1% PVP
5. 0.2M Na_2PO_4 (pH 8.0)
6. 2% SDS + 1% PVP + 0.5% PYRO
7. 0.2M Na_2PO_4 (pH 8.0)
8. 0.2M Na_2PO_4 + 0.5% PVP
9. 0.2M Na_2PO_4 + 1% PVP + 0.5% PYRO
10. 0.1M Na_2PO_4
11. 0.1M Na_2PO_4 + 1% PVP
12. 0.1M Na_2PO_4 + 1% PVP + 0.5% PYRO

For large scale extraction buffer systems, different pyrophosphate concentrations of buffer systems were used to optimize the pyrophosphate concentration. 5 ml of 0.2M Na_2PO_4 (pH 8.0), 100 mM EDTA and 1% SDS solution was used as extraction buffer. 2 μ g of CFAB soil was added to the

solution with 0.1 g PVP and different amount of pyrophosphate solution and 1 μ l of the RNA probe (160,000 cpm). 10 ml of 1:1 phenol/chloroform 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 10,000 rpm at 4°C for 15 minutes. The supernatants were removed to new tubes and 5 μ l of the solution was taken from each tube to measure the recovery rate.

Buffer systems tested for maximal prep of RNA from soil samples included:

O.2M Na ₂ PO ₄ + 100mM EDTA +1% SDS	CAFB soil	PVP	5% PYRO solution	dH ₂ O
5 ml	2 g	0.1 g	1 ml	4 ml
5 ml	2 g	0.1 g	2.5ml	2.5 ml
5 ml	2 g	0.1 g	5ml	0 ml
5 ml	2 g	0.1 g	1ml	5 ml

RNA soil extraction

50 ml of uninduced and induced F1 cells were grown as above. The soil RNA extraction was performed according to a modified hot phenol method (25). 20 g of CAFB soil was added to 20 ml of each sample. Cells were collected by centrifugation at 6,000 rpm for 10 minutes. The cells were then washed with 20

ml of 120mM NaHPO₄ buffer, pH 5.2 and shaken on a rotary shaker for 5 minutes. After centrifugation at 6,000 rpm for 10 minutes, the supernatant was decanted and discarded. The procedure was repeated 3 times. The pellet was resuspended in 5 ml of Tris lysis buffer, 50mM Tris, pH8.0, 0.25 mM EDTA, pH 8.0 and 20% sucrose containing 5 mg/ml lysozyme. Following incubation at room temperature for 10 minutes, the soil mix was centrifuged at 6,000 rpm for 10 minutes and the supernatant was discarded. The pellet was resuspended in a 10 ml of extraction buffer, 0.2M NaHPO₄, 100mM EDTA, 1% SDS pH 8.0 pre-warmed at 60°C in a reusable, siliconized 25 ml glass centrifuge tubes. 5 ml of phenol, pH 5.2, and 5 ml of chloroform were then added and the tubes were heated for 5 minutes at 60°C. The tubes then shaken for 5 minutes on a wrist action shaker and centrifuged at 10,000 rpm for 15 minutes. The extraction procedures were repeated once and the supernatant was transferred to a new tube. 10 ml of ice-cold isopropanol and 1 ml of 3M acetic acid, pH 5.2, were then added to the tubes and allowed to sit at -80°C for 30 minutes. The tubes were then centrifuged at 10,000 rpm for 30 minutes at 4°C. The slightly brown colored pellet was then dissolved in 100 µl of DEPC water and treated with 2 µl of RNase-free DNase I (Boehringer Mannheim, Germany). 0.1 volume of Mg/DTT, was added to the solution and incubated at 37°C for 30 minutes. The RNA solution was further purified using RNeasy columns according to the manufacturer's protocol.

4. RESULTS

Growth curves of *P. putida* F1 under induced and uninduced conditions

The growth curves of induced and uninduced *P. putida* F1 are shown in Figure 3. Uninduced *P. putida* F1 was grown in YEPG medium. Induced *P. putida* F1 was grown in MSB medium saturated with toluene vapor. The RNA was extracted when the OD₆₀₀ value reached 0.6. The lag phase of the induced sample was longer than that of uninduced sample. This might be due to the change of the physiological state of the cells in order to fit the new environment (MSB medium).

Time-course analysis of *tod* gene induction and quantitation of *tod* transcripts

Transcription of *tod* genes was induced by exposure of *P. putida* F1 to toluene vapors. Samples were taken from 0 to 24 hrs to detect *tod* transcript induction. The *tod* gene expression was induced after 3 hours. After 24 hour period, sufficient induction was reached (Figure 4). Quantitation of *tod* transcripts was accomplished by comparison with *tod* DNA standards. 0.01 to 10 ng of *todC1* fragment DNA were loaded to the same blot as the standards (Figure 5). The *tod* transcripts were almost undetectable in the uninduced sample. The amount of *tod* transcripts was determined to be 10⁷ transcripts (0.4 µg mRNA)/10 µg of total RNA in the induced sample when OD₆₀₀ was $\cong$ 0.6.

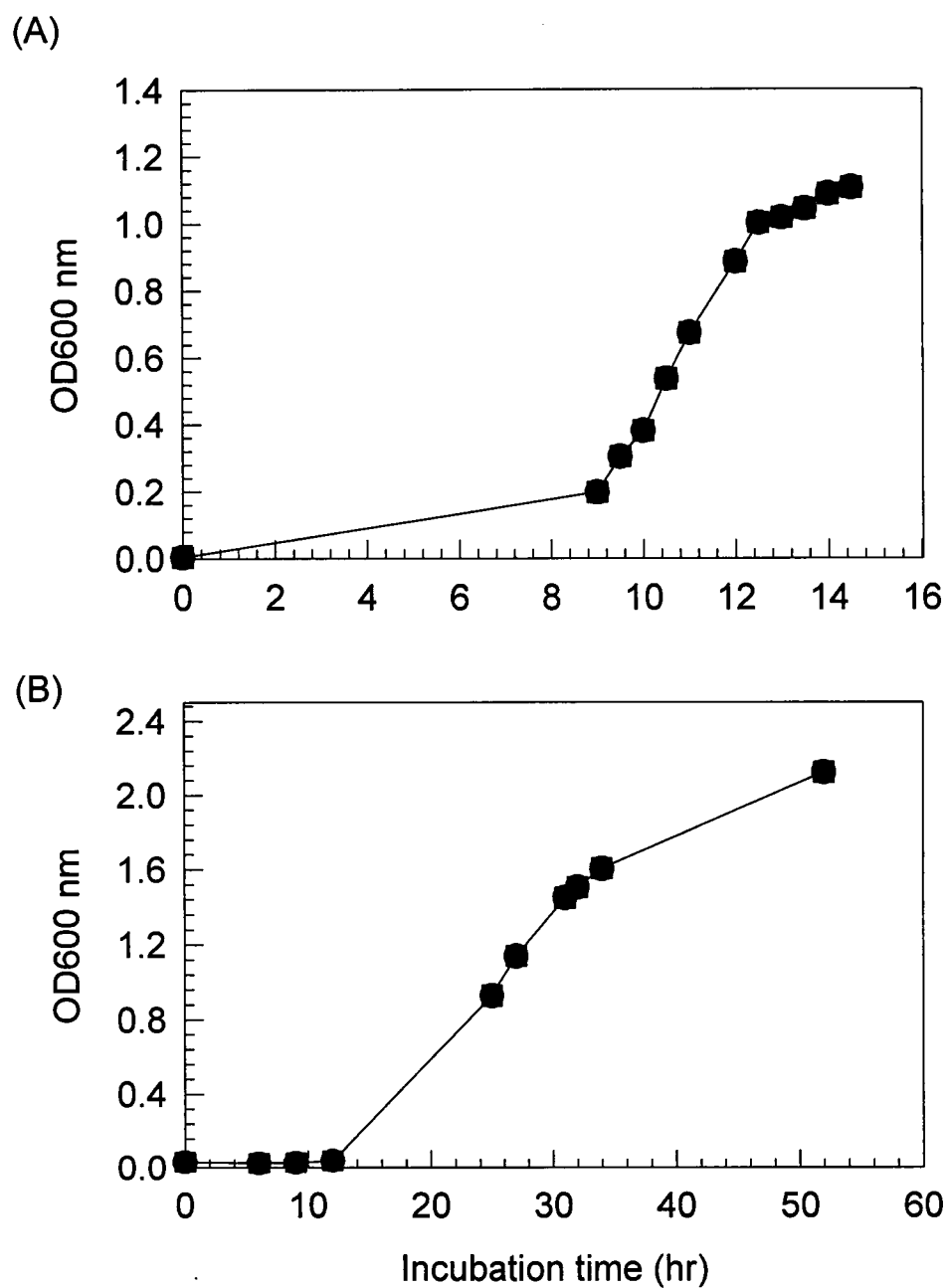


Figure 3. Growth curve of *P. putida* F1. (A) Growth curve of *P. putida* F1 cells in YEPG medium at 26°C. (B) Growth curve of *P. putida* F1 cells in minimal salt basal medium (MSB) with toluene vapor at 26°C.

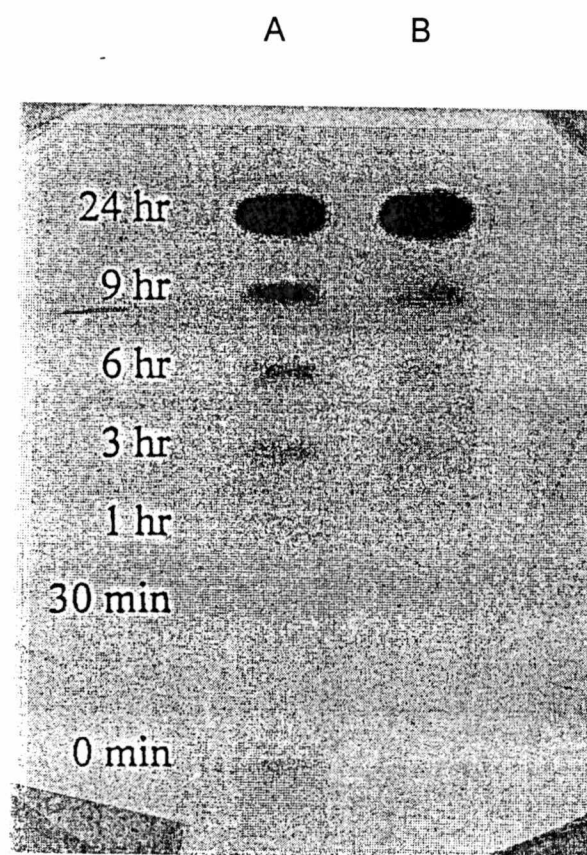


Figure 4. Time-course analysis of *tod* transcripts induction. Total RNA of toluene induced *P. putida* F1 cells were isolated at different time period, 0 min, 30 min, 1 hr, 3 hr, 6 hr, 12 hr and 24 hr. 10 ug of duplicate total RNA (lane A, B) of each sample were analyzed by RNA slot blot hybridization. A ^{32}P -labeled *tod* C1 fragment was used to probe the *tod* transcripts.

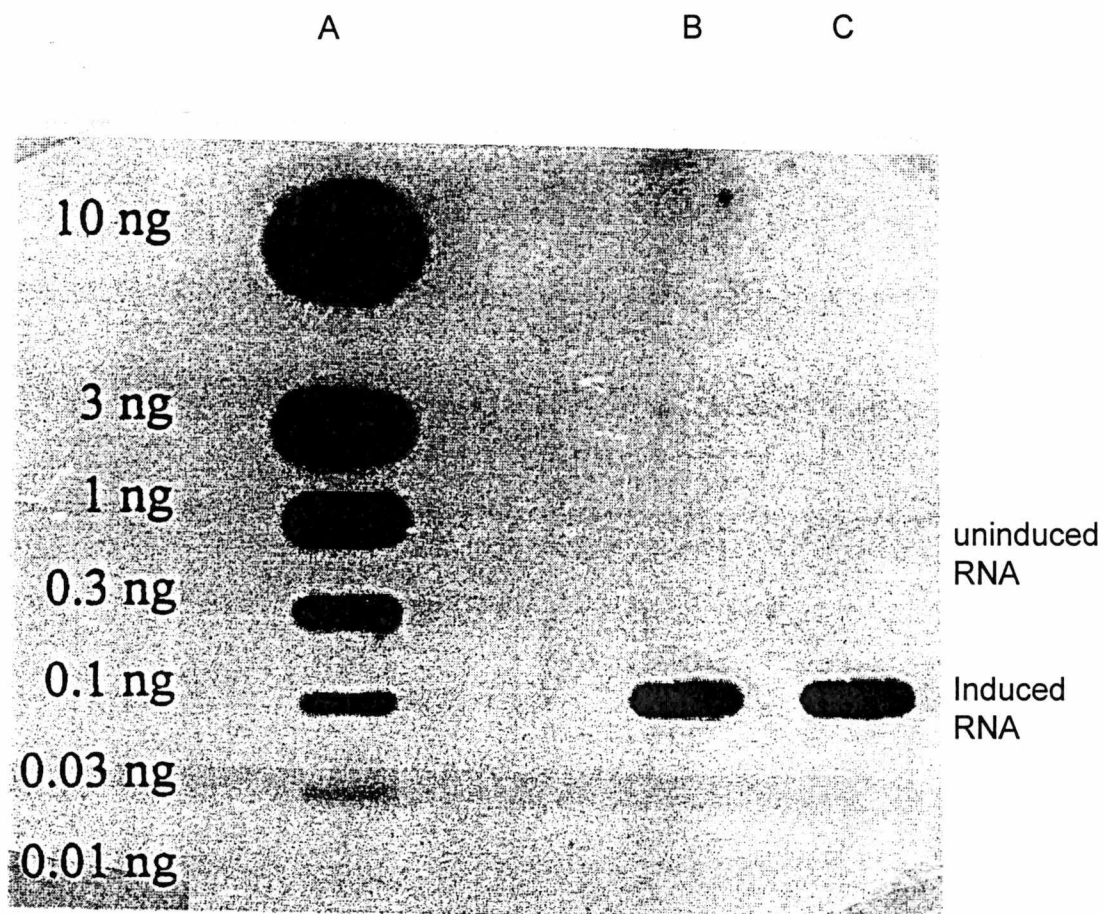


Figure 5. Quantitation of *tod* transcripts from toluene induced and uninduced *P. putida* F1 cells. *P. putida* F1 cells were inoculated until OD_{600} reached 0.6. (A) 10, 3, 1, 0.3, 0.1, 0.03, 0.01 ng of *tod* DNA were used as a standard. (B, C) 10 μ g of duplicate total RNA from induced and uninduced total RNA from *P. putida* F1 was loaded to the RNA blot. The specific ^{32} P labeled *todC1* fragment was used to hybridize the blot.

Fingerprinting of mRNA from pure culture and soil samples

Differential display of RNA from pure culture *P. putida* F1 cells was successful and resulted in a 382-bp *todC1* fragment of the expected size in induced samples (Figure 6). Results indicate that the expected *todC1* fragment was only detected in induced samples. Some minor differences of band patterns between toluene-induced and uninduced samples were also detected. There were an average of 70 bands per lane on sequencing gel. Identification of the *todC1* fragment was done by sequencing and comparison with the current GenBank database. Sequence analysis revealed a 98% homology with the toluene dioxygenase *todC1* fragment.

Soil samples were prepared by adding *P. putida* F1 cells to soil microcosms and the RNA was subsequently extracted by the modified hot phenol method. Identical amounts of soil extracted and pure culture derived RNA were used as templates for DD-RT-PCR. RNA fingerprints of the RNA from pure culture and soil samples were almost identical (Figure 6). Observations revealed that the *todC1* band was fainter in the soil sample versus the pure culture sample. This may be due to the fact that the recovery rate of different RNA varies. Moreover, fine soil particles or humic acids may also interact with the RNA, therefore, occupying the binding sites resulting in less efficient amplification of cDNA.

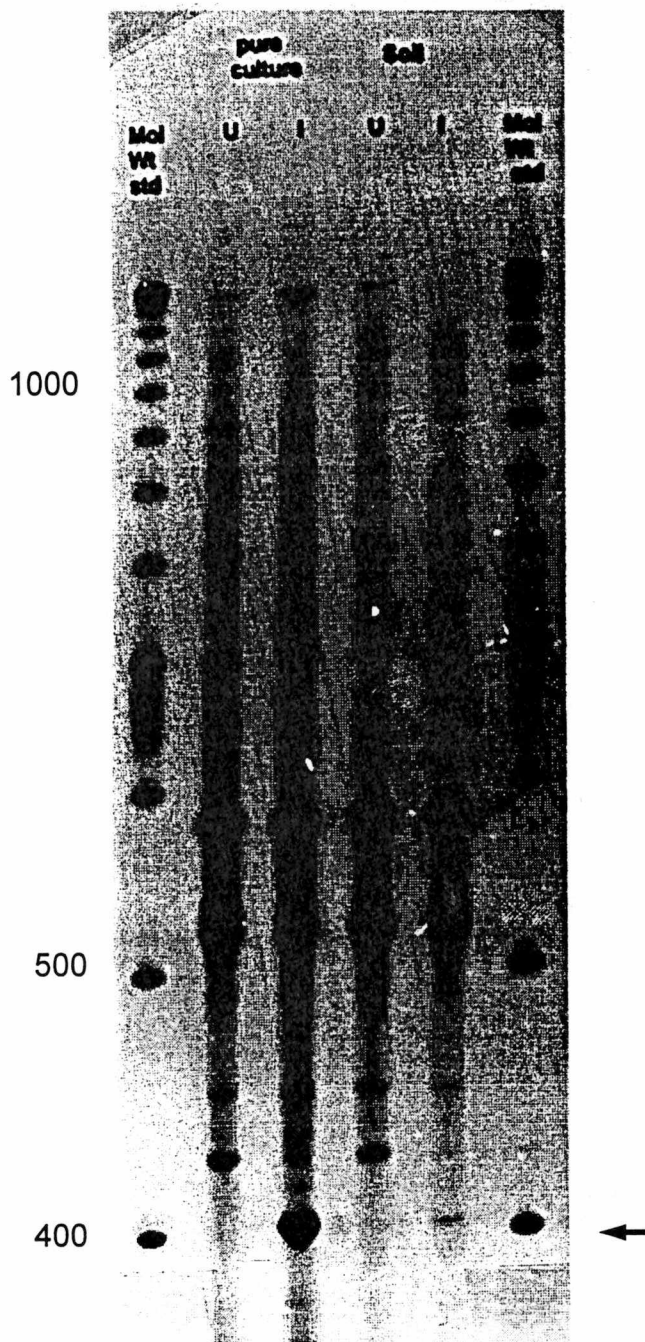


Figure 6. Differential display of toluene induced and uninduced RNA from *P. putida* F1 isolated from pure culture and soil samples. Total RNA from uninduced and toluene induced pure culture and soil samples were used for RNA fingerprinting. Expected differential *todC1* fragment was indicated by an arrow.

Optimization of RNA fingerprinting conditions from pure culture

Several parameters were varied in parallel using the same primers to allow optimization of the reaction conditions: I) template concentrations: 150 ng, 15 ng, 0.15ng, 0.015ng, and 0.0015ng. II) Magnesium concentration: 8, 4, 2, and 1 mM. III) dNTP concentrations: 200, 20, 2, and 0.2 μ M. IV) primer concentration: 2, 0.2, 0.02, and 0.002 μ M. V) annealing temperature: 50, 40, and 30°C. VI) primer lengths: 10 mer and 13 mer.

When a series of decreasing template concentrations were used, the *todC1* could still be detected when the total RNA was lowered to 0.015 ng. Thus, the *tod* mRNA seemed to be induced by at least 10^4 fold in the presence of toluene in MSB medium. In agreement with other labs, when the template concentration was lowered, the band patterns were similar; but the sensitivity of the reaction to lower abundance messages was much less (10,24,49). It also decreased the background, thus only the abundant cDNA bands showed up. RNA at a concentration of 0.015 ng could be detected with only a slight loss of sensitivity (Figure 7).

The optimal magnesium concentration was about 1 to 1.5 mM when a primer concentration of 0.2 μ M was used (Figure 8). The effect of changing dNTP concentration was considerable (Figure 9). Above 1 mM, the band number and intensity dramatically decreased. The optimized primer concentration was about 0.02 μ M to 0.2 μ M. Above 20 μ M, mis-priming greatly

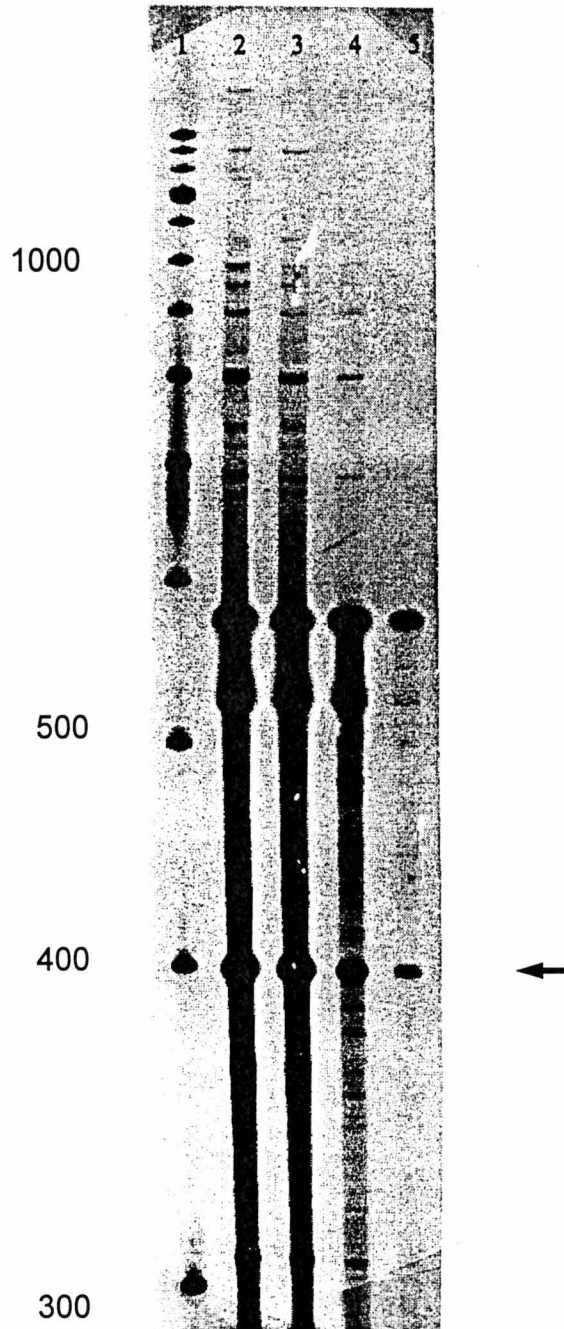


Figure 7. Sensitivity of differential display as a function of amount of input RNA. Lane 1: end-labeled 100 bp ladder. Total toluene induced RNA were 15 ng (lane2), 1.5 ng (lane 3), 0.15 ng (lane 4), 0.015 ng (lane 5). Predicted *todC1* fragment is indicated by arrow.

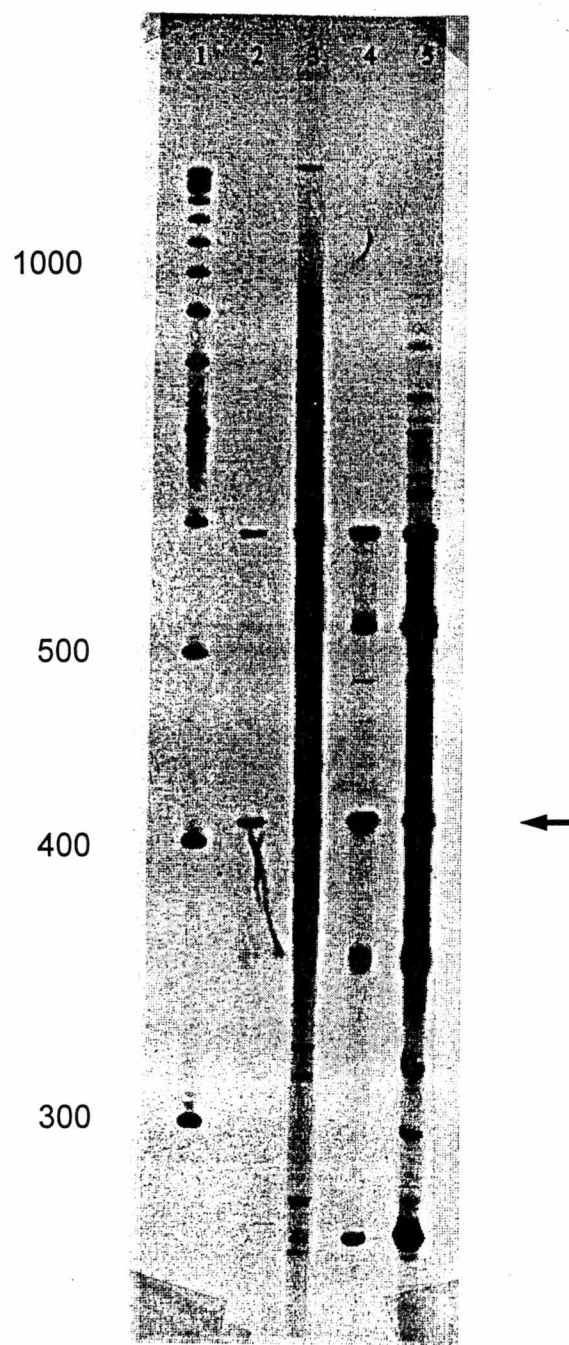


Figure 8. Effect of magnesium concentrations on the complexity of band patterns. Lane 1: end-labeled 100 bp ladder. $MgCl_2$ concentrations were 8 mM (lane 2), 4 mM (lane 3), 2 mM (lane 4), and 1 mM (lane 5). The *todC1* fragment is indicated by the arrow. Lane 1: end-labeled 100 bp ladder.

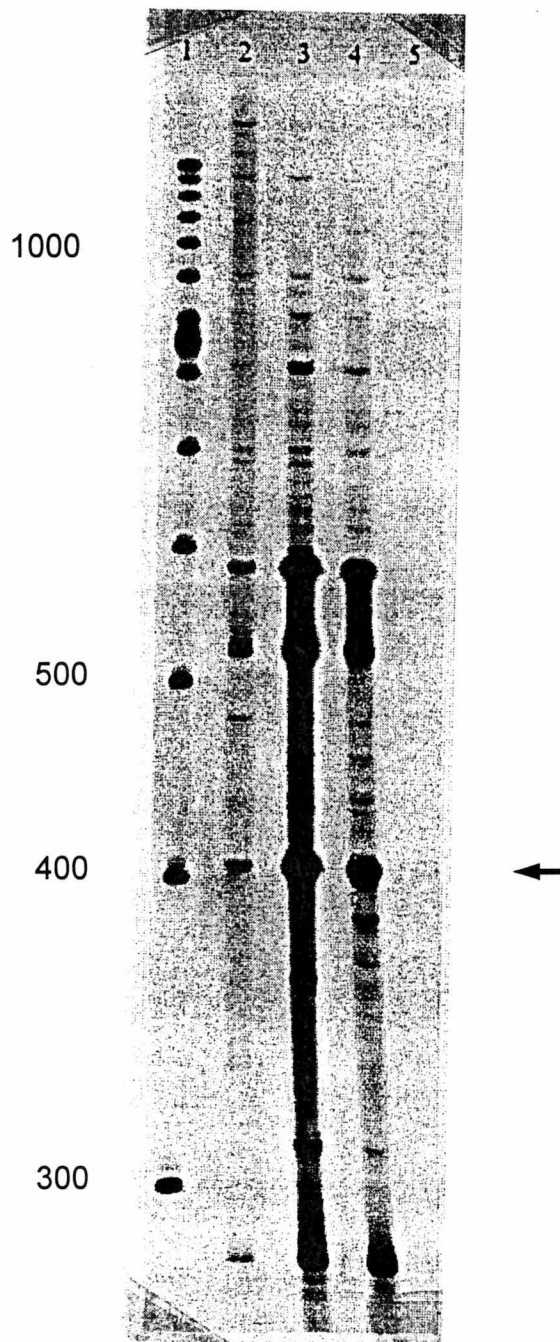


Figure 9. Effect of nucleotide concentrations on the complexity of band patterns. Lane 1: end-labeled 100 bp ladder. Nucleotide concentrations were 200 μ M (lane 2), 20 μ M (lane 3), 2 μ M (lane 4), and 0.2 μ M (lane 5). The amount of total RNA was 150 ng for the RT-PCR reaction. The *todC1* fragment is indicated by the arrow.

increased. When primer concentrations were lower than 0.02 μ M, the bands' intensity also dramatically decreased (Figure 10). When the annealing temperature was increased from 30 to 50°C, only a slight band pattern change was observed (Figure 11). The primer length also had a great influence on fingerprinting band patterns (Figure 12); more bands per lane were obtained by using longer primers.

Fingerprinting of RNA using non-specific arbitrary primers by enrichment of the *tod* transcripts in RT step.

Prior to the use of arbitrary primers for both the RT and PCR steps, a preliminary experiment was performed by using a *tod* specific primer for the RT reaction and arbitrary primers (70.03, 80.02 and 80.05) were used to examine the arbitrary primer effect in the PCR step. The DD-RT-PCR was run under the same conditions as above. The RT reaction was performed using *todC1* antisense primers (13mer) to enrich *tod* transcripts followed by the addition of a non-specific primer in the PCR reaction. Theoretically, the more primers used in PCR step will result in more band amplification. However, there were more bands per lane when only one non-specific primer was used than when two specific primers were used in the PCR condition (Figure 13). This may be due to the fact that these non-specific primers have been screened to obtain better amplification results. Several differential bands were detected. Two of them were verified as differential bands. Sequence results revealed that both

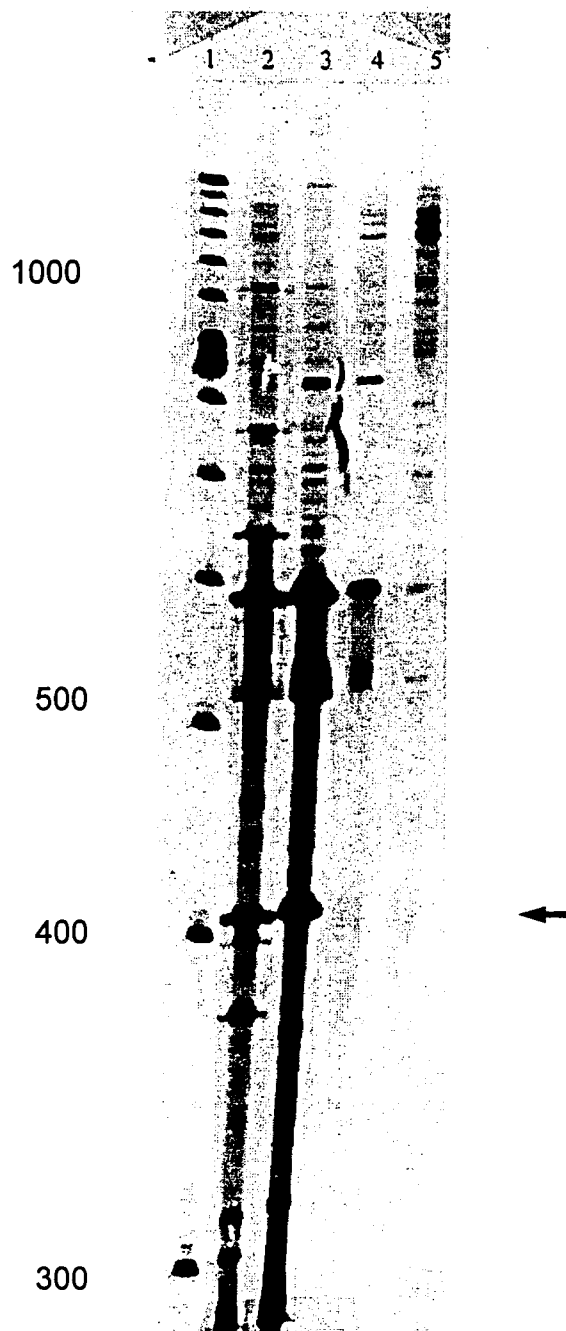


Figure 10. Effect of primer concentrations on the complexity of band patterns. Lane 1: end-labeled 100 bp ladder. Primer concentrations were 2 μ M (lane 2), 0.2 μ M (lane 3), 0.02 μ M (lane 4), and 0.002 μ M (lane 5). Total toluene induced RNA was 150 ng for the RT-PCR reaction. The *todC1* fragment is indicated by arrow.

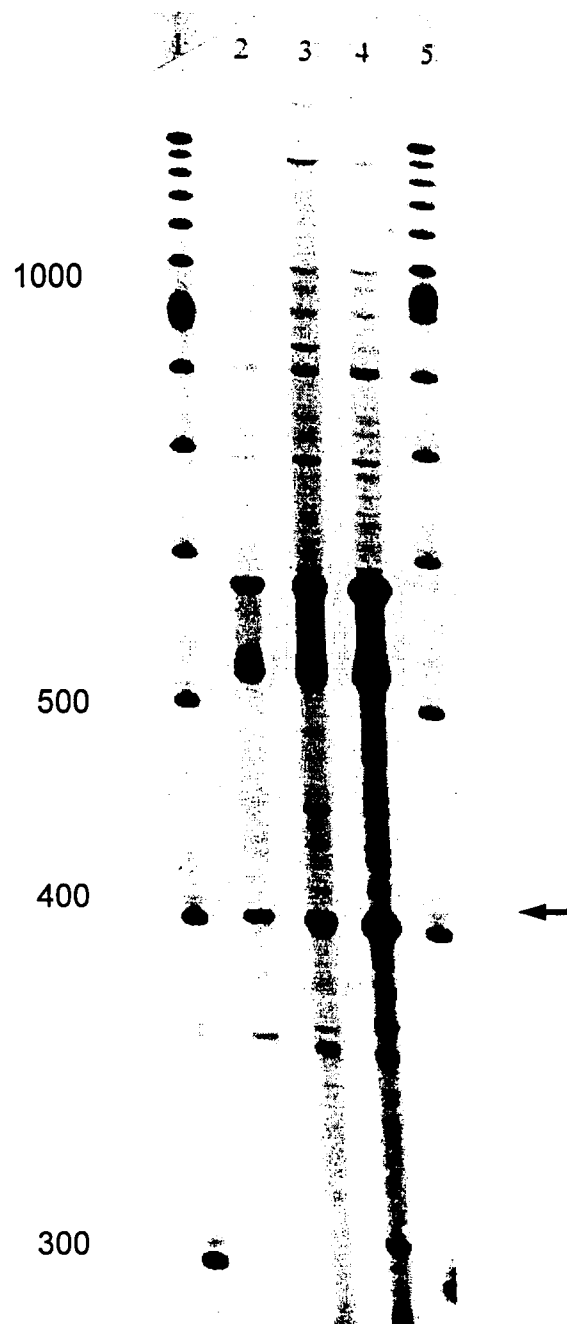


Figure 11. Effect of annealing temperature on the complexity of band patterns. Lane 1: end-labeled 100 bp ladder. Annealing temperatures were 30°C (lane 2), 40°C (lane 3), and 50°C (lane 4). The amount of total RNA was 150 ng for the RT-PCR reaction. *todC1* fragment is indicated by an arrow.

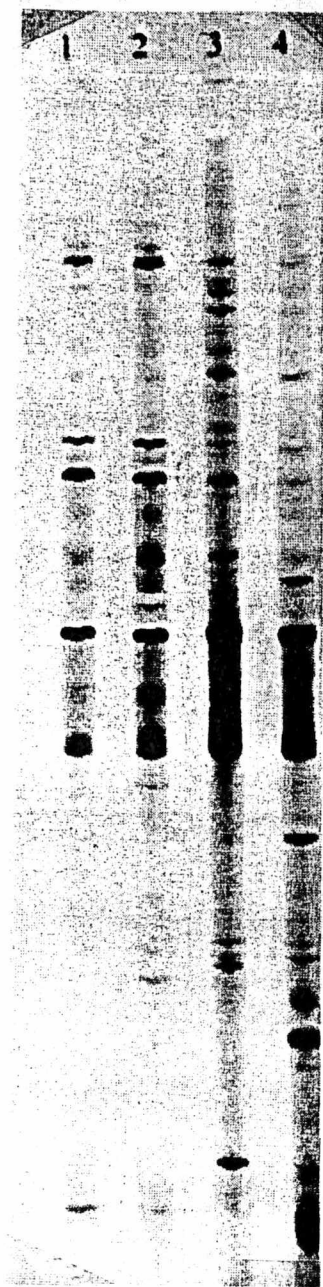


Figure 12. Effect of primer length on the complexity of band patterns. The primers' lengths were 10 mer (lane 1 and 2), and 13 mer (lane 3 and 4). RNA fingerprinting was derived from uninduced total RNA (lane 1 and 3) and toluene induced total RNA (lane 2 and 4). The *todC1* fragment is indicated by an arrow.

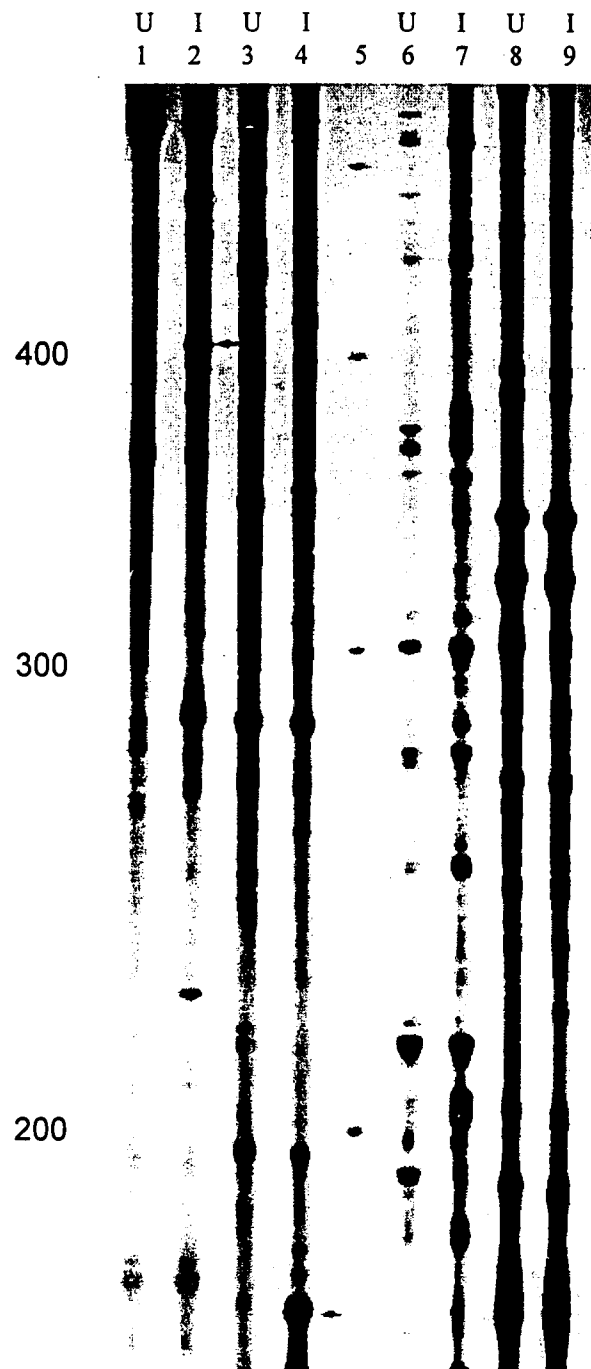


Figure 13. Differential display using specific and non-specific short primers. RNAs from uninduced (U) and toluene induced (I) *P. putida* F1 cells were reverse transcribed to cDNA using specific *todC1* antisense primer (13mer: GTGCTCGACCATG). Four different short primers were separately added to the PCR reaction to fingerprint the cDNA sample. Lane 1,2: *todC1* sense primer (CACATGCTCGAC). Lane 3,4: 70-03 primer (ACGGTGCCTG). Lane 5: 100 bp ladder. Lane 6,7: 80-02 primer (CGCCCAAGCC). Lane 8,9: 80-05 (ACCCAGCCG) primer. Arrows indicate true differential bands.

differentially expressed genes were partial *todC1* fragments, regardless of the primer used. The specific *todC1* sense and antisense primers flanked bases 1172 to 1553 and amplified a 382-bp cDNA fragment. The non-specific primer, 70.03, flanked bases 1035 to 1203 and amplified a *todC1* 169-bp cDNA fragment.

Fingerprinting of RNA using non-specific arbitrary primers

Non-specific arbitrary primers were used in both the RT and PCR steps to verify the differential display technique. The 70.3 primer was used in the RT reaction followed by adding either a 70.3/SD or both primers in the PCR reaction. After electrophoresis, several differential bands were detected (Figure 14). Three of these were cut out, eluted from the gel, and subsequently used as probes to verify the differential bands. Only one of them was verified to be a truly differentially expressed. Sequencing revealed that the differentially expressed gene has a 99% homology with *todC2* gene.

PCR reamplification

After detection of differential bands on the sequencing gel, the differential bands were cut out and eluted into TE buffer. Aliquots of the PCR products were amplified using the same conditions as the first PCR reaction. Reamplification of the differential bands resulted in expected bands as shown in Figure 15A. The PCR products were then used for the purpose of cloning.

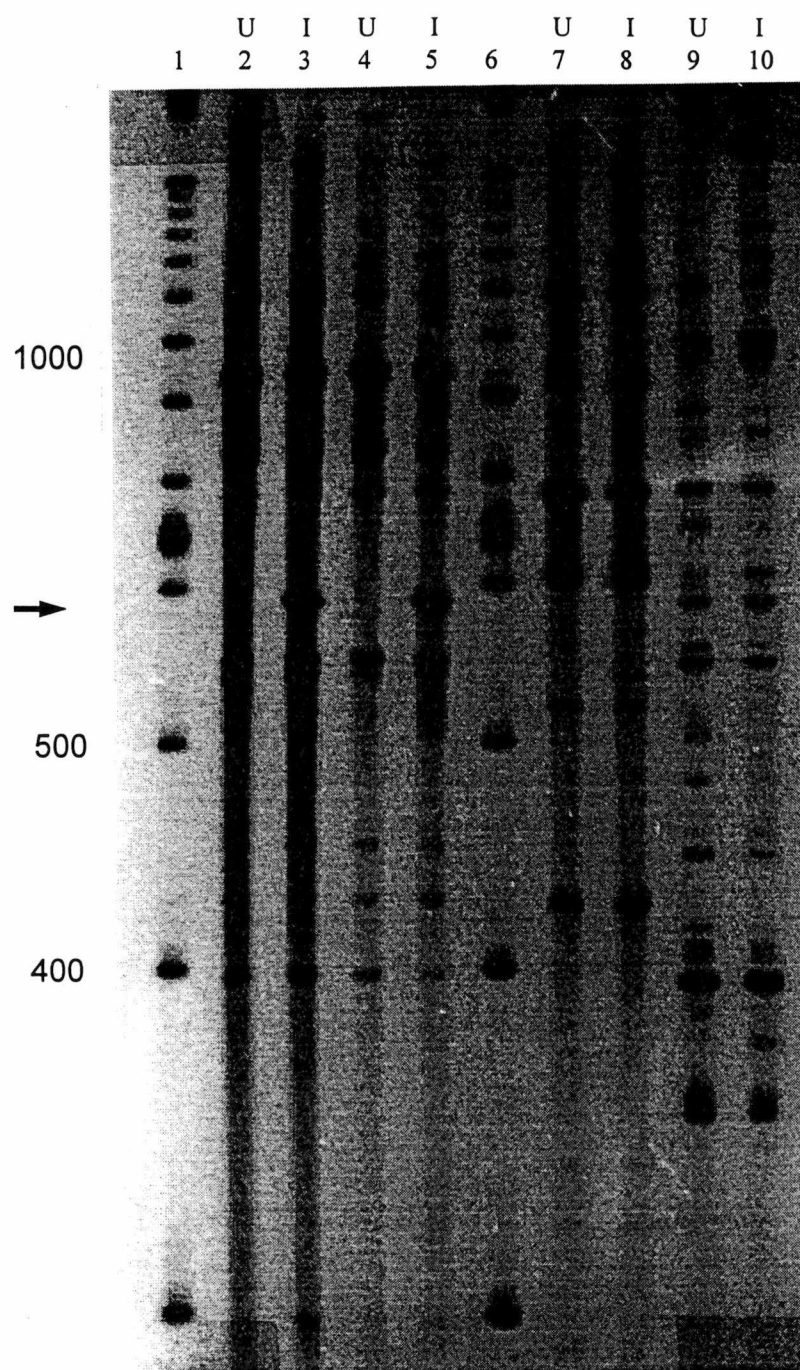
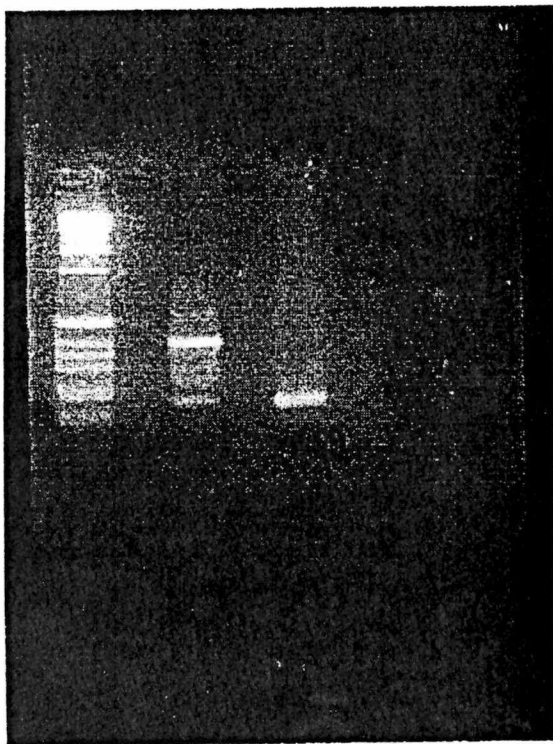


Figure 14. Fingerprinting of RNA using arbitrary primers. RNAs from uninduced (U) and toluene induced (I) *P. putida* F1 cells were reverse transcribed to cDNAs using 70.3 arbitrary primer. Either 70.3 or SD or both primers were added to fingerprint the cDNAs samples. Lane 1,6: 100 bp ladder. Lane 1,2 3, 4: different batch of uninduced and induced RNA using both 70.3 and SD primers in PCR reaction. Lane 7,8: 70.3 primer. Lane: 9, 10: SD primer. Arrow indicates true differential bands.

(A)

1Kb
ladder 410 170



(B)

1Kb 410.a 170.c
ladder clone clone

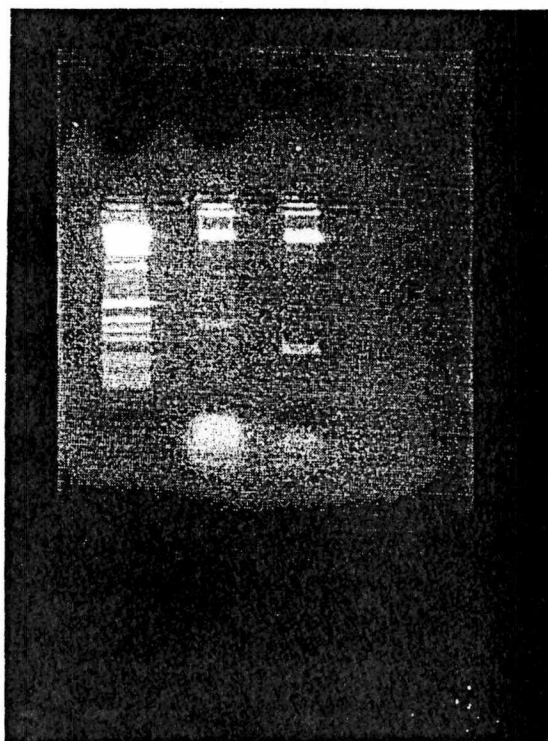


Figure 15. Reamplification of differential bands and restriction enzyme digestion of the cloned plasmid. (A) 410 and 170 differential bands were reamplified and run on agarose gel. (b) The 410 and 170 reamplified bands then were cloned to the plasmid and digested with EcoRI.

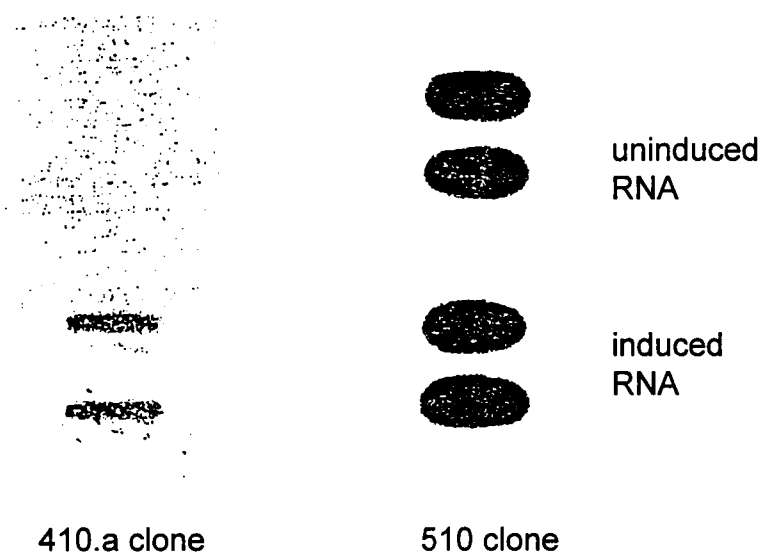
Verification of differential bands and clones

P. putida F1 RNA slot blots were probed with radiolabeled probes amplified from the differential bands from the sequencing gel. Bands 410 and 170 showed a difference in intensity between the induced and uninduced samples. The reamplified PCR products then were directly cloned into the pCRII vector (Invitrogen). 10 colonies were selected randomly to screen for the insert. The inserts were restricted with EcoRI from the plasmid (15B) and then were labeled by the random primer method (Stratagene) to screen true differentially expressed inserts. After the inserts were confirmed to be differentially expressed, the inserts were sequenced. The 410.a insert was verified to be differentially expressed (Figure 16A). Sequencing and GenBank data search showed that the specific band had a 98% homology with *todC1* sequence (Figure 17, 18).

The insert of clone 170.c also showed a difference between induced and uninduced samples (Figure 16B). After sequencing and GenBank data search, the data revealed that the differential gene had a 99% homology with the *todC1* partial sequence (Figure 19). Sequence results revealed that the amplification of partial *todC1* gene was due to mismatch amplification. Two mismatches were tolerated.

When non-specific primers were used to perform the DD-RT-PCR, several differential bands were detected and only one of them was verified to be a truly differentially expressed. Sequencing revealed that the differentially expressed

(A)



(B)

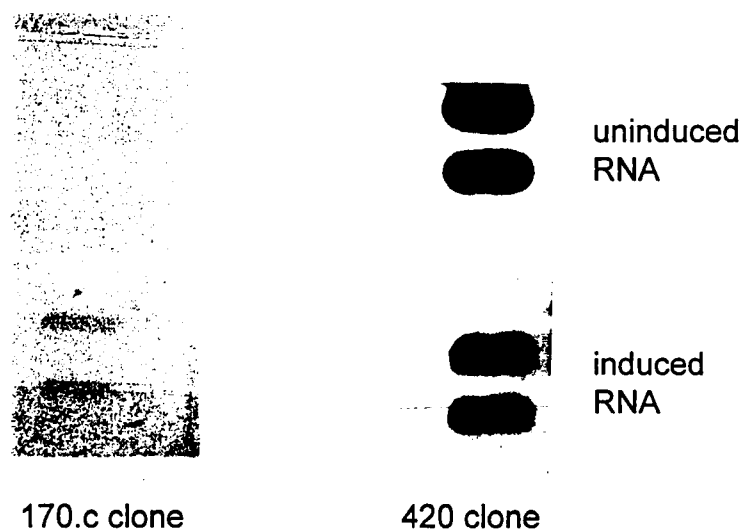


Figure 16. Confirmation of 410.a and 170.c clones by slot blot analysis. Total RNA was isolated from induced and uninduced *P. putida* F1 cells. 10 ug of duplicate total RNA was loaded onto each blot. (A) Blot was hybridized with ^{32}P -labeled 410.a clone inserted fragment. Identical blot was hybridized with 510 clone inserted fragment. Clone 510 contained rDNA insert. (B) Blot was hybridized with ^{32}P -labeled 170.c clone inserted fragment. Identical blot was hybridized with 420 clone inserted fragment. Clone 420 contained rDNA insert.

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
250,329 sequences; 353,753,039 total letters.

			High	Smallest	
			Score	Sum	
Sequences producing High-scoring Segment Pairs:				Probability	
				P(N)	N
gb M17904 PSEBDO	Pseudomonas putida benzene dioxygen...	1738	3.6e-145	2	
gb J04996 PSETODC1C	P.putida toluene dioxygenase (iron-...	1738	5.0e-145	2	
gb U15298 PSU15298	Pseudomonas sp. chlorobenzene dioxy...	1288	9.3e-106	2	
gb L04642 PSEBEDC12A	Pseudomonas putida oxygenase alpha ...	1297	2.6e-100	1	
dbj D32142 RERBPHA1	Rhodococcus sp. genes for biphenyl ...	1031	3.9e-78	1	
dbj D17319 PSEBPHABCC	Pseudomonas sp. genes for biphenyl ...	752	6.2e-55	1	
gb M83673 PSEBPHABC	P.pseudoalcaligenes dioxygenase (bp...	731	3.6e-53	1	
gb M86348 PSEBPHA	Pseudomonas sp. biphenyl dioxygenas...	724	1.4e-52	1	
gb U47637 CTU47637	Comamonas testosteroni biphenyl dio...	707	1.2e-50	1	
emb X80041 RGBPHA	R.globerulus genes bphA1-A4	676	2.7e-47	1	
gb U27591 RSU27591	Rhodococcus sp. strain M5 biphenyl/...	676	2.7e-47	1	
dbj D37828 PSECUMA	Pseudomonas fluorescens cumA1, cumA...	662	5.5e-46	1	
gb U51165 COU51165	Cycloclasticus oligotrophus XYLX (X...	515	3.6e-33	1	
emb Z37966 ECDIGOXAB	E.coli gene for digoxigenin alpha a...	302	4.0e-15	1	
emb Z61172 HS47B11F	H.sapiens CpG DNA, clone 47b11, for...	118	0.9999	1	

Figure 17. Comparison of cloned cDNA sequences (410.a) against GenBank database. Sequence results reveal that the cDNA sequences have a high sequence homology with benzene dioxygenase and toluene dioxygenase.

```

Query:      1  CACATGCTCGACCGCACCGAGGCCGGCACCGAAGCGATCCCGGGCGTGCAGAANTGGGTC 60
               |||
Sbjct: 1172  CACATGCTCGACCGCACCGAGGCCGGCACCGAAGCGATCCCGGGCGTGCAGAAGTGGGTC 1231

Query:      61  ATTCCCTGTAACTTGGAAATTCGCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGG 120
               |||
Sbjct: 1232  ATTCCCTGTAACTTGGAAATTCGCCGCAGAGCAGTTTTGCAGCGACATGTACCATGCCGGG 1291

Query:     121  ACGACCTCGCATCTGTCTGGCATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGAC 180
               |||
Sbjct: 1292  ACGACCTCGCATCTGTCTGGCATCCTGGCAGGCCTGCCAGAAGACCTTGAAATGGCCGAC 1351

Query:     181  CTTGCTCCGCCGACAGTTGGCAAGCAGTACCGTGCGTCATGGGGCGGACATGGAAGTGGC 240
               |||
Sbjct: 1352  CTTGCTCCGCCGACAGTTGGCAAGCAGTACCGTGCGTCATGGGGCGGACATGGAAGTGGC 1411

Query:     241  ATCTATGTGCGCGACCCCCAATCTGATGCTTGCCATCATGGGGCCAAAGGTCACCAGCTAC 300
               |||
Sbjct: 1412  TTCTATGTGCGCGACCCCCAATCTGATGCTTGCCATCATGGGGCCAAAGGTCACCAGCTAC 1471

Query:     301  TGGACCGAACGCCCCGCGTCGGAAAAGGCGGCCGAACGTCTGGGTAGCGTTGGGAGCG 358
               |||
Sbjct: 1472  TGGACCGAAGGCCCCGCGTCGGAAAAGGCGGCCGAACGTCTGGGTAGCGTTGGGAGCG 1529

Query:     353  GGAGCGCGGCTCGAAACTCATGGTCGAGCACA 384
               |||
Sbjct: 1522  GGAGCGCGGCTCGAAACTCATGGTCGAGCACA 1553

```

Figure 18. Comparison of nucleotide sequences of the cDNA clone (410.a) against toluene dioxygenase partial sequences. Query: cloned cDNA sequences. Sbjct: *P. putida* toluene dioxygenase (*todC1*) partial sequence. Cloned cDNA sequence has a 98% homology with the *todC1* partial sequence. The underlined region in the sequence corresponds to the primers (*todC1* antisense and sense primer) used for the DD-RT-PCR.

```

Query:   169  TACGGTGCCTG
          ||| |||||
Sbjct:  1035  TCGCGTGCCTG

Query:   165  GTGCCTGAACAAGAAGGAATGGAGCCCGCTGAAGGCCCGGGTAGAAACCTACAAGGGCCT 106
          |||||
Sbjct:  1039  GTGCCTGAACAAGAAGGAATGGAGCCCGCTGAAGGCCCGGGTAGAAACCTACAAGGGCCT 1098

Query:   105  GATTTTCGCCAACTGGGATGAGAACGCTGTAGACCTCGACACGTATCTGGGCGAGGCGAA 46
          |||||
Sbjct:  1099  GATTTTCGCCAACTGGGATGAGAACGCTGTAGACCTCGACACGTATCTGGGCGAGGCGAA 1158

Query:    45  GTTCTACATGGACCACATGCTCGACCGCACCGAGGCAGGCACCGT 1
          |||||
Sbjct:  1159  GTTCTACATGGACCACATGCTCGACCGCACCGAGGCCGGCACCGA 1203

```

Figure 19. Comparison of nucleotide sequences of the cloned cDNA (170.c) against toluene dioxygenase partial sequences. Query: cloned cDNA sequences. Sbjct: *P. putida* toluene dioxygenase (*todC1*) partial sequences. The cloned cDNA fragment has a 100% homology with the *todC1* partial sequences except the primer sequences. The underlined region in the sequences corresponds to the primer sequences (70.3) used for the DD-RT-PCR.

gene has a 99% homology with *todC2* gene. Six mismatches were tolerated when the 70.3 primer was used in the RT step. Three mismatches were tolerated when the SD primer was used in the PCR step. The two primers flanked bases 2072 to 2614 and amplified a *todC2* 543-bp cDNA fragment (Figure 20).

Optimization of extraction buffer for direct extraction of RNA from soil sample

Different concentrations of SDS, PVP, phosphate glass/sodium pyrophosphate (PYRO), and Na_2PO_4 were used to optimize the extraction buffer for the soil samples. A *nahA* fragment, from NAH7 plasmid of *P. putida* G7 strain, linked to an upstream T7 promoter, was *in vitro* transcribed to mRNA containing $\alpha\text{-}^{32}\text{P}$ -UTP. The radioactive mRNA was then added to the extraction buffer along with soil to determine the recovery rate. The results were shown in Table 3. The best buffer was 2% SDS + 1% PVP + 0.5% PYRO buffer and 0.1 M Na_2PO_4 + 1% PVP + 0.5% PYRO buffer. The recovery efficiency was about 70%.

Further manipulation of the pyrophosphate concentration to enhance extraction was also done. Results were shown in Table 4. The recovery rate only slightly decreased when the pyrophosphate concentration was increased. SDS and pyrophosphate were chosen as the large scale buffer system instead of the Na_2PO_4 buffer because the SDS solution had an additional RNase

Figure 20. Comparison of nucleotide sequences of the cloned cDNA (580.5) against *todC2* partial sequences. Query: cloned cDNA sequences. Sbjct: *P. putida todC2* partial sequences. The cloned cDNA has a 99% homology with the *todC2* partial sequences except the primer sequences due to mismatch amplification. The underlined region in the sequences corresponds to the primer sequences used for the DD-RT-PCR.

Table 3. Optimization of the extraction buffer for soil samples for small scale sample.

Buffer systems	radiolabled nahA mRNA added	Recovery (cpm)	Recovery efficiency
2% CTAB (w/v)	16845 cpm	251	1.5%
2% CTAB + 1% PVP	"	368	2.2%
2% CTAB + 1% PVP + 0.5% PYRO	"	428	2.5%
2% SDS	"	131	0.8%
2% SDS + 1% PVP	"	187	1.1%
2% SDS + 1% PVP + 0.5% PYRO	"	12100	71.8%
0.2M NaPO ₄ (pH 8.0)	"	1885	11.2%
0.2M NaPO ₄ + 1% PVP	"	83	0.5%
0.2M NaPO ₄ + 1% PVP + 0.5% PYRO	"	5260	31.2%
0.1M NaPO ₄	"	1260	7.4%
0.1M NaPO ₄ + 1% PVP	"	8690	51.6%
0.1M NaPO ₄ + 1% PVP + 0.5% PYRO	"	12258	72.7%

Table 4. Optimization of the extraction buffer for phosphate glass/sodium pyrophosphate (PYRO) concentrations.

Buffer systems 0.1M NaPO ₄ + PYRO	radiolabled <i>nahA</i> mRNA added	Recovery (cpm)	Recovery efficiency
0.2% PYRO	16845 cpm	9848	58.4%
0.4% PYRO	"	9369	55.6%
0.8% PYRO	"	10191	60.4%
1.6% PYRO	"	10227	60.7%

inhibitory effect (6). Optimization of soil extraction buffers for large scale RNA sample was also done. The data is shown on Table 5. The optimal pyrophosphate concentration was determined to be 0.5%.

Table 5. Optimization of soil extraction buffer system for large scale RNA sample.

O.2M Na ₂ PO ₄ + 100mM EDTA +1% SDS	CAFB soil	PVP	5% PYRO solution	dH2O	radiolabled <i>nahA</i> mRNA added	Recovery (cpm)	Recovery efficiency	after chloroform extraction recovery rate
5 ml	2 g	0.1 g	1 ml	4 ml	168415 cpm	135068	80.2%	76.6%
5 ml	2 g	0.1 g	2.5 ml	2.5 ml	"	120585	71.6%	62.3%
5 ml	2 g	0.1 g	5 ml	0 ml	"	116543	69.2%	51.5%
5 ml	2 g	0.1 g	1 ml	5 ml	"	135742	80.6%	77.0%

5. DISCUSSION

This thesis demonstrates the viability of DD-RT-PCR to detect and isolate functional genes from microorganisms in soil microcosms. This was accomplished by fundamental developmental studies using RNA from *P. putida* F1. The fundamental studies optimized the differential display reaction conditions, reamplification conditions and screening procedures for total RNA from *P. putida* F1 cells. Assessment of the use of non-specific arbitrary primers in the DD-RT-PCR has also been accomplished. The method developed to directly fingerprint RNA in soil microcosms may be applied to the contaminated field soil samples and allow direct study of gene expression in the naturally contaminated environment without traditional enrichment and cultivation steps. Therefore, it provides a fast approach to elucidate the functional genes associated with degradative activity as well as other stress inducible genes in environmental microbial populations. The cloned cDNA can be used as a source for the development of new gene probes that can monitor the active genes or bacterial activities. Gene probes that can monitor the transcriptional level will help to elucidate the gene regulation involved in degradative pathways.

The information from the results and further work to develop the method in soil microbial community analysis requires a further understanding of factors affecting the methods as currently applied toward the quality of target RNA.

RNA integrity

RNA integrity plays an important role in the success of the differential display. If enough RNA is available, a denaturing gel should be run to determine whether the RNA is degraded or not before beginning the differential display experiment. Several reasons exist as to why the integrity of RNA is so important. For example, if the RNA is slightly degraded at the 3' end and originally there were two identical or almost identical annealing sites near the 3' end of rRNA, there will be two bands for the two annealing sites. Now because of the degradation of 3' end, one annealing site would be eliminated, resulting in the detection of only one band on the gel. Since this is rRNA, the band pattern will be strong enough to be easily detected, creating a false positive result. The best way to avoid the rRNA problem may be to purify the mRNA. This could potentially be achieved by running the total RNA through affinity column containing the 16S and 23S conserved sequences.

RNA abundance

The abundance of rRNA always causes some degree of problem; elimination of rRNA or selection of suitable primers for the amplification of the prokaryotic mRNA seems more important because there is no unique sequence such as a poly-A tail in prokaryotic mRNA. Since there is no systematic method to purify the mRNA from the prokaryotic RNA pools, the best way to get gene

information from the RNA pool is to choose a primer set which can distinguish the induced and uninduced samples.

In eukaryotic cells, there are about 15,000 different species of mRNA transcribed in a specific cell (50). The number of primer combinations to amplify all of the messages is about 240-312 depending on the bands' number visualized on the sequencing gel (28), when poly-dT primer was used in the RT step. Although one can not use poly-dT primers for differential display of prokaryotic RNA because of lacking poly-A tail in prokaryotic RNA, differential display technique may still be a good way to isolate the differentially expressed gene because its mRNA species are far less than eukaryotic cells (Table 6). Compared with eukaryotic mRNA, an average of 15,000 different kinds of RNA (47,50), prokaryotic cells only have 400 to 2,300 different kinds of mRNA (30,66). This may be an advantage for the differential display technique because fewer primer combinations would be needed to amplify all the mRNA in the cell. Theoretically, if we expect 100 bands on the sequencing gel, because mRNA is only about 4% of the total RNA, 4 bands will be amplified from the mRNA pool. Therefore, 100 primer combinations will amplify all the gene information. If rRNA can be eliminated by using rDNA column, then only four to 23 primer combinations are needed to amplify all abundant gene information in prokaryotic cells.

Theoretically fewer primer combinations are needed to amplify all mRNAs from the proKaryotic RNA pool; however, the probability to detect abundant RNA

species is still greater than that for rare mRNA species because DDRT-PCR is biased toward abundant mRNA (14). The reason for such a bias is because rare mRNA after RT-PCR amplification usually is still not sensitive enough to be visualized on the sequencing gel because of its low level of expression (13). Therefore, for a microbial community in soil samples, the diversity of the microbial population will not have greater a influence on DD-RT-PCR because only abundant mRNA will be detected i.e. only the mRNA from most active microorganisms will be detected. Thus, DD-RT-PCR may "simplify" the diversity of RNA species and provide an amenable method to detect catabolic genes in a microbial community due to the genes' high level of expression.

To our knowledge, there is no report about the mRNA abundance of an individual gene relative to total prokaryotic mRNA (47,66). In the case of *P. putida* F1, after toluene induction, the *tod* operon is more expressed (at least 10^4 fold). The mean mass percentage of *tod* transcripts is about 0.004% of percentage of eukaryotic intermediate mRNA (24). Thus, the *tod* transcripts should belong to the intermediate or abundant class of mRNA. It is conceivable that in other strains, which use other organic compounds as energy sources, the operon should be abundantly expressed. Therefore, by using the differential display technique and choosing a suitable arbitrary primer set, it should be total RNA (0.4 ng/10 μ g of total RNA) which is equal to the mean mass possible to detect novel genes which are responsible for the degradation of the organic compounds.

Table 6. Overall RNA composition in *E. coli* cell

RNA	Weight per cell (μg) $\times 10^{15}$	Weight percentage of total RNA	Molecular weight	Number of molecules per cell	Different kinds of molecules
23S rRNA	31.0	52.6%	1.0×10^6	18,700	1
16S rRNA	16.0	27.1%	5.0×10^5	18,700	1
5S rRNA	1.0	1.7%	3.9×10^4	18,700	1
tRNA	8.6	14.6%	2.5×10^4	205,000	60
mRNA	2.4	4.1%	1.0×10^6	1,380	400

*Adapted from Physiology of the bacteria cell, a molecular approach, Neidhardt, C. F. et al 1990 (66).

Quantitation of mRNA

Because the difference in intensity of a particular differentially amplified product between samples generally corresponds to the actual ratio of RNA abundance (60), one can measure the induction level of the transcript by serial dilution of the template. PCR amplification of a series template dilution appeared to give the same band intensity (Figure 5). This may be due to the fact that the PCR reaction has reached the plateau phase which usually gives the same amount of amplified fragments regardless of the concentration of initial transcript in the samples (18).

In some cases the basal level of the *tod* mRNA was detected; however, most of the time the basal level was not detected. This may be due to the different physiological state of the cells or due to limited sensitivity of the blot.

Fingerprinting of prokaryotic RNA from pure culture and soil samples

Differential display has primarily been applied to eukaryotic cells, and until now only one published paper has dealt with the use of the differential display technique with prokaryotic mRNA (99). This may be due to the fact that the false positive results are too high because of the dominant ribosomal RNA and the use of arbitrary primers for prokaryotic RNA. Theoretically, RNA fingerprinting products may be derived from the rRNA; however, because the

rRNA is usually not differentially expressed, the rRNA problem can be minimized if special care is taken or if suitable reaction conditions are used.

Using the original procedure of Liang and Pardee (50) for fingerprinting prokaryotic RNA, faint bands or a high false positive result rate were always obtained in the current study. Thus, the procedures and reaction compositions were modified in order to fingerprint prokaryotic total RNA by using arbitrary primers, resulting in a reproducible PCR reaction for differential display of prokaryotic cells (Figure 6). These results suggest that differential display may be used to detect and isolate genes which involve the degradation pathway in environmental strains if a suitable primer is chosen for the differential display. This may facilitate the application of the differential display technique in the prokaryotic cells.

Successful application of the DD-RT PCR to soil samples relies on obtaining intact RNA from soil samples. However, because of the instability of mRNA and the presence of humic acids and fine soil particles, acquisition of intact RNA has been difficult. Although intact RNA has been obtained from sediment samples for hybridization (89), successful RT-PCR is still difficult because of the humic acids associated with other factors. In this study, successful DD-RT-PCR has been achieved by using RNA directly extracted from soil samples. This demonstrates the DD-RT-PCR technique can be directly applied to soil samples to obtain the active genes information, therefore, may provide a fast way to characterize contaminated sites.

In the current protocol, the average number of bands per lane was about 70. The length of amplification products which range from 100 bp to 2 Kb, was similar to Sokolov's and Averboukh's results (7,83). However, other labs have observed that only bands smaller than 600 could be found on sequencing gels (50).

Based on current findings, the primer which can be used to efficiently amplify cDNAs, can also be used to efficiently amplify those cDNA from other strains. Therefore, after initial selection of primer, the primer might be suitable to be used to distinguish the differential gene messages.

Optimization of RNA fingerprinting conditions

dNTP concentrations played a very important role in the differential display technique. In current protocols, above 200 μ M, the incorporation rate of radiolabeled dNTP was very low, therefore, the differential band intensity was also lowered. Lower than 2 μ M, all bands were not amplified (Figure 9, lane 5). This may be due to depletion of dNTP.

Primer concentration also had great influence on the banding pattern. The best primer concentration was determined to be in the range of 0.2 to 2 μ M. Above 20 μ M, the non-specific priming increased, therefore, increasing the background (Figure 10). Conversely, too low primer concentration decreased the band intensity. This presumably was due to low access of primers.

Originally it was the intent to design a specific primer to amplify the *todC1* genes. Theoretically, at 50°C for a 13 mer, only *todC1* would amplify specifically. However, it was shown that other than *tod* genes were also amplified. In the original design, it was intended that the primers specifically amplify the *tod* gene, and sequences which might amplify the rRNA were eliminated as much as possible. Therefore, any cDNA fragment other than *todC1* gene fragment which appears on sequencing gel was presumably 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 mis-priming events. Conversely, low annealing temperatures will allow more mis-priming to occur, resulting in massive non-specific priming and high background. With prokaryotic RNA, because few poly-A mRNAs are present, and also because arbitrary primers are used in the PCR reaction, the rRNA and tRNA will also be amplified contributing to the background. The standard method used in other labs is usually two low stringency cycles followed by 40 high stringency cycles. The high stringency annealing temperature depends on the length of the primer, template, and GC % of the primer. Thus the best optimal reaction temperature should be determined empirically.

Because short primers were used in the DD-RT-PCR, a low annealing temperature (40°C) was performed in the standard method (50). The current data showed that the annealing temperature had little influence on band patterns. When the annealing temperature was increased from 30 to 50°C, only

a slight band pattern change was observed (Figure 11). This may be because of the addition of PCR enhancers, such as triton and dimethyl sulfoxide (DMSO) in the PCR reaction, which reportedly increases the PCR efficiency or specificity (26). Moreover, it may be also due to the template or the particular primer used (59). This situation was preferable because if the annealing temperature range was broad, it was more suitable for the differential display of prokaryotic RNA.

The primer length also had a great influence on fingerprinting band patterns (Figure 12). In this particular case, more bands were detected by using longer primers when all the parameters were the same. The results showed that the 13 mer resulted in more bands than the 10 mer (Figure 12). This may be due to the annealing temperature which may be too high for the 10 mer, so the binding of the 13 mer could amplify more efficiently. This may also suggest that longer primers could be used in the RT and PCR step for the prokaryotic RNA.

Fingerprinting of RNA using non-specific arbitrary primers

Non-specific primers were used to access the use of arbitrary primers in DD-RT-PCR technique. When only one primer is used to amplify the cDNA, the amplified cDNA must be palindromic. This can be determined by sequencing the cDNA. Theoretically, the chance to amplify palindromic cDNA is less than that of using two primers in the PCR reaction. In current results, the selected arbitrary primers can amplify more bands per lane on the sequencing gel. This may be

due to the fact that primers which show poor amplification of cDNA has been eliminated. Five differential bands were eluted and reamplified including $\alpha^{32}\text{p}$ -dCTP in the PCR reaction. After initial screening, only two of them showed differential expression; the others didn't show any signal on the slot blot. The two differentially expressed cDNAs were cloned and sequenced. Sequence results reveal one (170.c) has 100% identity with the *todC1* partial sequence (Figure 18). The other one seemed to be an artifact since the primer sequence was different from the primer used in the PCR reaction. This phenomena was also observed by Guimaraes et. al. (28). Further investigation may be needed.

When two primers are used to amplify the cDNA in the PCR step, there would be 3 different events at the end of the cDNA sequence. If the cDNA is palindromic, it will contain either one of the primers. If the cDNA is not palindromic, it will have both primers at the end. When 70.3 and SD primers were used in the PCR reaction, one differential band was detected in different batches of RNA samples (Figure 14). Sequence results revealed that it is the *todC2* partial sequence amplified by both primers. Although Ling et al showed that up to four mismatches were tolerated during the DD-RT-PCR procedures (49), the data reveals that six mismatches were tolerated in current protocols. This may be due to the secondary structure of the *tod* RNA which may favor the primer to anneal to this particular area regardless of the six mismatches.

These results demonstrate the potential use of the short arbitrary primers to detect and isolate functional genes regardless of whether the primers are a

perfect match or not, because mismatches are tolerated during the low annealing temperature PCR reaction. Initial screening of better arbitrary primers which can efficiently anneal RNA and cDNA may be needed to obtain better results.

Verification of differential bands and clones

Our observation as well as others (99) showed that sometimes a single band on a differential display gel may contain more than one DNA species, as demonstrated by sequencing. This may be due to internal contamination or internal amplification of the differential bands. In this case a screening procedure must be done. Different screening methods can be used (see literature review) depending on the user's convenience.

Slot blots were used routinely to screen the differential bands because of its sensitivity. Although northern blots can avoid non-specific artifacts, the sensitivity is far less than that of slot blots due in part to low transfer efficiency to the membrane. Therefore, slot blots were the method of choice when screening differential bands.

A serious encountered difficulty was a high false positive result rate and the failure to clone bands clearly visible as differentially expressed on the gels. Twelve differential bands were eluted and used as probes to screen the true differential bands. Initially five differential bands were verified as differentially expressed. However, after cloning, only three (410.a, 170.c and 580.5) showed

differential expression. The other two didn't show any difference between induced and uninduced samples. This may be due to the fact that the slot blot was not sensitive enough to detect the rare mRNA message or the obtained clone may be a false positive. This may be due in part to the fact that true differentially expressed cDNA concentrations were far less than other cDNAs which had a similar molecular weight.

Although false positive results can be decreased by special care, some unchangeable parameters will still cause the false positive results such as tube variation. Thus, screening of true differentially expressed PCR products is necessary. Since the screening procedure is time consuming and labor demanding, more sensitive and fast ways to screen the differential cDNA bands or clones need to be developed.

Optimization of extraction buffer for direct extraction of RNA from soil samples.

The phosphate glass/sodium pyrophosphate seemed very important for the isolation of the RNA. The phosphate backbone of the pyrophosphate solution may mimic the phosphate bond of the RNA, saturating the binding site on fine soil particles or competing to bind to the soil particles.

The buffer system seems to be good to free the RNA from the soil particles. However, because of the high phosphate salt concentration, the salt co-precipitated along with RNA, thus making isolation of RNA more difficult.

Further modification of the procedure needs to be done to reduce salt precipitation. This may be accomplished by dialysis, but special care need to be taken because RNases are ubiquitous.

Conclusion

P. putida F1 can use toluene as a sole carbon source when grown in MSB medium. During uninduced state (no toluene), the *tod* operon expression is very low. Conversely, when the cells were inoculated in MSB medium with saturated toluene vapor, *P. putida* F1 highly expressed the *tod* transcripts in order to synthesize enough enzyme to degrade toluene to get the necessary nutrition for survival. Based on this, it was demonstrated that the differential display technique is an elegant technique to allow isolation of genes associated with degradation pathways if suitable primer sets are chosen. Therefore, This modified differential display technique may be very useful for detection of the novel genes associated with the uncharacterized compounds and mixtures of pollutants since there is no need for any specific gene information.

On the other hand, because of the bias of the differential display technique to abundant RNA, it may still be difficult to access all the genes which are involved in the change of bacterial physiological state response to a change in the environment. The expression of the regulatory genes may be at too low a level to be visualized with this method. However, the technique is appropriate for the isolation of catabolic genes that code for enzymes that metabolize the

substrate (pollutants) and are essential for the survival of the organism. Moreover, this technique may be applied to other conditions such as the response of *Salmonella typhimurium* to oxidative stress (99) where in the alternative state of the cell that mRNA level is highly expressed. Therefore, DD-RT-PCR may provide a fast way to develop new functional gene probes which enable the characterization of unknown strains or diagnosis of the "health" of microbial communities in response to chemicals and other stressors.

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VITA

Born December 26, 1968 in Changhau, Taiwan. The author graduated high school from Lilin high school in June 1987. He then studied bioengineering at Tatung Institute of Technology, Taipei, Taiwan and graduated with a B.S. in June 1991. He joined the National Army in September 1991 and then he worked as a technician at Academic Sinica, Botany, Taiwan in August 1993 under the direction of Dr. Lin, Yao-Hua.

He enrolled at the University of Tennessee in the Fall of 1994. His graduate career started at the Center Environmental Biotechnology under Dr. Gary S. Sayler. He received his M.S. in Biotechnology, Life Sciences in December 1996.

Following graduation he attended the Ph.D program in Environmental Toxicology, Ecology and Evolutionary Biology under the direction of Dr. Gary S. Sayler.