Differential display: a whole organism approach to studying phenol metabolism in *Trichosporon cutaneum*

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ABSTRACT

Differential display: a whole organism approach to studying phenol metabolism in *Trichosporon cutaneum*

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Currently, little is known about the mechanisms by which fungi respond to and degrade toxic chemicals. Their responses are defined, in part, by which genes are turned on or off. *Trichosporon cutaneum* catabolizes a variety of carbon sources including phenol and was selected for this study. In this study, differential display, a genomic based technique, is used to define the response of the filamentous fungus, *Trichosporon cutaneum,* to the aromatic compound, phenol. Messenger RNA (mRNA) was purified from cells grown in phenol or succinate medium, reverse transcribed using various primers and amplified via polymerase chain reaction (PCR) using arbitrary primers and radioactive nucleotides. PCR products generated from each mRNA population were electrophoresed on denaturing polyacrylamide gels. The population of radioactive species from the two samples was compared and PCR products differing in intensity between the two growth conditions were isolated, reamplified, cloned and sequenced. Northern blot and dot blot analyses enabled confirmation of differences detected. Using this approach, genes defined by sequence similarity as a potential transcription factor and the F subunit of vacuolar ATPase were shown to be upregulated while those genes encoding a basic amino acid permease, KDEL ER lumen retention receptor were shown to be down regulated in cells grown in phenol media. A number of genes of unknown function also were shown to be differentially expressed. In the cases tested, differential expression was confirmed by Northern blot or dot blot analyses.

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bp - base pair

DEPC - diethylpyrocarbonate

DNA - deoxyribonucleic acid

g - gram

Kbp - Kilobasepair

IPTG - Isopropyl-β-D-thiogalactoside

µL - micro liter

µM - micromolarity

mRNA - messenger RNA

MOPS - [N-morpholino]propanesulfonic acid

NaCl - sodium chloride

NB - Nutrient Broth

OD - optical density

PCR - Polymerase chain reaction

RNA - ribonucleic acid

RPM - revolutions per minute

rRNA - ribosomal RNA

TBE - Tris/Borate/EDTA

TE - Tris/EDTA

YPD -Yeast extract/peptone/dextrose

INTRODUCTION

1. Environmental organopollutants

For several decades now, the improper disposal, misuse and accidental release of toxic organic and inorganic compounds into the environment have resulted in widespread pollution of soils, groundwater and marine environments. The scale of production and release to the environment of these compounds is staggering. In the U.S.A. alone, an estimated 80 billion pounds of organic compounds are produced annually by the chemical, agricultural, oil, paper, textile, aerospace and other industries (Gadd, 2001) (see Table 1 for examples) and a significant amount of these compounds are released into the environment (Table 2).

Table 1: Production of organic compounds in the United States in 1996.

Compound	Production (kg x 10^6)
Ethylene	22,300
Propylene	11,400
Styrene	5,390
1,2-Dichloroethane	5,140
Ethylbenzene	4,700
Ethylene oxide	3,280
p-Xylene	2,800
Cumene	2,670
1,3-Butadiene	1,740
Acrylonitrile	1,530
Benzene	960
Isopropyl alcohol	628
Aniline	489
o-Xylene	402
2-Ethylhexanol	345

(Alexander, 1999)

Table 2: Relative amounts of organic compounds released into the environment in the United States in 1996.

Compound	Amount released ($kg \times 10^6$)
Methanol	111
Toluene	66.2
Xylene (all isomers)	43.4
n-Hexane	35.1
Methyl ethyl ketone	31.8
Dichloromethane	26.0
Glycol ethers	19.9
Styrene	19.0
Ethylene	15.5
Acetonitrile	13.1
n-Butanol	12.6

(Alexander, 1999)

A significant number of these chemicals are xenobiotics, *i.e.*, having structures that are unrelated to those formed by natural processes. These synthetic molecules degrade slowly, persist and accumulate in the environment where they may present a long term hazard. Most of the major organic pollutants are composed of one (aromatic) or more (polyaromatic) rings. Some major organic pollutants which have received considerable press coverage include organochlorines (such as DDT), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs, such as benzo(a)pyrene), organophosphorus compounds (*e.g.*, Parathion) and rodenticides (such as warfarin) (Walker, 2001).

Multicellular organisms have a limited ability to degrade xenobiotics (Walker, 2001). Extensive information on the toxicological properties and mode of action of many

of these compounds is still unknown. Many of these compounds may be directly toxic or become hazardous following biotransformations (the conversion by existing enzymes of the original compound into a new compound). For instance, benzo(a)pyrene, is converted to an isomer of 7, 8-diol-9, 10-oxide that interacts with guanine residues of DNA. DNA adducts can become fixed as mutations leading to cancer (Walker, 2001). Due to the evident toxicological consequences of many of these organic compounds, the removal of these organic pollutants is important.

2. Treatments of pollutants

Only 10% of the waste produced in the U.S. is believed to be disposed of in an environmentally safe manner (Gadd, 2001). Once an aromatic compound has been released into the environment, there are a number of traditional physical methods that can be used to reduce or eliminate its toxicity. These include vapor extraction, stabilization and solidification, soil washing, soil flushing, critical fluid extraction, chemical precipitation, thermal desorption, incineration and excavation and burial in a chemically secure landfill (Gadd, 2001). However, many of these physical treatments do not actually destroy the chemicals but instead transfer them to another matrix, phase or location. In addition, these treatments are costly to maintain, develop and may create other environmental problems. It has been estimated that one trillion dollars is spent in decontaminating toxic waste sites in the United States alone using traditional waste disposal methods (Gadd, 2001). The cost for incineration is estimated at $250-$800 per cubic yard of contaminated soil, while placing these chemicals in landfills cost $150-$250 per cubic yard (Gadd, 2001). Clearly, other more environmentally friendly and cost-effective treatment methods such as bioremediation need to be explored.

3. Bioremediation

Most organic compounds can be subjected to enzymatic attack through activities of living organisms. While an organism might convert a xenobiotic compound into something toxic, other organisms may break it down into non-toxic products. The pollutant or a product from its metabolism may be sequestered within an organism or even become a source of carbon or energy for the organism (Crawford and Crawford, 1996). Biodegradation is defined as the biologically catalyzed reduction in complexity of chemicals (Crawford and Crawford, 1996) while bioremediation refers to the productive use of biodegradative processes to remove or detoxify pollutants that have been released into the environment (Crawford and Crawford, 1996). Application of bioremediation technology is more cost-effective compared to the alternatives described above. For instance, cost is estimated at $40-$100 per cubic yard of contaminated soil (Crawford and Crawford, 1996).

However, one major drawback to applying bioremediation technology is the large amount of preliminary information required to support process design. Information on waste characteristics and microbial physiology must be known for process design in order for bioremediation to be successfully applied (Timmis *et al.*, 1994). There is no one organism that is capable of treating all of the different wastes. The understanding of how an organism will utilize a particular chemical is important because the organism may change the chemical to a more toxic compound than its precursor and thereby inhibit the growth of the organism and subsequent detoxification of the compound. Furthermore, adequate nutrients and an environment promoting growth and survival of the organism are important considerations since certain chemicals may not provide a suitable energy

source or may not be present at an appropriate concentration. Another consideration is the uptake and bioavailability of the compound to the microorganism. Xenobiotics often have low solubility in aqueous environments and are adsorbed into particulate matter, making them less accessible to microbial attack (Crawford and Crawford, 1996). In addition, chemically contaminated sites may have inhibitory mixtures, sites that contain not only the target toxic compound but also other chemicals that may be biochemically incompatible with the effective catabolism of the target compound and hence, may poison the process. Moreover, cellular responses, such as tolerance mechanisms, to a certain chemical which occur within an organism are important for designing effective bioremediation organisms. For instance, it was found that the microbial biomass, indicated by the phospholipid phosphate concentration was increased as a response to polyaromatic compounds (Langworthy *et al.*, 2002). Despite the information required to develop bioremediation strategies, it seems to be a more effective choice than alternative techniques for reduction or removal of aromatic pollutants from the environment.

4. Aromatic degradation in bacteria

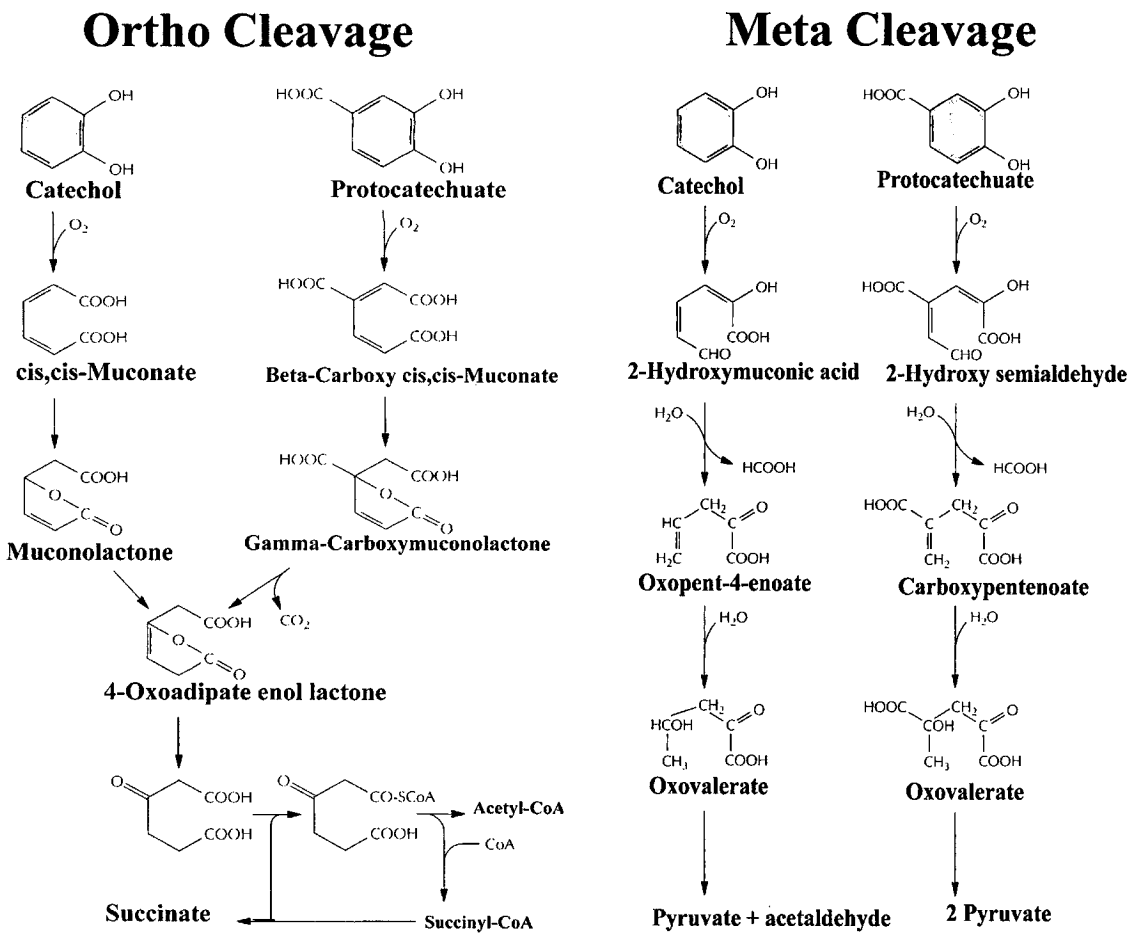
4.1 Enzymes involved in degradation

Individual genes and enzymes involved in bacterial degradation of aromatic compounds have been well characterized. The central chemical challenge of aromatic catabolism is to break open the relatively stable benzene ring (Dagley, 1986). Once the ring has been cleaved, the resulting products can be broken down and provide energy to the organism. Bacteria able to break open aromatic rings such as phenol or alkyl-substituted phenols use three catabolic strategies. Two of these strategies involve an initial conversion of phenol to a catechol (o-dihydroxybenzene) by a monooxygenase

such as phenol hydroxylase, followed by aromatic ring cleavage by one of two types of catechol dioxygenases. The ring cleavage product then can be channelled through a central series of reactions leading to glycolytic and Krebs cycle intermediates. These ring cleavage enzymes have associated transition metals which activate molecular oxygen for insertion into an aromatic substrate. Ring cleavage enzymes have specific substrate requirements. For example, the aromatic rings must contain two hydroxyl groups that are either next to or across from one another (Dagley, 1986, reviewed in Powlowski and Shingler, 2004). The two-hydroxyl groups destabilize the ring to the point that it can be attacked by a ring cleavage dioxygenase. These dihydroxylated rings can be opened in one of two locations, either between the two hydroxyl groups (intradiol or *ortho* cleavage) or adjacent to one of the two hydroxyl groups (extradiol or *meta* cleavage). The two types of cleavage pathways are illustrated in Figure 1.

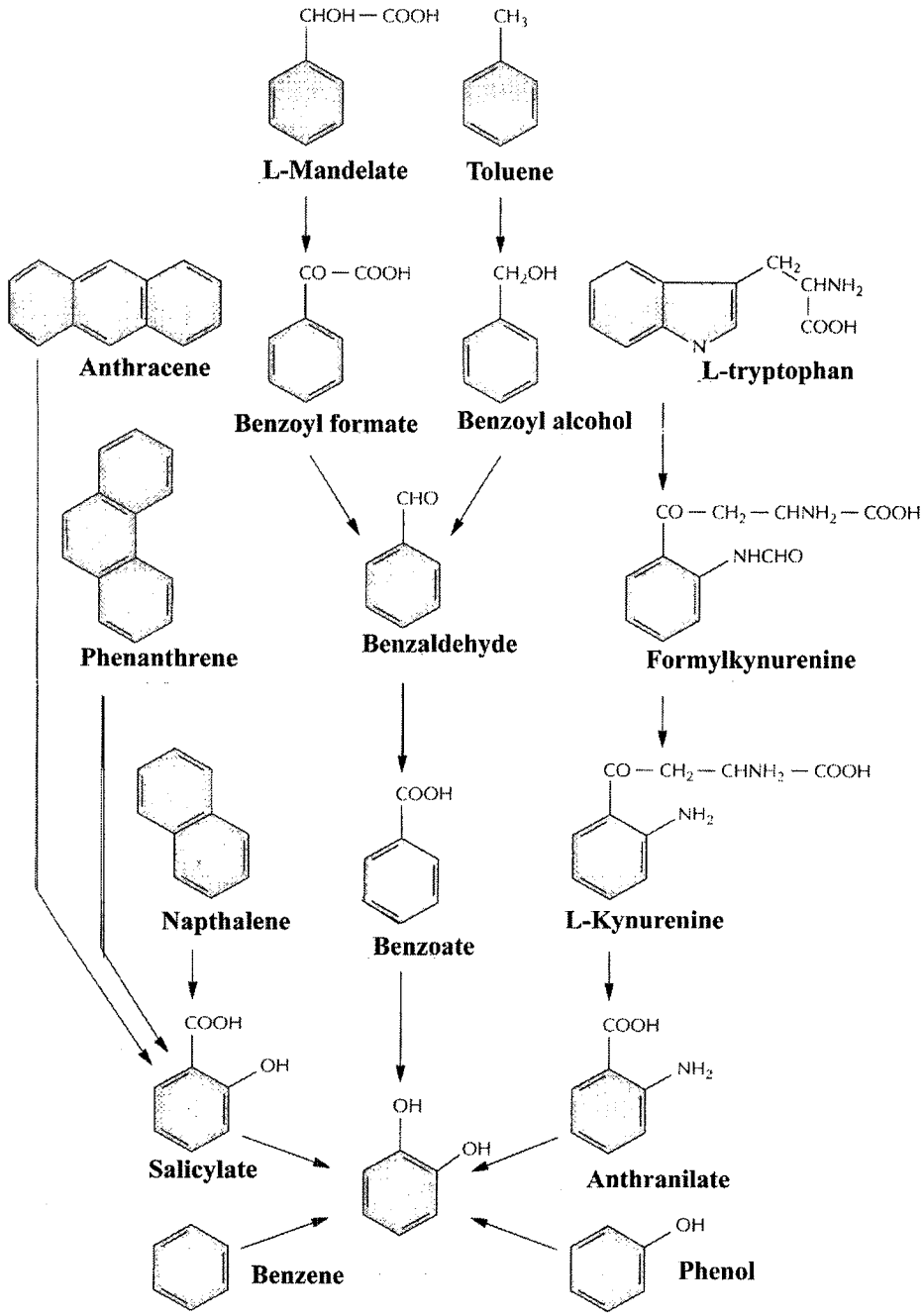
The requirement for two hydroxyl groups on the aromatic ring, combined with the narrow substrate specificities of the ring cleavage enzymes dictates that the degradation pathways of aromatic compounds be convergent. Hence, a wide range of hydroxylating enzymes, flavoproteins and metal containing proteins, process various aromatic molecules towards a relatively few ring-fission substrates such as catechol (Figure 2) which then can be degraded by "*ortho*" or "*meta*" cleavage pathways to succinate or pyruvate, respectively. Other central degradation pathways include ring fission dioxygenases for protocatechuate, 3,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,3-dihydroxybenzoate and 3,4-dihydroxyphenylacetate (Dagley, 1986).

Figure 1: *Ortho* and *meta* cleavage of catechol and protocatechuate.



(Adapted from Dagley *et al.*, 1986, Powlowski and Shingler, 2004)

Figure 2: Convergent catabolism of aromatic compounds to catechol

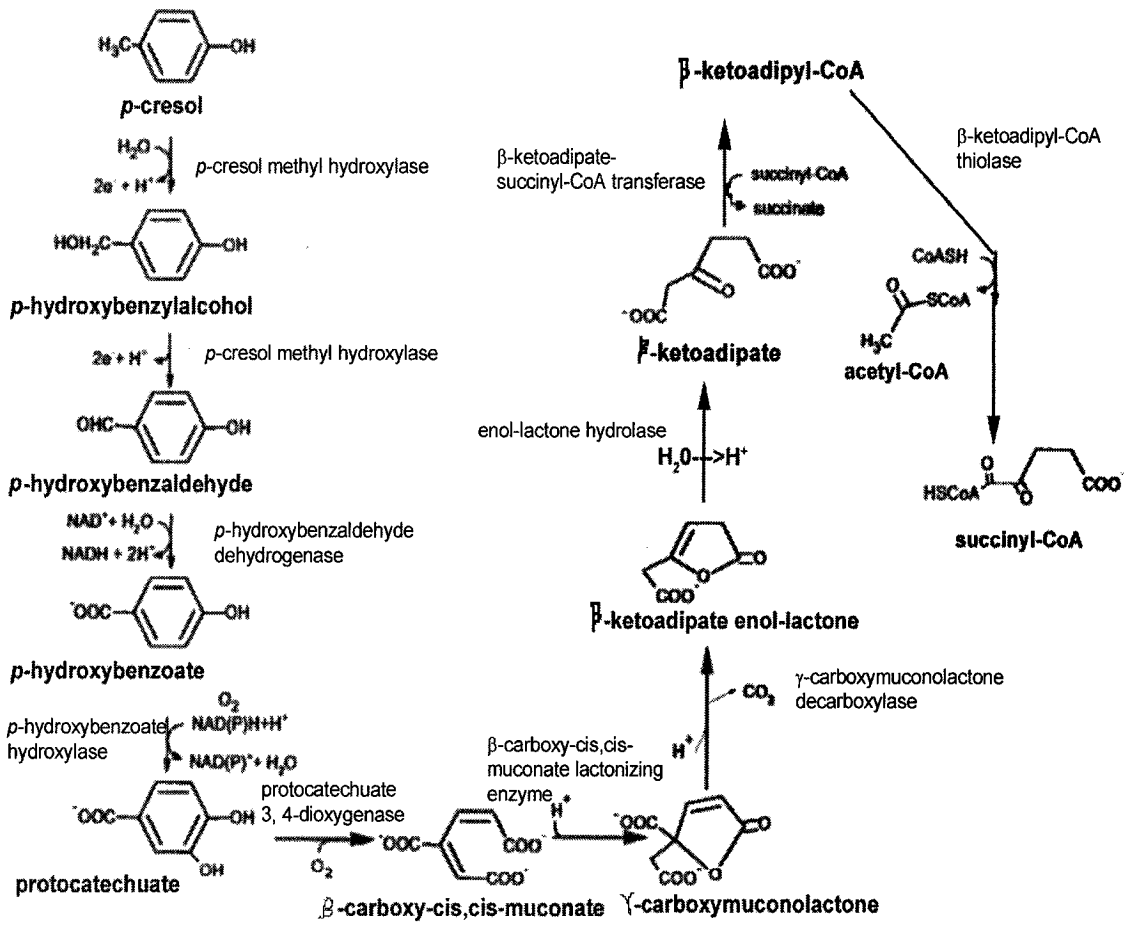


Many aromatic compounds are degraded to a few compounds such as catechol (Alexander, 1999).

For alkyl-substituted phenols, another strategy for degradation is used by bacteria. The alkyl-substituted phenols are oxidized by hydroxylases and converted to

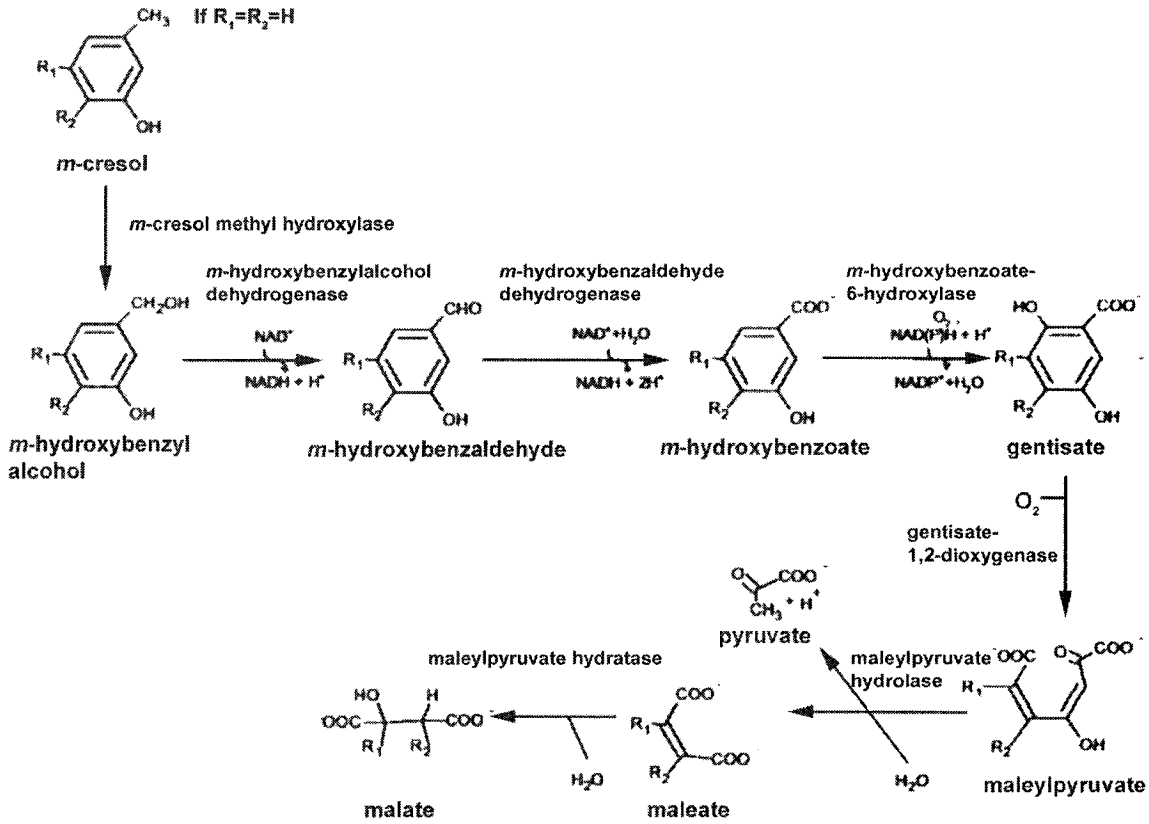
protocatechuate and gentisate, followed by degradation via the cresol/protocatechuate (pca) or gentisate pathways (Figures 3 and 4). For instance, *p*-cresol is hydroxylated and converted to protocatechuate (a substrate for protocatechuate-3,4-dioxygenase which cleaves by the *ortho* pathway) and degraded to succinate (Figure 3). A compound such as *m*-cresol is converted to gentisate, a substrate for gentisate 1,2-dioxygenase which cleaves via the meta cleavage pathway (Figure 4).

Figure 3: Cresol/protocatechuate pathway



(reviewed by Powlowski and Shingler, 2004).

Figure 4: Gentisate pathway



The compound, *m*-cresol is broken down using the gentisate pathway (Powlowski and Shingler, 2004).

4.2 Uptake of aromatic compounds in bacteria

An important aspect of aromatic degradation to be considered is the cellular entry of aromatic compounds. The uptake of aromatic compounds has been studied extensively in bacteria. While aromatic compounds can be imported by passive diffusion across biological membranes (Kashnet, 1985), active uptake mechanisms are also used to transport aromatic compounds into the cell (Nichols and Hardwood, 1997). These highly specific active transport systems contribute to the ability of bacteria to grow on micromolar concentrations of compounds in soil (Nichols and Hardwood, 1997). One

such transport system includes the well-studied permease designated PcaK, identified in *Pseudomonas putida.* It is a member of a major facilitator super family (MFS) of transport proteins (Nichols and Hardwood, 1997) and was found to transport aromatic compounds such as 4-hydroxybenzoate (4-HBA). This permease was shown to be regulated co-ordinately with genes encoding enzymes of the degradative, β-ketoadipate pathway. Uptake of compounds is tightly regulated and very specific to certain classes of molecules. For instance, the PcaK transport mechanism permitted 4-HBA uptake but was inhibited by benzoate (Nichols and Hardwood, 1997). Once a compound is taken up by a cell, intracellular enzymes can act upon it.

4.3 Organization and regulation of genes involved in aromatic degradation in bacteria

The genes involved in producing proteins responsible for the degradation of aromatic compounds are regulated, *i.e*, the genes are only expressed when substrates are available. As for most metabolic pathways, the genes are clustered into operons in bacteria and are under the control of one or more regulatory proteins.

In most cases, a single protein senses the compound and regulates the expression of the genes involved in its metabolism. Many of the regulatory proteins involved in the degradation of aromatic compounds belong to the XylR/DmpR aromatic-responsive subgroup of σ^{54} dependent activators (reviewed by Powlowski and Shingler, 2004). These activators are members of the AAA^{+} family of mechanoenzymes and control transcription by activation of σ^{54}RNA polymerase. They use ATP hydrolysis to promote σ^{54}RNA polymerase and DNA interactions to initiate transcription. They have a distinct

domain structure and a common σ^{54} activation domain that contains the characteristic signature GAFTGA motif (Powlowski and Shingler, 2004).

There are also two-component regulatory systems made up of a sensory histidine kinase and a response regulator (Lau *et al.*, 1997). The *todST* system for activation of expression of the toluene degradation (*tod*) pathway in *Pseudomonas putida* F1 involves the detection of aromatic compounds by the sensory histidine kinase (TodS protein) and subsequent phosphorylation and activation of the *todT* gene product, a response regulator which then binds to a palindromic sequence in the *tod* control region and activates the *tod* degradative pathway (Lau *et al.*, 1997).

5. Genetic manipulation and engineering of organisms to detoxify waste

Organisms found to degrade a wide variety of xenobiotics were used to develop bioremediation processes. One of the earliest attempts included induction of naturally occurring organisms to degrade aromatic compounds. The Exxon Valdez oil spill resulted in the release of 41 million litres of oil in Prince William Sound, Alaska in March 1989 and resulted in the contamination of approximately 2000 km of shoreline. Nitrogen-containing fertilizers were used to induce growth of hydrocarbon-degrading bacteria which consumed some of the hydrocarbons (Bragg, 1994). As mentioned in section 3, there are many factors that need to be considered for effective bioremediation. Once information is available, the ability of microorganisms to treat toxic wastes can be altered in different ways to make them more efficient and to better fit the different pollutant environments. Some examples of these alterations will be discussed below.

5.1 Increasing enzyme levels

One way to increase the efficiency of bioremediation is to increase the amounts of the enzymes of interest that are produced. This can be done, for instance, by the modification of the regulatory proteins to allow for greater expression of pathway gene products. For example, three to eight fold higher levels of transcription of the TOL operon in *P. putida* over wild type were seen after mutations had been introduced into the SylS regulatory protein (Ramos *et al.,* 1988). Another way to increase the levels of degradative enzymes is to alter the regulatory circuits. Global regulatory circuits link the expression of catabolic operons to metabolic activities to ensure the energetic and nutrient needs of the organism are met and are efficient. Chemically contaminated sites often contain multiple chemical compounds which may repress the expression of genes of certain biodegradative pathways. For instance, expression of proteins of the TOL plasmid catabolic operons in wild type *E. coli* and *P. putida* were repressed by glucose. Expression of TOL plasmid catabolic pathway genes was made possible in mutants defective in the cya-crp cAMP-mediated control circuit of catabolite repression (Duetz *et al.*, 1994, Timmis *et al.,* 1994). Foreign transcription signals also can be used to increase enzyme levels. This is important especially due to the fact that the concentrations of aromatic compounds in a polluted place are not evenly distributed and the concentration of these xenobiotics may not be sufficient for the production of degradative enzymes or growth of the organism. The gene of toluene 4-monooxygenase (T4MO) that degrades toluene, phenol and trichloroethylene from *P. mendocina* KR1 was cloned under the control of a starvation-induced *groEL* promoter (Timmis *et al.,* 1994). This allowed the organism to grow under very low concentrations of these compounds. Moreover, some

compounds do not induce the production of degradative enzymes. For instance, some benzoate analogues can be metabolized by *xyl* pathway enzymes but do not induce production of these enzymes since they competitively inhibit the activation of XylS, a regulator of the TOL operon. Mutant bacteria were made to produce XylS regulatory proteins that were activated by various benzoate analogues such as 4-ethylbenzoate and activated the production of degradative enzymes (Timmis *et al.,* 1994, Ramos *et al.*, 1987).

Increasing the level of transcription of a gene or operon cloned in a new organism may be only the first step in increasing the level of the required proteins. It is possible that the translation of proteins is rate-limiting. This can be addressed by optimizing the translation initiation region of the relevant gene. This was demonstrated in the modification of the 5' end of the DNA sequence of the cloned parathion hydrolase of *P. diminuta* which was poorly expressed in *E. coli.* The engineered enzyme had activities 100-fold greater in comparison to enzymes made by other organisms (Timmis *et al.*, 1994, Serdar *et al.*, 1985).

5.2 Modifying enzymes

The enzymes of degradation pathways can be improved to be more stable and efficient. Naphthalene dioxygenase (NDO) was engineered, by conversion of a histidine residue to cysteine in the ferredoxin component (shown to be highly conserved in other ferredoxin sequences), to have greater stability (Timmis *et al.*, 1994, Murdock *et al.,* 1992). Yet another way to increase the efficiency of particular enzymes is to create multifunctional enzymes. Catabolic pathways consist of multiple enzymatic reactions where the enzyme binds a substrate and converts it to a product. The release of a reaction

product into the cell by one enzyme results in its dilution. The dilution of intermediates can be offset by linking different enzymes together so that intermediates are passed from one active site to another. Many naturally occurring enzyme complexes are multifunctional such as the *dmpFG* gene product of *Pseudomonas CF600* involved in phenol degradation (Manjasetty *et al.*, 2001).

5.3 Expanding pathways

The substrate spectrum of a pathway can be extended to include previously nonmetabolized target substrates. This involves direct selection of mutants able to grow on target compounds or alteration of substrate specificities of regulatory proteins. However, some xenobiotics are not readily biodegradable by known biological processes since microorganisms never have evolved the capability to degrade these structures. Genetic engineering and manipulation of enzyme specificities can alleviate these blocks in the degradative pathways (Timmis *et al.,* 1994). Gene fusions of different pathways have been demonstrated. For instance some PCB-degrading strains can metabolize a specific compound but will degrade other compounds to chlorobenzoate which can inhibit the degradative process. A recombinant bacterium containing PCB and chlorobenzoate degradation pathways was constructed (Havel *et al.,* 1991). In addition, fusions of *bph* (PCB-degrading) and *tod* (toluene degrading) operons were made (Ramos *et al.,* 1987). Thus, PCB degrading strains KF707 and *P. putida* KF715 were able to grow on toluene and benzene (Furukawa *et al.*, 1993). Another example includes the introduction of *bph* genes into the toluene degrading strain of *P. putida F1* which conferred the ability to grown on polychlorinated biphenyls (Furukawa *et al.,* 1993).

In addition, directed evolution can be used to create enzymes or pathways to utilize the pollutant of interest (Wackett, 1998). There are many techniques that are available such as DNA shuffling. An illustration of directed evolution included subjecting the gene encoding for the enzyme catalyzing the dechlorination of the herbicide atrazine to iterative DNA shuffling. By using a plate screening assay, a mutated atrazine chlorohydrolase that catalyzed a more rapid dechlorination of atrazine was obtained. The mutant gene sequences were sequenced and found to encode 11 amino acid changes (Wackett, 1998). Thus, to optimize the potential of microorganisms to clean up our environment, an understanding of how they break down different compounds and their response to these chemicals is needed.

6. Aromatic degradation in fungi

Traditionally, research and development of bioremediation technology has involved the use of the metabolic capabilities of many prokaryotic organisms and less information is available for eukaryotic organisms. In many successful bioremediation processes developed, whole organisms have been used in preference to their isolated enzymes (Gadd, 2001). Since the mid-1980s, many eukaryotes have been investigated for their potential to detoxify pollutants such as polyaromatic compounds for use in bioremediation strategies (Sampaio, 1999, Gadd, 2001). For instance, one study found 332 strains of heterobasidiomycetous yeasts representing 200 species with the ability to utilize 20 low molecular weight aromatic compounds (Sampaio, 1999). Fungi are able to metabolize and grow using recalcitrant plant biomolecules, polycyclic aromatic hydrocarbon compounds, nitro aromatics, chlorinated aromatics, BTEX (Benzene/Toluene/Ethylbenzene/Xylenes) compounds, as well as miscellaneous dyes,

pesticides, effluent components and in the presence of even cyanide (Gadd, 2001). In addition, fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium* were studied for the ability to secrete enzymes such as ligninases that subsequently degrade large aromatic structures such as plant cell walls and a diverse group of environmental pollutants (Gadd, 2001). The release of enzymes by fungi into the medium is advantageous since it minimizes the need for uptake pathways and unnecessary energy expenditure. Fungi are generally more tolerant to high concentrations of pollutants and have been found to degrade a wider variety of chemical types in comparison to bacteria (Gadd, 2001). The branching filamentous mode of growth can allow for efficient colonization and exploration of contaminated soil and other solid substrates (Gadd, 2001). Some fungi already have been used in bioremediation technologies (Gadd, 2001).

6.1 *Trichosporon cutaneum*, a model organism for bioremediation

Trichosporon cutaneum is a filamentous fungus that has been isolated from a wide variety of sources including soil, pulp and paper waste and sewage (Wolf, 1996). It belongs to the genus, *Trichosporon* Behrend and was described as early as 1890 (Wolf, 1996). The genus includes yeasts that are characterized by budding cells of various types, a more or less developed pseudomycelium or a true mycelium and arthrospores. *Trichosporon cutaneum* is related to basidiomycetes with a high G+C content (57% to 64%) and has been shown to catabolize a wide variety of carbon sources, including disaccharides, cellulose, starch and straight chain and cyclic hydrocarbons such as phenol, protocatechuate, homoprotocatechuate and gentisate (Anderson and Dagley, 1980, Anderson and Dagley 1981, Powlowski and Dagley, 1985, Sparnins *et al.*, 1979, Gaal and Neujahr, 1979, Sampaio, 1999). The cell wall of the oleaginous yeast

Trichosporon cutaneum has been studied (Depree *et al.*, 1993). *Trichosporon cutaneum* has been used as a model organism for many different purposes (Table 3) such as phenol degradation or creating biosensors and industrial production.

Table 3: Different applications of the fungus, *Trichosporon cutaneum*

Strain	Collection number	Use
T. cutaneum	DSM 70698	Bioreactor studies Molecular genetics
T. cutaneum	ATCC 46490	Phenol degradation Phenol biosensor
T. cutaneum	ATCC 58094	Degradation of aromatic compounds
T. cutaneum	ATCC 20509[a]	Lipid production from whey
T. cutaneum	ATCC 62975	Peroxisome biogenesis
T. beigelii	CBS 5790	Production of polysaccharidases
T. pullulans	ATCC 10677	Production of amylases, cellulase, and xylanase

(Wolf, 1996)

6.2 Uptake of aromatic compounds in *Trichosporon cutaneum*

Transport and hydrolysis of disaccharides such as cellobiose, maltose, lactose, sucrose, melibiose and trehalose by *Trichosporon cutaneum* has been studied (Mortberg and Neujahr, 1986). *T.cutaneum* seems to have two different pathways for the uptake of phenol (Mortberg and Neujahr, 1985, Mortberg *et al.*, 1988). One is an inducible, high affinity system that is sensitive to protonophores. In this system, uptake is by a phenol-proton symport, where one proton is cotransported with every phenol molecule. Although, ATP is not directly involved in phenol transport, it is needed for the generation

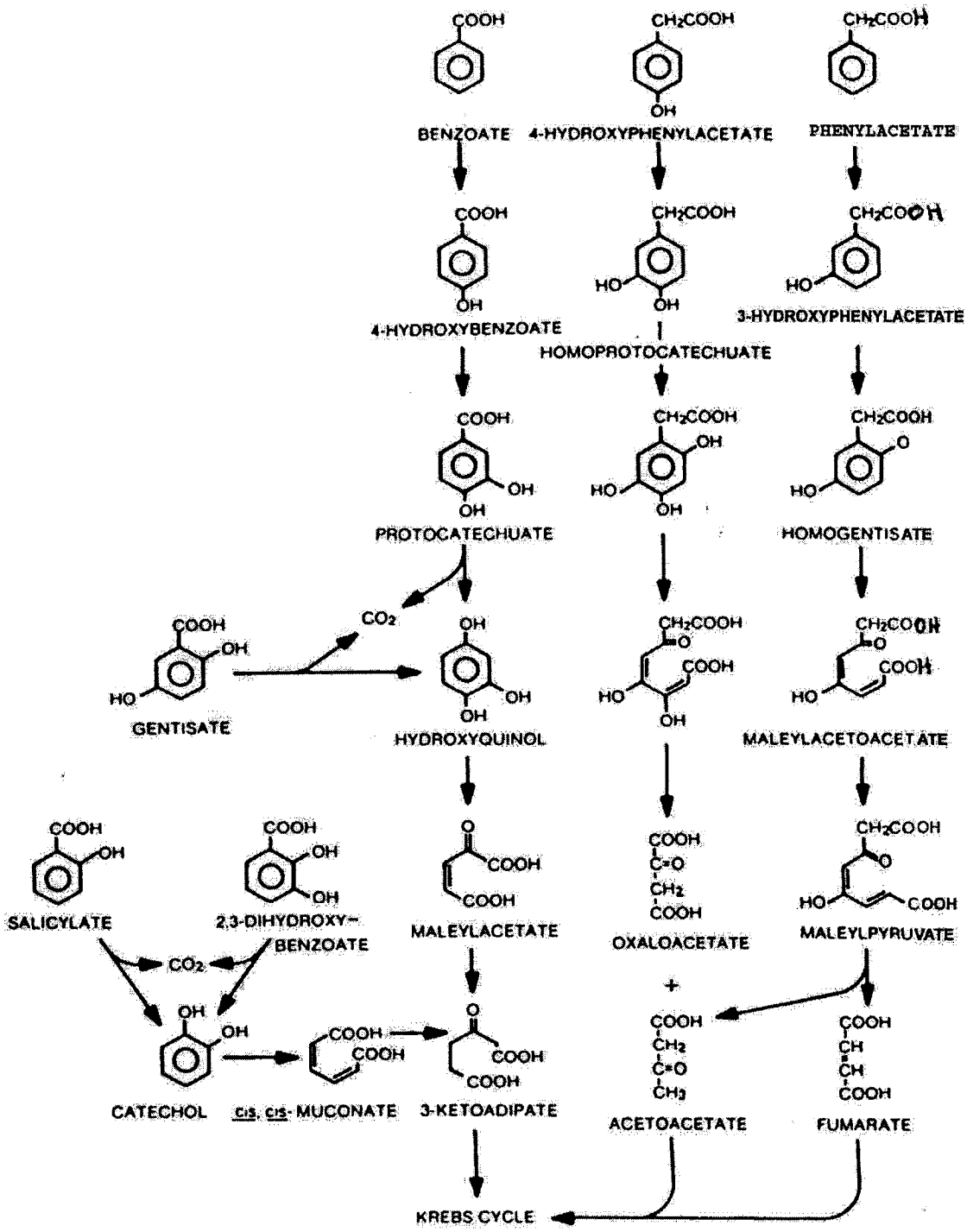
of a proton gradient. This uptake pathway is induced co-ordinately with phenol hydroxylase but can operate independently of phenol metabolism. The pH dependence of uptake indicated that phenolate is essentially formed during the uptake process. The second phenol uptake pathway is a low affinity constitutive system. It has a different specificity and pH optimum then the symport system and it is not sensitive to protonophores (Mortberg *et al.,* 1988).

6.3 Degradation of aromatic compounds in *Trichosporon cutaneum*

The overall metabolic scheme for breaking down aromatic compounds in *T. cutaneum* is similar to that of bacteria. A wide variety of aromatic compounds have been shown to be metabolized into a few compounds that are the substrates of ring fission dioxygenases such as catechol dioxygenase and hydroxyquinol dioxygenase (Figure 5). The organism makes a large number of hydroxylases which prepare these compounds for ring cleavage enzymes.

There are important differences between how aromatic hydrocarbons are metabolized in bacteria and in *T. cutaneum*. *T. cutaneum* seems to be versatile in its catabolic ability. Although, it lacks a number of hydroxylases and ring cleavage enzymes that are present in bacteria, *Trichosporon cutaneum* can nevertheless metabolize protocatechuate, homoprotocatechuate and gentisic acids (Anderson and Dagley, 1980). The ability to use these compounds is attributed to the introduction of a third hydroxyl group into the various dihedral phenols before ring fission (Figure 5).

Figure 5: Catabolism of aromatic compounds by *Trichosporon cutaneum*



(reviewed by Wolf, 1996).

Moreover, *T. cutaneum* uses catabolic enzymes with somewhat broader substrate specificities than those of bacteria and has a wider response to certain derepressors of the enzymes of aromatic catabolism (Anderson and Dagley, 1980).

Trichosporon cutaneum has been found to utilize only the *ortho* cleavage pathway and does not appear to use the *meta* cleavage pathway, used by many bacteria (Neujahr, 1990). In addition, *T. cutaneum* has enzymes with different actions or specificities on the intermediates than their bacterial counterparts (Figure 6).

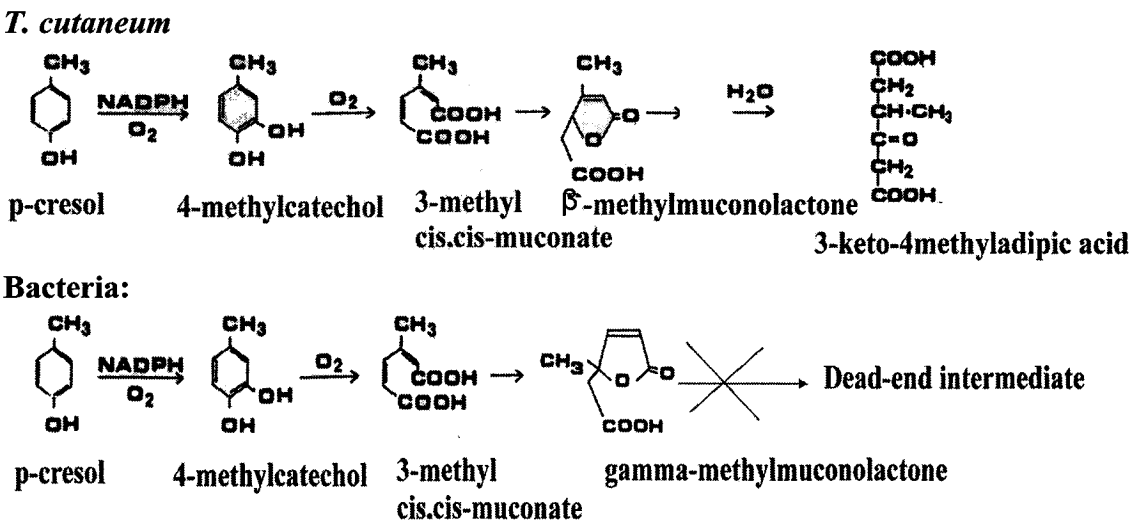


Figure 6: Breakdown of *p*-cresol by bacteria and *T. cutaneum*
(Powlowski and Dagley, 1985).

Cresols are degraded in bacteria such as *Pseudomonas* spp., by two separate and distinct catabolic routes that use *meta*-fission reactions (reviewed in Powlowski and Dagley, 1985). The degradation of methylated compound by the *meta* cleavage pathway in bacteria leads to the formation of a muconate lactone, γ-muconolactone, a dead-end intermediate. However, the cis, cis-muconate lactonizing enzyme in *T. cutaneum* is a novel isomerase and lactonization occurs at a different position, making an intermediate,

β-muconolactone that can be further degraded (Figure 6, Powlowski and Dagley; 1985, Powlowski *et al.,* 1985).

In response to certain compounds, *T. cutaneum* produces isozymes with narrower substrates specificities than those of bacteria (Powlowski and Dagley, 1985). For example, when *T. cutaneum* is grown on phenol and methylated phenols, it produces different hydroxylase isozymes, one which has a greater activity on phenol and another that has a greater activity on methylated phenol (Powlowski *et al.*, 1985). In contrast, bacteria produce a single phenol hydroxylase that has a broader specificity in response to both types of phenol (Powlowski *et al.,* 1985). The production of isozymes is advantageous since the fungus can be more efficient in degrading certain compounds. Furthermore, the ability to produce isozymes results in the creation of multiple catabolic pathways, making the organism more versatile (Timmis *et al.*, 1994). The production of isozymes can be important for survival if the isozymes are under different regulatory controls. In addition, where carbon sources are limiting, the ability of different regulatory proteins to recognize small amounts of specific classes of compounds will increase the potential survival of the organism. It results in an increase in the range of chemicals that the fungus can degrade (Timmis *et al.*, 1994). In conclusion, *T. cutaneum* is an excellent model organism for genome level studies of aromatic degradation because of the range of the compounds it can use.

7. High throughput analysis of differential gene expression

For effective bioremediation, there are many factors that need to be considered and explored. Characterization of each individual gene and gene product involved in the catabolic pathway of aromatic compounds is time consuming. Thus, techniques that look at the global response of an organism under a certain environment and specific conditions would provide the much desired "big" picture from which further research can develop. In order to adapt to a particular environment, organisms express certain genes while repressing others. Classically, genes that were expressed under different conditions were observed using protein-based approaches. One such approach included two dimensional gel electrophoresis (Jamieson *et al.*, 1994, Godon *et al.,* 1998). However, there are techniques that examine the expression of different gene products by looking at the level of transcription. High throughput analysis of differential gene expression has been applied successfully to many areas in molecular cell biology, including cell differentiation, development, physiology and pharmacology. Many comparative techniques including, expressed sequence tag (EST) sequencing, differential display (DD), PCR-based subtractive cloning, microarrays and serial analysis of gene expression (SAGE) have been developed to analyse differential gene expression. Each technique has its own advantages and disadvantages (Table 4).

Table 4: General attributes of major techniques used to study gene expression

	Minimum RNA requirements	Throughput	Sequencing requirements	Cloning requirements	Bioinformatics requirements
EST sequencing	1.0–5.0 µg polyA RNA	Low	High	Full-length cloning may be required for novel genes of interest	Target databases: standard Search protocols: standard Volume: high
RT-RDA	10–100 ng polyA RNA	Medium	Low	Full-length cloning required for novel genes of interest	Target databases: standard Search protocols: standard Volume: low
SAGE	1.0–5.0 µg polyA RNA	High	High	Full-length cloning required for novel genes of interest	Target databases: specialized Search protocols: specialized Volume: high
Microarray hybridization	1.0 µg or more polyA RNA	High	Low	Full-length cloning may be required for novel genes of interest	Image analysis required Target databases: standard Search protocols: standard Volume: low
Differential display	10–100 ng polyA RNA	High	Medium	Full-length cloning required for novel genes of interest	Target databases: standard Search protocols: standard Volume: low

(Carulli *et al.*, 1998)

7.1 Expressed sequence tag (EST) sequencing

EST sequencing was developed by Adams *et al.* (1991) and involves production of cDNA libraries from tissues of interest and selection and sequencing of clones at random. Each sequencing reaction produces approximately 300 base pairs of sequence that represents a unique tag for a particular transcript. An EST sequencing project is very simple since it requires only a cDNA library, automated DNA sequencing and standard bioinformatics. It can be accomplished using normalized or non-normalized libraries. With a normalized library, every transcript is represented equally and redundant sequencing of highly expressed genes is minimized. The advantage of a non-normalized

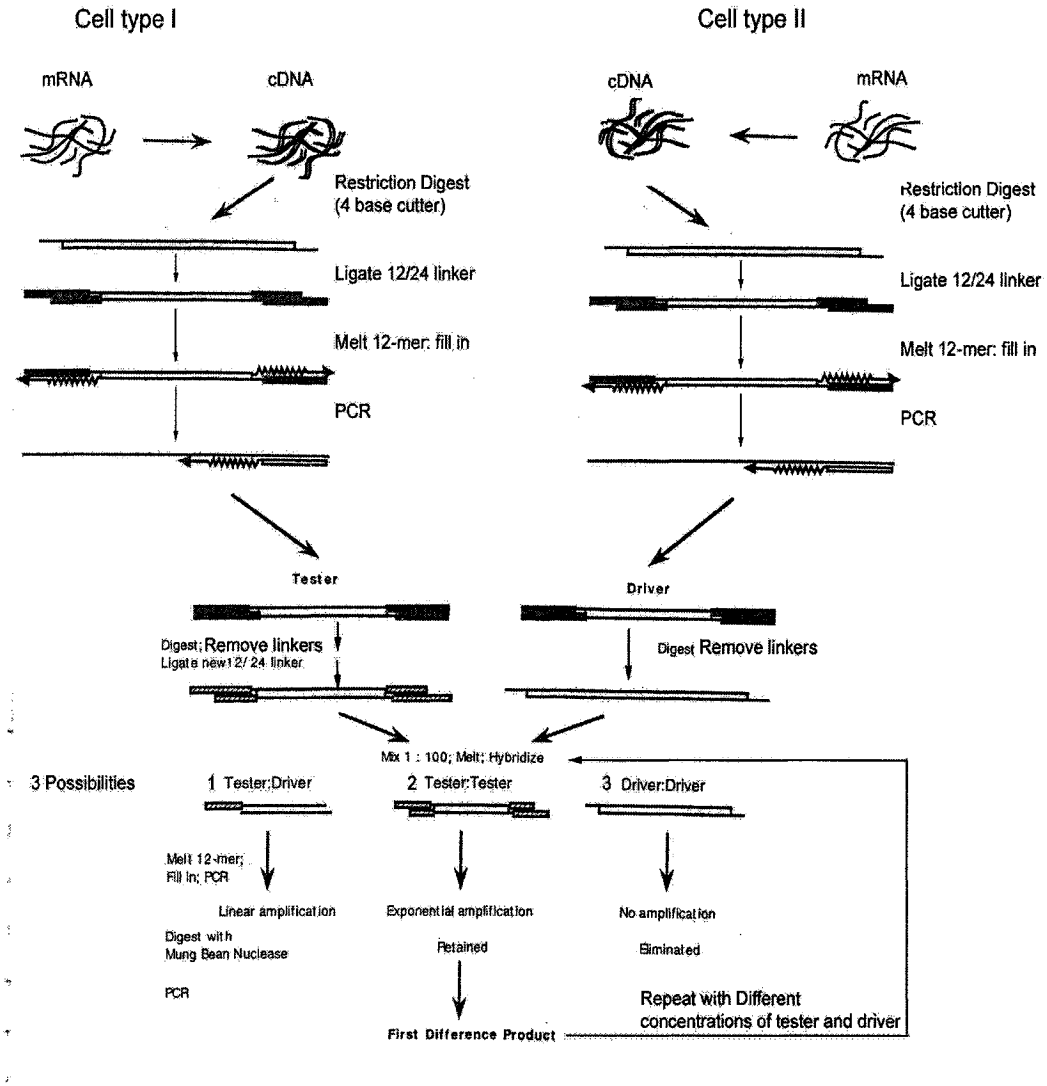
library is that the transcript abundance is reflected in the frequency of clones in the library and thus, can be used to identify highly expressed genes in different samples. The number of new genes found and the statistical significance of transcript abundance are related to the number of clones sequenced. The disadvantages of this technique include the large amount of RNA that is required to obtain good EST libraries and the cost of sequencing.

7.2 Subtractive cloning/ representational differential analysis

A polymerase chain reaction (PCR)-based subtractive cloning method, known as Reverse Transcriptase Representational Difference Analysis (illustrated in Figure 7) can be used to study gene expression. RDA removes fragments common to both populations and leaves only cDNAs that are differentially expressed (Hubank and Schatz, 1994, Carulli *et al.*, 1998). In this approach, double stranded cDNA populations are created from the two cell populations of interest. Linkers are ligated to the ends of the cDNA fragments and the cDNA pools are amplified by PCR. The cDNA pool from which unique clones are desired is designated the "tester". The cDNA pool produced from the control cells is known as the "driver" pool and it is used to subtract away shared sequences. After the initial PCR amplification, the linkers are removed from both cDNA pools and unique linkers are ligated to the tester sample. The tester then is hybridized with a vast excess of driver cDNA and amplified by PCR. There are three possibilities of annealing between the two cDNA pools which would allow for amplification by PCR. The strands of cDNA from driver pools will anneal together and thus, they will not be amplified by PCR as they do not contain the unique linkers that were ligated to the "tester" cDNA pool. Strands of the tester pool may anneal with the strands of the

"driver" pool and will result in linear amplification. The strands of the "tester" cDNA pool also may anneal back together which results in exponential amplification. The PCR products are isolated, cloned and sequenced.

Figure 7: Schematic of steps involved in reverse transcriptase representational difference analysis



(Hubank and Schatz, 1994)

The primary limitation of this method is that it can not be used to detect subtle differences in gene expression. For instance, a gene that is expressed at a higher rate in

one condition may not be detected since many rounds of subtraction could have removed its presence. Hence, most cDNAs that are identified have significant differences in gene expression. Reproducibility and whether the products found are indeed unregulated, is a concern since comparisons are made between two samples and the subtractions are based on a series of sensitive biochemical reactions. These reactions may not go to completion in the different steps (Carulli *et al.,* 1998) and some products found may not in fact be unregulated but rather, produced as a result of incomplete or inefficient subtraction. In addition, false positives are increased due to the sensitivity of PCR.

7.3 Serial analysis of gene expression (SAGE)

In this method, a unique sequence tag of 13 or more bases is generated for each transcript in the cell or tissue of interest (Carulli *et al.*, 1998). Double stranded cDNA is prepared from the different cells or tissues of interest. The cDNA is digested with a restriction enzyme that has a four base recognition site. Linkers are ligated to the cDNA pool and the cDNA pool amplified via PCR. The linkers contain a recognition site for a type II restriction enzyme such as *Bem*is which digests DNA at a site 20 nucleotides away from the recognition site. The cDNA pools are digested with the type II restriction enzyme to yield 13-20 bp cDNA fragments. These fragments are uniquely defined by the original four-base cutter and the adjacent DNA sequence. These sequence tags are then ligated in a series of steps. A SAGE library is made and each clone contains multiple short, unique tags that are ligated together. Each tag represents a unique gene. Transcript profiles are then created by sequencing each library. The relative abundance of each gene is determined by counting or clustering sequence tags. For known genes, the identity can be determined by standard database searches. For unknown genes, the

SAGE tag can be used to obtain a cDNA clone by PCR or hybridization based methods. An advantage of SAGE includes the high throughput processing that can be achieved and the ability to accumulate and compare SAGE tag data from a variety of samples. The disadvantages are related to the technical difficulty of generating good SAGE libraries as well as the analysis. A large amount of RNA is required to produce a good SAGE library. The genome must be sequenced in order to use this on a large scale. The quality of the SAGE tags depends on the quality of RNA and upon the series of biochemical reactions. Any inefficiency such as mispriming or incomplete reactions in the cDNA synthesis will result in artefacts. In addition, highly specialized bioinformatics are required to analyze SAGE data.

7.4 Microarrays

Microarrays involve the placement of cDNAs or gene specific oligonucleotides on glass slides at a density of greater than 1000 clones per cm^2. Differential hybridization is performed using fluorescent labelled cDNA made from RNA derived from two different samples. The intensity of the hybridization is recorded and the relative level of expression of each gene is represented by the intensity of the hybridization signal and compared. Microarrays can evaluate a large number of genes with only one labelling reaction per RNA sample. A significant advantage of microarray is the ability to analyse the same set of genes under different conditions (Carulli *et al.*, 1994).

The cost of using microarrays is relatively high and a large amount of RNA required. The degrees of differences in gene expression that can be evaluated are dependent on the number of genes spotted onto a slide. In addition, when using microarrays, a single population of RNA is used in every experiment and many

experiments must be performed in order to attain statistical data for that one sample of RNA before different RNA samples can be compared. There are also multiple steps which may have an effect on the reproducibility of microarrays such as isolation of RNA.

Once a signal is detected on a microarray, the hybridization signal is related to the nature of the gene. However, cross hybridization is an issue in microarrays. This may be attributed to the fact that a cDNA probe used for microarrays is so complex because it consists of as many as 10,000 different RNA species ranging from a few to thousands of copies per cell. In addition, the fact that there are eukaryotic genes which are parts of families with many conserved sequences among them increases this problem. Furthermore, highly specialized bioinformatics personnel and equipment are required to analyze and design microarray experiments. Moreover, chief problems involved in microarray are reproducibility, probe sensitivity, non-linearity with signal detection, probe cross-hybridization due to homologous cDNA sequences and data management (Liang, 2002).

7.5 Differential display

Only 10 to 15% of genes are estimated to be "turned on" (expressed as mRNAs for protein synthesis) at any given time in cells (Liang, 2002). Differential display integrates three of the most simple, powerful and commonly used biological methods, RT-PCR, cDNA cloning and DNA sequencing to identify these genes. The mRNA is extracted from cells and converted to cDNA using reverse transcriptase with various reverse transcriptase primers. The resulting subpopulations of cDNA are amplified and labelled with either isotope or fluorescent dyes by polymerase chain reaction (PCR) in the presence of a second set of primers. The length of the arbitrary primers is designed to

recognize 50-100 mRNAs under any given PCR condition (Liang, 2002, Liang and Pardee, 1992). As a result, mRNA 3'termini defined by any given pair of anchored primer and arbitrary primer are amplified and displayed on a denaturing polyacrylamide gel. Comparison of cDNA patterns, side by side, between or among relevant RNA samples reveals differences in gene expression. Differentially expressed cDNA bands can be cloned and sequenced for further characterization.

Differential display visualizes the mRNAs in subsets directly after their amplification and labelling by either isotopes or fluorescence. Highly specialized personnel are not required to use this technique. It can be used for prokaryotic or eukaryotic organisms. One of the major advantages of differential display is that there is no requirement for knowledge of mRNA sequences which makes gene screening systematic and non-biased. Differential display is sequence-dependent and capable of detecting both known and novel genes for any living organism (Liang, 2002, Fleming et al., 1998). For instance, when differential display was used for the study of aromatic metabolism of pentachlorophenol in *Coriolus versicolor* (Iimura *et al.*, 1997), three cDNA fragments representing previously unidentified products were generated. Another advantage of differential display is that very little RNA is required to perform differential display. There are fewer steps involved in differential display in comparison to other techniques which allows for comparison of different RNAs from different samples and better reproducibility. The cost requirement of differential display is significantly lower than any other technique since the amount of sequencing is limited to only potential candidates and expensive equipment is not required. Differential display does not require

highly specialized bioinformatics personnel since it uses simple bioinformatics search tools.

For differential display, hundreds of PCR reactions must be prepared and their products separated on a gel matrix for each RNA sample tested to ensure that most of the mRNAs are detected. A disadvantage of DD is that the identity or nature of the gene may not be known until it is amplified, cloned and sequenced.

8. This work

Many eukaryotes have been investigated for their potential to detoxify pollutants (such as aromatic and polyaromatic compounds) for use in bioremediation strategies. One such organism of interest is *Trichosporon cutaneum* which can catabolize many aromatic compounds. For the development of an effective bioremediation strategy, information pertaining to the cellular responses, not just the enzymes that catabolize the aromatic compounds is of critical importance. Despite the research on characterization of some of the enzymes involved in degradation, genetic information in *T. cutaneum* is lacking. In this project, a high throughput strategy using a genomic technique, differential display, has been developed to study genes expressed as a result of phenol metabolism in *Trichosporon cutaneum.* This approach identified genes that are up regulated and down regulated and showed differential gene expression of low abundance genes. Furthermore, Northern blots confirmed the expression patterns of the differential display products tested and a Dot blot procedure was adapted as a high throughput method to evaluate the gene expression patterns of some of the differential display products.

MATERIALS AND METHODS

1. Strains

Trichosporon cutaneum (ATCC 58094) was purchased from the American Type Culture Collection. *Escherichia coli* strains XL2-Blue and XL10 were purchased from Stratagene and JM109 was purchased from Promega. The relevant genotypes are listed in Table 5.

Table 5: Strains

Strain	Organism	Genotype	Reference
ATCC 58094	*Trichosporon cutaneum*		American Type Culture Collection
XL2-Blue	*E. coli*	*sup E44 hsd R17 recA1 gyrA46 thi relA1 lac⁻ F'*[*pro AB*$^+$ *lacI*$_q$*lac ZΔM15::Tn*10*(tet*^{R}*)*]	Stratagene
XL10	*E. coli*	Tet^{r}Δ(*mcr*A)183Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *supE*44 thi-1 *rec*A1 *gyr*A96 *rel*A1 lac The [F' *pro* AB$^+$ lacI^{q}ZΔM15 *Tn*10(Tet^R)Amy Cam^r]	Stratagene
JM109	*E. coli*	*endA1, recA1, gyrA96, thi, hsdR17 (*r_k^-*,*m_k^+*), relA1, supe44, (lac-proAB, [F', traD36, proAB, lacI*$_q$ *M15*]	Promega

2. Growth media and solutions

Recipes for growth media, including solutions used in their preparation, are listed in Appendix 1. Rich media such as YT, YPD, NB were autoclaved for 30 to 45 minutes prior to use. Solutions that include potassium phosphate solution, 1 M magnesium sulphate and 100X CAYE were sterilized by autoclaving for 20 minutes. The succinic acid solution and trace metals were filter sterilized through 0.45 μm filters (Nalgene). The growth conditions were based upon previous work by Tara Hooper and Sparnins *et*

al. (1979). For growing *T. cutaneum*, special care had to be taken in preparation of the glassware since soap residue seemed to affect the growth rate in minimal medium. Fernbach flasks were autoclaved for 45 minutes containing 1.5 L of distilled water and then the water was discarded. To each flask, 50 mL of freshly prepared non-sterile 10 X minimal medium and 442 mL of distilled, deionized water (for succinate medium) or 440 mL of distilled water (for phenol medium) were added and autoclaved for an additional 30-45 minutes. Using sterile technique, the remaining sterilized components for phenol or succinate media were added.

3. Propagation of *T. cutaneum*

3.1 Growth of *T. cutaneum* in minimal medium

Initially, a single colony from a YPD or NB plate was inoculated into 100 mL or 200 mL of rich medium and grown for twenty-four to forty hours with shaking at 225 RPM at 30°C to generate an inoculum. Growth rates of *T. cutaneum* with phenol or succinate in minimal medium were achieved by the addition of cells from the inoculum in rich medium into 500 mL of the appropriate minimal medium (Appendix 1) followed by growth for up to thirty hours with shaking at 225 RPM and 30°C until an OD_{600} of 1.6 was obtained. An additional 1.5 mL of 0.75 M succinate solution (Appendix 1) or 3.2 mL of 0.50 M phenol solution (Appendix 1) was added, to the appropriate medium when the OD_{600} had reached 0.4 to 0.5

3.2 Growing *T. cutaneum* for RNA isolation

T. cutaneum was first grown in 200 mL of rich medium (NB or YPD) from a single colony from a YPD plate to an OD_{600} of 0.90 and collected by centrifugation in a sterile 250 mL centrifuge bottle using the Beckman JA-14 rotor at 4500 x g for 10

minutes. The supernatant was discarded and the cell pellet was resuspended in 200 mL of cold 0.050 M potassium phosphate solution. The cells then were centrifuged again as above and resuspended in 20 mL of cold 0.050 M potassium phosphate solution. Based upon the calculated doubling times, an appropriate amount (0.35 mL for phenol or 0.135 mL for succinate medium) of these cells was added to a Fernbach flask containing 500 mL of growth medium and incubated at 30°C with shaking (225 RPM) such that an OD_{600} of 0.1 would be achieved after a growth time of 16 hours. An additional 1.5 mL of 0.75 M succinate solution or 3.2 mL of 0.50 M phenol solution was added to the appropriate medium when the OD_{600} had reached 0.4 to 0.5 and the cells were subsequently harvested at mid-log phase (after half a doubling, at an OD_{600} of approximately 0.6 to 0.8 by centrifugation in 500 mL centrifuge bottles at 5000 xg for 15 minutes using the Beckman JA-10 rotor. The supernatants were discarded and the cell pellets frozen in liquid nitrogen and stored at -80°C. This generally provided approximately 0.5 g of cell pellet.

4. RNA Isolation

Total RNA was isolated according to the instructions provided with the Trizol Reagent (Invitrogen). A cell pellet was weighed while still frozen, placed in a previously chilled mortar and ground under liquid nitrogen. Trizol (10 mL/g of cells) then was added to the mortar and the grinding continued. The Trizol-ground cell mixture was transferred to a 50 mL Oak Ridge tube and allowed to thaw at room temperature for a few minutes with occasional vortexing. This mixture was vortexed for 1 minute and centrifuged at 12 000 xg for 15 minutes. The supernatant was transferred to a chloroform-washed Oak Ridge tube and the pellet was discarded. The solution was

passed through a syringe with a 25 1/2 gauge needle many times and 2 mL of chloroform, per 10 mL of Trizol used, was added to the Trizol mixture. This mixture was shaken for 2 minutes by hand and centrifuged at 12 000 x g for 30 minutes. The lower organic phase containing cellular debris, DNA and proteins was discarded. The top aqueous layer containing the RNA was transferred to another chloroform-washed Oak Ridge tube and 1 volume of acid phenol (Ambion) was added. The solution was vortexed as above for 1 minutes and then centrifuged at 12 000 xg for 10 minutes. The aqueous phase was transferred to another chloroform-washed Oak Ridge tube and one half volume of isopropanol was added and the mixture was vortexed. This mixture was incubated at –80°C for 30 minutes and then centrifuged at 12 000 xg for 20 minutes. The supernate was removed and the pellet was washed with 5 mL of 70% ethanol and centrifuged for 10 minutes as above. The supernatant was removed and the pellet was allowed to dry. The pellet was resuspended in 480 µL of RNAse free water and 20 µL of RNASecure reagent (Ambion). The RNA Secure reagent (25X) is added to a final concentration of 1X and the RNA sample is incubated at 60°C for 10 minutes. The RNA was transferred from the Oak Ridge tube to an RNAse free microfuge tube and stored at –80°C. RNA was quantitated by measuring OD_{260} and OD_{280} values.

4.1 Removal of DNA from total RNA

Contaminating DNA was removed from the total RNA according to the manufacturer's instructions provided with the DNA-free kit (Ambion). RNA was diluted to a final concentration of 0.5 mg/mL using diethylpyrocarbonate (DEPC)-treated water, heated at 65°C for 15 minutes and then cooled on ice for 2-5 minutes. DNA-free 10X DNAseI buffer was added to the RNA to a final concentration of 1X and 3 µL of DNaseI

(2 U/μL) was added. The reaction was incubated at 37°C for 30 minutes and then cooled on ice. A change in the protocol included using 1 volume of acid phenol to remove the DNaseI from the RNA instead of using the DNaseI removal reagent. The aqueous phase containing the RNA was precipitated at -80°C for 30 minutes by addition of 1/10 volume of 5.0 M ammonium acetate and 2.5 volumes of ethanol. The ammonium acetate used was treated with RNA Secure reagent (Ambion). RNA Secure (25X) was added to the RNA sample to a final concentration of 1X and the sample was then heated at 60°C for 10 minutes. The mixture was centrifuged for 30 minutes at 4°C at 13 000 xg in an Eppendorf microfuge. The supernatant was removed and the pellet was washed by adding 1 volume of 70% ethanol followed by centrifugation at 14 000 xg for 5 minutes as above. The 70% ethanol was removed and the pellet was allowed to dry. The pellet was resuspended in 500 μL of RNAse-free water and treated with RNA Secure reagent as described above. RNA was quantitated by measuring OD_{260} and OD_{280} values. The RNA was transferred to an RNAse free microfuge tube and stored at –80°C.

5. Isolation of mRNA

Isolation of mRNA was done according to protocols provided by the poly A kit (Amersham Biosciences), poly(A) isolation kit (Qiagen) and the poly(A) Purist kit (Ambion). As the poly(A) purist kit from Ambion proved to be the most efficient in removal of contaminating ribosomal RNA, this was the final method of choice. In order to maximize the yield and purity of the mRNA produced, certain modifications were made. The RNA samples were precipitated after storage at -80°C for 30 minutes using 1/10 volume of 5.0 M ammonium acetate and 2.5 volumes of 99 % ethanol by collected , by centrifugation for 30 minutes at 14 000 xg in an Eppendorf microfuge. The

ammonium acetate was treated with RNA Secure reagent (Ambion) as described in section 4.1. The supernatant was removed and the pellet was washed by adding 1 volume of 70% ethanol, followed by centrifugation for 5 minutes at 14 000 RPM. The 70% ethanol was removed and the pellet was allowed to dry. The pellets were resuspended with 750 µL of DEPC-treated water, and 750 µL of 2X binding buffer (Ambion) was added. The RNA sample was transferred to an Eppendorf tube containing oligo (dT) cellulose (Ambion) and mixed. The sample then was denatured for 10 to 20 minutes at 75°C and agitated gently for 90 minutes (instead of 30 minutes) at room temperature. The sample was centrifuged at 4000 xg in a microcentrifuge for 5 minutes at room temperature. The supernatant was removed and stored on ice. To the oligo (dT) cellulose, 0.5 mL of Wash solution I was added, vortexed briefly to mix and the entire mixture transferred to a 2 mL microfuge tube containing a spin column for centrifugation at 4000 xg for 5 minutes at room temperature. The oligo (dT) cellulose from the spin column was resuspended in 0.5 mL of Wash solution I and centrifuged again as before. These steps were repeated an additional two times. The oligo (dT) cellulose was washed four times with Wash solution 2 in the same fashion. To elute the mRNA, the spin column was placed in another 2 mL microfuge tube and 200 µL of the RNA storage solution at 65 to 80°C was added and vortexed thoroughly to mix. It was then incubated at 65 to 80°C for 10 minutes and centrifuged at 5000 xg for 3 minutes. This was repeated with an additional 200 µL of RNA storage solution. The mRNA (400 µL) then was precipitated using 5 to 10 µg of carrier glycogen (Ambion), 1/10 volume of 5.0 M ammonium acetate and 2.5 volumes of 99% ethanol, as previously described, and resuspended in 10 µL of DEPC-treated water. The supernatant, previously retained after

the RNA was purified by the oligo (dT) cellulose and stored on ice, was precipitated. The mRNA purification procedure was repeated once more using the same resin. The mRNA was quantified by comparing the OD_{260} and OD_{280} values and by the use of the Agilent Bioanalyzer, an instrument used to quantify and evaluate the integrity of the mRNA.

6. Evaluation of integrity of RNA

RNA was evaluated on 0.8% agarose 1x TBE (0.05 M Tris, 0.05 M Boric acid, 0.005 M EDTA) gels that were electrophoresed at 120 V for 45 to 60 minutes with post staining with ethidium bromide at a concentration of 0.5 mg/mL in 1xTBE.

In addition, RNA was separated on formaldehyde agarose denaturing gels according to Sambrook *et al.* (1989) and the Poly(A) purist kit (Ambion). The formamide, water and formaldehyde were deionized using AG 501-X8 and Bio-Rex MSZ 501 (D) mixed bed resin (Biorad) according to the manufacturer's instructions. For every 100 mL of sample to be deionized, 5 g of resin was added, stirred for one hour, and filtered. Unused formaldehyde was stored at -20 °C or -80 °C. RNA samples for gels were prepared in RNAse free microfuge tubes. Each sample included 2 µL of RNA (4-20 µg/µL), 2 µL of 10X MOPS electrophoresis buffer (0.2 M MOPS, 20 mM sodium acetate, 10 mM EDTA, pH 7.0), 4 µL of formaldehyde, 10 µL of formamide and 50 µg/mL ethidium bromide. The 10X MOPS electrophoresis buffer was prepared according to Sambrook *et al.* (1989). The samples were denatured for 20 minutes at 65°C and 3 µL of 10x formaldehyde loading dye (50 % glycerol, 10 mM EDTA, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) was added. Formaldehyde was included in the running buffer at a concentration of 0.22 M (Sylvers and Serges, 1993).

Electrophoresis typically was carried out at 86 V for 2.5 hours. The RNA was visualized quickly on a UV-transilluminator and the gel photographed.

7. Oligonucleotides used for reverse transcription and polymerase chain reactions (PCR)

The following oligonucleotides (Table 6) were used in these experiments. They were purchased from Biocorp, Inc (Montreal, Quebec). Oligonucleotides RT1, RT2, RT3, RT4 and AP1, AP2, AP3, AP4, AP6 were used for differential display. Oligonucleotides AP1, AP2, AP3, AP4 were modified from Melin *et al.* (1999). PH52 and PH31 were used to amplify, by PCR, a portion of the gene coding for phenol hydroxylase. Oligo DRT was used for probe synthesis for use in dot blots.

Table 6: Oligonucleotides used

Name	Primer description	Sequence
RT1	Reverse Transcription primer 1	5'-$(T)_{12}$VG-3'
RT2	Reverse Transcription primer 2	5'-$(T)_{12}$VC-3'
RT3	Reverse Transcription primer 3	5'-$(T)_{12}$VA-3'
RT4	Reverse Transcription primer 4	5'-$(T)_{12}$VT-3'
AP1	Arbitrary Primer 1	5'-CGTACGTCAA-3'
AP2	Arbitrary Primer 2	5'-GACTGAACGT-3'
AP3	Arbitrary Primer 3	5'-AGGTCGAGCT-3'
AP4	Arbitrary Primer 4	5'-GTTCCAGTCG-3'
AP6	Arbitrary Primer 6	5'-ACCTTGGCAG-3'
PH52	Primer for the 5' portion of the phenol hydroxylase gene	5'-CGACGTCCTCATCGTTGGTG-3'
PH31	Primer for the 3' portion of the phenol hydroxylase gene	5'-GCACAAGACCGAGAACAGCC-3'
DRT	OligodT-*Not*I primer for dot blots	5'-ACCTGGAAGAATTCGCGGCAGGAA $T_{(18)}$-3'

8. Reverse Transcription

The reverse transcription reaction was optimized for *T. cutaneum* RNA. The reverse transcription reactions were performed according to a modified procedure of the manufacturer (Invitrogen) using either a combination of Superscript II and Thermoscript, or Thermoscript alone. The reverse transcription reactions were performed in reactions of a final volume of 20 µL. In an Eppendorf tube, the following were combined: 500 ng or 1 µM (final concentration) of one reverse transcription primer (RT1, RT2, RT3 or RT4, Table 6), 200 ng of mRNA (Section 6), 2 µL of 10 mM dNTP mix (each dNTP was at a concentration of 10 mM), 1µL of RNA Secure (Ambion) and DEPC-treated dH_2O to a total volume of 12 µL and denatured for 15 minutes at 75°C prior to cooling on ice. If Thermoscript was used alone, 4 µL of 5X cDNA synthesis buffer (Invitrogen), 1µL of 0.1 M DTT (Invitrogen), 2 µL (20 u/µL) of RNAse inhibitor (superRNAsin, Ambion) and 1 µL (15 U/µL) of Thermoscript were added to the reaction, followed by incubation at room temperature for 10-15 minutes and incubation at 60 to 65°C for one to two hours. If both enzymes were used, the following were combined in an Eppendorf tube: 500 ng or 1 µM (final concentration) of one reverse transcription primer (RT1, RT2, RT3 or RT4, Table 6), 200 ng of mRNA (Section 6), 2 µL of 10 mM dNTP mix (each dNTP was at a concentration of 10 mM), 1µL of RNA Secure (Ambion) and DEPC-treated dH_2O, to a total volume of 10 µL. The sample was denatured for 15 minutes at 75°C and then cooled on ice. SuperscriptII, 1 µL (200 U/µL), 4 µL of 5X cDNA synthesis buffer, 2 µL of 0.1 M DTT, 2 µL (20 U/µL) of RNAse inhibitor (superRNAsin, Ambion) was added and incubated at 25°C for 10 minutes. The reaction temperature was increased by 5°C and the samples were incubated for 10 minutes. This step was repeated until the

temperature reached 50°C. At this point, 1 μL (15 U/μL) of Thermoscript was added and the reaction was incubated for 30 minutes. The reaction temperature then was increased again by 5°C increments with the reaction incubated for 30 minutes at each step (until 65°C was reached). The former procedure generated longer cDNAs, while the latter generated products with sizes less than 2 kbp, but allowed for higher incorporation of radioactive label. The former procedure was used to produce cDNAs for differential display. In either case, the samples then were placed on ice and centrifuged briefly to remove condensation from the lids of the tubes.

To check the efficiency of the reverse transcription reaction, 50 μCi of radioactive dATP ($\alpha^{32}P$) was added to duplicate, parallel reactions. An aliquot was evaluated by electrophoresis using alkaline agarose gels as described in Section 13.2.

9. Polymerase chain reaction (PCR)

The PCR reactions for differential display were based on the protocols of Melin *et al.,* (1991). In brief, 2 μL or 10% of a reverse transcription reaction was used in a PCR reaction with a final volume of 20 μL. The reaction mix included components at a final concentration of 1x PCR buffer with ammonium sulphate (MBI), 1.5 mM of $MgCl_2$, 2 μM reverse transcription primer (used for cDNA synthesis), 2 μM of an arbitrary primer (AP1, AP2, AP3, AP4, AP6, Table 6), 25 μM of each dNTP, 5 μCi of radiolabelled dATP and water to a volume of 19.5 μL. The PCR program was 1 cycle at 94°C for 5 minutes, followed by 40 cycles of 94°C for 15 seconds, 42°C for 120 seconds, 72°C for 22 seconds and a final cycle of 72°C for 20 minutes. Taq polymerase (2.5 units, MBI) was added during the first 5 minute cycle. The PCR products were separated by

electrophoresis on denaturing acrylamide gels (Section 11) and differential display products of interest were identified and extracted (Section 12).

PCR products representing differential display products of interest that were eluted from acrylamide gel fragments were reamplified according to modifications of the procedures by Melin *et al.* (1991). The PCR procedure was the same as described above with the following changes: the annealing temperature was changed to 38 °C from 42 °C and the primer concentrations reduced to final concentrations of 1 μM of the reverse transcriptase primer and 0.2 μM of the arbitrary primer (since a specific product was now being amplified, these changes increased the yields of the amplified fragments). The yields ranged from 0.5-10 μg. The PCR products were precipitated as described previously and redissolved in 20 μL of water.

A portion of the phenol hydroxylase gene known to be expressed when *T. cutaneum* was grown with phenol as a carbon source was cloned and used as a control in some experiments. The sequence of phenol hydroxylase (Accession #L04488) is shown in Figure 8. The sequence that is amplified by the primers is shown in grey and the sequence where the primers anneal is bolded. The PCR should produce a fragment of 622 bp.

Figure 8: Sequence of phenol hydroxylase of *Trichosporon cutaneum*

The sequence that is amplified by the primers is shown in bold and the regions defining where the primers anneal is underlined.
(www.ncbi.nlm.nih.gov)

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ACCESSION   L04488, GI:170524
   1 atgaccaagt acagcgaatc ctactgcgac gtcctcatcg ttggtgccgg ccccgccggt
  61 ttgatggccg cccgcgtcct ctcagagtac gtgcgccaga agcccgacct caaggtccgc
 121 atcatcgaca agcgctcgac caaggtctac aatggccagg cagacggtct ccagtgccgt
 181 accctcgagt ctctaaagaa ccttggtctt gccgacaaga tcctctcgga ggcaaacgac
 241 atgtcgacga tcgcgctcta caaccccgac gagaatggac acattcgtcg caccgaccgc
 301 atcccagaca ccctcccccgg catctcgcgc taccaccagg tcgtgctcca ccaaggccgg
 361 attgagaggc acatcctcga ctcgattgcg gagatttcgg acacccgtat caaggtcgag
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 421 cggccgctca tccccgagaa gatggagatc gacagctcca aggctgagga ccccgaggcc
 481 taccccgtca cgatgactct ccgctacatg agtgaccacg agtcgactcc tctacagttc
 541 ggGCACAAGA CCGAGAACAG CCTCTTCCAC TCCAACCTCC AGACCCAGGA GGAGGAGGAT
 601 GCCAACTACC GCCTCCCCGA GGGCAAGGAG GCGGGCGAGA TCGAGACCGT TCACTGCAAG
 661 TACGTTATCG GCTGTGACGG TGGCCACTCA TGGGTCCGCC GCACTCTCGG CTTCGAGATG
 721 ATTGGCGAGC AGACCGACTA CATCTGGGGT GTTCTTGACG CTGTCCCGGC CTCCAACTTC
 781 CCCGACATTC GCTCGCCGTG CGCCATCCAC TCTGCCGAGT CTGGCTCGAT CATGATCATC
 841 CCGCGCGAGA ACAATCTCGT CCGCTTCTAC GTTCAGCTCC AGGCCCGCGC TGAGAAGGGC
 901 GGGCGCGTCG ACCGCACCAA GTTTACTCCC GAGGTCGTCA TTGCCAACGC AAAGAAAATC
 961 TTCCACCCCT ACACCTTTGA TGTCCAGCAG CTCGACTGGT TTACTGCCTA TCACATTGGC
1021 CAGCGTGTTA CTGAGAAGTT CTCGAAGGAC GAGCGCGTGT TCATCGCCGG TGACGCTTGC
1081 CACACCCATT CGCCCAAGGC CGGCCAGGGC ATGAACACGT CAATGATGGA CACCTACAAC
1141 CTCGGCTGGA AGCTCGGTCT CGTACTCACT GGCCGTGCCA AGCGCGACAT CCTCAAGACG
1201 TACGAGGAGG AGCGCCACGC ATTCGCACAG GCCCTCATCG ACTTTGACCA CCAGTTCTCG
1261 CGCCTCTTCT CGGGCCGCCC GGCTAAGGAC GTGGCCGATG AGATGGGCGT CTCGATGGAC
1321 GTGTTCAAGG AGGCATTCGT CAAGGGCAAC GAGTTCGCCT CGGGCACCGC TATCAACTAC
1381 GACGAGAACC TCGTGACCGA CAAGAAGAGT TCCAAGCAGG AGCTTGCCAA GAACTGCGTT
1441 GTCGGAACCC GCTTCAAGTC GCAACCCGTT GTCCGCCACT CTGAGGGCCT CTGGATGCAC
1501 TTTGGCGACC GCCTCGTCAC CGACGGCCGA TTCCGCATCA TTGTCTTCGC CGGCAAGGCT
1561 ACCGATGCCA CCCAGATGTC CCGCATTAAG AAGTTTTCCG CCTACCTCGA CTCGGAGAAC
1621 TCGGTCATCT CGCTCTACAC CCCCAAGGTC TCTGACCGCA ACTCGCGCAT CGACGTCATC
1681 ACCATTCACT CCTGCCACCG CGATGACATC GAGATGCACG ACTTCCCCGC ACCGGCTCTC
1741 CACCCCAAGT GGCAATATGA CTTCATCTAC GCCGACTGCG ACTCATGGCA CCACCCCCAC
1801 CCCAAGTCCT ACCAGGCCTG GGGCGTCGAC GAGACCAAGG GTGCCGTCGT GGTCGTCCGC
1861 CCAGACGGCT ACACCTCGCT CGTGACCGAC CTCGAGGGCA CCGCCGAGAT TGACCGCTAC
1921 TTCAGCGGTA TCCTTGTCGA GCCCAAGGAG AAGTCCGGAG CCCAGACCGA GGCCGACTGG
1981 ACCAAGTCAA CTGCATAAGC AGTGCCGTAT AGATGGCAGG GAGCAGGGGA CGGGATTTTG
2041 TATCGCTTAG CAGTGCGGGA TTATGATAGG ATAGCAGTAC TTTTCTCGGG GGCAATGAAC
2101 GCATGACTCT T
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The gene was made by amplifying 50 ng of genomic DNA from *T. cutaneum* in a PCR reaction with components at a final concentration of 1x PCR buffer with ammonium sulphate, 200 μM of each dNTP, 100 pmoles of primers PH52 and PH31 (Table 7), 1.5 mM $MgCl_2$, 2.5 units of Taq (MBI). The PCR conditions were one cycle of 10 minutes at 94°C, 2 minutes at 52°C, 1.5 minutes at 72°C, 40 cycles of 30 seconds at 94°C, 2 minutes at 52°C, 1.5 minutes at 72°C and finally one cycle consisting of 30 seconds at 94°C, 2 minutes at 52°C and 20 minutes at 72°C. The PCR products were separated electrophoretically as described in Section 11.2 and the phenol hydroxylase fragment was extracted as indicated in Section 14.

10. Preparation of molecular weight markers

Markers used for differential display gels were prepared using the 100 bp DNA ladder (MBI) or Lambda (*Eco*RI/*Hind*III digested) molecular weight markers (Promega). The ladder was first dephosphorylated with calf intestinal alkaline phosphatase according to instructions provided by the supplier (MBI). The reaction was in a volume of 50 µL consisting of 2 µL (0.5 mg/mL) of 100 bp ladder, 5 µL of 10X CIAP buffer, 42 µL dH_2O and 1 µL (10 u/µL) of CIAP and was incubated for 2 hours at 37 °C. The reaction was extracted once with one volume of phenol, precipitated using 3 M sodium acetate and ethanol and resuspended in 7 µL of dH_2O. Sodium acetate was prepared and used for precipitation as described in Sambrook *et al.,* 1989. The dephosphorylated marker then was labeled by polynucleotide kinase, PNK (MBI) with radioactive ATP (γ-P^{32}). To the ladder, 2 µL of 10X reaction buffer A, 50 or 100 µCi of radioactive ATP specific activity [3000 Ci/mmol], 1 µL of PNK (10 u/µL) and dH_20 was added to a final volume of 20 µL and incubated for 2 hours at 37°C. It then was precipitated at -80°C for 30 minutes, twice using half a volume of 7.5 M ammonium acetate and 2.5 volumes of 99 % ethanol to remove unincorporated dNTPs, and resuspended in 20 µL. About 1 µL was used for scintillation counting to determine incorporation of the radiolabel, and 400 CPM of the molecular weight markers was loaded per differential display gel.

11. Electrophoresis

11.1 Separating differential display products on denaturing acrylamide gels.

The sequencing gels were made by a modified protocol from Sambrook *et al.* (1989). Sequencing plates were thoroughly cleaned using granular soap (Fisher) and distilled water, rinsed with 99% ethanol and allowed to dry. The gel mix was made by

heating 99.2 g of urea (Bioshop), 40 mL of 5x TBE (made using 18.3 megaohm water), 30 mL of a 40% acrylamide, bisacrylamide, mix (19:1) (Bioshop) and water to a volume of 200 mL. The urea gel mix was placed in a filtering flask and degassed under vacuum for 30 minutes and used immediately. The remaining solution was stored at 4°C and reheated under hot water to dissolve the urea when needed. In order to cast a gel, 30 mL of the gel mix was mixed with 60 µL of TEMED and 120 µL of 20 % ammonium persulfate to create a plug at the bottom of the sequencing plates. This assured that there would be no subsequent leakage. To 100 mL of sequencing gel mix, 100 µL of TEMED and 200 µL of 20% ammonium persulfate were added and the gel mix cast between 38 x 52.4 cm glass plates separated by 0.4 mm spacers (coated with petroleum jelly). The comb (0.4 mm) was inserted and more gel mix was added to ensure the formation of the wells. The gel polymerized within 30 minutes and it was allowed to stand for 4 hours. The PCR reactions (20 µL) were transferred from PCR tubes to Eppendorf tubes, boiled for 10 minutes, cooled to room temperature, centrifuged at 14 000 xg for 5 seconds at room temperature and 5 µL of loading dye (50% deionized formamide, 0.025% xylene cyanol and 0.025% bromophenol blue) was added. The wells of the sequencing gel were thoroughly flushed with the running buffer (1X TBE, made using deionized 18.3 megaohm water). Into each lane, 12.5 µL of the reaction mixture was loaded. Electrophoresis was carried out at 2000 V for 6.5 or 13 hours. The gel was transferred onto 3 MM and Whatman No 1 paper, dried without heat for 4 hours using a gel drier and exposed to X-ray film overnight or 14 hours. The autoradiograms then were developed.

11.2 Agarose gel electrophoresis

DNA that was extracted and amplified was further purified by using agarose gel electrophoresis according to Sambrook *et al,* (1989), and by extracting the fragments of interest (Section 13). DNA samples in a final volume of 6 or 12 μL of 1X loading dye (Promega) were loaded on 50 mL agarose 1X TBE gels containing 1μg/mL ethidium bromide with wells made using a 3 mm by 5 mm by 1 mm comb. Electrophoresis was carried out for 45 to 60 minutes at 120 V. DNA samples that were diluted to 25 μL were loaded on 100 mL 1X TBE agarose gels (containing 1 μg/mL ethidium bromide) with 5 mm by 8 mm by 1 mm comb and the gels were electrophoresed for 2 hours at 86 V. DNA fragments that ranged from 500 bp to 5000 bp were separated electrophoretically on 0.7 to 1 % agarose gels while DNA fragments that ranged in sizes from 50 bp to 1000 bp were separated on 1.5 to 2.0% agarose gels.

11.3 Alkaline agarose gel electrophoresis

Alkaline agarose gel electrophoresis typically was used to evaluate the size of single stranded cDNA after reverse transcription and was performed according to the protocol of Bluescript II-cDNA library kit (Stratagene). Gels were made by dissolving 0.8 g of agarose by heating in 72 mL of DEPC-treated water and allowed to cool to 55°C. Approximately, 8 mL of 10x alkaline agarose running buffer (0.3 M NaOH, 0.02 M EDTA) were added to the cooled agarose, mixed, and poured into the gel trays. The DNA to be evaluated was dissolved in a volume of 6 μL and diluted with an equal volume of 2x alkaline agarose loading buffer (20 % glycerol, 4.6% of saturated bromophenol blue and 0.025 M NaOH) and loaded into the wells of the gels. About 1 μg

of Lambda (*Eco*RI/*Hind*III digested) molecular weight markers (Promega) were also prepared in the same manner. The gels were electrophoresed for 12 hours at 23 V or 100 mA until the bromophenol blue had traveled a distance of about 80 % of the gel. The gels then were stained with 100 mL of 1x alkaline agarose buffer containing 1 μg/mL of ethidium bromide. This allowed for visualization of the molecular weight markers using a UV-transilluminator and photographs were taken. Scotch TM tape was placed on the lane containing the molecular weight markers and the position of each marker was noted using a ball point pen. The gel then was covered with Saran wrap and dried for four to five hours with no heat using a gel drier. The molecular weight markers were further marked using radioactive ink (made by diluting old [^{32}P]dCTP) and the gel was exposed to X-ray film overnight.

12. Isolation and gel extraction of differential display products from denaturing acrylamide gels

The procedures of Bewsey *et al.* (1991) and Melin *et al.* (1999) were modified to be used with acrylamide gels. Gel slices containing fragments of interest, located by autoradiography, were excised from the acrylamide gel and boiled for 20 minutes in Eppendorf tubes with 200 μL of dH_2O. Subsequently, 500 μL of phenol was added to each tube with shaking for 4 hours at 4°C. The fragments then were placed at -80°C for 30 minutes, and then incubated at 37°C for 10 minutes. An additional 500 μL of phenol was added to each tube and the tubes were vortexed, placed in -80°C for 30 minutes, and then incubated at 37°C for 10 minutes. Finally, 50 μL of 3 M sodium acetate was added to each tube, the tubes were vortexed and then centrifuged for 5 minutes. The aqueous phase was collected and extracted two times with equal volumes of phenol. The aqueous

phase was collected and one volume of chloroform was added. The tubes were vortexed, centrifuged for another 5 minutes, and the aqueous phase collected and extracted two times with an equal volume of ether. After the ether phase had been discarded, the sample was precipitated at -80°C for 30 minutes using 5 to 10 μg of glycogen, one half volume of 7.5 M ammonium acetate and 2.5 volumes of 99% ethanol as described in Section 5. The differential display products then were dried in a speed vac and resuspended in 20 μL of water. An aliquot of this (usually 10 μL) was amplified in a PCR reaction as described (Section 9) using the same primers as used for differential display.

13. Phenol freeze fracture

DNA fragments of interest were extracted from agarose gel slices using the protocol of Bewsey *et al.* (1991). In summary, after electrophoresis the DNA fragment of interest was excised from the gel and placed in a 1.5 mL Eppendorf tube to which 500 μL of phenol was added. The remaining steps are as from section 12 (above) except vortexing for 1 minute replaced the boiling and shaking steps and no glycogen was used in the precipitation.

14. Cloning and sequencing of differential display products

14.1 Preparation of pBluescript (BSKSII) for cloning (T-tailing)

Plasmid pBluescript II was first digested with *Eco*RV to produce blunt ends. The restriction digestion reaction contained 20 μg of pBluescript, 1x *Eco*RV buffer (Promega), water, 0.8μL of 0.5 mg/mL BSA (Promega), 4 μL of *Eco*RV (10 u/μL) in a

total volume of 40 µL. The digest was incubated at 37°C for 3 hours. A small portion of the digest (5 µL) was evaluated on a 1% agarose gel as described in Section 11.2 to check for complete digestion. If the digestion was not complete, the sample was electrophoresed on a 1.0 % agarose gel and the fragment of interest was extracted as described in Section 13. A thymine nucleotide was added to the 3' termini of the vector by a reaction containing 10 µL (5 µg) of the linearized plasmid, 5 µL of 10x Taq buffer (MBI), 30 µL of sterile water, 0.5 µL of 10 mM dTTP and 0.5 µL of Taq polymerase (5 u/µL) in a PCR tube and incubating at 72°C for two hours. This was followed by phenol extraction and ethanol precipitation as described previously.

14.2 Ligation of differential display products

Ligations were performed according to the protocol supplied with T4 DNA ligase (MBI). Differential display products were cloned into the T-tailed pBluescript BSKS II in a ligation reaction of a final volume of 20 µL which included 10-50 ng of T-tailed BSKSII, a varied amount of insert, 0.5 mM ATP, 1 µL (5 u/µL) of T4 DNA ligase and water. The reaction was incubated for 4 to 8 hours at 16°C or overnight at 4°C.

14.3 *E. coli* transformations

Ligation mixtures (2 µL to 5 µL) or 50 ng of plasmid DNA were incubated for 30 minutes on ice in Eppendorf tubes with 45 µL of XL-2 Blue competent cells (prepared by Dr. Pamela Hanic-Joyce). The cells then were heat shocked at 42°C for 1.5 minutes, an additional 900 µL of YT (Appendix 1) was added and the cells incubated at 37°C with

shaking for 45 minutes. The cells were centrifuged at 13 000 xg for 1 minute, 800 µL of the aqueous phase was removed and the pellet was resuspended in the remaining liquid. The transformed cells were plated on YT agar plates containing 50 µg/mL ampicillin. X-gal and IPTG were added to the plates according to Sambrook *et al.* (1989). The plates were incubated at 37°C overnight. In order to increase transformation efficiency, commercial cells, XL-10 gold (Stratagene) and JM109 (Promega) also were used according to the manufacturer's instructions.

14.4 Preparation of plasmids

A single colony was inoculated into a 50 mL Falcon screw cap tube containing 5 mL of YT with ampicillin (50 µg/mL) and incubated overnight at 37°C with shaking at 225 rpm. Small plasmid preparations were made using the protocol supplied with the commercial Spin Prep Plasmid Kit (Novagen) with the following modifications: plasmid was eluted with 2 aliquots of 30 µL of prewarmed (60°C) elution buffer C at step 12 and the tubes were incubated for 5 minutes at 65°C, prior to centrifugation at 12 000 xg giving 60 µL of plasmid DNA at a concentration of 1-2 µg/µL. If higher amounts of plasmid were needed, large plasmid preparations were done according to the DNA template preparation procedure in the Promega protocols and applications guide (1991). The DNA pellets were resuspended in 200 µL of sterile dH_2O to give a final concentration of plasmid of 1-2 µg/mL.

14.5 Screening and confirmation

The cloning of products was confirmed by gel electrophoresis (as described in section 11.2) of plasmid DNA digested by *Pvu*II (MBI) on 0.8% agarose gels. The reaction consisted of 0.5 µg to 2 µg of prepared plasmid DNA (Section 14.4) in a restriction digest with 1x Buffer G^+ (MBI), 0.5µL (10 u/µL) of *Pvu*II and dH_20 in a volume of 20 µL and was incubated for 2 hours at 37°C. Alternatively, plasmid DNA was digested by *Eco*RI (MBI) and *Sal*I (MBI) in a 20 µL reaction which included 1x Buffer O^+ (MBI), 0.5 µg to 2 µg of plasmid DNA, dH_20 and 0.25 µL of *Eco*RI (10 u/µL) and *Sal*I (10u/µL).

15 Characterization of differential display products

15.1 Sequencing and sequence analysis of differential display products

Samples were sequenced by the sequencing center at the Centre for Structural and Functional Genomics at Concordia University or by Bio S and T, Inc. Sequences were compared to the NCBI (www.ncbi.nlm.nih.gov) databases using Basic Local Alignment Search Tool (BLAST). The blastn, tblastp and tblastx programs were used for sequence analysis (Altschul *et al.*, 1997).

15.2 Northern analysis

15.2.1 Northern transfer

Either 20-40 μg of total RNA or 1 μg of mRNA, from different cell preparations, were separated by electrophoresis on a formaldehyde gel as described in section 6. The gels were visualized and photographed. The RNA was transferred overnight onto Hybond XL membranes (Amersham Biosciences) by upward capillary transfer as described by the manufacturer and Sambrook *et al.* (1989). The membrane had been pretreated for 30 minutes in DEPC-treated water and DEPC-treated 20X SSC (3 M NaCl, 0.3 M Sodium citrate, pH 7.0). The membrane was placed on a stack of 3 mm electrophoresis paper (Canadawide Scientific Inc) that was immersed and soaked in 20 X SSC. The formaldehyde agarose gel containing the RNA was washed with DEPC-treated water and placed on top of a stack of 3 MM paper soaked in 20X SSC with the back of the gel facing up. The membrane was placed on top of the gel and a glass pipet (baked for 12 hours at 200°C) was rolled on top to remove any air bubbles that were trapped between the gel and the membrane. The wells were marked on the membrane using a ball point pen. Two or three 3 MM papers, pre-soaked in 20X SSC were placed on top of the membrane and then two dry 3 mm papers were placed on top. Each time, a glass pipet was rolled on top to ensure that there were no air bubbles present. Paper towels were added on top to a height of 14 cm along with a 0.5 kg weight. Transfer was allowed to proceed overnight up to 18 hours. The membrane was removed and crosslinked at 1200 J/cm^2 using the autocrosslink feature, available on a Fisher UV-crosslinker. In

order to check for efficiency of transfer, the RNA were visualized either by observation under a hand held UV lamp or by staining the membrane with methylene blue solution (0.02% w/v methylene blue in 0.3M sodium acetate (pH 5.5) (Sambrook *et al.,* 1989) until the RNA bands appeared. Staining with methylene blue revealed the RNA bands within 5 to 15 minutes. These membranes were typically scanned and the RNA markers were marked using a ball point pen. Later, the gels were destained for 2 to 6 hours using DEPC treated 0.3 M sodium acetate or water with many changes in destaining solution. In addition, the formaldehyde gels were visualized by post-staining for 5 to 10 minutes in a plastic container with 100 mL of 1X MOPS running buffer containing 1 μg/mL of ethidium bromide to confirm the efficiency of transfer.

15.2.2 Pre-hybridization

The membranes were individually placed in hybridization bottles (Fisher) and washed 3 times with 200 mL of 4X SSC (0.6 M NaCl, 0.06 M sodium citrate, pH 7) for 2 hours while the membranes were rotating at room temperature. Commercial hybridization buffer (2 mL per 100 cm^2), Ultrahybe (Ambion), was added and incubated at 65°C for a few minutes. The temperature was then lowered to 42°C and the membranes were allowed to prehybridize for 2 to 6 hours.

15.2.3 Probe preparation

The probes were made to a specific activity of 1x 10^9 CPM/μg using the Decaprime II kit (Ambion) using radiolabelled dCTP (MP Biomedicals, specific activity

of 3000Ci/mmole). The probe was made using purified DNA. In order to synthesize probes from differential display products or particular controls, plasmids containing these clones were digested with *Eco*RI and *Sal*I. The restriction digestion reaction contained 10 μg of plasmid pBluescript, 1x O^+ buffer (MBI), water, 1 μL of *Eco*RI and *Sal*I (10 u/μL) in a total volume of 20 μL. The digest was incubated at 37°C for 3 hours. To the digest, 4 μL of 6X loading dye (MBI) was added and the sample was then loaded on a 2 % agarose 1X TBE gel containing 1μg/mL ethidium bromide with wells made using a 5 mm by 3 mm comb. Electrophoresis was carried out for 45 to 60 minutes at 120 V. The fragments of interest were extracted as described in Section 13 and an aliquot was evaluated on a 50 mL 2% agarose 1X TBE gel containing 1μg/mL ethidium bromide as described in section 11.2. Quantification of the probe was done according to the Decaprime II Kit protocol (Ambion). Labelling of the probes of interest was done according to specifications of the Decaprime II Kit protocol (Ambion). An aliquot, 2 μL of the reaction was combined with 198 μL of H_2O containing 100 μg of carrier DNA (herring sperm DNA, prepared by Dr. Pam Hanic-Joyce). The tube was vortexed and 100 μL of this reaction was taken and counted using a scintillation counter. The remainder was precipitated using 2 mL of cold 10% trichloroacetic acid (TCA), filtered through GF/C filters (Fisher) and the radioactivity was subsequently measured using the scintillation counter. The trichloroacetic acid was prepared according to Sambrook *et al.* (1989). About 25-50% of radiolabelled dCTP was incorporated into the probe generated. The probe then was precipitated three times using 10 μg of glycogen, one half volume of 7.5 M ammonium sulfate (prepared as described in Sambrook *et al.* (1989) and 2 volumes

of ethanol as previously described to remove unincorporated nucleotides and resuspended in 25 μL TE (10 mM Tris, 1mM EDTA).

15.2.4 Hybridization and autoradiography

The probe was added at a concentration of 10^6 CPM/mL of hybridization buffer used. The probes were denatured for 10 minutes at 94°C and then placed on ice for 2 minutes. The probes were added with an additional amount of hybridization buffer (2 mL per 100 cm^2) of Ultrahybe or hybridization buffer (50% deionized formamide, 5x SSC, 5x Denhardt's solution, 0.1mg/mL herring sperm DNA, 0.5% sodium dodecyl sulphate or SDS) specified by Sambrook *et al.* (1989). When the hybridization buffer from Sambrook *et al.* (1989) was used, the DNA probe and herring sperm DNA were denatured together and then added to the hybridization buffer. The hybridization buffers were thoroughly heated at 65°C to remove or dissolve any particulate matter. The membranes were hybridized for 12 to 24 hours at 42°C. The membranes were rinsed with 200 mL of 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 0.5-1 hr at 42°C. The membranes then were washed 3 to 4 times for 0.5-1 hr at 42°C with 200 mL of 2X SSC containing 0.1% SDS and then 3 times for 0.5-1 hr at 42°C with 200 mL of 0.1XSSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7) containing 0.1% SDS. The membranes were dried, wrapped in Saran wrap and exposed to X-ray film.

15.3 Dot Blots

Dot blots were made according to a modified protocol of Hames and Higgins (1987). Plasmid DNA containing selected differential display products was diluted to concentrations of 100 ng/μL and 10 ng/μL using water and 5 μL of loading dye. Hybond XL membranes were used for the dot blots. The membranes were immersed in 20X SSC for 30 minutes and then allowed to dry. The plasmid DNA was denatured at 94°C for 20 minutes, placed on ice and centrifuged briefly. Each clone at the appropriate concentrations in a volume of 1 μL was spotted onto the prepared Hybond XL membranes in triplicate using a multi channel pipettor. The membranes were placed for 5 minutes in a dish containing 3 MM paper soaked in 0.5 M NaOH and 1.5 M NaCl. The membranes then were transferred for 5 minutes to a dish containing 3 MM paper soaked with 0.5 M Tris-HCl, (pH 7.4). The membranes then briefly were dried in air and crosslinked as described in Section 15.2.1.

15.3.1 Probe preparation from mRNA

Probes were made by reverse transcribing mRNA isolated from cells grown as described previously (Section 8) with phenol or succinate as a carbon source. A different preparation of mRNA was used to make each probe. The reverse transcription reaction was done according to a modified procedure of the manufacturer using Thermoscript (Invitrogen). In brief, 500 ng (1 μL) of 50 μM of the primer, *Not*I-dT_{18}, 200 ng of

mRNA, 2 μL of a dNTP mix consisting of 12 mM dGTP, 8 mM dATP and 8 mM dTTP and 80 μCi of dCTP in a total volume of 12 μL was denatured by heating for 15 minutes at 75°C and then placed on ice for 2 minutes. Then 5 μL of 4X cDNA synthesis buffer (Invitrogen), 1 μL of 0.1M DTT and 1μL (15 units) of Thermoscript was added and the reaction was incubated at 25°C for 10 minutes and 65°C for 2 hours. At this point, 3 μL of 12 mM dCTP, 2.5 μL of 4X cDNA synthesis buffer, 0.5 μL of 1M DTT, 1μL of the dNTP mix consisting of 12 mM dGTP, 8 mM dATP and 8 mM dTTP 2.5 μL dH_2O and 0.50 μL of Thermoscript were added and the reaction was incubated for an additional 2 hours. The reverse transcription reactions then were cooled on ice. An aliquot of the reverse transcription reaction was quantified as described in section 15.2.3. An aliquot (3 μL) was taken and evaluated on an alkaline agarose gel as described in section 11.3. The remainder of the reverse transcription reaction was precipitated as described in section 12 using glycogen, 7.5 M ammonium acetate and 99% ethanol and resuspended in 50 μL of TE.

15.3.2 Hybridization and autoradiography

Each membrane was placed in a roller bottle (Fisher) and treated as described in Section 15.2.2. The hybridization and probe addition, added at a concentration of 10^6 CPM/mL of hybridization buffer used, was carried out as described in Section 15.2.4. Every probe synthesized by reverse transcription was used in a separate hybridization reaction. Probes were made using mRNA isolated from 3 different preparations of cells grown either in phenol or succinate and used to evaluate the abundance of differential display products. Autoradiography was carried out at 4°C without an intensifying screen.

RESULTS

1. Growth of *T. cutaneum* in minimal and rich media

Representative growth curves of *Trichosporon cutaneum* grown from a single colony in rich medium (Nutrient Broth) or minimal medium containing succinate or phenol is shown in Figure 9. Additional succinate or phenol was added at an OD_{600} of 0.4. The doubling time was determined to be approximately 3.0 hours. *T. cutaneum* was grown many times and the observed growth rates were noted. The doubling times for cells grown in succinate or phenol media were determined to be approximately 2.5 hours and 3.3 hours, respectively.

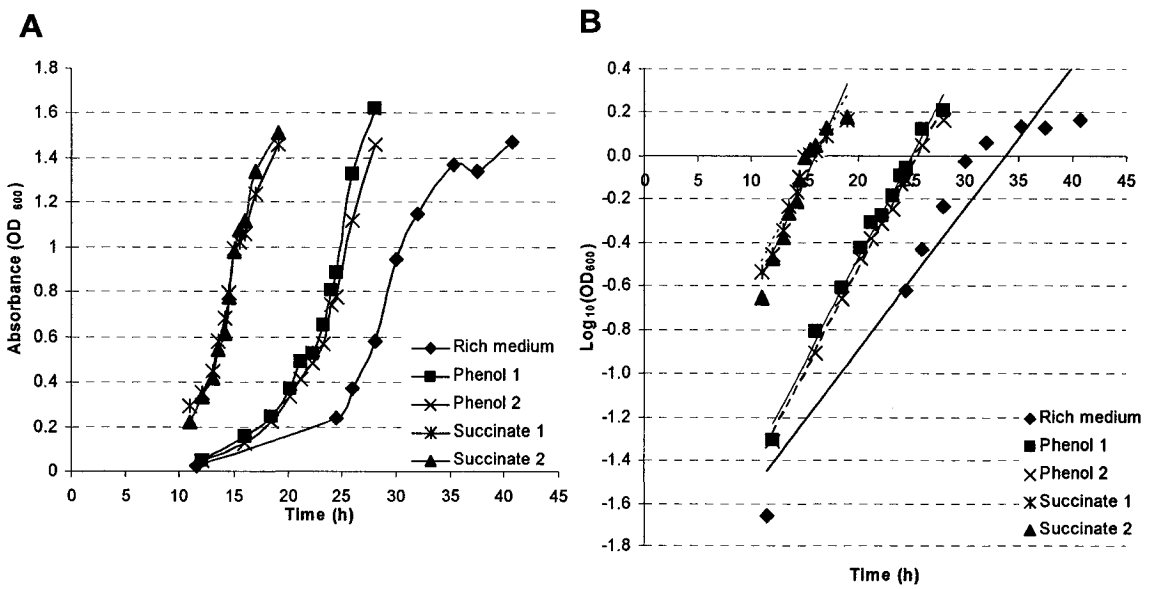


Figure 9: Growth rates of *T. cutaneum*
A) Growth rates for *T. cutaneum* cultures grown in rich medium (—◆—), succinate (—✳—, —▲—) or phenol (—■—, —×—) medium. Additional succinate or phenol was added at an OD_{600} of 0.4. B) Log_{10} plots of data from panel A. Rich medium (◆), in phenol (■, ×) or succinate (✳, ▲).

2. Quantification and evaluation of integrity of RNA

RNA was extracted from *T. cutaneum* grown in phenol or succinate medium and typical yields were 1-3 mg of total RNA per gram of cells with an OD_{260}/OD_{280} ratio near 2. Messenger RNA (mRNA) was purified from total RNA and typical yields were 5-15 µg of mRNA per 1.5 mg of Total RNA used for each purification. The OD_{260}/OD_{280} ratio was near or greater than 2. Samples of purified total RNA and mRNA were routinely evaluated on gels to confirm RNA integrity. A representative gel of total RNA is shown in Figure 10. The two distinct bands present, represent the 28S ribosomal RNA (rRNA) and the 18S ribosomal RNA. Hence, total RNA was used for further experimentation or purification when both rRNA bands were present, with the 28S rRNA showing approximately double the intensity (lane 2 in Figure 10). (Degraded RNA would not show these two major bands).

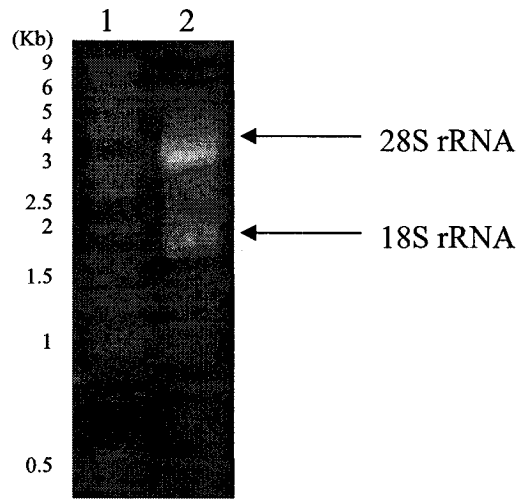
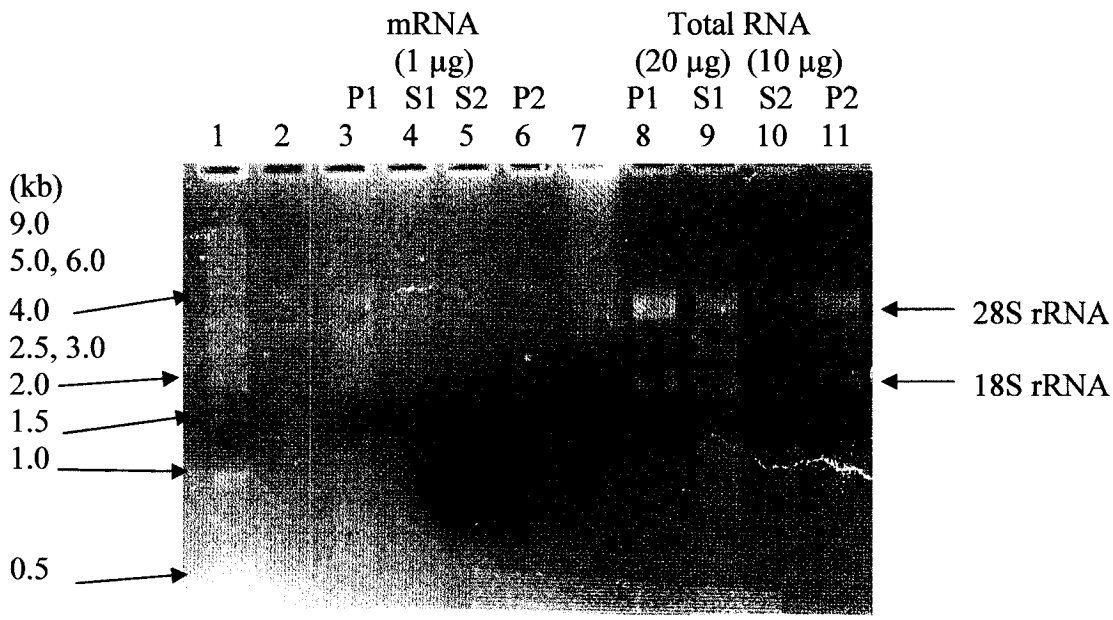


Figure 10: Total RNA samples evaluated by denaturing gel electrophoresis
A sample of total RNA (lane 2) obtained from cells grown in phenol medium was electrophoresed under denaturing conditions as described in section 6. Lane 1 contains RNA millennium markers (Ambion).

Purification of the mRNA involved the removal of the contaminating DNA by DNaseI treatment and the rRNAs using an oligo dT resin. The mRNA was routinely evaluated by denaturing agarose gel electrophoresis (Figure 11). The mRNA in lane 3 which was purified from total RNA (shown in lane 11) isolated from cells grown in phenol medium contains very little of the 28S and 18S ribosomal bands.

Figure 11: Denaturing agarose gel representing mRNA and total RNA



Samples of mRNA purified from cells grown in phenol (P1, P2, lanes 3, 6) or succinate (S1, S2, lanes 4, 5) and samples of total RNA from cells grown in phenol (P1, P2, lane 8, 11) or succinate (S1, S2, lane 9, 10) were electrophoresed under denaturing conditions as described in section 6. Lane 1 contains RNA millennium markers (Ambion) and lane 2 and 7 are empty. The positions of 28S and 18S rRNAs are indicated by arrows.

3. Reverse transcription (quality control)

Differential display and production of probes to be used for reverse Northern dot blots involved the conversion of mRNA to cDNA. In order to evaluate the efficiency of the reverse transcription reactions, radioactively labelled dCTP was included in the reverse transcription reactions. The products of reverse transcription were separated by denaturing agarose gel electrophoresis and evaluated by phosphoimaging or exposure to X-ray film. An example of the efficiency of the reverse transcription reaction is illustrated in Figure 12.

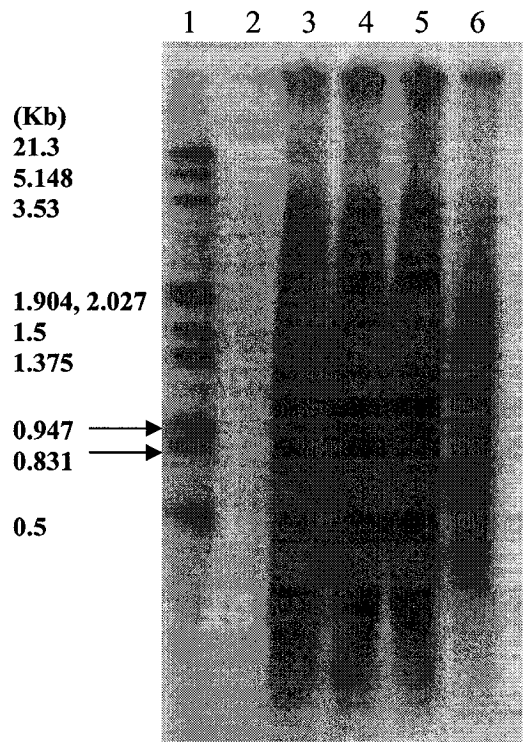


Figure 12: Phosphoimage of reverse transcriptase products separated by alkaline agarose electrophoresis

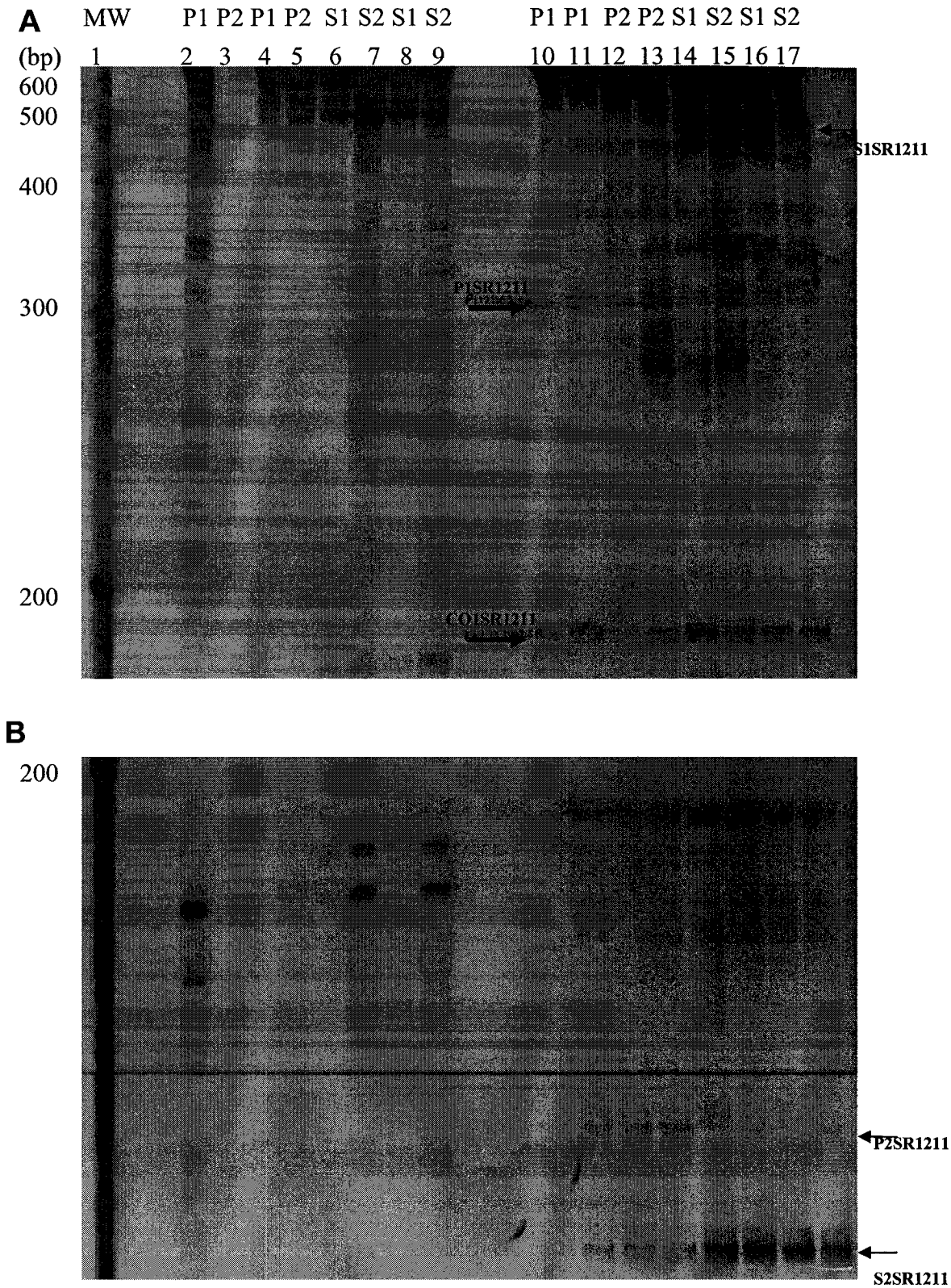
Reverse transcriptase products of mRNA purified from phenol (lanes 3, 4) or succinate (lanes 5, 6) grown cells were separated by alkaline agarose gel electrophoresis and visualized by phosphoimaging. The image was developed after two days of exposure. Labelled lambda DNA digested with *Eco*RI and *Hind*III (500 CPM) was loaded in lane 1. A control reaction of phenol mRNA with no reverse transcriptase was loaded in lane 2.

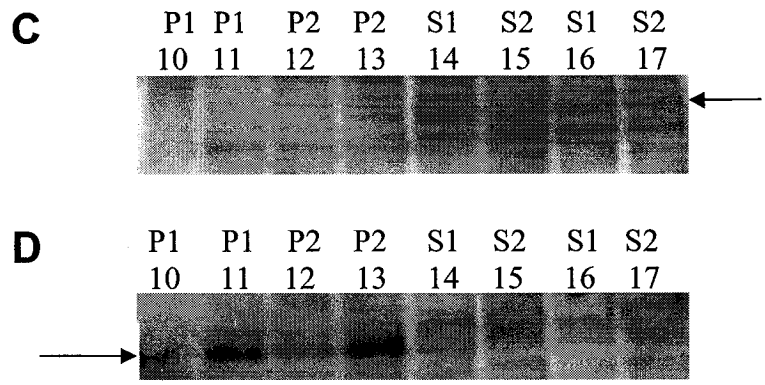
The dark streak in lanes 3, 4, 5 indicates that the reverse transcription reaction produced cDNAs in the range of 0.5 to 4 kb. The reverse transcriptase reaction shown in lane 6 indicates that cDNA production was not as efficient as in the other reactions so it was not used.

4. Differential display

Initially, differential display was carried out mRNA purified from total RNA extracted from two different cultures of cells grown in phenol (P1, P2) or succinate (S1, S2) medium (Figure 13). In order to demonstrate reproducibility, differential display was repeated using two additional biological samples, P3 and P4 for cells grown in phenol and S3 and S4 for cells grown in succinate medium (Figure 14). Differential display was carried out with primers RT1 and AP1 (50% G+C) or AP2 (60% G+C).

Figure 13: Autoradiogram showing differential display products produced using primers RT1 and AP1 or AP2 from mRNA purified from cells grown in phenol (P1, P2) or succinate (S1, S2)





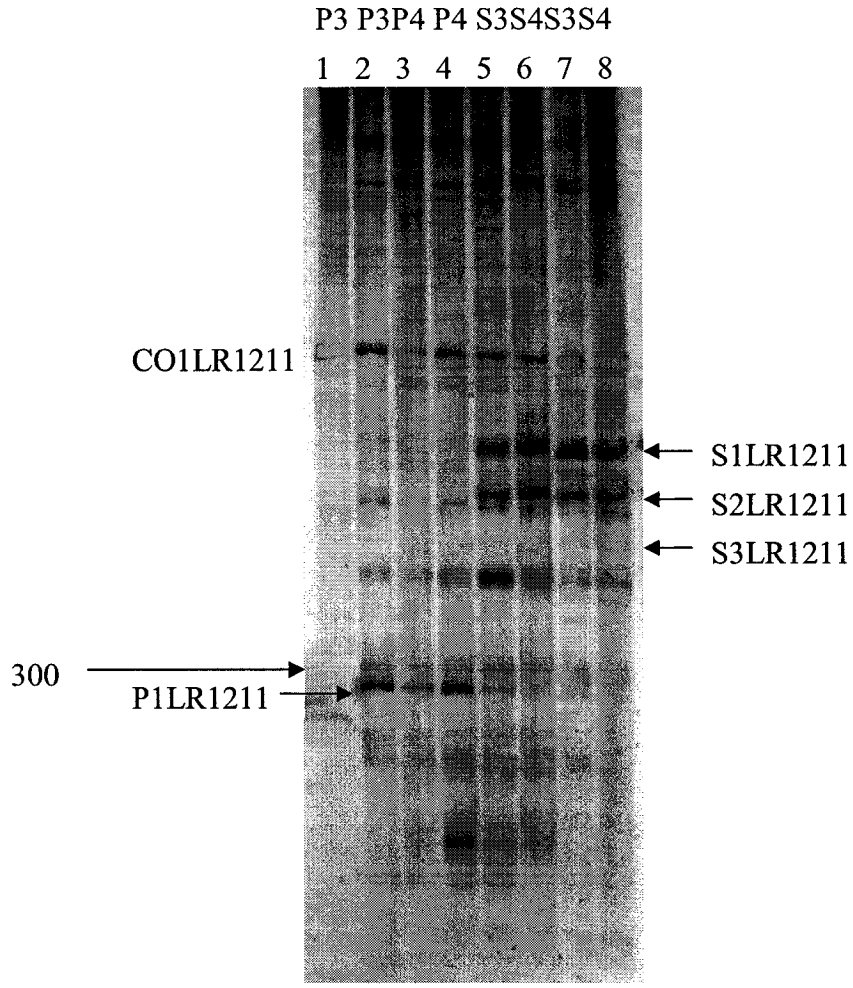
A) An autoradiogram of electrophoresed differential display products, amplified by primers RT1 and AP1 or AP2. Lane 1, 500 CPM of radioactively labelled 100 bp molecular weight markers; lanes 2-5 and 10-13, differential display products from two phenol samples (P1 and P2); lanes 6-9 and 14-17, differential display products from two succinate samples (S1 and S2). Primer pair RT1 and AP1 was used in lanes 2-9 and primer pair RT1 and AP2 was used in lanes 10-17. The gel was electrophoresed for 6.5 hours and exposed to X-ray film for 8 hours. Differentially expressed differential display products were identified and labelled.

B) An autoradiogram of differential display products, amplified by primers RT1 and AP1 or AP2. The samples were loaded as listed in panel A. Differentially expressed differential display products were identified and labelled.

C) An enlargement of a region containing differential display product S1SR1211 (down regulated when cells are grown in phenol medium).

D) An enlargement of a region containing differential display product P1SR1211 (upregulated when cells are grown in phenol medium).

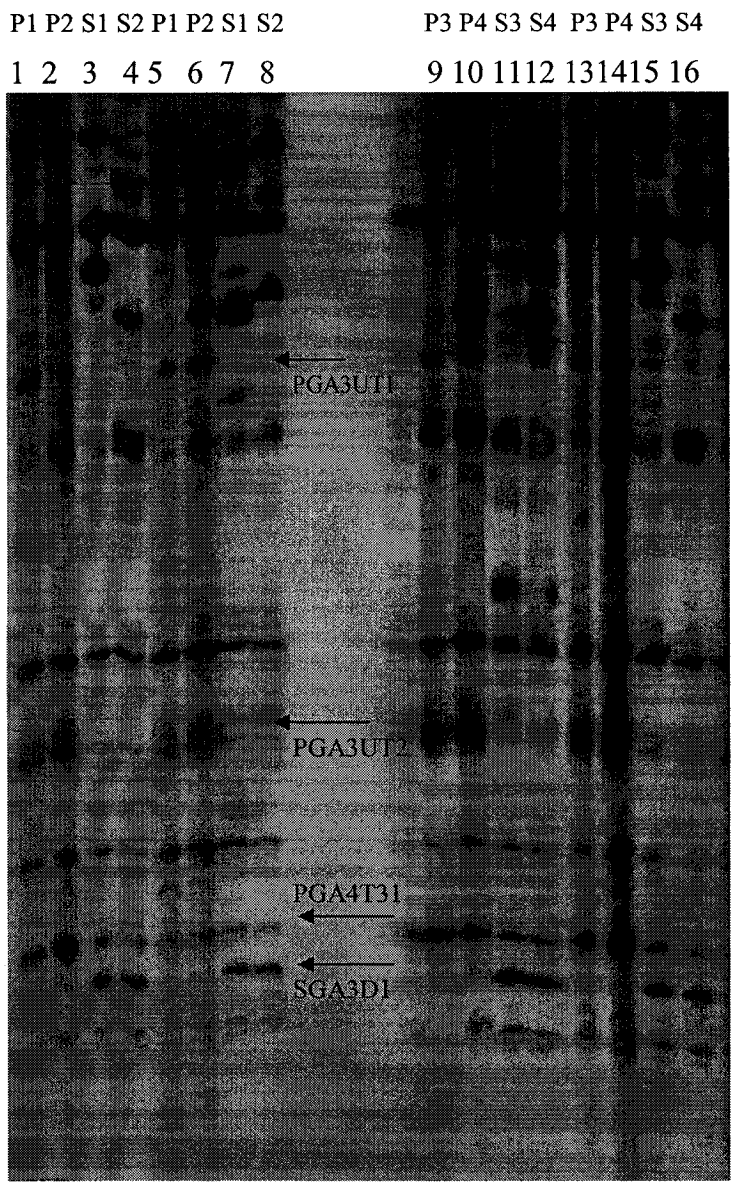
Figure 14: Portion of an autoradiogram showing differential display products produced using primers RT1 and AP2 from mRNA purified from cells grown in phenol (P3, P4) or succinate (S3, S4)



Lanes 1-4, differential display products from two phenol samples (P3 and P4); lanes 5-8, differential display products from two succinate samples (S3 and S4). Primer pair RT1 and AP2 was used for amplification of each sample. The gel was electrophoresed for 13 hours and exposed to X-ray film for 8 hours. The number 300 represents the location of the 300 bp molecular weight marker. Differential display products are identified and labelled.

The results of differential display using primers RT1 and AP3 (which has 60% G+C content) are shown in Figure 15. In order to show reproducibility, differential display products were produced on different days from mRNA purified from independent preparations of cells grown with phenol (P1, P2, P3, P4) or succinate (S1, S2, S3, S4) and separated on the same denaturing acrylamide gel.

Figure 15: Autoradiogram showing differential display products produced using primers RT1 and AP3 from mRNA purified from cells grown in phenol (P1, P2, P3, P4) or succinate (S1, S2, S3, S4)

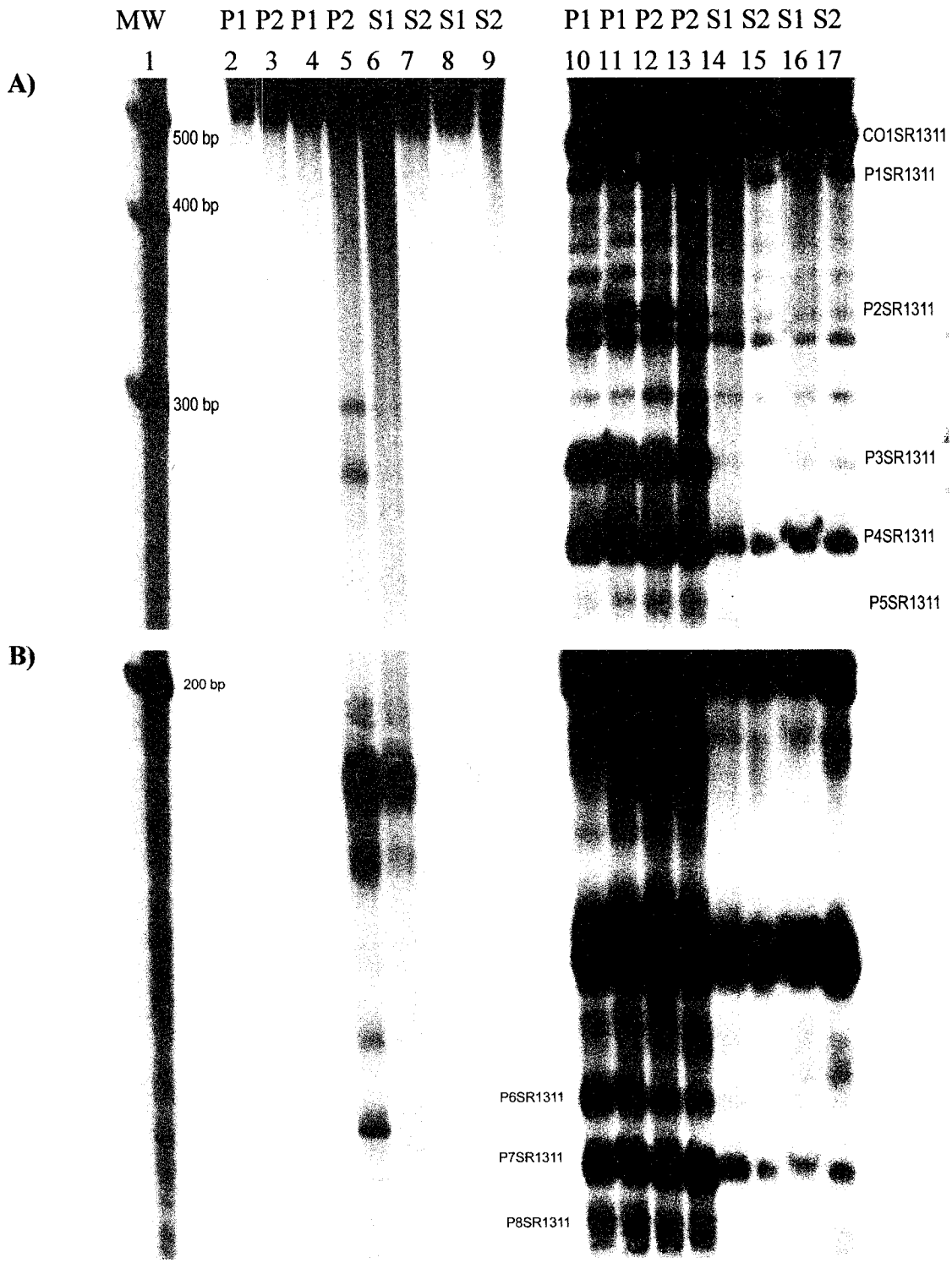


Lanes 1, 2, 5, 6, 9, 10, 13, 14, differential display products from four phenol samples (P1, P2, P3, P4); lanes 3, 4, 7, 8, 11, 12, 15, 16, differential display products from four succinate samples (S1, S2, S3, S4). Primer pair RT1 and AP3 was used for amplification of each sample. The gel was electrophoresed for 6.5 hours and exposed to X-ray film for 8 hours. Differentially expressed differential display products were identified and labelled.

The results of differential display using primers RT1 and AP4 or AP6 (which has 60% G+C content) were produced from mRNA isolated from cells grown in phenol (P1,

P2) or succinate (S1, S2) medium are shown in Figure 16. Differential display products produced from cells grown in phenol (P3, P4) or succinate (S3, S4) medium using primers RT1, AP4 or AP6 were separated on the same denaturing acrylamide gel for 13 hours and the autoradiogram is shown in Figure 17.

Figure 16: Autoradiogram showing differential display products produced using primers RT1 and AP4 or AP6 from mRNA purified from cells grown in phenol (P1, P2) or succinate (S1, S2)

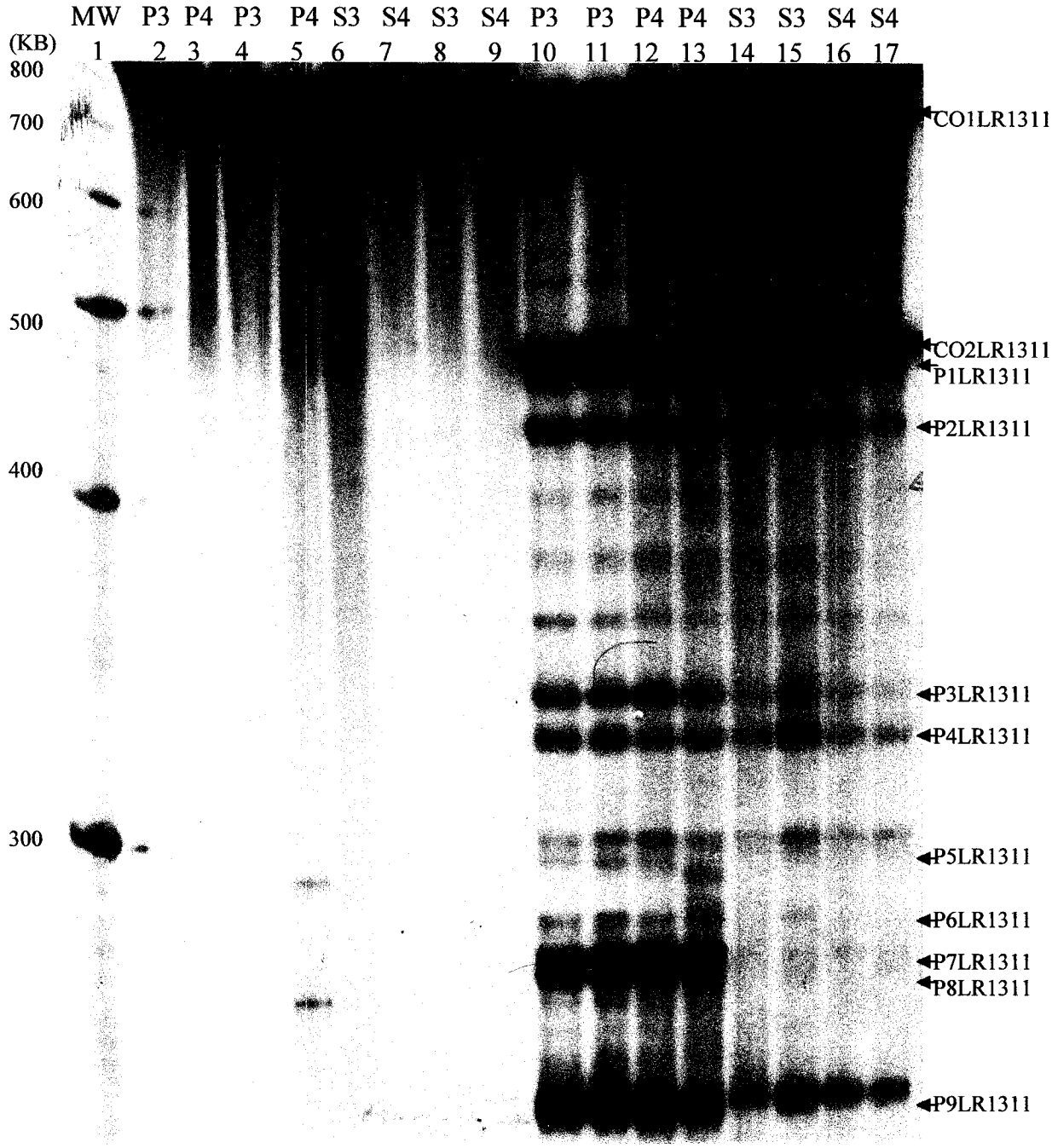


A) An autoradiogram of electrophoresed differential display products, amplified by primers RT1 and AP4 or AP6. Lane 1, 500 CPM of radioactively labelled 100 bp

molecular weight markers; lanes 2-5 and 10-13, differential display products from two phenol samples (P1 and P2); lanes 6-9 and 14-17, differential display products from two succinate samples (S1 and S2). Primer pair RT1 and AP4 was used in lanes 2-9 and primer pair RT1 and AP6 was used in lanes 10-17. The gel was electrophoresed for 6.5 hours and exposed to X-ray film for 8 hours. Differentially expressed differential display products were identified and labelled.

B) An autoradiogram of differential display products, amplified by primers RT1 and AP4 or AP6. The samples were loaded as listed in panel A. Differentially expressed differential display products were identified and labelled.

Figure 17: Autoradiogram showing differential display products produced using primers RT1 and AP4 or AP6 from mRNA purified from cells grown in phenol (P3, P4) or succinate (S3, S4)



An autoradiogram of electrophoresed differential display products, amplified by primers RT1 and AP4 or AP6. Lane 1, 500 CPM of radioactively labelled 100 bp molecular weight markers; lanes 2-5 and 10-13, differential display products from two phenol samples (P3 and P4); lanes 6-9 and 14-17, differential display products from two succinate samples (S3 and S4). Primer pair RT1 and AP4 was used in lanes 2-9 and primer pair RT1 and AP6 was used in lanes 10-17. The gel was electrophoresed for 6.5

hours and exposed to X-ray film for 8 hours. Differentially expressed differential display products were identified and labelled.

5. Isolation of PCR products and amplification of differential display products

After comparing the differential display autoradiograms and locating the products of interest, these regions of the gels were excised and the DNA was extracted. Once extracted, the differential display product was amplified, evaluated on agarose gels, excised and cloned. Amplification of certain fragments excised from a typical differential display gel is shown in Figure 18. According to the differential display gel, fragments P1SR1311, P2SR1311and P3SR1311 were approximately 450 bp, 350 bp and 300 bp, respectively. After excision and amplification, they gave products of the expected sizes (Fig 18). A non-differentially expressed product, CO1SR1311 of 500 bp did not produce a product on this first attempt at reamplification, but produced a product when the reaction was repeated (data not shown).

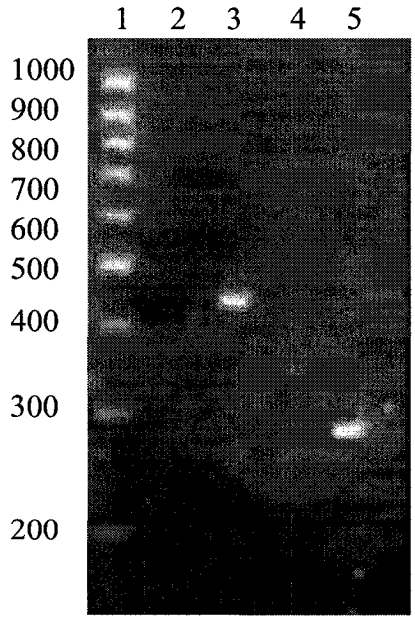


Figure 18: Ethidium bromide stained agarose gel containing excised and amplified differential display products
Lane 1, 100 bp Ladder; lane 2, CO1SR1311; lane 3, P1SR1311; lane 4, P2SR1311; lane 5, P3SR1311.

6. Characterization and identification of differential display products

6.1 Sequence analysis

Non-differentially expressed and differentially expressed products were identified by comparison of lanes containing differential display products produced from cells grown in phenol or succinate. These products were excised, extracted, amplified, cloned into plasmid pBluescript and sequenced. The sequences are listed in Appendix 2. In order to identify these differential display products, the sequences were compared to NCBI databases (www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST). Each of blastn, tblastp and tblastx was used for sequence analysis (Altschuhl *et al.*, 1997). Table 7 includes sequence analysis and proposed identities for differentially expressed products observed to be upregulated in cells grown in phenol medium. Table 8 lists sequence analysis and proposed identities for differentially expressed products observed to be downregulated in cells grown in phenol medium and upregulated in cells grown in succinate medium. Table 9 includes sequence analysis and proposed identities of non-differentially expressed products. A summary of the sequence analysis of the isolated differential display products is listed in Table 10. Identities were assigned based on the highest sequence similarity and lowest E value indicated. Most products which remain unidentified had sequences which were similar to unassigned open reading frames in organisms so that the identification of these products was not possible. In cases where the best e value was to a hypothetical protein or to unassigned open reading frames, the first match to any protein of known function is listed.

Table 7: Differential display products found to be upregulated in phenol medium

		Nucleotide database		Protein database		Translated database		
Clone	Size (bp)	Alignment with nucleotide data base	Organism (E value)	Alignment with protein database	Organism (E value)	Alignment with translated database	Organism (E value)	Proposed Identity
PGA3UT1	514	Transposase like carrier protein**	Thermophilic bacterium (E=0.014)**	Sporulation control protein (spo0M) ***	*Bacillus subtilis* (E=3e-13)***	(glpF) Glycerol facilitator***	*Thermus aquaticus* (E=1e-13)***	Glycerol facilitator***
PGA3UT2	593	No significant similarity*	-	Hypothetical protein	Various	Hypothetical protein	Various	Unidentified
P1SR1211	294	No significant similarity*	-	No significant similarity*		Hypothetical protein	*Homo sapiens* (E=0.071)***	Unidentified
				Hypothetical transmembrane 4 superfamily protein	*Streptomyces avermitilis* (E=0.28)	Tartrate dehydrogenase **	*Pseudomonas syringae* (E=1.3) **	
P1LR1211	300	No significant similarity*	-	Transcription factor Rfef***	*Emericella nidulans* (E=4e-06)***	Transcription factor Rfef***	*Emericella nidulans* (E=0.028)***	Transcription factor***
				Hypothetical protein	*Aspergillus nidulans* (E=4e-06)	Transcriptional regulator (MerR family)	*Pseudomonas putida* (E=1.7)	
P1SR1311 P1LR1311	427	Vacuolar ATPase***	*M.sexta* (E=1e-05)***	Vacuolar ATPase ***	*Neurospora crassa* (E=1e-30) ***	Vacuolar ATP synthase subunit F (vma-7) gene ***	*Neurospora crassa,* (E= 4e-24) ***	Vacuolar ATPase***
					Schizosaccharomyces pombe) (E=2e-26)			

*No significant similarity to anything in the database (E > 0.1)

**Clones were most similar to unassigned open reading frames/hypothetical proteins, alignments listed are for any protein identified

***Closest alignment listed, highest E value and similarity seen

N/A-not applicable

Table 7 (continued)

		Nucleotide database		Protein database		Translated database		
Clones	Size of fragment (bp)	Alignment with nucleotide database	Organism (E value)	Alignment with protein database	Organism (E value)	Alignment with translated database	Organism (E value)	Proposed Identity
P2SR1311	336	No significant similarity*	-	Not much similarity* Leucine rich protein DNA polymerase elongation subunit (family B)**	*Pseudomonas aeruginosa* (E=1.1)**	pteA5 "modular polyketide synthase", hypothetical protein in human, Drosophila***	*Streptomyces avermitilis* (E= 0.093)***	Unidentified
P6SR1311	100	No significant similarity*	-	No significant similarity*		No significant similarity		Unidentified
P2LR1311	403	No significant similarity*	-	Hypothetical protein, related to triose phosphate/3-phosphoglycerate phosphate translocator***	*Neurospora crassa* (E= 0.004)***	Hypothetical protein, related to triose phosphate/3-phosphoglycerate phosphate translocator***	*Neurospora crassa* (E=0.007) ***	Unidentified
P3LR1311	336	No significant similarity*	-	No significant similarity *	-	modular polykeytide synthase*** argininosuccinate synthase	*Streptomyces avermitilis* (E=0.087)*** *Caulobacter crescentus* (E=0.12)	Unidentified

*No significant similarity to anything in the database (E > 0.1)
**Clones were most similar to unassigned open reading frames/hypothetical proteins, alignments listed are for any protein identified
***Closest alignment listed, highest E value and similarity seen
N/A-not applicable

Table 7 (continued)

		Nucleotide database		Protein database		Translated database		
Clones	Size of fragment (bp)	Alignment with Nucleotide DB	Organism (E value)	Alignment with Protein DB	Organism (E value)	Alignment with Translated DB	Organism (E value)	Proposed Identity
P2SR1211	134	No significant similarity* 16S ribosomal RNA	*Nematostella vectensis* (E=3.2)***	No significant similarity *	-	No significant similarity *	-	16 S R.RNA
P5LR1311	400	No significant similarity*		No significant similarity*	-	No significant similarity*	-	Unidentified
P3SR1311	276	28S rRNA***	Various organisms (E=1e-135)***	28S rRNA	Various organisms	28S rRNA	Various organisms	28S rRNA
P4SR1311	275							
P7SR1311	137	26S rRNA***	*Trichosporon asahii* (E=4e-37)***	No matches	No matches	rRNA	*Trichosporon mycotoxinivorans* (E= 1e-09)***	26S rRNA
P8SR1311	114							
P6LR1311	375							
P7LR1311	350							
P8LR1311	325							
P9LR1311	300							
P4LR1311	425	5.8S rRNA***	*Cryptococcus neoformans* (E=e-136)***	cytochrome P450 monooxygenase	*Zea mays* (E = 2e-12)	N/A	N/A	5.8S rRNA

*No significant similarity to anything in the database (E > 0.1)
**Clones were most similar to unassigned open reading frames/hypothetical proteins, alignments listed are for any protein identified
***Closest alignment listed, highest E value and similarity seen
N/A-not applicable

Table 8: Differential display products found to be downregulated in phenol grown cells

		Nucleotide database		Protein database		Translated database		
Clones	Size of fragment (bp)	Alignment with Nucleotide DB	Organism (E value)	Alignment with Protein DB	Organism (E value)	Alignment with Translated DB	Organism (E value)	Proposed Identity
S1SR1211	500	No significant matches*		ERD2 protein	*Scherffelia dubia* (E=5e-31)***	KDEL ER lumen retention receptor***	*Drosophila melanogaster* (E=1e-35)***	KDEL ER lumen retention receptor
				KDEL ER lumen retention receptor***	*Caenorhabiditis elegans* (E=6e-30)	KDEL ER lumen retention receptor	*Caenorhabiditis elegans* (E=6e-30)	
S1LR1211	371	No significant matches*		Basic amino acid permease gene***	*Aspergillus fumigatus* (E=9e-07)***	Basic amino acid permease gene***	*Aspergillus fumigatus* (E=1e-06)***	Basic amino acid permease gene
S2LR1211	359	No significant matches*		No significant matches*		No significant matches*		Unidentified
				Unknown protein	*Arabidopsis thaliana*	ORF	*Homo sapiens* (E=0.068)*	
				Zinc finger**	(E=0.96)**	rpb1 RNA polymerase**	*Pleistophora hippoglossoides os* (E=1.1)**	
SGA3D	600	No significant matches*		No significant matches*		No significant matches*		Unidentified

*No significant similarity to anything in the database (E > 0.1)
**Clones were most similar to unassigned open reading frames/hypothetical proteins, alignments listed are for any protein identified
***Closest alignment listed, highest E value and similarity seen
N/A-not applicable

Table 9: Differential display products found to have no change in expression

		Nucleotide database		Protein database		Translated database		
Clones	Size of fragment (bp)	Alignment with Nucleotide DB	Organism (E value)	Alignment with Protein DB	Organism (E value)	Alignment with Translated DB	Organism (E value)	Proposed Identity
CO1SR1211	200	No significant similarity*	-	Hypothetical protein, similar to aldose epimerase***	*Arabidopsis thaliana* (E=(9e-16)***	Hypothetical protein, similar to aldose epimerase**	*Arabidopsis thaliana* (E=9e-16)**	Aldose epimerase
CO1LR1311	750	Mitochondrion rRNA***	*T. papilionaceus* (E=5e-42)***	No significant similarity*	-	Mito rRNA	*T. papilionaceus* (E=1e-22)***	rRNA
CO2LR1211	468							

*No significant similarity since E > 0.1

**Clones were open reading frames/hypothetical proteins, alignments listed are for any protein identified

***Closest alignment listed highest E value and similarity seen

N/A-not applicable

Table 10: Summary of the sequence analysis of differential display products

Clones	Regulation	Size (bp)	Reverse primer	Forward primer	Proposed Identity	E values
P1SR1211	Upregulated (phenol)	294	RT1	AP2	Unidentified	-
P2SR1211	Upregulated (phenol)	134	RT1	AP2	Unidentified	-
P1LR1211	Upregulated (phenol)	300	RT1	AP2	Transcription factor	E=4e-06
PGA3UT1	Upregulated (phenol)	514	RT1	AP3	Glycerol facilitator	E=1e-13
PGA3UT2	Upregulated (phenol)	593	RT1	AP3	Unidentified	
P1SR1311	Upregulated	427	RT1	AP6	Vacuolar	E= 4e-24
P1LR1311	(phenol)	428			ATPase	
P2SR1311	Upregulated (phenol)	336	RT1	AP6	Unidentified	-
P6SR1311	Upregulated (phenol)	100	RT1	AP6	Unidentified	-
P2LR1311	Upregulated (phenol)	403	RT1	AP6	Unidentified	-
P3LR1311	Upregulated (phenol)	336	RT1	AP6	Unidentified	-
P5LR1311	Upregulated (phenol)	400	RT1	AP6	Unidentified	-
S1SR1211	Upregulated (succinate)	500	RT1	AP2	KDEL ER lumen retention receptor	E=6e-30
S1LR1211	Upregulated (succinate)	371	RT1	AP2	Basic amino acid permease gene	E=9e-07
S2LR1211	Upregulated (succinate)	359	RT1	AP2	Unidentified	
CO1SR1211	No change	200	RT1	AP2	Aldose epimerase	E=9e-16
CO1LR1311	No change	750	RT1	AP6	28 S rRNA	
CO2LR1211		400		AP2		
P3SR1311	Upregulated	276	RT1	AP6	28S rRNA	E=1e-135
P4SR1311	(phenol)	275				
P7SR1311	Upregulated	137	RT1	AP6	26S rRNA	E= 1e-09
P8SR1311	(phenol)	114				
P6LR1311		375				
P7LR1311		350				
P8LR1311		325				
P9LR1311		300				
P4LR1311	Upregulated (phenol)		RT1	AP6	5.8S rRNA	E=e-136

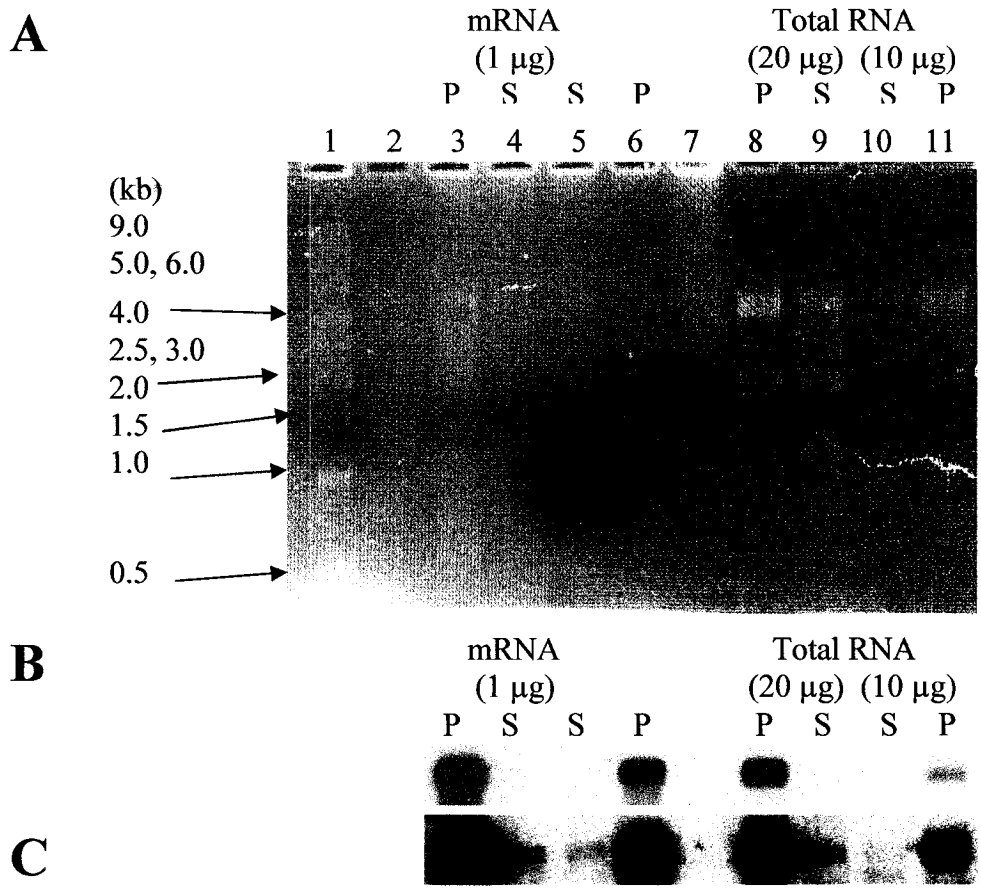
6.2 Northern blots

Differential display is designed to detect differences in mRNA levels. In order to verify that differential display does detect these differences in gene expression in *T. cutaneum* under the conditions used here Northern blot analysis was performed using some of the differential display products.

6.2.1 Northern blot analysis using phenol hydroxylase

A portion of the phenol hydroxylase gene was used as a positive control for the Northern analysis. It has been shown that phenol hydroxylase is involved in phenol metabolism and it should be upregulated in cells growing with phenol (reviewed in Powlowski and Shingler, 2004). As expected, when a portion of the phenol hydroxylase gene was used as a probe, a strong signal of expected size, 2 kb, was seen both in total RNA and in mRNA isolated from phenol grown cells (Figure 18) with less signal seen in RNA isolated from succinate grown cells.

Figure 19: Northern Blot using a portion of the phenol hydroxylase gene as a probe

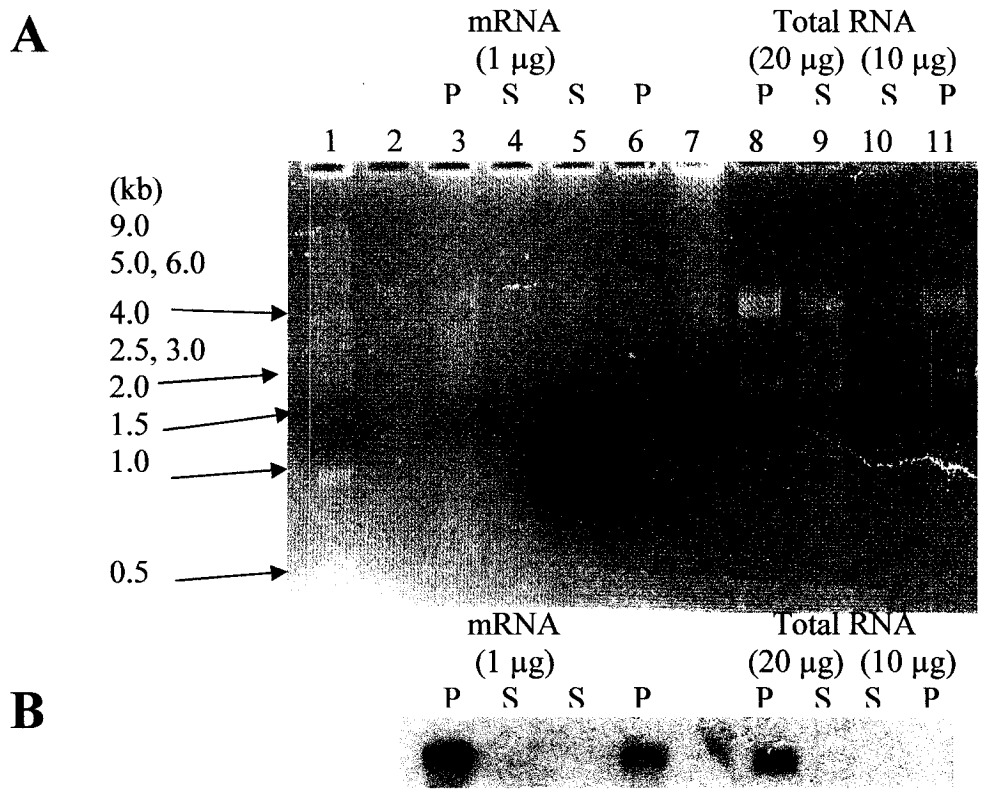


A) RNA was loaded on a denaturing agarose gel, electrophoresed and stained with ethidium bromide: 2 μg of RNA millenium molecular weight markers (lane 1); 1 μg of mRNA from two cultures of cells grown with phenol (lanes 3 and 6) or succinate (lanes 4 and 5), 20 μg (lane 8) or 10 μg (lane 11) of total RNA extracted from cells grown in phenol medium or 20 μg (lane 9) or 10 μg (lane 10) of total RNA extracted from cells grown in succinate medium. A portion of the autoradiogram representing the expression of phenol hydroxylase in cells grown with phenol or succinate in minimal medium is shown after exposure of the blot probed with a portion of the phenol hydroxylase gene to X-ray film for 2 days (B) or 7 days (C).

6.2.2 Northern blots of differential display products

From differential display, an unidentified differential display product, P3LR1311 (Figure 17), was observed to be upregulated in phenol grown cells. This gene expression pattern was confirmed by Northern analysis and the autoradiogram using P3LR1311 as a probe of RNA isolated from cells grown in succinate or phenol medium is shown in Figure 20. A strong signal of a size of 2.2 kb was seen both in total RNA and in mRNA isolated from phenol grown cells (Figure 20) and little signal was seen in RNA isolated from succinate grown cells.

Figure 20: Northern Blot using differential display product, P3LR1311 as a probe

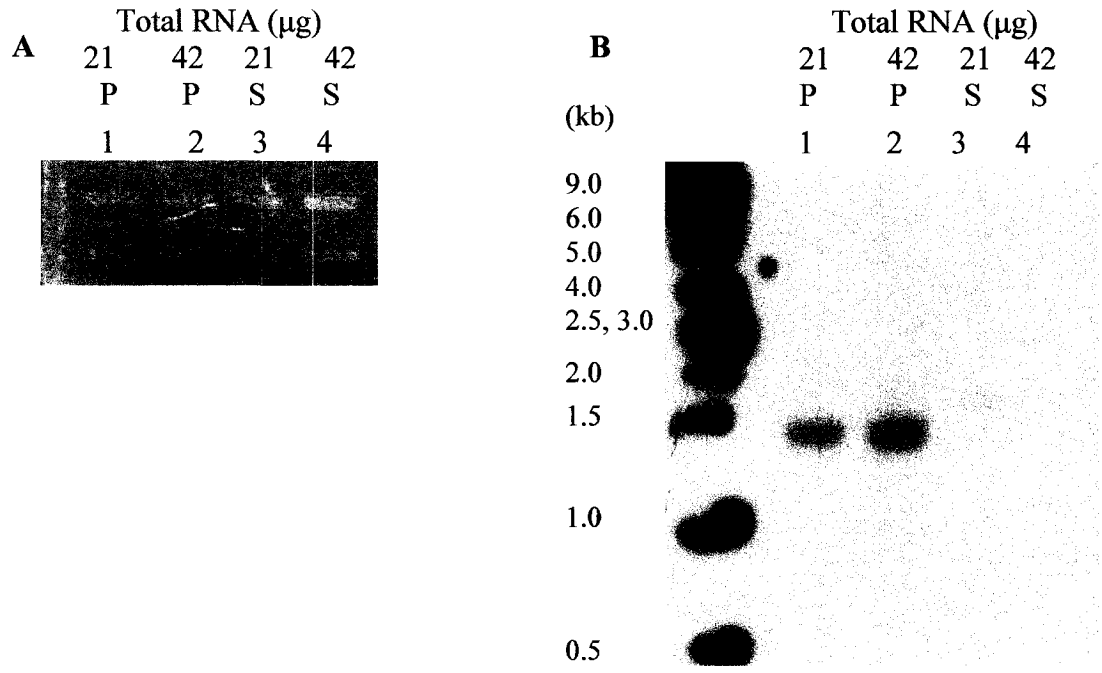


A) RNA was loaded on a denaturing agarose gel, electrophoresed and stained with ethidium bromide: 2 µg of RNA millenium molecular weight markers (lane 1); 1 µg of mRNA from two cultures of cells grown with phenol (lanes 3 and 6) or succinate (lanes 4 and 5), 20 µg (lane 8) or 10 µg (lane 11) of total RNA extracted from cells grown in phenol medium, 20 µg (lane 9) or 10 µg (lane 10) of total RNA extracted from cells grown in succinate medium. A portion of the autoradiogram representing the expression

of differential display product P3LR1311 in cells grown with phenol or succinate in minimal medium is shown after exposure of the blot probed with differential display product P3LR1311 to x-ray film for 2 days (B).

From differential display, a differential display product, P1LR1211 (Figure 14), identified as a transcription factor, was observed to be upregulated in cells grown in phenol media. This was confirmed by Northern analysis and the autoradiogram using differential display product, P1LR1211 as a probe of RNA isolated from cells grown with succinate or phenol as a carbon source is shown in Figure 21. A strong signal of a size of 1.5 kb was seen both in total RNA and in mRNA isolated from phenol grown cells which indicate that the differential display product is upregulated in cells grown with phenol in minimal medium.

Figure 21: Northern blot using differential display product, P1LR1211 as a probe

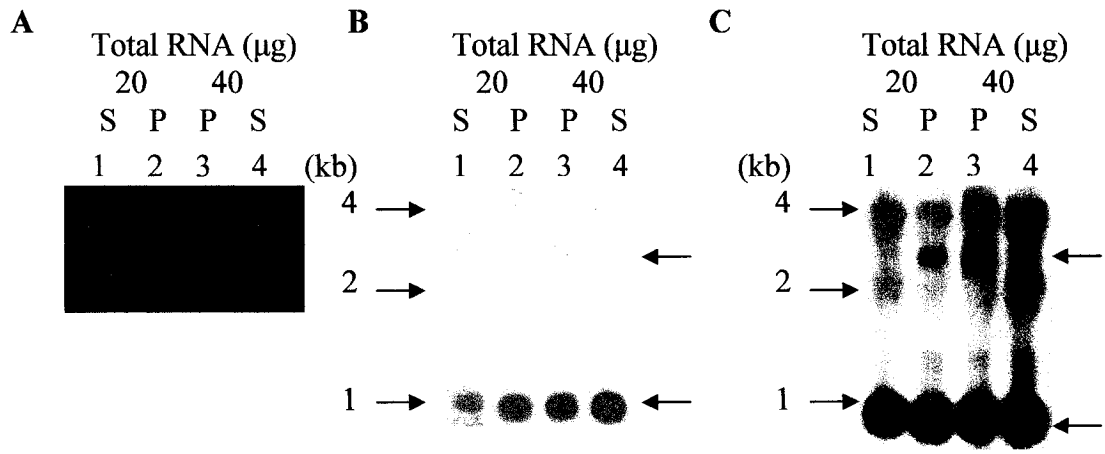


A) RNA was loaded on a denaturing agarose gel, electrophoresed and stained with ethidium bromide: 21 µg (lane 1) or 42 µg (lane 2) of total RNA extracted from cells grown in phenol medium or 21 µg (lane 3) or 42 µg (lane 4) of total RNA extracted from cells grown in succinate medium. Autoradiogram representing the expression of

differential display product P1LR1211 in cells grown with phenol or succinate in minimal medium is shown after exposure of the blot probed with differential display product P1LR1211 to x-ray film for 2 days (B).

From differential display, a differential display product, P1SR1311/P1LR1311 (Figure 17) was observed to be upregulated in cells grown in phenol medium. This was seen by Northern analysis (Figure 22) using differential display product, P1SR1311 as a probe of RNA isolated from cells grown with succinate or phenol as a carbon source. A strong signal of a size of 2.5 kb was seen in the lanes containing total RNA purified from phenol in comparison to a weak signal in the lanes containing total RNA from succinate grown cells. In addition, a strong signal of a size of 1 kb of was present in all the lanes. When different exposures were compared, no difference in intensity between the two conditions was seen. This mRNA of 1 kb is not differentially expressed in either condition.

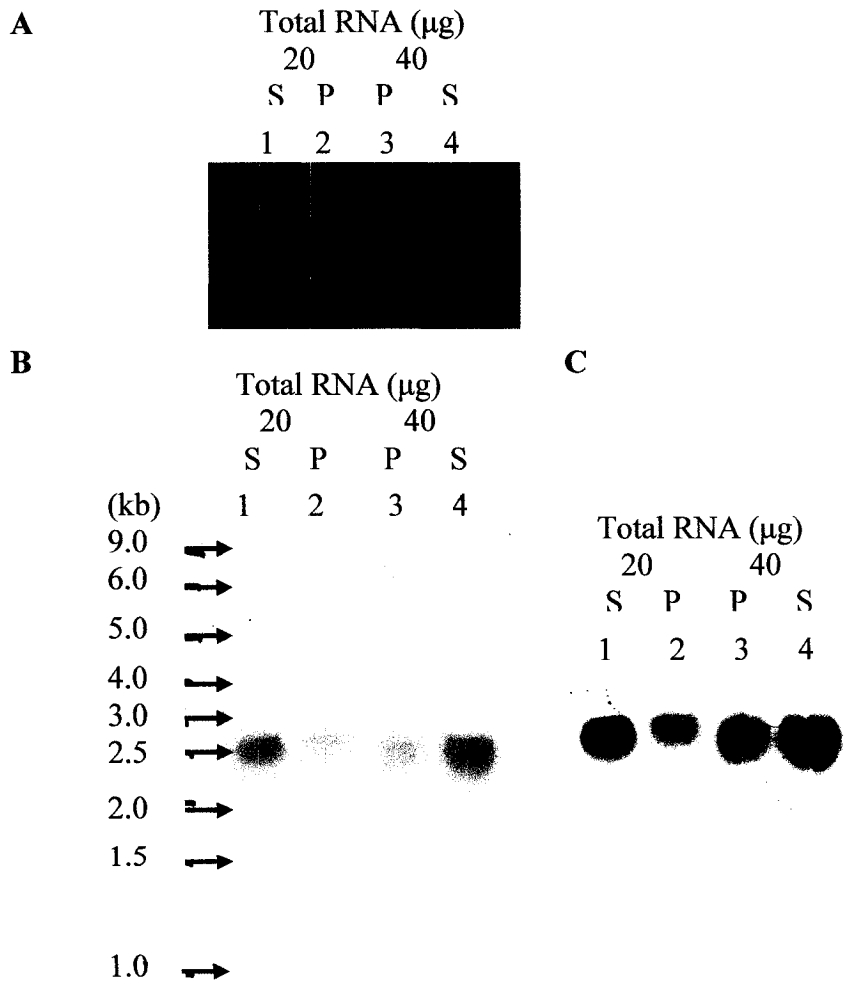
Figure 22: Northern blot using differential display product, P1SR1311 as a probe



A) Ethidium bromide stain of RNA electrophoresed under denaturing conditions: 20 µg (lane 1) or 40 µg (lane 4) of total RNA extracted from cells grown in succinate medium or 20 µg (lane 2) or 40 µg (lane 3) of total RNA isolated from cells grown in phenol medium. Autoradiogram of Northern blot probed with P1SR1311 as a probe is shown after exposure to X-ray film for (B) 2 hours and (C) 12 hours using an intensifying screen.

From differential display, differential display product, S1LR1211 (Figure 14) was observed to be downregulated in phenol grown cells. The Northern blot using S1LR1211 as a probe of RNA isolated from cells grown with succinate or phenol as a carbon source is shown in Figure 23. The intensity is higher in lanes containing RNA isolated from cells grown with succinate (lanes 1, 4) in comparison to that of phenol (lanes 2, 3). Autoradiography indicates that the differential display product is upregulated in cells grown in succinate medium. The size of the product is approximately 2.5 kb.

Figure 23: Northern blot using differential display product, S1LR1211 as a probe

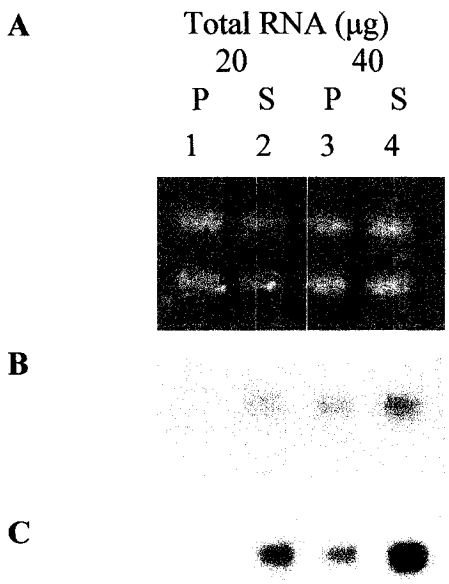


A) Ethidium bromide stain of RNA electrophoresed under denaturing conditions: 20 µg (lane 1) or 40 µg (lane 4) of total RNA extracted from cells grown in succinate medium or 20µg (lane 2) or 40 µg (lane 3) of total RNA isolated from cells grown in phenol

medium. Autoradiograms of a Northern blot probed with S1LR1211 as a probe after exposure to X-ray film for (B) 8 hours and (C) 2 days using an intensifying screen.

Differential display product S2LR1211 (Figure 14) was found to be downregulated in phenol grown cells. The Northern blot using S2LR1211 as a probe of RNA isolated from cells grown with succinate or phenol as a carbon source is shown in Figure 24. The resulting products indicate that the differential display product is upregulated in cells grown in succinate media. The size of the product is approximately 1.5 kb.

Figure 24: Northern blot using differential display product, S2LR1211 as a probe



A) Ethidium bromide stain of RNA electrophoresed under denaturing conditions: 20 μg (lane 1) or 40 μg (lane 3) of total RNA isolated from cells grown in phenol medium or 20 μg (lane 2) or 40 μg (lane 4) of total RNA extracted from cells grown in succinate medium. A portion of the autoradiogram of the Northern blot using S2LR1211 as a probe is shown. It was exposed to the X-ray film for overnight (A) and 4 days (B) using an intensifying screen.

6.3 Dot blots

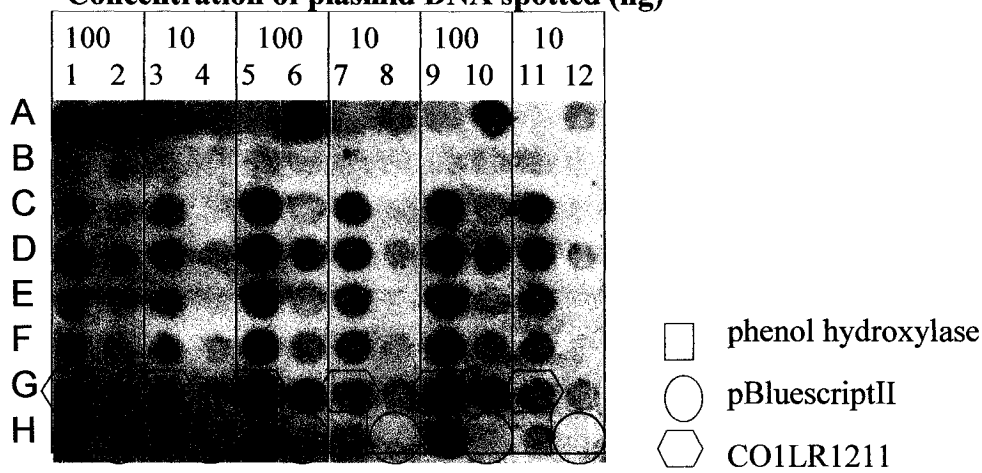
Differential display products were subjected to dot blot analysis to confirm the gene expression patterns observed by differential display and to develop a high throughput method to analyze expression. Plasmid DNA containing differential display products as well as positive and negative controls was spotted on blots and probed with cDNA synthesized from mRNA purified from independent preparations of cells grown in phenol or succinate. The identity of the DNA arrayed on the membranes used for dot blot analysis is listed in Appendix 3. Autoradiograms of dot blots probed with cDNA generated from cells grown in phenol or succinate medium are shown in Appendix 4 and Appendix 5. The positive control used in the dot blot analysis was phenol hydroxylase (PH), a gene product which is known to be involved in phenol metabolism (Powlowski *et al.,* 1985) which has been shown to be upregulated by Northern analysis (Figure 19). A negative control in this experiment was plasmid pBluescript II that does not contain any differential display product. Another control was the non-differentially expressed product, CO1LR1211, a potential aldose epimerase used to normalize the intensity of the spots observed. The results of dot blot analysis for these controls are shown in Figure 25.

The relative expression levels of differential display products in RNA from different growth conditions were evaluated by probing the dot blots with cDNA synthesized from cells grown in phenol or succinate medium. Products that displayed differential expression, indicated by dot blot analysis are indicated (Figure 26).

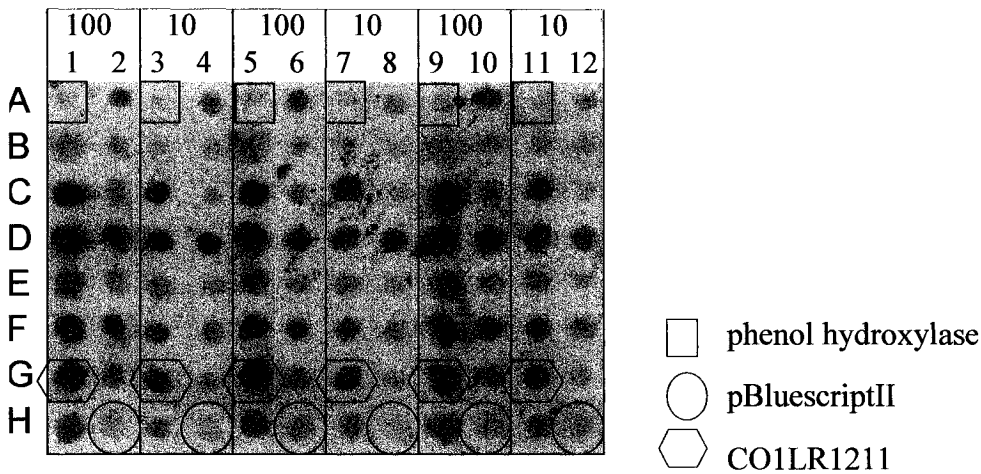
Table 11 summarizes the analysis of differential display products by Northern analysis and by Dot blot analysis.

Figure 25: Results of dot blot analysis of control samples, probed with cDNA synthesized from RNA from phenol or succinate grown cells.

I Dot blot probed with cDNA from phenol grown cells



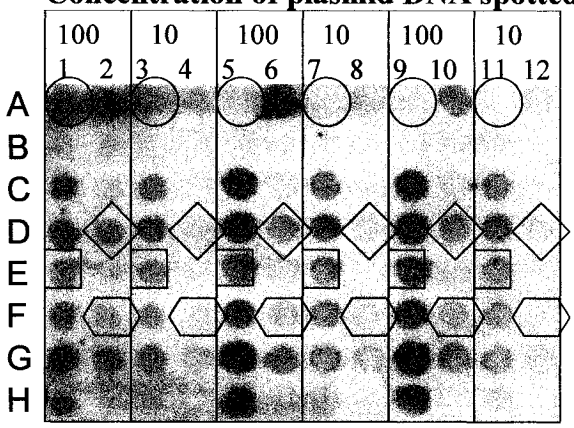
II Dot blot probed with cDNA from succinate grown cells



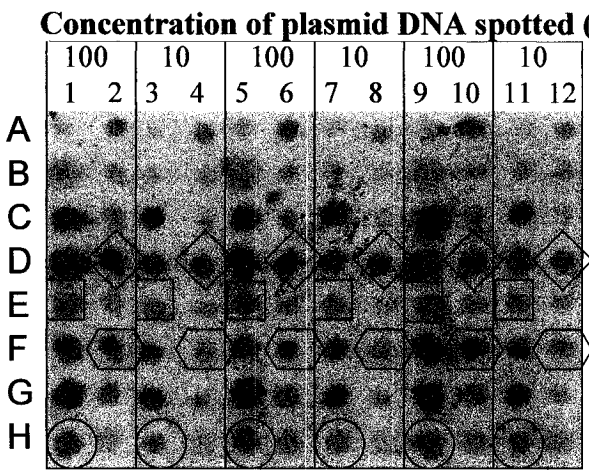
Autoradiogram of dot blots containing plasmid DNA probed with cDNA synthesized from mRNA purified from cells grown in phenol (I) or succinate (II) medium. The autoradiograms were exposed for 30 days at 4°C without an intensifying screen. The identity of each spot is listed in Appendix 3 but the positive control phenol hydroxylase (□), the negative control pBluescript (○) and the non-differentially expressed product, CO1LR1211 (⬡) are indicated.

Figure 26: Dot blot analysis of differential display products, probed with cDNA synthesized from RNA from phenol or succinate grown cells.

I Dot blot probed with cDNA from phenol grown cells



II Dot blot probed with cDNA from succinate grown cells



□ Upregulated differential display product, P2LR1311
○ Downregulated differential display product S1LR1211
⬡ Downregulated differential display product S1SR1211
◇ Down regulated differential display product S2LR1211

Autoradiogram of dot blots containing plasmid DNA probed with cDNA synthesized from mRNA purified from cells grown in phenol (I) or succinate (II) medium. The autoradiograms were exposed for 30 days at 4°C without an intensifying screen. The identity of each spot is listed in Appendix 3 but the upregulated differential display product, P2LR1311 (□), the downregulated differential display product S1LR1211 (O), downregulated differential display product S1LR1211 (⬡) and down regulated differential display product S2LR1211 (◇) are indicated.

Table 11: Evaluation of differential display products from Northern and dot blot analysis

Differential display product	Observed/implied gene expression profile from differential display	Results of Northern analysis	Results of dot blot analysis	Expression pattern with respect to phenol media
BSKSII	N/A	-	little or no signal	N/A
CO1LR1211	no change in expression	-	intense signal in either blot	no change in expression
Phenol hydroxylase	upregulated	upregulated	upregulated (intense signal in phenol blots and less signal in succinate blots)	upregulated
P1LR1211	upregulated	upregulated	intense signal in either blot	upregulated
P1LR1311	upregulated	upregulated	intense signal in either blot	upregulated
P1SR1211	upregulated	-	intense signal in either blot	undetermined
P2LR1311	upregulated	-	upregulated (intense signal in phenol blots and less signal in succinate blots)	upregulated
P2SR1211	upregulated	-	intense signal in either blot	undetermined
P2SR1311	upregulated	-	very little signal in either blot	undetermined
P3LR1311	upregulated	upregulated	intense signal in either blot	upregulated
P4SR1311	upregulated	-	very little signal in either blot	undetermined
P5LR1311	upregulated	-	intense signal in either blot	undetermined

Table 11 (continued)

Differential display product	Observed/implied gene expression profile from differential display	Results of Northern analysis	Results of dot blot analysis	Expression pattern with respect to phenol media
P6SR1311	**upregulated**	-	**intense signal in either blot**	**undetermined**
S1LR1211	**downregulated**	**downregulated**	**downregulated signal in succinate dot blots and less signal in phenol blots**	**downregulated**
S1SR1211	**downregulated**	-	**downregulated signal in succinate dot blots and less signal in phenol blots**	**downregulated**
S2LR1211	**downregulated**	**downregulated**	**downregulated signal in succinate dot blots and less signal in phenol blots**	**downregulated**

N/A - Not applicable
"-" - experiment was not conducted

DISCUSSION

An effort has been made in this study to utilize for the first time, differential display to study differential gene expression in *Trichosporon cutaneum*. This would enable the isolation of more genes expressed as a result of aromatic metabolism in *T. cutaneum*. The characterization of the products isolated using this approach would help to better understand the events involved in aromatic metabolism in *T. cutaneum* and allow for further development of this organism toward an application for bioremediation purposes.

Phenol was chosen as an aromatic carbon source because it is one of the simplest aromatic compounds and has a reactive electron-donating hydroxyl substituent (Powlowski and Shingler, 2004).

Growth conditions

The doubling time of *T. cutaneum* was found to be 3.5 hours in rich medium, 2.5 hours in succinate medium and 3.5 hours in phenol medium. The cells grown in rich medium seem to have a longer doubling time than those grown in minimal medium containing succinate. This may be due to the lack of trace metals in the rich medium. When cells were grown in phenol or succinate medium, additional phenol or succinate was added during the incubation time to ensure enough carbon was present. If no additional carbon source was added, the growth rate of these cells decreased (results not shown). Cells grown in phenol or succinate medium were isolated for RNA extraction at points in exponential growth phase. In order for samples to be directly compared, cells were harvested at the same growth phase, indicated by cell density. Moreover, samples

taken at different time points during the growth of *T. cutaneum* would reveal information about the genes expressed when growing using an aromatic carbon source.

Differential display

Differential display was used in this study because it has been shown to detect subtle differences in gene expression, to detect novel genes (knowledge of mRNA sequences is not required) and to require a limited number of steps in comparison to other techniques (Liang, 2002). Furthermore, it requires less RNA and it is a more cost effective approach since sequencing is limited to only potential candidates and expensive equipment and highly specialized bioinformatics personnel are not required (Liang, 2002).

The first step of differential display involves converting the RNA extracted from cells to cDNA using reverse transcriptase and various reverse transcription primers. Typically, total RNA is used for differential display experiments. In this study, it was found that mRNA provided more defined differences in gene expression than did total RNA (data not shown). Therefore, mRNA was used for the differential display experiments described here. The reverse transcription primers used in this study were composed of 5'-(T_{18})VN-3' (V=A or C or G, N = A, C, T, G). A basic difficulty in using differential display with *T. cutaneum* was that full length cDNA was not produced. This is attributed to the fact that mRNA is single stranded and can form secondary structures which inhibit the production of cDNA by the reverse transcriptase enzyme. The formation of secondary structures is more problematic in organisms that have a high G+C content such as *Trichosporon cutaneum* which has a G+C content of 57% to 64% (Wolf, 1996). The production of full length cDNA by reverse transcription was difficult for this

reason. To obviate this, many different reaction conditions were tested along with different reverse transcriptase enzymes (results not shown) until the optimal reaction conditions described were found. Perhaps the use of higher concentrations of mRNA would improve the number of differential display products produced and thus, increase the number of differentially expressed genes detected using differential display. In addition, different reverse transcriptase enzymes could be investigated to further optimize this technique.

For PCR amplification of the reverse transcriptase products generated here, it was found that primers with a low G+C content (*e.g.*, AP1) yielded few defined products while primers with a G+C content greater than 60 % (*e.g.*, AP2, AP3, AP6) yielded more products. This trend was noted by other researchers using differential display (reviewed by Liang, 2002). On average any successful set of reaction conditions generated up to 50 PCR products of which six to fifteen were clearly distinguishable PCR and showed similar or different levels of expression in the different assay conditions (see Figures 13, 14, 15, 16, 17). Moreover, these procedures proved to be highly reproducible. Furthermore, experiments could be repeated using freshly prepared biological samples. When four samples of RNA from cells grown in the same carbon source were subjected to differential display and compared, the differential display products produced. This clearly can be seen in a differential display gel containing differential display products from phenol grown cells (Figure 15, lanes 1, 2, 5, 6, 9, 10, 13 and 14). The differential display patterns produced from cells grown in succinate also were similar as can be seen in Figure 15 (lanes 3, 4, 7, 8, 11, 12, 15, and 16).

A problem that is typically found in using any genomic based technique such as differential display is the production of false positives and artefacts. PCR tends to produce artefacts from rRNA or to create products from non-specific binding. A false positive is a gene that is not differentially expressed but appears to be when evaluated by differential display. In the past, the selection of differential display products by comparison of single samples from each condition may have led to selection of false positives (Imura *et al.,* 1997, Cui *et al.* 2001). In this work, one way that the selection of false positives was minimized was by selecting differentially expressed products based on the comparison of eight differential display reactions (two technical replicates of two biological samples for each condition, *i.e.*, phenol or succinate). It was found that when mRNA was purified once using the polyA purist kit, the ribosomal RNA contamination was 10 to 20% (evaluated by the Agilent RNA detection system). This contamination level by ribosomal RNA was less than the other kits which typically had about a 50 % contamination rate. Another point of improvement would be to design primers that do not amplify rRNA sequences.

It has been suggested that differential display shows a strong bias towards high copy mRNAs (Bertioli *et al.*, 1995). However, differential display can detect differences of rare or low abundance genes. The bias towards high copy mRNAs is due to the labelling involved in differential display. The amount of dNTPs used in the differential display reaction is lower than most typical PCR reactions to allow for incorporation of radiolabelled nucleotides. This low level of dNTPs causes a bias towards abundant mRNAs and may not allow detection of genes with lower differentially expressed patterns. Thus, the amount of dNTPs was varied in this study to maximum the

differential display products produced (results not shown). Yet, another way to alleviate this problem is not to label the products in the PCR reactions, but instead to increase the amount of dNTPs to produce the maximum number of products and post-label the differential display reactions using for instance, polynucleotide kinase to label of the phosphate at the 5' end. One also could use fluorescence detection methods to increase sensitivity.

For differential display, hundreds of PCR reactions must be prepared and their products separated on a gel matrix for each RNA sample tested to ensure that most of the mRNAs are detected. Most of the reactions produce fifteen to fifty PCR products of which six to fifteen are clearly distinguishable (Figures 13, 14, 15, 16 and 17). This agrees well with the mathematical estimation that any two primers would amplify up to 100 products (Liang, 2002). These numbers are based on purely mathematical models based on a few organisms and still have to be verified by experimental work (Liang, 2002).

There are some changes that have been introduced recently in the design of primers. The new reverse transcriptase primers include some additional nucleotides at their 5' ends and have one varied nucleotide at the 3' end, instead of 2. Thus, a primer for reverse transcription, for example, would include 5'-ACGC(T_{18})G-3', 5'-ACGC(T_{18})C-3' or 5'-ACGC(T_{18})A-3'. These changes increase the annealing temperature at which the PCR is performed in order to reduce the production of non-specific products. The length of the arbitrary primers have been varied in lengths from 13 bp to 25 bp to increase the amount of differential display products produced (Liang, 2002). Currently, researchers are using bioinformatics tools to rationally design the

arbitrary primers by, for example, designing them to amplify specific targets such as transcription factors (Liang, 2002). Thus, the products they produce are for specific genes. This would allow for more specific amplification of cDNAs in the differential display reactions. In the past, the use of a few arbitrary primers was the norm but currently with automation and kits designed for common organisms, a study of a particular system now can include a minimum of one hundred arbitrary primers. The number of arbitrary primers used by researchers vary from a minimum of five to three hundred (Liang, 2002). These arbitrary primers should be designed with typical parameters of G+C content and so as not to produce primer dimers. Using the primer pairs developed in this study twenty eight differentially expressed products were generated and cloned. Of these, fifteen were assigned putative functions while the other remain unidentified due to low similarity with any known sequence.

Characterization and analysis of differential display products

A problem associated with any genomic technique is that the identity or nature of a gene may not be known until the PCR product is reamplified, cloned and sequenced. The cellular responses and interactions between different gene products are very complex. A potential problem encountered in this study was in the characterization of differential display products and the determination of their roles. From this work, the products that were generated by differential display included eleven products that were seen to be upregulated, four that were down regulated and one that showed no change in expression. Sequence comparisons of these products allowed potential identities to be assigned based on sequence similarity.

Differential display product, PGA3UT1 is a 510 bp fragment and seems to be upregulated in the presence of phenol (Figure 15). It was identified as a glycerol facilitator, a protein that creates a channel involved in glycerol transport, based on its 59% amino acid sequence similarity and 45% amino acid sequence identity to a 52 amino acid region predicted from the glpF gene of *Thermus aquaticus*. Mortberg *et al.* (1988) have shown that phenol is taken up into cells by two mechanisms, either by a constitutive low-affinity pathway or a highly phenol specific uptake pathway. The connection between either of these systems and the potential glycerol facilitator identified here remains to be determined.

Differential display product P1SR1311 is 428 bp in length and was up regulated in phenol grown cells (Figure 17). The differential display product was confirmed by Northern analysis to be upregulated (Figure 22). It was identified as subunit F of a vacuolar ATPase (V-ATPase), based on 77% sequence similarity and 62% sequence identity over a 122 amino acid region to a V-ATPase of *N. crassa*. V-ATPases have been shown to be required for various stress tolerance systems. For instance, a V-ATPase has been demonstrated to be required for salt tolerance in *S. cerevisiae* (Finbow *et al.*, 1997). V-ATPases are involved in many complex cellular responses such as cation influx, efflux and sequestering of cations in the vacuole by generating a proton motif force. Subunit F was shown to have multifunctional roles in membrane transport (Finbow *et al.*, 1997). V-ATPases regulate cytosolic pH and allow for the accumulation of amino acids in the vacuole. Apart from a general role of these proteins in membrane transport, the specific connection between this protein and phenol metabolism remains unclear.

Differential display product P1LR1211 of 300 bp was found to be up regulated in phenol grown cells (Figure 14_. It was assigned an identity of a putative transcriptional regulator based on sequence similarity to Rfef of *Emercella nidulans*. The functional role of this portion in the organization of cellular responses can not be hypothesized at this time. However, one way to determine its specific role would be to either silence this gene or delete it and look for a phenotype or use differential display to evaluate what genes are being expressed differently as a result of the deletion.

Differential display product S1SR1211 of 500 bp was identified as a KDEL (lysine-aspartate - glutamate - leucine) receptor. The transport of newly synthesized proteins from the ER includes proteins with a KDEL sequence motif. When these KDEL proteins leave the ER, they are recognized by the KDEL receptor and transported back to the ER. Most ER proteins containing a KDEL signal function as chaperones for protein folding and assembly (Aoe *et al.*, 1998). The KDEL retrieval system also is implicated in a pathway of retrieval of protein toxins into the ER for degradation and as a pathway for toxins to infiltrate the cell. Why the gene encoding this receptor would be down regulated when cells are grown on phenol is not clear. Perhaps fewer proteins are targeted to the ER. One could study this cellular targeting by the use of specific tags such as green fluorescent protein (GFP) which allow for visualization and localization of proteins.

Differential display product S1LR1211 of 371 bp was identified as a basic amino acid permease. The translated sequence of S1LR1211 over its first 60 predicted amino acids had an amino acid sequence similarity of 59% and an amino acid sequence identity of 39% to a basic amino acid permease of *Aspergillus fumigatus*. Furthermore,

similarities also were noted to other lysine specific permeases in other yeasts. Basic amino acid permeases are known to be induced under growth in acidic medium, anaerobiosis and in the presence of high concentration of basic amino acids such as lysine (Steffes *et al.*, 1992). Perhaps, as with some of the other proteins we have seen, this protein is activated as part of the stress response.

The most exciting aspect of this research and one of the main reasons for carrying out the experiments in this study is the potential to find new proteins that have not been characterized as part of a response to phenol. With this in mind, most of the products found in this research were most similar to hypothetical ORFs (open reading frames) or had no significant sequence similarity to anything in the databases searched. The finding of novel or unidentified products is exciting and differential display is certainly a technique that can be used for gene discovery.

For example, differential display product S2LR1211 of 359 bp shows a 60% sequence similarity and 40% identity to a zinc finger (C3HC4-type RING finger) protein of *Arabidopsis thaliana*. The implications of this may involve it being a transcription factor.

It is interesting that genes coding for enzymes involved in phenol catabolism were not identified in this study. This may be due to the arbitrary primers used. For example, because the reverse transcriptase primer used in this study ends in TVG, phenol hydroxylase would not be expected to be isolated as its mRNA ends with $TT(A)_{22}$-3' (Kalin *et al.*, 1992). In addition, mRNAs may be produced for short periods of time or only at specific time points after exposure to phenol, as thus far, the time points have not been assayed.

Northern blots

Differential display is able to detect genes that are upregulated or down regulated. Northern blot analysis was used as a secondary technique to confirm the gene expression patterns detected by differential display. RNA was isolated from cells grown in phenol or succinate medium in the same manner as the RNA which was used for differential display. Probes were made using the differential display products. Phenol hydroxylase was used as a positive control for the Northern blots to ensure that the cells were expressing genes as a result of phenol metabolism. A strong signal of expected size was seen both in total RNA and in mRNA isolated from phenol grown cells with little signal observed in RNA isolated from succinate grown cells (Figure 19).

Differential display product P3LR1311 was observed by differential display to be upregulated in phenol grown cells (Figure 16) and Northern analysis confirmed this apparent upregulation. A strong signal of 2.2 kb was seen both in independent preparations of total RNA and mRNA isolated from phenol grown cells (Figure 20) with little signal on the Northern blot in RNA isolated from succinate grown cells. These data are consistent with the hypothesis that the differential display product is upregulated in cells grown in phenol medium. This also indicates that the full length mRNA for this product is 2.2 kb.

Another differential display product, P1LR1211, a putative transcription factor was observed by differential display to be upregulated in cells grown in phenol medium (Figure 13). As expected, a strong signal of approximately 1.5 kb was seen in independent preparations of total RNA and mRNA isolated from phenol grown cells.

This supports the differential display data that it is upregulated in cells grown in phenol media. This also indicates that the full length mRNA coding for this protein is 1.5 kb.

Differential display product P1SR1311, subunit F of a vacuolar ATPase was observed by differential display to be upregulated in independent preparations of cells grown in phenol medium (Figure 15). The Northern blot (Figure 22) of independent samples of RNA isolated from cells grown in succinate or phenol medium using differential display product, P1SR1311 as a probe shows a strong signal of a size of 2.5 kb in the lanes containing RNA from phenol grown cells in comparison to a weak signal in RNA isolated from succinate grown cells. However, a strong signal around 1 kb also was seen in all samples of RNA, isolated from cells grown in phenol or succinate medium. The intense band observed at 2.5 kb in the Northern analysis indicates that the differential display product is upregulated in cells grown in phenol medium. On the other hand, the presence of the intense band of 1 kb may be due to the fact that different ATPases are abundant in the cell and may contain conserved sequences. Thus, the intense signal seen in RNA isolated from phenol or succinate grown cells may be due to recognition of another related ATPases (Conesa *et al.*, 2001). The mRNA coding for subunit F of V-ATPase is about 1 kb in size in yeast (NCBI nucleotide database, www.ncbi.nlm.nih.gov). In order to study the expression of this particular differential display product, Southern blot analysis of digested genomic DNA could be used to identify the conserved sequences of this particular V-ATPase. Once this information is known, probes without these conserved sequences can be used for further study of the expression pattern of this differential display product.

Differential display product, S1LR1211 was observed by differential display to be down-regulated in phenol grown cells. It has been assigned an identity as a basic amino acid permease. As expected, the Northern blot analysis (Figure 23) shows the presence of an intense band of 2.5 kb in lanes containing RNA from independent cultures grown in succinate medium and a low intensity band in the lanes containing RNA isolated from cells grown with phenol. The presence of a band with higher intensity in lanes containing RNA from succinate grown cells indicates that the differential display product is down regulated in cells grown in phenol medium. The identity of this differential display product was assigned based on high sequence similarity to a partially sequenced cDNA product of basic amino acid permease from *Aspergillus fumigatus*. This cDNA has a high sequence similarity and is related to the *APL1* gene for basic-amino-acid permease of *S. cerevisiae* (GI: 496659). The *APL1* gene is around 1724 bp which agrees reasonably with the size of the intense band seen on the Northern blot of around 2.5 kb. Why this gene should be down regulated in phenol grown cells is unknown.

Differential display product S2LR1211 (Figure 13) was observed by differential display to be downregulated in cells grown in phenol medium. The Northern blot using S2LR1211 as a probe of RNA isolated from cells grown with succinate or phenol as a carbon source is shown in Figure 24. This differential display product was shown to be downregulated because of the presence of a high intensity band of 1.5 kb in the lanes containing RNA from cells grown with succinate in comparison to a lower intensity band in RNA from cells grown with phenol. The size of the product is approximately 1.5 kb. This product has not yet been identified.

Although, the amount of RNA loaded on the gels for Northern hybridization were quantitated by absorbance readings, by the Agilent Bioanalyzer system and by observing the intensities of the rRNA stained with ethidium bromide, it would have been desirable to have a control to quantify and reaffirm the quantity of RNA loaded prior to Northern analysis. Some investigators use house keeping genes such as actin whose expression is believed to be unchanged under certain conditions. This particular control for the Northern analysis was unavailable due to the lack of genomic sequence information for *T. cutaneum*. Ideally, this control would be a gene product that does not change in expression due to the use of the different carbon sources. From differential display, one potential candidate would be CO1LR1211, a potential aldose epimerase which seems to be non-differentially expressed. Once this differential display product has been studied further, it may be a valuable asset as a control for the Northern analysis.

For the samples tested, the Northern analysis of differential display products confirmed the observed expression patterns seen by differential display. The Northern data show clearly that differential display does evaluate the differential expression of genes.

Dot blot analysis

As described previously, the expression profiles of differentially expressed products can be supported by Northern analysis. However, this relies on the use of one Northern blot for every differential display product (Zegzoutis *et al.*, 1997). The use of this technique is impractical when a small amount of RNA is available and in the case where many products are to be analyzed and it can be labour intensive, costly and time consuming. A more high-throughput approach was needed to confirm the remaining

differential display products and a dot blot, reverse Northern technique was developed. This technique, once perfected, may not only allow for the simultaneous and quick analysis of many differential display products to confirm whether they are differentially expressed but also reduce further analysis and characterization of false positives. Moreover, this procedure could be used for evaluation of differentially expressed display products prior to subsequent cloning and thus, decrease unnecessary cost and loss of time. Furthermore, analysis of these differential display products on dot blots using RNA isolated from cells from different experimental conditions such as cells receiving different challenges (*e.g.*, phenol vs. catechol vs. heat shock, etc.) would allow genes specific for defined parts of the response pathway to be identified.

For dot blot analysis, plasmid DNA containing differential display products as well as positive and negative controls were spotted on blots and probed with cDNA synthesized from mRNA purified from independent preparations of cells grown in minimal medium with phenol (phenol cDNA) or succinate (succinate cDNA). A selected number of differential display products were analyzed by dot blot analysis to develop this procedure. Plasmid DNA containing clones of interest and controls were used in preference to spotting the products of interest alone because they could be quantitated more simply. In addition, the samples were spotted at different concentrations to take into account potential saturation effects of autoradiography. The identity of the DNA arrayed on the membranes used for dot blot analysis is listed in Appendix 3. One of the problems encountered in this experiment involved the production of probe with high specific activity. This was subsequently solved by first removing non-labelled dCTP from the reverse transcription reaction, allowing the reverse transcription reaction to

proceed and then adding non-labelled dCTP to permit further synthesis of the cDNA. A further improvement in the production of probes with high specific activity involved the use of Thermoscript (Invitrogen) polymerase with incubation of the reaction with increasing increments of 5°C. This procedure allowed for greater incorporation of labelled nucleotides and produced longer cDNAs.

The autoradiograms of the dot blots probed with cDNA generated from cells grown in phenol or succinate medium are shown in Appendix 4 and 5. The positive control used in the dot blot analysis was phenol hydroxylase, a gene product which is involved in phenol metabolism (Powlowski *et al.,* 1985) which was shown to be upregulated by Northern analysis (Figure 19). A negative control in this experiment was plasmid pBluescript that does not contain any differential display product. Another control was a non-differentially expressed product, CO1LR1211, a potential aldose epimerase used to normalize the intensity of the spots observed. The results of dot blot analysis for these controls are shown in Figure 25. It was expected that phenol hydroxylase would produce a high intensity signal when the dot blots were probed with phenol cDNA in comparison to blots probed with succinate cDNA. This was seen in the dot blot analysis, shown in Figure 25. Plasmid lacking a differential display product should show little or no signal when probed with cDNA produced from RNA from either growth condition. There was little or no signal on the spots in the blots probed with phenol cDNA or succinate cDNA (Figure 25). In addition, the differential display product, CO1LR1211 used as control for no change in expression in either growth condition did have consistent signals in both sets of dot blots (Figure 25).

The other differential display products were analyzed within the framework of these controls. Differential display product P2LR1311, a product observed to be upregulated in phenol grown cells, displayed a high intensity signal in dot blots probed with phenol cDNA and a lower intensity signal in blots probed with succinate cDNA (Figure 26). Similarly, differential display products S1LR1211, S2LR1211 and S1SR1211, observed by differential display to be downregulated in phenol grown cells showed strong signals in the dot blots probed with succinate cDNA than with phenol cDNA (Figure 26). Differential display product, S1LR1211, S2LR1211 were found by Northern analysis to be down regulated in phenol grown cells. The dot blot analysis agreed with the observation that S1SR1211 was downregulated in phenol grown cells.

However, the expression patterns observed for other differential display products, seen to be upregulated in cells grown with phenol in minimal medium could not be confirmed. Differential display products P1SR1211, P3LR1311, P1LR1211 and P6SR1311 produced high intensity signals on dot blots, probed with phenol cDNA or succinate cDNA. Differential display products P3LR1311 and P1LR1211 were confirmed by Northern analysis to be upregulated in phenol grown cells but produced signals of equal intensity in dot blots probed with either succinate cDNA or phenol cDNA. One reason differences could not be detected may be due to the masking of the signal produced by hybridization of more abundant but constitutively expressed mRNAs and mRNAs transcribed from related families. This may be true in some cases but it is not the case for P3LR1311 and P1LR1211 which showed by Northern analysis dramatic differences in gene expression in phenol grown cells in comparison to succinate grown cells. A plausible reason for the lack of detection in the dot blot assay would be that

there might not be enough DNA spotted on the blots to compensate for the abundance and complexity of the cDNA probes used. For example, consider the case where a spot contains 100 molecules of the DNA of a differential display product that was found to be upregulated. If the succinate probe has at least 50 molecules of that cDNA, the signal seen will be high enough to be similar to a signal produced by hybridization of 100 molecules of that differential display product by a phenol cDNA probe containing 10 000 molecules of that particular cDNA. In addition, the dot blot procedure only detected differences in products whose gene expression pattern in one condition differed in expression in the other condition by a significant amount. This can be seen in comparison to samples analyzed by Northern blots of any of the samples. For example, phenol hydroxylase produces a less dramatic difference signal in dot blot analysis (Figure 25) in comparison to the results of the Northern analysis (Figure 19). The difference in intensities noted by the Northern analysis is more dramatic than that seen in the dot blot analysis. An improvement that can be made is to use multiple and higher concentrations of the same DNA on the dot blots. In addition, the apparent upregulation of differential display products, P2SR1211, P4SR1311, P5LR13111, P2SR1311, P1SR1311 could not be confirmed since they produced very little signal in both dot blots probed with phenol cDNA or succinate cDNA. One reason may be that there is not enough DNA present on the blot to allow for the differences to be seen. In addition, this was not surprising since differential display is able to detect subtle differences of genes expressed at low abundance. This also was seen by other researchers (Zegzouti *et al.*, 1997). Most researchers who encountered such clones used real time polymerase chain reaction (RT-PCR) as a method to evaluate the gene expression pattern (Zegzouti *et al.*, 1997). A

further improvement that could be made would be to include a quality control step with the use of either labelled oligonucleotides that hybridize to part of the plasmid DNA or labelled random oligonucleotides to ensure that each of the spots on the dot blots contains a particular amount of DNA, prior to hybridization of the dot with the particular probe. Furthermore, a problem with the use of autoradiography is saturation effects and the time required to obtain results. An improvement would include the use of fluorescence labelling to synthesize the probes. This would eliminate saturation effects and decrease the time required to obtain results to minutes rather than months.

Results of differential display, Northern and dot blot analysis

Differential display has been used to study aromatic metabolism. Previously, genes involved in toluene induction in *Pseudomonas putida* F1 were studied by differential display (Fleming *et al.,* 1998). Differential display allowed for the identification of a new salicylates-inducible naphthalene dioxygenase in *Burkholderia cepacia* JS150 (Fleming *et al.,* 1998). This technique also was used to study aromatic metabolism of pentachlorophenol in the fungus, *Coriolus versicolor* (Iimura and Tatsumi, 1997).

The work described in this thesis was done as a preliminary study for the applicability of differential display as a tool for analyzing the genes in *T. cutaneum* that are expressed in phenol and succinate grown cells. Differential display can detect genes that are up regulated and down regulated, as well as the differential expression of genes that are expressed at a low abundance. As used in these experiments, differential display is a highly reproducible technique both for products generated in different replicates of the same sample (*e.g.*, phenol) or for differences displayed between samples (*e.g.*, phenol

vs. succinate). Sequence analysis of the products isolated by differential display allowed some differential display products to be assigned identities based on high sequence similarity. However, identification of these products is rather limited since the genetic, sequence information and characterization of many fungi are only recently being investigated and being compiled.

Northern blot analysis was used as a secondary technique to confirm the gene expression patterns detected by differential display. Some differential display products were tested by Northern analysis and the observed gene expression profiles were confirmed. For example, P3LR1311, P1LR1211, P1SR1311 were confirmed by Northern analysis to be upregulated in phenol grown cells and S1LR1211 and S2LR1211 were confirmed by Northern analysis to be downregulated in cells grown in phenol medium.

A more high-throughput approach, a dot blot, reverse Northern technique also confirmed the differential expression of some isolated products. For example, P2LR1311 was confirmed by dot blot analysis to be upregulated in phenol grown cells and S1SR1211, S1LR1211 and S2LR1211 were confirmed by dot blot analysis to be downregulated in cells grown in phenol medium. Dot blot analysis revealed expression profiles of differential display products that were not tested by Northern analysis. The upregulation of a differential display product, P2LR1311 was confirmed by a high intensity signal in dot blots probed with phenol cDNA and a lower intensity signal in blots probed with succinate cDNA (Figure 26). Similarly, downregulated differential display products, S1LR1211, S2LR1211, S1SR1211 were shown to be downregulated by dot blot analysis since strong signals were seen in the dot blots probed with succinate cDNA than with phenol cDNA (Figure 26).

However, there appear to be some limitations to the dot blot approach as the expression patterns observed for some of the differential display products (P3LR1311, P1LR1211, P5LR1311, P6SR1311) could not be confirmed using dot blot analysis even though, Northern analysis of products P3LR1311 and P1LR1211 indicated that they were upregulated in phenol grown cells.

The expectation of this project was to develop a high throughput method to evaluate genes expressed as a result of exposure of *T. cutaneum* to phenol. Differential display can be used to detect differences in gene expression and the ability of differential display to detect genes that are upregulated or downregulated in *T. cutaneum* as a response to growth using phenol as a carbon source was confirmed by Northern analysis and dot blot analysis of products isolated by differential display. Thus, high throughput analysis of *T. cutaneum* as a response to growth using phenol as a carbon source could be accomplished by using differential display with many different primers to identify differentially expressed genes and using a reverse Northern dot blot technique to quickly analyze and confirm the gene expression patterns detected. Once confirmed, the differential display products can be cloned, sequenced and be used to identify the full length cDNAs to conduct more research to characterize the differential display products found. This information someday may be used for development of an efficient bioremediation technology. Thus, this study has developed and validated the applicability of differential display as a tool for high throughput gene expression analysis of *T. cutaneum.*

Future Work

In order to further characterize the differentially expressed products isolated, the full length cDNAs must be found. This can be done with either the use of a cDNA library or by using RACE (Rapid Amplification of cDNA Ends) such as RLM-RACE (Ambion). In addition, comparisons of gene sequence to proteomic based approaches that include two dimensional gel electrophoresis, mass spectrometry and analysis by comparisons to EST libraries would help establish functional roles for many of the hypothetical proteins. In addition, producing knockouts of these differentially expressed products or transferring the genes encoding for these products into other organisms would help establish potential roles of these differentially expressed products.

The experiments described here represent the first attempt to use differential display to investigate differential gene expression in *T. cutaneum*. In order to further characterize aromatic metabolism in *T. cutaneum*, many more differential display reactions must be performed with many more arbitrary primers. The primer pair length should be varied and have a similar G+C content of 60%. The use of different reverse transcriptases may be investigated to further improve the differential display technique. Perhaps labelling the differential display products differently may improve the number of products observed. On the other hand, alternate points of isolation should be considered or varied amounts of phenol should be used so as to gain more insight into the cellular responses. Further research in *T. cutaneum* should involve studying the growth of *T. cutaneum* at different points, challenging with other aromatics like *p*-cresol and comparing by dot blot the differences in gene expression patterns.

The dot blot technique can be used to screen differential display products prior to cloning. It can be improved by using fluorescence detection methods and by using differing amounts of DNA on the blots and by using a control to evaluate the presence of DNA on the blots. This technique once perfected may allow for the simultaneous and quick analysis of many differential display products to confirm whether they are differentially expressed and also may reduce further analysis and characterization of false positives. Moreover, this procedure could be used for evaluation of differentially expressed display products prior to subsequent cloning and thus, decrease unnecessary cost and loss of time. Furthermore, analysis of these display products on dot blots using RNA isolated from cells from different experimental conditions such as cells receiving different challenges (e.g., phenol vs. catechol vs. heat shock, etc.) would allow genes specific for defined parts of the response pathway to be identified.

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APPENDICES

Appendix 1: Growth media and solutions

Organism	Medium or solution	Recipe	Reference
E. coli	YT	0.8% bacto-tryptone (Bioshop) 0.5% yeast extract (Bioshop) 0.5% NaCl (Fisher) (Plates: 1.5% Agar)	Sambrook *et al.*, 1989
E. coli	Ampicillin	50 mg/mL Ampicillin (Bioshop)	Sambrook *et al.*, 1989
E. coli	Isopropyl-β-D-thiogalactoside (IPTG)	1 M IPTG (Bioshop)	Sambrook *et al.*, 1989
E. coli	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside	20% X-gal in dimethylformamide (DMF)	Sambrook *et al.*, 1989
T. cutaneum	YPD	1% yeast extract (Bioshop) 2% peptone (Bioshop) 2% dextrose (Fisher) (Plates: 2% Agar)	Sambrook *et al.*, 1989
T. cutaneum	Nutrient Broth (NB)	0.8 % Nutrient broth (DIFCO)	Sparnins *et al.*, 1979
T. cutaneum	potassium phosphate solution	0.050 M potassium phosphate monobasic (Bioshop), (adjusted to pH 7.0 using NaOH)	Sparnins *et al.*, 1979
T. cutaneum	10X minimal medium	20.0 g/L NH_4Cl (Aldrich) 19.7g/L Na_2HPO_4 (Bioshop) 49.2 g/L KH_2PO_4 (Bioshop) (adjust to pH 6.4 using NaOH)	Sparnins *et al.*, 1979

Appendix 1 (continued)

Organism	Medium or solution	Recipe	Reference
T. cutaneum	1 M magnesium sulphate	49.3 g of $MgSO_4 \cdot 7H_2O$ (Sigma) in 200 mL dH_20, (adjusted to pH 7.0 using NaOH)	Sparnins *et al.*, 1979
T. cutaneum	100X CAYE	50 g/L casamino acids (Bioshop) 50 g/L yeast extract (Bioshop)	Sparnins *et al.*, 1979
T. cutaneum	0.75M Succinic acid solution	40.5 g of succinate (Sigma) per 200 mL dH_2O (adjusted to pH 7.0 using NaOH)	Sparnins *et al.*, 1979
T. cutaneum	0.50 M Phenol solution	11.76 g of phenol (Fisher) dissolved to 250 mL using sterile dH_2O in a sterile flask	Sparnins *et al.*, 1979
T. cutaneum	1000 X Trace metals	0.71 g/L disodium EDTA (Bioshop) 0.23 g/L $ZnCl_2$ (Sigma) 1.3 g/L $FeSO_4 \cdot 7H_2O$ 0.62 g/L $MnCl_2 \cdot 4H_2O$ (Sigma) 0.10 g/L $CuSO_4 \cdot 5H_2O$ 0.050 g/L $CoNO_3 \cdot 6H_2O$ (Sigma) (acidified by a few drops of sulphuric acid)	Powlowski and Shingler (1990)

Appendix 1 (continued)

Organism	Medium or Solution	Recipe	Reference
T. cutaneum	succinate medium	For 500 mL: 50 mL of 10X minimal medium 5 mL of 100X CAYE 0.5 mL of 1000x trace metals 0.432 mL of 1 M magnesium sulphate 2.0 mL of 0.75 M succinic acid solution	Sparnins *et al.*, 1979
T. cutaneum	phenol medium	For 500 mL: 50 mL of 10X minimal medium 5 mL of 100X CAYE 0.5 mL of 1000x Trace metals 0.432 mL of 1 M magnesium sulphate 3.2 mL of 0.50 M phenol solution	Sparnins *et al.*, 1979

Appendix 2: Sequences of differential display products

PGA3UT1 (T3)

AGGTCGAGTCCACCTGCGATATCAAGACGGGTAACAAACGTATAATGAACGC
TGTGTCCGGAAATCGGAAGACTTTTTGGTAATTCATATGTAAAAGGCAGCAC
CTTTTTCTCGCCAGCCTGAATAACAAAGGCAGAAGCAACCGGAATTGTTGCC
ACTGTTTTTGTCTGCACTTGACCGTTATGGTTTAATGTTAAACGAAGTTCTAC
ATCAATTTTGTTGATATGCTGTTCTACTGCCCCTCCCTCAATAAAAAATTCAC
CACTAATGGTCTCTCCCAAACGAACTTGGGAATGATGCAGCATCAGATTAAT
ATTGGCAGAGCCAATCCCAAGTTTCGATAAAAGTTTTTTAAACATCCCTTTCA
TCTCTCACCCCTTATTCATTAATATCCATTGGTGACATACTCCCACCACCNTA
CGCTTCGCCTAGAGGTGGGGGCTTCTTCGGGTNAATCCCATCCTCATTGATGG
GGAAGNTTTGAACCNGAAGCGNANCCCCCCGGTNGTNGCCC

PGA3UT1 (T7)

AGGTCGAGTCAAGGAGTCTCTATCGCTTTCTGAACGGACATTCCGCTGTGAAT
GTGGATTCGAGAGCGACAGGGACGTCAATGCGGCCATCAATATCAAACATGA
GGGCATGAAACGATTGGCGATCGTCTAATTTGTCCTCGAACCGTGGGGCACA
CGGGGATCGCTCGGTCAACTTCCCATCATGAGATGGGATTACCCGAGAAGCC
CCCACCTCTAGGCGAAGCGTAGGTGGTGGGAGTATGTCACCAATGGATATTA
ATGAATAAGGGGTGAGAGATGAAAGGGATGTTTAAAAAACTTTTATCGAAAC
TTGGGATTGGCTCTGCCAATATTAATCTGATGCTGCATCATTCCCAAGTTCGT
TTGGGAGAGACCATTAGTGGTGAATTTTTTATTGAGGGAGGGGCAGTAGAAC
AGCATATCAACAAAATTGATGTAGAACTTCGTTTAACATTAAACCATAACGG
TCAAGTGCAGACAAAAACAGTGGCAACAATTCCGGTTGCTTCTGCCTTTGTTA
TTCAGGCTGGCGAGAAAAAGGTGCTGCCTTTTACATATGAATTACCAAAAAG
TCTNCCGAA

PGA3UT2:

TTTTTTTTTTTTTTTTTTTTTTTTTTTTCGNTAAAGNTAATTGACCGGNAGTTC
AATCAACAGTTTCACAGGTTGTTAAATGGATGTTANTTCAACGCCTCAAAGA
ANNCAATTCACTAATGNATCCTTCCGCAGGNTTCACCTACGGNAAACCTTGTT
CCAAAAAAAAAAAAAAGGGGGGGGGNAGGGCAAAAAAAAAAAAAAATCAA
CCTTATCAATACCGTCAACCTCAAGGGGGGGCCCGGTACCACCTTTTGTTCCC
TTTATTGAAGGGTTAATTTCAACCTTGGCTTAATCATGGTCATACCTGTTTCCT
GTNTNNAAATTGTTATCCGCTCACAATTCCACACAACTATACAACCCGGAAA
CCNNTAAAGTGTAAACCCTGGGGTGCCTAATGNATTGACCTAACTCACTTTA
ATTGCTTTGCCCTCACTGCCCGCTTTCCATTCGGGAAAACCTGTCTTGCCACC
TGCTTTAATGAATCGGCCAACNCCCGGGGAGAGGCGGTTTGCGTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCNTTCGGGCTGCGG
GCAACCGGTATCAC

PGA3UT2 (T3)

AGGTCGAGTCGGCTCTGAACGGAAAGGATGCCGAGCTGGTCAAGTCGCTGCA
GGAGCGCCTAGTCAAGCGTGCTACTGCTGAGGGCCGCGAGAGCTGGCTGTCA

GACTGGTGGCTTTCGTACTCGTATATGTCATACCGTGATCCCGTCGTTCCATT
CTCGAGTTACTTTTATCTACACAAATCTGTGCCCAAGACAACCACTGGTGTCA
GCCGCGGCGCACAACTGCTCAAAGCCATGATGGCTTTCCGTGAAATGATTGT
TTCTNGAGACCCTCTCCCCCGAAAAGACCCAANGAACTNGGTTNNAATNGTN
NCCTGTTANCCCCNGTANCACAGTTGNGATTGTTTTAAAACTCCCGTTGNCCC
CCANNNTCCCNNGTTGAACCCCGGGCNNAAGGGAATTGTTGGGCCCCAAAA
NTTCNNTNCNCAAACCNCCCCNNNCNNNNCCCNNNTCANCCCNTTGNNTNGG
TTTNNNTTTGATTNCCCCGCCNNNTTGGGNNTNNCACCTTTCCNNNNTNNNNG
NTTGGGNAAATTCNTTTNNTTACAACCCCANNGGTTNTNNAAACNTTNGGGN
NNAACAGGGGAAGNCNNNNNNTNNGGCTTGANANN

PGA3UT2 (T7)
AGGTCGAGTCCAGATTCAACACAATAATACCGCTCTCCACTAGCTCCAGCAG
CTTACGGTTGTGCTCACCAGTGGCACCCGCTAACAAGGCCTCGCGATTTTTGA
CCCATTCGTCTCTGTTGGCAACAGTAAATGAACCGATGGGGACAGGGTTGAC
CTGTTGAGTGCGCTCGTCCTCCACGATGCGCTGAAGCTGCAGCTCGATCTCAG
CCACGCTAAGCTCCTTGCCAGTCAACTTGTTGACAAGATCCACAATAAAGAA
GTGACCATTGCGGATCACAACAACATGCTGATGCGTGGCGGGGTCGTATGAC
TTGGCCACATCCTCGCCGGTCACAGGAATGTGGCACGAGTTAAACATCCACT
TGTACGGGTACATGCACATGAAACCAGTCTTGGTCTTTTCGGGGGAGAGGGT
CTCAGAAACAATCATTTCACGGAAAAGCCATCATGGCTTTGAGCAGTTGTGC
GCCGCGGCTGACACCAGTGGGTTGTCTTGGGCACAGATTTGTGTAGATAAAA
GTAACTCGAGAATGGAACGACGGGATCACNGTATGACATATACGAA

PGA4T31
TTTTTTTTTTTTTTTTTTTTTTTTTTTTCGNTAAAGNTAATTGACCGGNAGTTC
AATCAACAGTTTCACAGGTTGTTAAATGGATGTTANTTCAACGCCTCAAAGA
ANNCAATTCACTAATGNATCCTTCCGCAGGNTTCACCTACGGNAAACCTTGTT
CCAAAAAAAAAAAAAAGGGGGGGGGNAGGGCAAAAAAAAAAAAAAA

CO1LR1211-F
SIZE: 411 BP
TGACTGAACGTGTGTATGCCAAGGTTCCTGGGCAGGAGGTGCGCGTCGACGA
CGGCCAGGGCGGCGGCTACAAGGTCCGCTTCCGTGGTTTCGAAGACTGCGTG
ATCTGGAACCCCGCTGCCGAGACCGGCAGCAAGATCGCCGACATGGAGGAC
GGCGGCTGGAACCGCTACGTCTGTGTCGAGCCTGGGTTCGTGAGCTAGTTCA
AGTCGCTCGAGCCCGGACAGAAGTTCCTCGGCCAAGAGACCATCACCCTCAT
CGAGCCTCCCCGGCTGTAACCGGTGGCGGCCACGGGCCACCATGGCTTAAGC
AAACTCGCGACAAACCAGTGTAATATGAAGGTAGTACCAGGGCTGCTCAGTC
TGTGCTGTAGCCTCGAAATGAACTGTGCAGATGCGAAAAAAAAAAAAAA

CO1LR1311-F
TTTTTTTTTTTTTCGCCAATGAAATGATACATCCCCCTCCCCTTTTTTTTTTTT
TTTTTTTTTGGTCAAAGACTTTCTAATTTATACTCATCAACTATTAACAATGAC
TTGCGCATAGTCTTAGAGACCGTTTTATTTATCAAGCTGTCTAACATAGCTTA

ACAACCCTTTCGCATTCGGCGAGAAGTATTATATCTTCTTTCTACGTTACTCAT
GTTAGCATAATCTCTTCAGTAATGTCCAAATAATGTAACACTATTCTTCTACC
ATACTGAATGCTCCTCTACCCTTCATTCTTCCTATATATATCCGAGATCCTACT
TTCATAACTCAGGCCACTTAATTAAGTGGAGTAAAATAGTAGTTTTCCTCTCA
TAAGAGGAAATATTTCAACAAATAAGAAAAATATAGACATAGTTTCGGTGAA
TGATCTAAGACCCGGGTATCTTCGGTGCGATGATTCACTAGACTACTAGGTTG
TTACACTTTCATTAAAAGATAGCAACTGCCAAGGTA

CO2LR1311:
TACCTTGGCAGTTGCTATCTTTTAATGAAAGTGTAACAACTTAGTAGTCTAGT
GAATCATCGCACCGAAGATAAACGGGTCTTAGATCATTCACCGAAACTATGT
CTATATTTTTCTTATTTGTTGAAATATTTCCTCTTATGAGAGGAAAACTACTAT
TTTACTCCACTTAATTAA
GTGGCCTGAGTTATGAAAGTAGGATAATAAATATATATAGGAAGAATGAAGG
GTAGAGGAGCATTCAGTATGGTAGAAGAATAGTGTTACATTATTTGGACATT
ACTGAAGAGATTATGCTAACATGAGTAACGTAGAAAGAAGATATAATACTTC
TCGCCGAATGCGAAAGGGTTGTTAAGCTATGTTAGACAGCTTGATAAATAAA
ACGGTCTCTAAGACTATGCGCAAGTCATTGTTAATAGTTGATGAGTATAAATT
AGAAAGTCTTTAACCAAAAAAAAAAAAAAA

P1SR1211 22:
TTGACTGAACGTGATAATGTAGAGAGTAGGGCGCGGGTAACCGCAGCATGCA
GCATACAGATTCGGACACGTGCGGGATGTGTCTGTGGAGTGCGACGTTGGGC
TCCTCCAGAGCCGTCGTTACTCCTGTTCCGCCTTAGCCGCCGTACCCTGTCCC
CGCTCGAGGCAGCTCGTCCACCATATCGTTCCGCTCGCACCCCGTTGCACCAG
CTCCACTGCACCAGCGCGAAGATGTCTAACACGTGCATACCACAGACAATGC
AGCTACAGTCCAACCTACGAAAAAAAAAAAAA

P2SR1211 32-M13F:
TGACTGACCGTGATAATGTAGAGAGTAGGTCGAGTCGGAGGGCTGGCAGCTG
AAGAGTAGAGTGTGAGGGAGCTGTGTTTGGTTGATAGGGGTTGATATCTGCA
GCTTTCGTTCAAGTGCGAAAAAAAAAAAAA

P1LR1211 61-M13F:
TGACTGAACGTCCAACTTCGAGAGCGAGCAGGCCGAGATGATGCGCAAGAC
GGGCATTGAGCTCACCCCCGAGACGTGGCTGGTCAAGCTGCTCATGGGCCCG
ATTGACACGTCGTACGACTCGAAGGACGAGGGCACTGGCCCGCCTCCCCGCC
GCTAGGCGCTTGCTTGACGCTAATGTGTGAAGAAGTACGGTATGAACAGTGT
CTAGTCAAGGCGGCCGCAGCTATAGTCTACTTACTCGTCCATAGTGATACCAG
TATGAATCCTTATTGCTCGAAAAAAAAAAAAA

P1LR1311 E-M13F
TACCTTGGCAGCTCCAACCTCCAACCCCAAGGGACGCAACCTACTTGCCGTT
ATCGGCGACGAGGACTCGGTCACTGGCCTCCTCCTCGCGGGCATTGGCGATG
TCAACGAGAAGCAGGACAAGAACTTCATGATCGTGGACGCGAAGACGCAGA
CGTCCGCCATCGAGGCTGCGTT

CCAGGAGTACACCGAGGACCGCAAGGATATTGCTATCCTCCTCATCAACCAG
CATATCGCCGAGCGGATACGCCCTACCGTTGACCGGTACCAGGCTGCCTTCC
CCGCGCTTCTCGAGATTCCCTCCAAGGAGCACCCGTATGACCCAACCAAGGA
CTCTGTCCTGAAGCGCGTGCAGAAGCTGCGCGGCGACTAAATAGTAGACCAG
CAGGATGATGTGATGTACAGTTTACTCGCGAAAAAAAAAAAAA

P2LR1311 J-M13F

TACCTTGGCAGAGGGTGTATCAACTAACGCCAGCTCCTTATCTCGGGCTCGGT
TCTTGCCTTTGGATCGGAGATCACCGCCCTGCAGGTCTTTGGCTACGGCATCG
CCCTGGGCGGTCTCGTGACCTTCAAGACGACTGGTGGCAAGTAAAACAGCCG
ACCTATTGTTCGACATTTA
GCAGTCGCCGTGGCACGACACGGGGCCTTGTTGAATGTCGACGCTTGCCGTC
GGCCATGGCCGGTGGCTTGCTCCTTCTCGACGAGGGGCATTGTTGTGGATATA
GAGAGAGATTGCTGGAGAGCAGAGGGCTACGCCGCACGTCGTTAGCCCTCGT
TCACTCATTTTAGTCTGTCATAGAACACTGCTTGCATGCTGTCTGGCGCGAAA
AAAAAAAAAAAAAAAA

P3LR1311 N-M13F

TACCTTGGCAGAGCGAGTGTGTCGGCGACACTTCGGCCGGGCTTCGTGGCTC
ACGTTCTCACAGCACTGACGTTCACGCAGAGCTCTGCAGCACCATGCCATGC
TCTTCGGCGTCGCTGGCTCGCCGGAAGGCCGCGGGTCTTCTGATGGCATACG
GGTAACTGCCCCACTCCTATC
TGCTCCACTCCCCGACTCCGACTTCGCCACCCCCATGCACGAATGCCTCATCT
TCCCTCGACCTCGACGTTGTGTACAGACAGACATTCATGCTTTACAGTTCTAA
TGTGGGTTTTGTAATATGCTGTTGTATCCTATGCCTGCCGAAAAAAAAAAAAA

P4LR1311-F

TTTTTTTTTTTTTCGTACCAGTTCTAAGTCGGTTGTTAATTGTATGCCGGACGC
CCGAAGACTGCCAAGAGCTTCCGATCGCCAGTCCGGGCCGGTCCGAGTAGGT
TGCCCCACTCAGTCCAGCCTAGTCCAGCTCACTTCTACTCAAGGCCCGACATA
CCCAACCCTTAGAGCCAATCCTTATCCCGAAGTTACGGATCTATTTTGCCGAC
TTCCCTTATCTACATTGTTCTATCAACTAGAGGCTGTTCACCTTGGAGACCTG
CTGCGGTTATGAGTACGACCAGGCGTGAGACTTATACATTCCGTCGGATTTTC
AAGGACTGTCAAGGACGCACCGGACACAACAGAAGTGTTGTGCTCTACCCGC
CATATAACCTTAGCTCCGGATAATCCGATTTCAAGGTCGTAAGCGGTTAAGG
AGAAAAGAGAACTCTTCCCAGGGCCCTTGCCAGCGTCTCCAACTTCATTTAC
GTTGCCGTTAAAGATCCACATCCTGGTTCCGGAATCTTAACCGGATTCCCTTT
CGATAGGCGACACGGAAAATCGTGTACTTTAAAACGGAGCTTCCCTATCTCTT
AGGATCGACTAACCCATGTCCAACTGCTGTTACCATGGAACCTTTCTCCACTT
CAGTCTTCAAGGTCTCACTTGAATATTTGCTACTACCACCAAGATCTGCACTA
GAGGCCGTTTCACCCAGCATCACTGCCAAGGTA

P5LR1311-F
TACCTTGGCAGACTCTTCCACCGCCGTCCCCGCACCGTCGCCGTCCCCGTGCG
CAGGAAGCGCTTCTTCGGCCTCTTTTAGAGTCTCGGATGACAACAACAGAGT
CTCGGAATAGACGCGGAGTGAAGACGTAGACGTTGATTCAGAAGTGATGTTC
ATTAATCTCTGGTGAAGCCCATGGCCGATGCCCAGGCCCTTCCAGTCGTTGTA
GCTTTCATGTATAAAGTTATGAGCGAAAAAAAAAAAAAA

P6LR1311-F
TACCTTGGCAGCGTCTCCAACTTCATTTACGTTGCCGTTAAAGATCCACATCC
TGGTTCCGGAATCTTAACCGGATTCCCTTTCGATAGGCGACACGGAAAATCGT
GTACTTTAAAACGGAGCTTCCCTATCTCTTAGGATCGACTAACCCATGTCCAA
CTGCTGTTACCATGGAACCTTTCTCCACTTCAGTCTTCAAGGTTCTCACTTGAA
TATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTTCACCCAGCATCAC
TGCCAAGGTA

P7LR1311-F
TACCTTGGCAGCGTCTCCAACTTCATTTACGTTGCCGTTAAAGATCCACATCC
TGGTTCCGGAATCTTAACCGGATTCCCTTTCGATAGGCGACACGGAAAATCGT
GTACTTTAAAACGGAGCTTCCCTATCTCTTAGGATCGACTAACCCATGTCCAA
CTGCTGTTACCATGGAACCTTTCTCCACTTCAGTCTTCAAGGTTCTCACTTGAA
TATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTTCACCCAGCATCAC
TGCCAAGGTA

P8LR1311-F
TACCTTGGCAGCGTCTCCAACTTCATTTACGTTGCCGTTAAAGATCCACATCC
TGGTTCCGGAATCTTAACCGGATTCCCTTTCGATAGGCGACACGGAAAATCGT
GTACTTTAAAACGGAGCTTCCCTATCTCTTAGGATCGACTAACCCATGTCCAA
CTGCTGTTACCATGGAACCTTTCTCCACTTCAGTCTTCAAGGTTCTCACTTGAA
TATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTTCACCCAGCATCAC
TGCCAAGGTA

P9LR1311-F
TACCTTGGCAGTGATGCTGGGTGAAACGGCCTCTAGTGCAGATCTTGGTGGT
AGTAGCAAATATTCAAGTGAGAACCTTGAAGACTGAAGTGGAGAAAGGTTCC
ATGGTAACAGCAGTTGGACATGGGTTAGTCGATCCTAAGAGATAGGGAAGCT
CCGTTTTAAAGTACACGATTTTCCGTGTCGCCTATCGAAAGGGAATCCGGTTA
AGATTCCGGAACCAGGATGTGGATCTTTAACGGCAACGTAAATGAAGTTGGA
GACGCTGCCAAGGTA

P1SR1311 85-T3:
TTTTTTTTTTTTTCGCGAGTAAACTGTACATCACATCATCCTGCTGGTCTACTA
TTTAGTCGCCGCGCAGCTTCTGCACGCGCTTCAGGACAGAGTCTTGGCTGGGT
CATACGGGTGCTCCTTGGAGGGAATCTCGAGAAGCGCGGGGAAGGCAGCCTG
GTACCGGTCAACGGTAGGGCGTATCCGCTCGGCGATATGCTGGTTGATGAGG
AGGATAGCAATATCCTTGCGGTCCTCGGTGTACTCCTGGAACGCAGCCTCGAT
GGCGGACGTCTGCATCTTCGCGTCCACGATCATGAAGTTCTTGTCCTGCTTCT

CGTTGACATCGCCAATGCCCGCAAGGAGGAGGCCAGTAACCGAATCCTCGTC
GCCGATAACGGCAAGTAGGTTGCATCCCTTGGGGTTGGAGGTTGGAGCTGCC
AAGGTA

P2SR1311 93-T3:
TTTTTTTTTTTTTCGGCAGGCATAGGATACAACAGCATATTACAAAACCCACA
TTAGAACTGTAAAGCATGAATGTCTGTCTGTACACAACGTCGAGGTCGAGGG
AAGATGAGGCATTCGTGCATGGGGGTGGCGAAGTCGGAGTCGGGGAGTGGA
GCAGATAGGAGTGGGGCAGTTACCCGTATGCCATCAGAAGACCCGCGGCCTT
CCGGCGAGCCAGCGACGCCGAAGAGCATGGCATGGTGCTGCAGAGCTCTGC
GTGAACGTCAGTGCTGTGAGAACGTGAGCCACGAAGCCCGGCCGAAGTGTCG
CCGACACACTCGCTCTGCCAAGGTA

P3SR1311 103-T3:
TACCTTGGCAGCGTCTCCAACTTCATTTACGTTGCCGTTAAAGATCCACATCC
TGGTTCCGGAATCTTAACCGGATTCCCTTTCGATAGGCGACACGGAAAATCGT
GTACTTTAAAACGGAGCTTCCCTATCTCTTAGGATCGACTAACCCATGTCCAA
CTGCTGTTACCATGGAACCTTTCTCCACTTCAGTCTTCAAGGTTCTCACTTGAA
TATTTGCTACTACCACCAAGATCTGCACTAGAGGCCGTTTCACCCAGCATCAC
TGCCAAGGTA

P4SR1311 117-T3:
TACCTTGGCAGCGTCTCCAACTTCATTTACGTTGCCGTTAAAGATCCACATCC
TGGTTCCGGAATCTTAACCGGATTCCCTTTCGATAGGCGACACGGAAAATCGT
GTACTTTAAAACGGAGCTTCCCTATCTCTTAGGATCGACTAACCCATGTCCAA
CTGCTGTTACCATGGAACCTTTCTCCACTTCAGTCTTCAAGGTTCTCACTTGAA
TATTTGCTACTACCACCAA
GATCTGCACTAGAGGCCGTTTCACCCAGCATCACTGCCAAGGTA

P6SR1311 135-T3:
TTTTTTTTTTTTTCGAAACATGACGGTTCATTAACCAACAACACTTGGCATCGC
CACCCACATGCTCCCCGAGATCCACGTCTATCCGCATCAGCGGCACAGCCCA
CAGCCCTCGTCTGCCAAGGTA

P7SR1311 145-T3:
GGTATCGATAAGCTTGATTACCTTGGCAGACGACTTGAATAGAACGGAGTGC
TGTAAGAGTAGAGTAGCCTTGTTGCTACGATCTGCTGAGGCTCAGCTCTTGTT
CTACAGATTTGTGCTTTCGAAAAAAAAAAAAA

P8SR1311 154-T3:
TTTTTTTTTTTTTCGCAAATCTGTAGAACAAGAGCTGAGCCTCAGCAGATCGT
AGCAACAAGGCTACTCTACTGCTTACAGCACTCCGTTCTATTCAAGTCGTCTG
CCAAGGTA

S1LR1211 73-M13F:
TGACTGAACGTGGGGATCCACGCGCGGAACCCGTTCGTGATCAGCACGAGCA
TGAACAGCGCGATGGTGTAGTACGCGAGGAAGGGCTGAAAGTAGCCGCGGT
ACGTGAACTTTGTGCGGTCGACGCCCTGGCGGAGGCACGCCTTGCGGAAGCG
GATGTAGGCCAAGTCTCTCCAGGTTGGAGGCCCTGGGTCGGGCGATGAGGTC
AACAAGGAGTCCAAGTGAGGCGTAGTGGTTTCTTAGACGATTCTAGTTTATTT
CCCGCTCTCTTGTCTCCACAATGCAGCGGCACGAGACAGCTAGGTCTCGTGTG
GCCCGATACCCGAACAGTTCCACAATGCAATCTTTTGTACGGTCGAAAAAAA
AAAAAA

S1SR1211 M13F:
TGACTGAACGTGAAGCGGTAGTTGAAGATGAGGGCGAGGAGCGCGCAGGGC
CCGAGGATGTAGGCAATGTTGAACGTGTCGAGGGCGGGGTCCTGCGTCGGCC
GGAAGCGGAACTTCATGAGGTACAGCACGTACGCGCTCGAGCCGATGAAGA
AGAGCTTCATCAGGCTGTTGTAGAGCGACACGTAGTTTGTGAAGAGGTCGAG
ATAGCGCATGACGAACACGAGGAGGTAGAGCGCCTGCGACTTGAAGCTGAT
GCCGCGGCATGACCGCGACGTCTGGATCTTGTGCAGGAGGATGAAGATTGAC
GCGAGGTGCGACAAGTCGCCAAAGAAGCGGAAGATGTTCATGGCGGCCGTG
GATGTGGGATGTCGAGTGATGAGATGGATGGGCGACATGTATGACGAAAAA
AAAAAAAA

SILR1211BOT-F
TGACTGAACGTGCCATACGACTACCCGAACCTCAACACGAAGAACTCTGCGA
CCTCGCCGTTCACGATCGTGTTCCAAATGACTKGCGCCAAGGCGGCTGGCAG
TGTGATCAACGGCAGTCATCCTGACCTCGGTACTCTCCGCCGGTAACCACGC
GCTCTTTGCTGGCGCACGCTGCTGTACACCCTGGGCAGCGAGGGCCACGCGC
CGCGGTTCTTTGCACGGCTGAATGGACAACGCGTTCCGTGGATCGCGACGAT
TGCGACCGCCCTTGTGGCCGGCCTGTGCTTTGGCAGCTCGTTCATCGGGGCCG
GGCAGCTCTGGAGCTGGCTCCAGAACCTCGTGGGCGTGAGCAACCAGCTGAG
CTGATCAGCATCGGCATTGCGAGCATCCGCTTCCGCCGCGCGCTCGGCCGGC
AGGGCAAGACCCACCTCCTCCCCTTCAAGAACTGGACGTACCCTTTCGGCCC
GTGGATCAGCGTCATCCTCGGCTCGGTCCTCGTCCTTGTGCAAGGCTGGAGCT
GCTTCAGTCCAAAGTTCAACGCCGTCGACTTTGT

S2LR1211-F
TGCTGAACCGTGTACAACCATCAGGACGCGGCTTGGCCGTTGGAGCTTG
GGATTCAACAGATAGCAGTTTGTATTAGAGTATCATTCACCTGATGCAGG
GGTAGCATAGTGCAGCCTCCTGGGAGCTTCCTGGACAATGTAGGAGCTCT
GTCGGGTTTCTGGGAGTTGAGGATAGAGGAGAGGAGGTTGGCGGCACGAG
GAGGTTGAGAAGGCAGGTCAGAGGTGGAGGCCCTATTCTGCAGCACACGC
TGCCGTATAGGGCCTCATACTGGCGCATACGATGGACAGGGTGCCTCTCG
ATCCATCTGTTTGGAACTTGCTCTGTATGCATTAGCATTGTTGCGAAAAA
AAAAAAAA

Appendix 3: Identity of DNA arrayed membranes used for dot blots analysis

Identity of DNA arrayed on dot blots, probed with cDNA synthesized from phenol grown cells

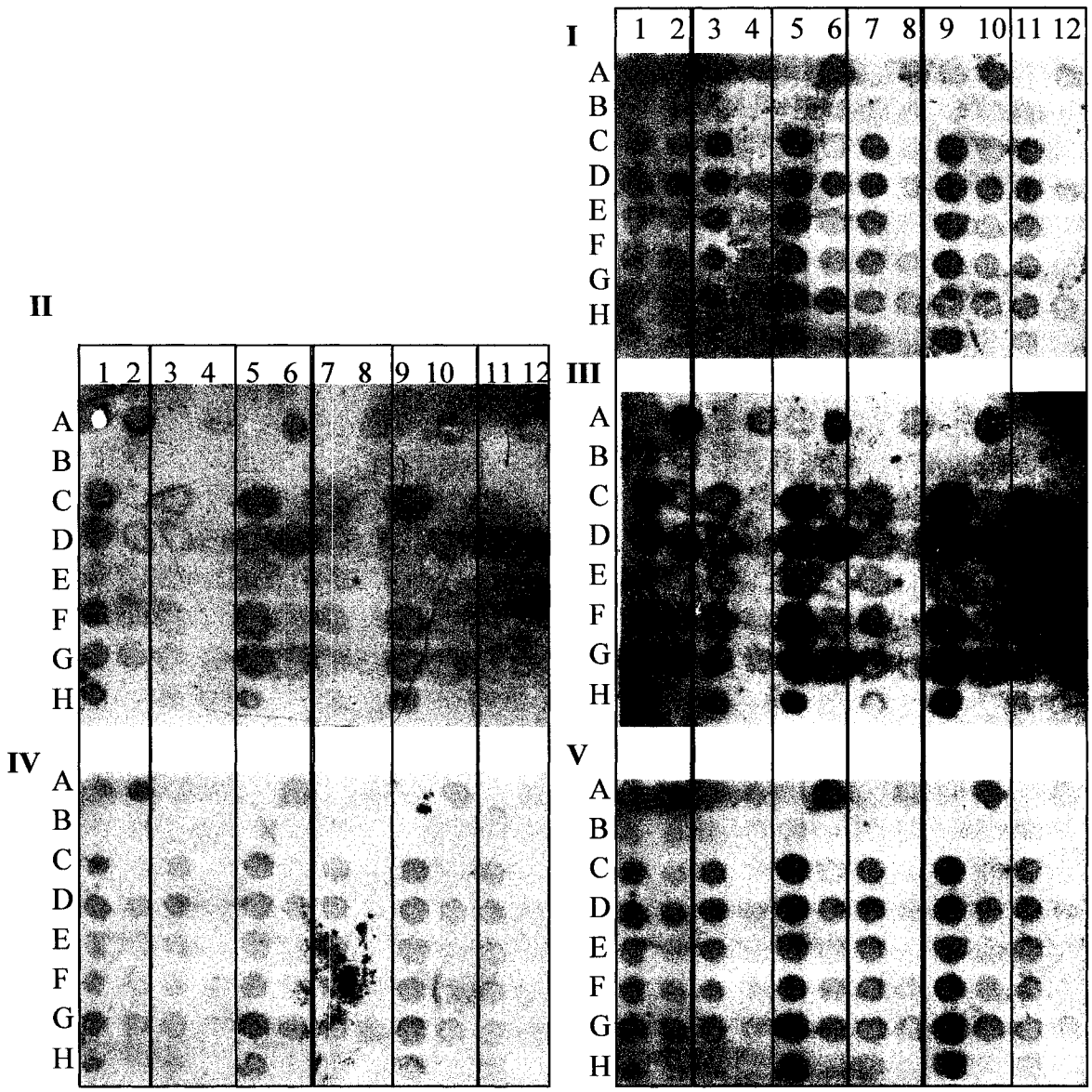
	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)
	100	100	10	10	100	100	10	10	100	100	10	10
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1LR1211	P6SR1311	S1LR1211	P6SR1311	S1LR1211	P6SR1311	S1LR1211	P6SR1311	S1LR1211	P6SR1311	S1LR1211	P6SR1311
B	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311
C	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311
D	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211
E	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311
F	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211
G	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311
H	Phenol hydroxylase	pBluescript II	Phenol hydroxylase	pBluescript II	Phenol hydroxylase	pBluescript II	Phenol hydroxylase	pBluescript II	Phenol hydroxylase	pBluescript II	Phenol hydroxylase	pBluescript II

Appendix 3 (continued)

Identity of DNA arrayed on dot blots, probed with cDNA synthesized from succinate grown cells

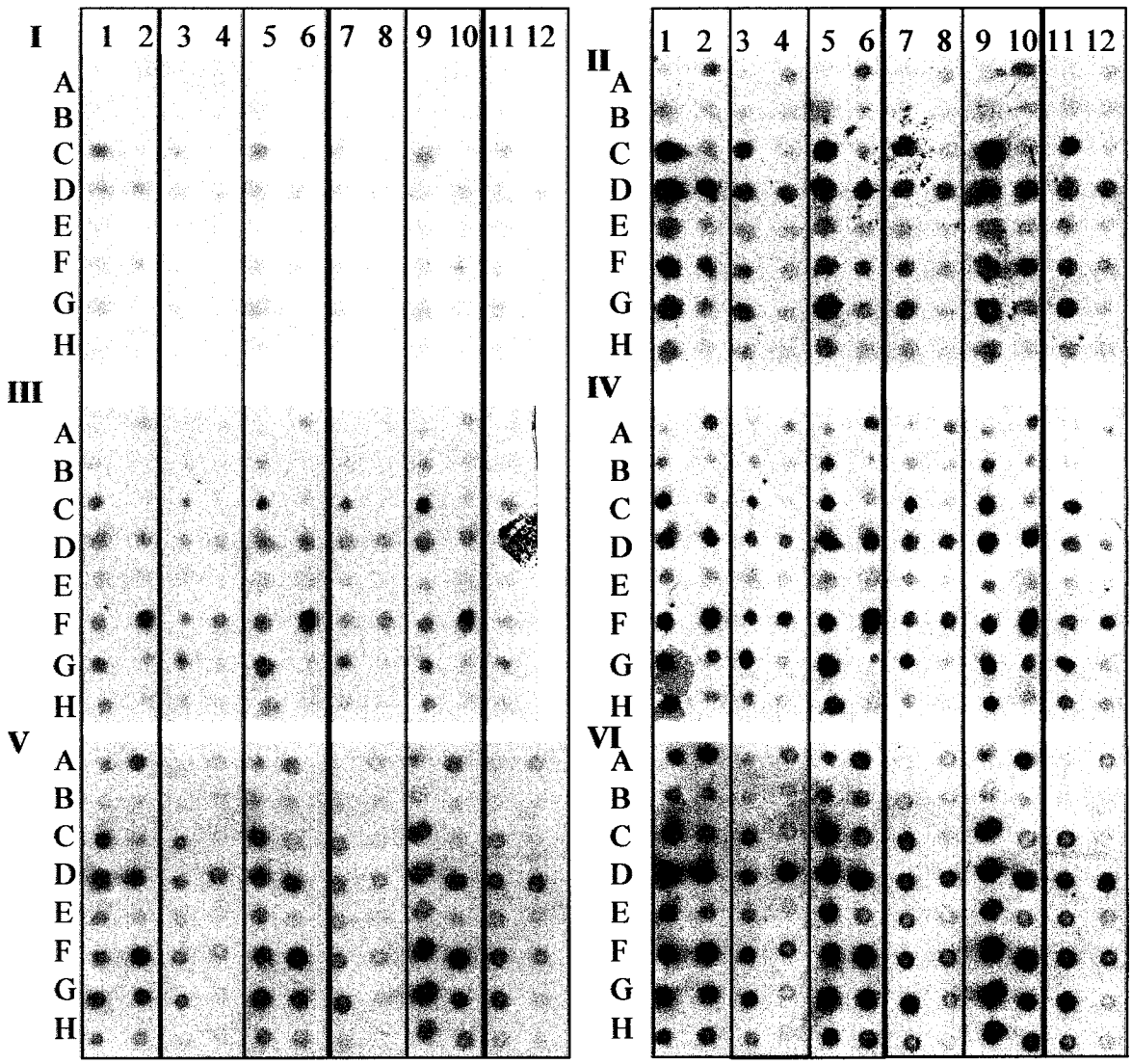
	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)	[DNA] (ng)
	100	100	10	10	100	100	10	10	100	100	10	10
	1	2	3	4	5	6	7	8	9	10	11	12
A	Phenol hydroxylase	P6SR1311	Phenol hydroxylase	P6SR1311	Phenol hydroxylase	P6SR1311	Phenol hydroxylase	P6SR1311	Phenol hydroxylase	P6SR1311	Phenol hydroxylase	P6SR1311
B	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311	P2SR1211	P4SR1311
C	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311	P1SR1211	P2SR1311
D	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211	P3LR1311	S2LR1211
E	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311	P2LR1311	P5LR1311
F	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211	P1LR1211	S1SR1211
G	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311	CO1LR1211	P1LR1311
H	S1LR1211	pBluescript II	S1LR1211	pBluescript II	S1LR1211	pBluescript II	S1LR1211	pBluescript II	S1LR1211	pBluescript II	S1LR1211	pBluescript II

Appendix 4: Results of dot blot analysis of dot blots probed with cDNA synthesized from mRNA purified from phenol grown cells



Dot blots were performed as stated in section 15.3. The blots were probed with cDNA synthesized from different preparations of mRNA. Blot I was probed with cDNA synthesized from mRNA preparation 1, blots II and III were probed with cDNA synthesized from mRNA preparation 2, blots IV and V were probed with cDNA synthesized from mRNA preparation 3. Blots I, III and V were exposed for 30 days while blots II and IV were exposed for 10 days. The clones were arrayed as indicated in Appendix 3.

Appendix 5: Results of dot blot analysis of dot blots probed with cDNA synthesized from mRNA purified from succinate grown cells



Dot blots were performed as stated in section 15.3. The blots were probed with cDNA synthesized from different preparations of mRNA. Blot I, II was probed with cDNA synthesized from mRNA preparation 1, blots III and IV were probed with cDNA synthesized from mRNA preparation 2, blots V and VI were probed with cDNA synthesized from mRNA preparation 3. Blots II, IV and VI were exposed for 30 days while blots I, III and V were exposed for 10 days. The clones were arrayed as indicated in Appendix 3.