Conjugal Transfer of a Transcriptional Fusion Plasmid Containing the Toluene 2-monoxygenase Gene Promoter From an E.coli host to a Parental Strain, Burkholderia Cepacia G4

Ross Martin Vander Noot
"Conjugal transfer of a transcriptional fusion plasmid containing the toluene 2-monooxygenase gene promoter from an E. coli host to the parental strain, Burkholderia cepacia G4."

Ross Martin Vander Noot
Advisor: Dr. Joseph Leahy

Honors Thesis
April 11, 2000
As the world continues to rely on petroleum based fuels for its energy source, a growing problem that must be faced is the danger of the environmental impact that occurs with the mishandling of these fuels. When spills and leaks occur, many chemicals are introduced not only into the oceans and rivers but also into drinking water. Many of the compounds that leak into the environment are carcinogenic or lethal even in small amounts. The BTEX group includes benzene, toluene, ethylbenzene, and xylene and is a major component of petroleum and gasoline spills (1). The BTEX group produces carcinogenic or lethal results even when exposure is limited. To humans and other wildlife, devastating effects such as immune system depression, bone marrow toxicity, and damage to the liver, nervous system, and kidneys occurs while some bacteria react to the substances in a way helpful to them (2). It has been shown that 54% of shallow ground water in non-rural areas is contaminated with one or more so called volatile organic compound, of which BTEX compounds are included (3). Toluene and xylenes are seen frequently in urban storm drains (4). Finding a way to break down these chemicals is of utmost important for the safety of the world’s population.

The Degradation of Toluene

Bacteria and fungi both can break down hydrocarbons into compounds that are less toxic or even into compounds such as carbon dioxide and water (5). The organisms then use these compounds for a carbon source and energy. By altering the microbial population of an environment containing a hydrocarbon spill, bioremediation may allow for a greater rate of the degradation of the dangerous compounds and allow for a great method for treating contaminated soil and groundwater. The enzymes produced by hydrocarbon degraders fall into the category of oxygenases (6,7). They use a NADH to reduce the oxygen to a hydroxyl group which is then incorporated into an aromatic ring or an alkyl group (8). Further steps allow for TCA cycle intermediates to be produced which are then used by bacteria. This allows for the utilization of a potential toxin as a carbon and energy source for metabolism of the cell.
Different organisms break down BTEX compounds under different pathways. The degradation of toluene has been studied in depth through a variety of techniques to determine enzymatic action and intermediate product. The toluene deoxygenases rely on their ability to destabilize the aromatic ring of the toluene hydrocarbon which then allows for the cleavage of the ring. Further steps may allow for the adding and degrading of side chains which will eventually result in an intermediate compound of a metabolic pathway. While several aromatic ring oxygenases exist, most fit into one of two distinct families. The soluble diiron monooxygenase family includes the methane and alkene monooxygenase and phenol hydroxylases. The toluene 2-, 3-, and 4- monooxygenases fall into this family. These enzymes are made up of three or four proteins. The toluene 2,3-dioxygenase belongs to the family of soluble aromatic-ring dioxygenases and is made up of three proteins. Pseudomonas putida F1 (9) utilizes a toluene 2,3-dioxygenase to produce cis-toluene dihydrodiol. This diol is then oxidized to form 3-methylcatechol. Pseudomonas mendocina KR1 (10) uses a toluene 4-monoxygenase to begin the process of degradation. Ralstonia pickettii PKO1 (11) uses both a toluene 3-monoxygenase and a phenol/cresol hydroxylase to carry out the same reactions that Burkholderia cepacia G4’s toluene 2-monoxygenase catalyzes. Burkholderia cepacia G4 (12) uses a 2-monoxygenase which is coded for on a plasmid. This oxygenase causes the aromatic ring of toluene to be broken down in two steps to 3-methylcatechol. This intermediate is further degraded, depending on which regiospecific monoxygenase is used. Figure 1 shows the pathways used by the four bacteria for the degradation of toluene. Burkholderia cepacia G4’s toluene 2-monoxygenase utilizes a single-component electron-transport chain (13). The electrons flow from NADH to the diiron center of the oxygenase through reductase. The other diiron monooxygenases use a two-component chain. The flow of electrons to the diiron oxygenase center ensures that the catalyzed reaction is greatly favorable towards the products and the reaction in the presence of the enzymes is not naturally reversible.
Fig. 1. Pathways for the aerobic metabolism of toluene by bacteria.
The study of cell cultures has shown that different bacteria species degrade toluene at different rates. Just as a connection exists between growth rate and promoter type, it has been found that a correlation exists between this rate of degradation and the type of promoter which activates the gene coding for the degradative enzyme. The genes of Burkholderia cepacia G4’s toluene 2-monooxygenase are found on a natural plasmid. The expression of this gene, with comparison made to R. pickettii PKO1’s toluene 3-monooxygenase, is regulated by sigma 54 type transcriptional activators. The tomA0A1A2A3A4A5 operon that encodes the 2-monooxygenase is regulated by a NtrC-like transcription activator, TomR, which is seen as a type sigma 54 activator. The tomR gene seems to be transcribed from the tom A0 promoter, along with the genes that encode for the catechol dioxygenase and meta-cleavage enzymes. Figure 2 shows the gene arrangement of the toluene 2-monooxygenase, along with the gene arrangements of the other oxygenases for comparison.

Understanding the Plasmid

By manipulating these genes to allow for the induction of a the gene, the corresponding protein’s actions within the cell can better be described and understood. The manipulation of the gene must occur in an environment in which the specific action of the gene can be seen. All other variables must be kept constant. In complex systems and organisms, this is often not possible. To alleviate this problem, the scientific world has turned to E. coli. This bacteria is the most studied and best understood of all bacteria because of its efficient growth rate and because of its ability to accept genes from other cells. This competence allows for genes of other prokaryote species to be inserted into the E. coli and transcribed as if it were a natural part of the bacteria’s genome. Likewise, DNA in E. coli plasmids can be moved to other bacteria. The genes are normally added to competent bacteria through the use of plasmids. These circular stretches of DNA are naturally found within bacteria and are passed from generation to generation through normal replication mechanisms. The plasmids can be passed from a donor bacteria to a
Fig. 2. Gene organization maps for the operons encoding the A) toluene 2,3-dioxygenase from *P. putida* F1, B) toluene 3-monoxygenase from *R. picketti* PKO1, C) toluene 2-monoxygenase from *B. cepacia* G4, and D) toluene 4-monoxygenase from *P. mendocina* KR1. Horizontal bars indicate the size and location of genes within operons. Similar shadings and hatchings represent homologous genes and gene products. Symbols: ■, α- and B-oxygenase subunits; □, γ-oxygenase subunit; ▪, ferredoxin; △, effector protein; ▲, oxidoreductase; ▲, assembly protein; ●, transport facilitator protein; ○, sensor protein; ○, regulatory protein; □, other proteins. Arrows indicate the direction of transcription for each operon. The locations of DNA fragments cloned for transcriptional fusion analysis or used as probes in Northern hybridization analyses are depicted beneath the gene maps, together with the names of the corresponding plasmid constructs. Fragments for promoter fusions which are depicted without terminal restriction sites were cloned directly as PCR products into a blunt-ended vector.
competent recipient bacteria through a variety of methods, both in natural environments as well as in the laboratory. This environment is manipulated to allow for a greater efficiency of the transferring of genes and allows for the screening of those bacteria that take up the plasmid of choice. The process of mating allows for the addition of a known plasmid to a competent cell.

**Manipulating the Plasmid**

A gene to be studied is first added to a plasmid. The plasmid that is taken up by the E. coli, or other competent cell, is created through the manipulation of an already existing natural plasmid. Many of these circular DNA pieces exist and have been completely described. The plasmid pKRZ1 contains the gene lacZ which encodes for the beta-galactosidase that will be used for the promoter measurements. Immediately upstream from this sequence is a restriction enzyme site that allows for the addition of the G4 oxygenase promoter region. This plasmid is placed in solution with competent E. coli so that, through transformation, the E. coli takes up the available plasmid. Lambda pir was then used to supply the protein required for proper replication of the pKRZ1 plasmid. The protein pi is encoded by the pir gene and is added to the E. coli genome through transduction of the prophage lambda. The plasmid contains several parts which are important in the study of the gene. An origin of replication is required for proper reproduction of the plasmid during normal cell division. The original plasmid must have sequences where enzymes will cut. Matching these cut sequences with the ends of the gene of study will allow the gene to be placed within the plasmid. Particular proteins known as restriction endonucleases will cut the DNA sequence at specific points, many of which cause sticky ends to form. Restriction endonucleases were originally found because of their ability to breakdown foreign DNA. While the first restriction endonuclease was found by Hamilton Smith in the bacteria Haemophilus influenzae, many more proteins of this family have been found (14). They all recognize specific sequences within double stranded DNA. When the protein cuts the DNA, a 5’ overhand or 3’ overhang (sticky end), or blunt end can be made. Sticky ends, as
opposed to blunt ends, contain a portion of DNA without a complimentary strand. This then allows for another sequence to be added to the plasmid at the point of the sticky end. Ligase will complete the addition by bonding the gene ends. This plasmid then contains the gene to be studied. The plasmid also contains antibiotic resistance so that screening for the bacteria with the plasmid can be completed easily and efficiently. Every step in the study will contain the antibiotic for which the plasmid allows resistance so that only that bacteria will grow. Plasmids often contain antibiotic resistance genes that then allow for this screening. When placed in an environment with the antibiotic, those cells that contain the resistance gene will survive, while those cells that did not take up the plasmid will die. Bacteria will release the plasmid if it is not needed because energy is required to transcribe the extra DNA. Maintaining the antibiotic environment will ensure that the bacteria retains the needed plasmid. Besides antibiotic isolation, other ways for screening are used, including the use of the lac genes. This gene causes tell-tale changes within the bacteria so that visual differences are noticeable within the bacteria. When a bacteria containing a plasmid with a beta-galactosidase gene is grown on X-Gal, the growth substrate is enzymatically altered such that the bacteria turns blue (15). Isolation streaking from the blue colony forming units (CFU’s) allows for the isolation of bacteria only with the plasmid.

**Transferring the Plasmid**

The plasmid with the gene must then be transferred to the cell type that naturally transcribes that gene. E. coli is almost exclusively used as the original host cell. E. coli’s genetic make-up is known and has been studied for years. Almost all techniques for bacteria, such as isolating DNA or purifying protein, have been set up for E. coli. In addition, the cellular membrane of gram positive bacteria often causes complications when manipulating the addition of a DNA strand. Using the gram negative E. coli eliminates those problems. Calcium chloride causes the bacteria to become more competent by creating pores within the cellular membrane. This process is completely natural and has only been enhanced by the scientific environment.
The moving of DNA from one bacteria to another, or from bacteria to a eukaryotic cell, has allowed for many of the advancements in microbiology. The transferring of genes can occur through a variety of methods, but the three most important include transformation, conjugation, and transduction (16). Transformation was the first method developed for transferring genes and was actually what led to the discovery that the genetic material of cells is DNA. In the process of transformation, DNA is acquired by direct uptake by the cell. DNA is first extracted from donor cells and then is added to cells that have been made more susceptible to uptake. Calcium chloride increases the competency of the cells, allowing the DNA to enter through pores and channels that are found in the cell wall or membrane. In most cases, plasmids are used as the gene carrier. Transduction utilizes induced viral infection to transfer genes. In generalized transduction, any chromosomal marker can be packaged into phage heads. Phage P1 attacks E. coli. During infection, bacterial DNA is occasionally incorporated into the phage head. When this phage then infects a new bacterial cell, the DNA from the original bacterial host is injected into the second cell. In this manner, one allele from the recipient cell can be exchanged for another allele from the donor cell. Homologous recombination occurs and the transferred segment of DNA is successfully incorporated into the recipients chromosome. Specialized transduction is different from generalized transduction in that only a limited set of genes can be transferred. The specificity of temperate phage integration allows for this specialized transduction. Lambda is a temperate phage which will only integrate at specific attachment sites in the cell, thereby allowing the specific placement of a gene within a bacterial chromosome. The current experiment employed conjugation for the transfer of genetic material. Conjugation was first discovered when DNA was seen transferring from cell to another through cell-cell contact. Further work found that factor F is a required component that allows for the unidirectional movement of genetic material. Understanding the F factor allows for the understanding of conjugation. The F factor is found on a small circular DNA plasmid that either replicates autonomously in the cell or
incorporates itself into the chromosome of the host. RP4 is a conjugate plasmid that contains the so-called fertility gene. The tra genes encode the proteins known as the F proteins, and without this plasmid the conjugation cannot take place. The beta-galactosidase promoter is upstream of the tra genes. Adding IPTG, which binds to the promoter, induces the tra genes and allows for the beginning of conjugation. The F factor codes for fibrous proteins known as F pili or sex pili. These proteins promote cell-cell contact and hold the cells in proximity of each other. Other F-encoded functions allow for the transfer of a copy of F DNA to a recipient cell. A single strand is transferred to a recipient cell through a pore in the cell wall and membrane and then a new strand is synthesized from this strand using normal replication systems. When individual cells with an integrated F factor is streaked for isolation, pure colonies form and the resulting strain will transfer chromosomal markers at a high frequency, because every cell in the colony will have F integrated at the same point in the chromosome. This type of strain is known as Hfr for high frequency of recombination. When F factors are transferred to a recipient cell, neighboring segments of the host gene are often excised as well. These F’ elements carry specific genes, many times including the lac genes. When reintroduced into a strain carrying the lac genes, F’ lac episomes can be successfully transferred from one strain to another through conjugation. Strains with F’ factors covering virtually the entire region of the E. coli chromosome have been collected so that laboratories can integrate specific genes, such as the lacZ gene.

**Measuring the Promoter**

The promoter activity is measured through the use of the promoter reporter vector pKRZ1 (17). The promoter region, PtomA0, of B. cepacia G4 is added upstream to the promoterless lacZ gene. This gene codes for beta-galactosidase. Measuring the activity of this enzyme is the same as measuring the activity of the promoter when it is attached to its actual gene. Therefore, you get accurate results of promoter activity without confounding effects of other proteins or conditions. After PCR amplification of previously constructed genes, the DNA
of the promoter is cloned into the pKRZ1 plasmid, and then transformed into the conjugative strain of E. coli SM10 (lambda pir). The E. coli is then mated with the native strain. This places the plasmid with the G4 PtomA0 promoter back into the G4 bacteria.

The lacZ gene encodes for beta-galactosidase, which will break down a number of chemicals including ONPG. When studying promoter regions of specific genes, the promoter is often linked to the beta-galactosidase gene so that when the promoter for your gene of study is activated, the beta-galactosidase gene is activated instead. This gene produces an enzyme which causes the break down of ONPG to produce a yellow color. The yellow absorbs maximally at 420 nm so that a UV spectrophotometer can be used to determine the amount to which the promoter is activated. The greater the degree of yellow, the more the gene has been turned on. This is the results of the process known as induction. In induction, synthesis of a specific inducible enzyme, in this case beta-galactosidase, occurs when cells are exposed to the substance, or substrate, upon which the enzyme acts to form a product. Formation of beta-galactosidase has been shown to be controlled by a so-called regulator gene presumed to specify a repressor protein that binds to the region of DNA responsible for directing the synthesis of the enzyme. If substrate is present, it acts as an inducer by combining with the repressor so as to prevent its binding to DNA. As a result, transcription proteins such as RNA polymerase are allowed to bind to DNA and the enzyme is synthesized. Such mechanisms are important in the cell because they prevent the synthesis of enzymes that a cell cannot use. Beta-galactosidase is needed only when it is present, and through the use of the inducer, it is only made when needed. The actions of these promoters are different depending on what type of promoter it is. A schematic view of positive control is seen in Figure 3. The organic substrate in this case is toluene. The toluene binds to the activator, which is a protein coded for downstream in G4, and thus allows the RNA polymerase to transcribe the lacZ gene.

Transcriptional activity is the distinctive step in determining the differing degrees to
- if organic substrate is not present

- if organic substrate is present

Fig 3. Schematic diagram of positive regulation
which the promoter causes gene expression. Toluene is a chemical which activates the PtomA0 promoter of G4 and so will be the inducer in the time course experiments. Differing concentrations of toluene will be used once the optimal induction time is determined. This differing in concentrations will allow for a wide angle of comparison to other bacteria with different promoter types. Corrected values for the induction will be found by subtracting the amount of beta-galactosidase activity with only pKRZ1 present within the cell. Statistical analysis can be done to determine differences in strains. This difference is expected to exist between the sigma 70 and sigma 54 promoter types. Figure 4 shows a comparison of the different types of organisms and the pathways used in comparison to the genetic organization of the operon.

**Growing the Bacteria Strain**

The time course experiments revolve around the activity of bacteria as it grows within a closed environment. As the density of bacteria increases, the expression of genes and proteins changes. These changes must be incorporated into any study that is determining gene induction, and so the density of the bacteria must be maintained throughout the course of the experiment. This is done by determining the density of the bacteria under specific times through optical density tests with a spectrophotometer. As the density increases, the absorption at 600 nm will increase. The growth pattern of bacteria is sigmoidal in shape. The lag-phase of the growth curve is the point at which the bacteria is half the density of its maximum. This density corresponds to an OD600 of 0.32. By conducting experiments with bacteria that live in an environment at OD600 equal to 0.32, the degree of protein expression will be maintained throughout the coursework. Figures 5, 6, and 7 show the log OD600 versus time for G4, G4 pKRZ1, and G4, pKRZ1:600 b.p. Ptom. The bacteria were grown and their optical density was taken until two semi-parallel lines were seen. This proves that the bacterial growth cycle was being maintained in the broth. Also, it gives an idea of when the mid-log will be. All inductions
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
<th>Gene Organization</th>
<th>Bacterial Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>toluene 2,3-dioxygenase</td>
<td></td>
<td>todC1C2BAD</td>
<td>Pseudomonas putida F1</td>
</tr>
<tr>
<td>toluene 4-monoxygenase</td>
<td></td>
<td>tmoABCDEF</td>
<td>Pseudomonas mendocina KR1</td>
</tr>
<tr>
<td>toluene 3-monoxygenase</td>
<td></td>
<td>tbuA1UBVA2C</td>
<td>Ralstonia pickettii PK01</td>
</tr>
<tr>
<td>toluene 2-monoxygenase</td>
<td></td>
<td>tomA1A2A3A4A5</td>
<td>Burkholderia cepacia G4</td>
</tr>
<tr>
<td>phenol hydroxylase</td>
<td></td>
<td>dmpKLMNOP</td>
<td>Pseudomonas sp. strain CF600</td>
</tr>
</tbody>
</table>

**Fig 4.** Comparative chart of oxygenase enzymes
Fig. 5: Growth Curve of G4

30°C, 200 rpm
Fig. 6: Growth Curve of G4 (pKRZ1)

30°C, 200 rpm
Fig. 7: Growth Curve of G4 (pKRZ1::600 bp PCR Ptom->SmaI)

30°C, 200 rpm
were done at mid-log to keep protein concentrations similar. After plotting growth curves, the growth rate for each bacteria was determined, as seen in Figures 8, 9, and 10.

**Inducing the Promoter**

Once the growth rates had been determined and the amount of time required to reach mid-log is set, induction curves can be completed. In a closed environment, induction of the bacteria occurs as a set amount of toluene is introduced. Set amounts of time are allowed to pass under specific conditions and then the amount of induction is determined by testing the amount of beta-galactosidase protein produced in that amount of time. The protein which is broken down by the beta-galactosidase enzyme, ONPG, will turn a yellow color and the amount of yellow will be a comparison for the amount to which the toluene induced the production of the protein compared to bacteria that were not exposed to toluene. Bacteria only exposed to DMF will also be tested to see its affect on beta-galactosidase protein production. Figures 11, 12, and 13 show the amounts of protein versus the density of the bacteria.

While the beta-galactosidase protein is not the actual protein or gene of interest, it allows an efficient way for testing the degree to which the promoter of the toluene degrader gene is activated. While testing the amount of toluene degrader is not possible, testing another gene, such as lacZ, is. The induction time course is completed simply to find the optimal time over which the promoter acts. Once this is determined, different concentrations of toluene can be added to the bacteria to determine concentration gradient changes which affect promotion of Ptom. All of this will be used to compare the activities of the different types of promoters found in the four bacteria strains tested.
Fig. 8: Growth Rate of G4
30°C, 200 rpm

y = 0.3468x - 2.1089
R² = 0.9871

y = 0.3032x - 2.1278
R² = 0.9997
Fig. 9: Growth Rate of G4 (pKRZ1) 
30°C, 200 rpm

\[ y = 0.1439x - 2.1298 \quad R^2 = 0.9977 \]
\[ y = 0.1541x - 2.3419 \quad R^2 = 0.9996 \]
Fig. 10: Growth Rate of G4 (pKRZ1::600 bp PCR Ptom->Smal)  

$y = 0.1318x - 1.641$  
$R^2 = 0.9981$ 

$y = 0.1371x - 1.4656$  
$R^2 = 0.9973$
Fig 11: Protein Density of G4
Protein Conc. vs. Log OD$_{600}$

$30^\circ$C, 200 rpm

$y = 0.2788x^3 + 0.6577x^2 + 0.6629x + 0.3307$

$R^2 = 0.9341$
Fig 12: Protein Density of G4 (pKRZ1)
Protein Conc. vs. Log OD$_{600}$

$y = 0.1627x^3 + 0.467x^2 + 0.5408x + 0.296$
$R^2 = 0.8308$

$30^\circ C, 200$ rpm
Fig 13: Protein Density of G4 (pKRZ1::Ptom)  
Protein Conc. vs. Log OD <sub>600</sub>  

Protein Conc. (mg/mL) vs. Log OD<sub>600</sub>  

30°C, 200 rpm  

Log OD<sub>600</sub>  

R<sup>2</sup> = 0.892  

R<sup>2</sup> = 0.9399
Discussion

It is known that toluene induces these promoter regions, but the actual effects of toluene on the speed of oxygenase protein production are not known. Several questions arise concerning what determines the rates at which hydrocarbons are degraded. The answers to these questions may include the inherent activity of the enzyme, the rates of transcription or translation, or the transport of the hydrocarbon or enzyme within the cell. The specific mechanisms which regulate the specific enzymes or the global effects on gene expression may be involved. Further questions include, “Do one of these regulation processes predominate as the factor controlling the rate of degradation, or do many processes play a role? Are the factors which predominate the same for all bacteria? For all types of hydrocarbon substrates? To what extent are the factors controlling degradation related to the general physiology of the cell, i.e. whether the strain is oligotrophic and adapted to low nutrient conditions, or whether it is copiotrophic and adapted to high nutrient conditions? To what extent do these factors relate to the phylogeny of the strain?” The knowledge provided from studying the answers to these questions will not only allow for the prediction of rates of hydrocarbon degradation in the environment but also may lead to the development of new strategies for bioremediation. The initial work in answering many of these questions begins with understanding which factors determine the rates at which bacteria, specifically B. cepacia G4, degrade hydrocarbons, specifically toluene. Comparing this strain to P. putida Fl, P. mendocina KR1, and R. pickettii PKO1 will allow for a complete comparison of different types of promoter regions. It is expected, because of the differences in cellular degradative activities as contrasted with the turnover rates of the different bacteria, that levels of gene expression rather than the inherent activities of the toluene oxygenases may be the key player in determining the toluene degradative activity. For this reason, the promoter activities of the different bacteria must be explored. This promoter activity can be assayed through techniques including plasmid integration, which then allows for the manipulation of genes.
Methods

Mating Procedure

LB, VBG + Km 100, VBG + Km 300, and VBG + Km 600 agar plates were made and allowed to cool at 65 degrees C in a water bath for two hours before adding IPTG. E. coli SM10((pir)-(pKRZ1-clone) was lawn streaked 6x on LBAp100. The recipient strain was streaked 14x onto TNA plates. A sterile spatula was used to collect cells from 2x E. coli plates and 7x recipient plates. A second collection from 4x E. coli plates and 7x recipient plates was collected. These were mixed well on the surface of separate LB + IPTG plates. A moist paper towel was placed in the bottom of a beaker and the plates were placed on the towel. The plates were incubated for 8 hours at 30 degrees C. A 10 mM MgSO4 solution was made and autoclaved before each use.

The mating paste was transferred to a 15 mL conical centrifuge tube and suspended with 1 mL of the 10 mM MgSO4. 0.1 mL of the undiluted mixture was plated onto each type of the VBG plates. A 1:10 dilution was also made by adding 0.2 mL of the mixture to 1.8 mL of 10 mM MgSO4. 0.1 mL of this dilution was plated onto each type of the VBG plates. These plates were incubated at 30 degrees C and observed for 2 to 3 days. Any of the colonies formed were streaked for isolation on a corresponding plate so that a colony formed on a VBG + Km300 plate would be streaked for isolation on another VBG + Km300 plate. The colonies were subcultured two more times on the same type of plate and then again on LB + Km75 plates. A subcultured colony was inoculated into 2 mL LB + Km75 for analysis.

Growth Curves for Protein Assays

The organism was plated out from a freezer stock for 3 days on BM + citrate (10(M) plates with the proper antibiotic, such as kanamycin for G4. 100 mL of BM + citrate (10mM) broth was prepared in 2x 500 mL Erlenmeyer flasks. Enough G4 was inoculated into the broth until a fairly turbid mixture was made and then incubated for 24 hours at 30 degrees C and 200 rpm. 5 mL of
the starter culture was subcultured into fresh medium and then incubated at 30 degrees C and 200 rpm. 3 mL samples were taken on a schedule for OD600 analysis in a spectrophotometer against a BM + citrate blank. The schedule was set for a sample every hour until the optical density was 0.1, every 30 minutes until OD was 0.2, every 15 minutes until OD is 0.32, and every 30 minutes until stationary phase defined as 2 successive points differing by less than 5%. A variety of dilutions were made with the stationary phase broth culture as described in Table 1.1

For OD600 in test tubes:

<table>
<thead>
<tr>
<th>Broth (mL)</th>
<th>Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.7</td>
<td>0.3</td>
</tr>
<tr>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>2.1</td>
<td>0.9</td>
</tr>
<tr>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>0.6</td>
<td>2.4</td>
</tr>
<tr>
<td>0.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

For Protein Assay in microfuge tubes:

<table>
<thead>
<tr>
<th>Broth (mL)</th>
<th>Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>1.2</td>
<td>0.60</td>
</tr>
<tr>
<td>0.9</td>
<td>0.45</td>
</tr>
<tr>
<td>0.6</td>
<td>0.30</td>
</tr>
<tr>
<td>0.3</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Broth (mL)</th>
<th>Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>1.2</td>
<td>0.60</td>
</tr>
<tr>
<td>0.9</td>
<td>0.45</td>
</tr>
<tr>
<td>0.6</td>
<td>0.30</td>
</tr>
<tr>
<td>0.3</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 1.1: Dilutions using Stationary Phase broth culture

The protein samples were vortexed, placed on ice for 30 minutes, and then microfuged in the freezer for 10 minutes. The sample was aspirated immediately afterward. 1 mL of 1N NaOH was added to each sample and vortexed to resuspend completely. The mixture was heated at 90
degrees C for 30 minutes and then frozen for assay at a later time. The protein assay was thawed and vortexed. 0.5 ml of each sample was diluted with 0.5 mL of distilled water and vortexed. 75 microliters of the diluted sample was aliquoted into 13x100 tubes. Bio-Rad assay was followed using 0.5 N NaOH instead of water for the standards. The dilutions of the samples were integrated and the protein concentrations multiplied by 1.33333.

**Beta-galactosidase Assays for Time Course Experiments with G4 (pKRZ1) strains**

The organism was plated from a freezer stock onto BM + citrate + kam75 and allowed to incubate at 30 degrees C for 3 days. 100 mL of BM + citrate (10mM) broth was prepared in 2x 500 Erlenmeyer flasks for starter culture and then kanamycin was added. 750 mL of pre-BM + citrate (10mM) broth was prepared in 2x 4 L Erlenmeyer flasks for log-phase cultures. 300 mL of pre-BM + citrate was prepared in 1x 500 mL Erlenmeyer flask for later addition to sidearm flasks. 6 sidearm flasks were sterilized. The starter broth was inoculated until fairly turbid with about 4 large loopfuls of bacteria. This was incubated for 24 hours at 30 degrees C and 200 rpm. The pre-BM + citrate was finished by adding remaining BM components and antibiotics. 40 mL of the starter culture was subcultured into 800 ml of fresh medium in a 4 L flask and incubated at 30 degrees C and 200 rpm. 3 mL samples were taken on a set schedule for OD600 analysis in a spectrophotometer against a BM + citrate blank. The sample was taken every 30 minutes until OD was 0.2 and then every 10 minutes until OD is 0.32. During this time, 50 mL of BM + citrate + Kan75 was added to each sidearm flask. When at log phase, OD is 0.32, a 25 mL pipette was used to transfer 50 mL of the broth into each sidearm flask. 20 microliters DMF was added to each sidearm flask. 3.0 microliters Neat Toluene was added to the induced flask. Different needles were used to ensure that toluene contamination did not occur in the control flasks. The flasks were incubated at 30 degrees C and 200 rpm for the required times of 4, 6, 8, or 12 hours. During this time, the UV spectrophotometer was warmed up, the water bath was warmed up, tubes were labeled, an ice bucket was obtained, and solutions were made. 50 mL of
Z buffer was made by adding 50 microliters of 1M MgSO_4 and 135 microliters beta-mercaptoethanol to 50 mL of Z buffer stock. 0.1% SDS was prepared by adding 0.1 mL stock 10% SDS to 9.9 mL distilled water. 1M Na_2CO_3 was prepared by adding 2.65 grams Na_2CO_3 to 25 mL of distilled water. 0.8 mL of distilled water was added to protein tubes. 1 protein tube was labeled for each time point. 0.5 mL of distilled water was added to the blank tubes, four for each time point. Three experiment tubes were used for each flask: citrate only, DMF only, and DMF + toluene. 15 minutes before the first sample, 4mg/mL ONPG was prepared in 0.1M phosphate buffer, pH 7.0. This was prepared fresh for every time point by adding 0.012 grams per 3 mL 0.1M phosphate buffer, pH 7.0 and then storing in the refrigerator. 0.5 mL of cells were pulled out of the flask with the specified needle and added to the experiment microfuge tubes. The experiment and blank tubes were placed on ice for 30 minutes in the fume hood with stoppers off. 0.4 mL of cells were added to the protein microfuge tubes and vortexed. 0.8 mL from the protein tubes was used to determine the OD600 in the UV spectrophotometer against a water blank. The protein concentration was determined against the appropriate calibration curve and then multiplied against the dilution factor. The experiment tubes were removed from the ice after 30 minutes and 0.5 mL Z buffer was added to the tubes. After vortexing, 10 microliters chloroform was added with a 10 microliters syringe and 15 microliters 0.1% SDS was added with a P20. The tubes were vortexed for 20 seconds and then placed in a 30 degrees C water bath for 5 minutes. 0.1 mL ONPG was added with a P200 and then mixed well. Taking note of the time, the tubes were incubated at 28 degrees C until a yellow color developed. The OD420 was not allowed to exceed 0.6. The induced tubes with DMF + Toluene were allowed to incubate for 75 minutes. The controls and negative tubes were allowed to incubate for 2 hours. The times were recorded in all cases. The reaction was stopped by adding 0.25 mL of Na_2CO_3. The tubes were stored in the refrigerator if necessary. The cells were placed on ice for 30 minutes and then
microfuged in the freezer for 10 minutes. 0.8 mL of the solution was immediately removed from
the tubes and transferred to the cuvettes. The OD420 and OD550 were measured in an UV
spectrophotometer.

**E. coli Transformation**

Growth conditions for the growing of cells are set up as follows. Preparation of competent cells:
E. coli DH5α - LB; E. coli SM10 (pir) - LBKm50. Selection for plasmids after transformation:
pBS - LBAp100; pKRZ1 - LBAp100

Two flasks were prepared each containing 50 mL of LB broth. 1x TSS Buffer was prepared and
kept refrigerated. One day prior to the actual procedure, the flasks were inoculated containing
50mL LB broth + antibiotic with a single colony isolate. They were incubate overnight at 37
degrees C and 250 rpm. The centrifuge head was refrigerated. On the procedure day the
centrifuge was turned on and 50 mL of pre-warmed LB broth with 500 microliters of the
overnight culture was inoculated. The cells were shaken cells at 37°C and 250 rpm for 1.5 hours.
During this time label fifty 1.5 mL tubes, get ice, and prepare cuvets. Cuvets were prepared as
follows: blank--2700µl D.I. water + 300µl LB broth, vortex; sample--2700µl D.I. water + 300µl
culture, vortex. Use the spectrophotometer and check culture for an OD600 of 0.3-0.4 (which
equals 0.03-0.04 after dilution. Continue to check new samples every 15 min. until this
absorbance is obtained. (Spec: set to infinity with left knob, insert blank and set to 0 with right
knob.) Immediately transfer the culture to 2 O.R. tubes. For the following steps, it is critical to
maintain cold temperatures. Centrifuge cells at 5,000 rpm for 10 min. At 4°C. Immediately
discard the supernatant in a waste beaker and aspirate the pellet. Add 5 mL of cold 1xTSS buffer
and suspend pellet by pipeting up and down and vortexing. Do this quickly! Aliquot 100µl to
microcentrifuge tubes an freeze at -80°C. (keep sample and aliquots on ice during this step.)

**Transformation Procedure**

Prepare a “wet” ice bath and thaw competent cells for approximately 15 minutes. Transfer 100
ul of thawed cells to a cold, labeled 1.5 mL microcentrifuge tubes. Add 30 µl of ligation mixture and mix gently with the pipet tip. (Do not pipet up and down.) Incubate mixture on ice for 30 minutes. Heat shock the mixture in a 37°C water bath for 5 minutes. Add 500 µl of pre-warmed LB broth to the mixture, and transfer it to labeled Falcon tubes. Grow cells for 1.5 hours in a 37°C incubator at 100 rpm. Plate 100 µl aliquots of cells onto 5 plates containing selective media. Incubate plates 37°C overnight.

**B-galactosidase Assays for Toluene Concentration Experiments with G4 (pKRZ1) Strains**

Plate out organism from freezer stock for 3 days on BM + citrate + Km75. Prepare BM+citrate+Km75 broth (92 ml dw + 0.294 g citrate + BM components => 100 ml) in 2x 500 ml Erlenmeyer for starter cultures, and add kanamycin. Prepare pre-BM+citrate+Km75 broth (920 ml dw + 2.94 g citrate) in 2x 4 L Erlenmeyer for log-phase cultures. Prepare pre-BM+citrate+Km75 broth (920 ml dw + 2.94 g citrate) in 1x 2 L flask for induction cultures. Sterilize 16X sidearm flasks and label them. Remember to change septa and parafilm the sidearms. Inoculate 100 ml BM+citrate+Km75 starter broths until fairly turbid (four large loopfuls). Incubate 24 h at 30°C and 200 rpm. [11 pm] Add BM components and kanamycin to complete preparation of 1 L BM+citrate+Km75 in the 4 L flasks. Subculture 40 ml of starter culture into the 1 L of BM+citrate+Km75 in the 4 L flasks. Incubate at 30°C and 200 rpm. [11 pm] Arrive 6:30 am. Add BM components and kanamycin to complete preparation of 1 L BM+citrate+Km75 broth in the 2 L flask. Take 3 ml samples for OD600 determination using the following schedule:

- every 30 min until OD is 0.2
- every 10 min until OD is 0.32

During this time dispense 50 ml each of BM+citrate+Km75 from 2 L flask into 16X sidearm flasks and label as follows:

- pKRZ1
- 0 uM
- 25 uM
- 50 uM
- 75 uM
- 100 uM
250 uM  
500 uM  
1000 uM  

**Ptom**  
0 uM  
25 uM  
50 uM  
75 uM  
100 uM  
250 uM  
500 uM  
1000 uM

When log-phase cultures are ready, use a sterile 25 ml pipet to transfer 50 ml broth to each of sidearm flasks. Add DMF and toluene to the flasks according to the table below.

<table>
<thead>
<tr>
<th>Final Toluene Concentration (µM)</th>
<th>Neat Toluene Added (µL)</th>
<th>0.94 M Toluene in DMF Added (µM)</th>
<th>DMF Added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>30</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>750</td>
<td>22.5</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>15</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>250</td>
<td>7.5</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>75</td>
<td>-</td>
<td>22.5</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>15.0</td>
<td>6.5</td>
</tr>
<tr>
<td>25</td>
<td>-</td>
<td>7.5</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>
Incubate at 30°C and 200 rpm for the required times (6, 8, 10, 12 hrs). During this time get ice, warm up UV Spec and water bath, label all tubes and cuvettes, prepare 50 mL of Z buffer in 50 ml cct. (Add 50 µL 1M MgSO4 and 135 µL B mercaptoethanol per 50 mL of Z buffer stock.) , prepare 10 ml of 0.1% SDS in 15 ml cct. (0.1 mL stock 10% SDS and 9.9 mL water), prepare 25 ml of 1M Na2CO3 in 50 ml cct. (2.65 grams/25 mL water), prepare and label microfuge tubes:

For Proteins (SINGLE TUBES - ADD 0.8 ML DW TO EACH TUBE)

- pKRZ1
  - 0 uM
  - 25 uM
  - 50 uM
  - 75 uM
  - 100 uM
  - 250 uM
  - 500 uM
  - 1000 uM

- Ptom
  - 0 uM
  - 25 uM
  - 50 uM
  - 75 uM
  - 100 uM
  - 250 uM
  - 500 uM
  - 1000 uM

SET UP CUVETTES

15 minutes before EACH sample, prepare 4 mg/ml ONPG in 0.1M phosphate buffer, pH 7.0. Prepare fresh for every time point. (0.012 g per 3 ml 0.1M phosphate buffer, pH 7.0) (It takes a while to dissolve.) Store in refrigerator. Withdraw 0.4 mL of cells and aliquot to protein microfuge tubes. Vortex. Take 0.8 mL from protein tubes and determine OD600 in UVSpec. Use water as a blank. [Determine protein concentration from appropriate calibration curve, then multiply to correct for dilution.] Place culture flasks on ice for 30 min in fume hood with stoppers off. Set
timer for 30 min. Withdraw 0.5 ml of cells from culture flasks and aliquot to ONPG tubes. Aliquot 0.50 ml Z buffer to tubes, vortex well. Add 10 ul chloroform with 10 ul syringe, 15 ul 0.1% SDS with a P20. Vortex 20 s, place tubes in 30C water bath for 5 min. Add 0.1 ml ONPG with a P200, mix well, and incubate at 28C until a yellow color develops. (Remember OD 420 cannot exceed 0.6) Induced tubes (DMF + Toluene) should go 75 minutes. Controls and negative tubes should be incubated for 2 hours. Record time of development in all cases. Stop the reaction by adding 0.25 ml of Na2CO3. Tubes may be stored in refrigerator if necessary. Place cells on ice for 30 min. Microfuge cells in the freezer for 10 min. Immediately remove 0.8 ml of solution from tubes and transfer to cuvettes (remember to check cuvettes for any scratches). Measure OD420 and OD550.

Units = \frac{\text{OD420} - (1.75 \times \text{OD550})}{(\text{min}) \times (\text{volume of cells}) \times (\text{mg/mL protein})}

Mating Plates

VBG + IPTG + Km 100, 300, & 600.

1.) 2 grams purified agar into 100 mL DI water. Autoclave for 35 minutes. Also make 36% glucose solution and autoclave for 35 minutes.

2.) Allow to cool in 55C water bath for 2 hours.

3.) Add the following in order:

   For VBG plates:
   
   2 mL 50X VB Salts Solution
   
   2mL 36% glucose
   
   0.5 mL stock IPTG

4.) Appropriate antibiotic concentrations:

   for Km 100 add 0.01 g Kanamyacin to 100mL
   
   for Km 300 add 0.03 g Kanamyacin to 100mL
   
   for Km 600 add 0.016 g Kanamyacin to 100mL
5.) Stock solutions:

- ITPG stock

0.24 g IPTG/10mL sterile water and mix well. Aliquot to 1.5 mL centrifuge tubes and freeze.

- 50X VB Salts:

  670 mL water

  10 g MgSO4-7H2O

  100 g Citric Acid

  500 g K2HPO4(dibasic)

  175 g NaNH4PO4-4H2O

  autoclave 15 minutes

- 36% glucose:

  add 36g glucose per 100mL solution and autoclave for 35 minutes.
References


10. Whited, G.M., and D.T. Givson. 1991. Toluene-4-monooxygenase, a three-component enzyme...


