

Appendix D - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL

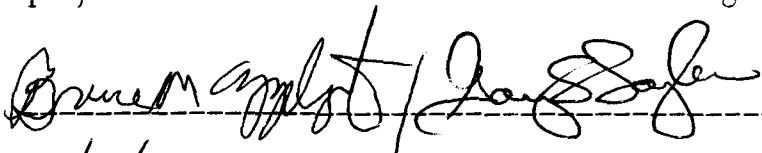
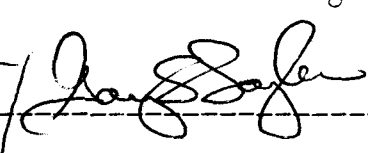
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PROJECT TITLE: Independent expression of the putative transport
protein TcdX in the bioluminescent reporter *Pseudomonas putida*
TVA8

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed:  /  Faculty Mentor

Date: 05/10/00

Comments (Optional):

THE INDEPENDENT EXPRESSION OF THE GENE *todX* IN THE BIOLUMINESCENT REPORTER

STRAIN *Pseudomonas putida* TVA8

Senior Thesis

Jennifer J. Rahn

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ABSTRACT

Previous work has identified *todX*, a gene in the *tod* operon, as coding for a putative transport protein TodX with homology to the passive transport protein FadL found in *Escherichia coli*. Since the expression of *todX* is controlled by the *tod* promoter, the presence of TodX is regulated by toluene. Therefore initial influx of toluene into the cell is by passive diffusion across the cell membrane. To examine whether the putative transport protein TodX would increase the influx of toluene into the cell, TodX was independently expressed in the bioluminescent reporter *P. putida* TVA8. This was accomplished by fusing the *todX* to the salicylate-inducible *nahRG* promoter cassette contained in a MiniTn5 transposon carrying a tetracycline resistance marker. The resultant *nahRG-todX* expresses *todX* in the presence of salicylate. An *E. coli* strain harboring the MiniTn5 *nahRG-todX* transposon was biparentally mated with *P. putida* TVA8. Transconjugants were selected on LB agar plates containing tetracycline 14mg/L and exposed to toluene vapor. Several transconjugants were isolated and examined for fitness and functionality. A strain designated *P. putida* TVA8 TodX was selected for further study. Growing cell assays were conducted with the TVA8 TodX strains where bioluminescence was measured at various concentrations of toluene and salicylate. The samples with TodX expressed prior to exposure to toluene did not show significantly higher bioluminescence or a faster light response compared to the parent strain TVA8. This data suggests that expression of TodX prior to toluene exposure does not increase the influx of toluene into the cell. The data also indicates passive diffusion across the cell membrane is not rate limiting in *tod* gene expression as assayed using the *tod-lux* gene fusion.

LITERATURE REVIEW

Pseudomonas putida F1

In the environment, there exist bacteria in the soil that can naturally degrade and survive on organic compounds as their sole carbon source. These bacteria have a unique adaptation and are usually found in areas that are contaminated with organic compounds from spills, waste dumps, or industrial processes. One such bacterium, *Pseudomonas putida* F1, has the ability to degrade the compound toluene. It has been isolated from the soil and can be successfully cultured in the lab. This organism and its unique ability to utilize toluene as the sole carbon and energy source are the beginning of the work described in this thesis.

Pseudomonas putida F1 degrades toluene through a multi-component enzyme system composed of four protein components encoded by the genes designated *todA*, *todB*, *todC1*, and *todC2*. These four proteins make up the enzyme called toluene dioxygenase. This enzyme acts by incorporating two atoms of molecular oxygen into the aromatic toluene ring to give cis-toluene dihydrodiol. This compound is further broken down by sequential action of the protein products of *todD*, *todE* and *todF*. The final result is 2-hydroxypenta-2,4-dienoate and acetate (11). Additionally, the genes *todFGHI* encode an enzyme that further degrades the first product in to TCA cycle intermediates (10).

The *tod* genes are organized as *FC1C2BADEGIH* and are arranged in an operon that is induced coordinately by toluene. A region downstream of *todH* and denoted *todS* and *todT* regulates expression of these genes (10). This system of degradation is very similar in structure to the sequence reported for benzene dioxygenase as well as cis-benzene dihydrodiol dehydrogenase both from *Pseudomonas putida* strains (12).

Lux fusions

Upon consideration of the application of species like *PpF1*, one can see immediate application in bioremediation. These bacteria could easily be used to clean up contaminated sites. However, another application is using bacteria to make biosensors. For this application, all that is needed is a way to quantify gene expression that is a result of analyte concentration. This has been done with the use of the *lux* genes. The *lux* genes are responsible for bioluminescence in the marine bacterium *Vibrio fischeri*. The *lux* cassette of genes contains *lux CDABE* genes (2). *LuxAB* encodes the luciferase enzyme responsible for

bioluminescence. Luciferase converts an aldehyde group to a carboxyl group with the use of molecular oxygen. This process regenerates a fatty acid (myristic) and it is the aldehyde that provides the substrate for light production (3). The *luxC* encodes for the reductase, *luxD* for the transferase and *luxE* for the synthetase enzymes (5). Together these genes *luxCDE* encode the fatty acid reductase enzyme complex (3). This *lux* gene cassette is then fused to the promoter for the degradation genes so that the *lux* genes are transcribed simultaneously with the degradation genes upon induction. The result is the production of light when the operon of choice is induced. If the whole *lux* cassette of *CDABE* is used, no substrate need be added because the substrate, aldehyde, is produced by the enzymes encoded by the *lux CDE* genes (2). These constructs can be made with virtually any inducible catabolic gene system. This thesis describes the use of a *lux* fusion with the genetic regulatory genes for the degradation of toluene. However, fusions have been made in strains to detect and degrade other compounds as well, such as naphthalene (5,7).

Bioluminescent Reporter TVA8

The bioluminescent reporter TVA8 was constructed by fusing the *tod* genes promoter to the *lux* genes and introducing that fusion into *PpF1*. The fusion was introduced directly into the *PpF1* chromosome by the use of a transposon. This eliminated the problems normally associated with having genes on a plasmid such as constant selective pressure to maintain the plasmid population in the cells as well as the negative effect of plasmid copy number. *PpTVA8* was compared to the parent *PpF1* strain to determine fitness. It performed equally well growing on toluene as the sole source of carbon as the parent *F1* thus verifying that the insertion of the *lux* gene fusion had not interrupted vital functional genes as well as confirming that bioluminescence did not adversely affect the cell. The strain was shown to be stable upon repeated platings and culture batches without antibiotic selection pressure proving that the transposon insertion was stable. Bioluminescence that was observed in response to toluene was correlated with cell number but also with toluene concentration (2). A linear correlation between bioluminescence and toluene degradation was observed (9). In addition to toluene, this strain showed response to benzene, m- and p-xylenes, phenol and JP-4 jet fuel (2).

Additional work with TVA8 also showed that it could be used to detect trichloroethylene (TCE). TCE is a pollutant with potential health hazards that is sometimes found in ground water supplies as a result of its improper disposal. The toluene dioxygenase degrades TCE but what was not known was

whether or not it would induce the *tod* operon (9). TCE is degraded by the *tod* operon but cannot be used as a sole carbon source. Therefore, it must be studied with toluene as a partner in co-metabolism (1). This work did show that TCE would induce the *tod* operon as determined by the bioluminescent response in TVA8 and in the parent F1 by mRNA analysis (9).

NahR

nahR is a regulatory gene found in the system responsible for the degradation of naphthalene. The *nahR* gene product (NahR) positively regulates *nah* gene expression in by the presence of the compound salicylate which is a degradation intermediate of naphthalene. It is proposed that when the NahR protein binds salicylate, it undergoes a conformational change that allows transcription to proceed at an increased rate from the promoter (8). The *lux* genes are used in situations studying the expression of different genes. In these cases, the promoter-less *lux* cassette is fused to the promoter of the gene to be studied so that the *lux* gene is expressed when the fused gene is expressed. In this case of this thesis, a similar situation is used in conjunction with the *tod-lux* fusion already in the chromosome of the TVA8 strain. In this study the *nahRG* promoter cassette is fused to a gene of the *tod* operon called *todX* (see next section). In this case, the objective was to express *todX* prior to induction via the *tod* promoter already in the chromosome. Constructs have also been made where *nahR* has been used to regulate or induce the *lux* genes and study direct biodegradation in that manner without coupling the *lux* genes with catabolic genes (3).

The *nahR* gene shows extensive homology to the gene *nodD* found in *Rhizobium*. In this organism, *nodD* is a transcriptional activator similar to *nahR*. This homology suggests an evolutionary relationship between *nodD* and *nahR*. It also suggests that the divergence between the two genes occurred with *nahR* requiring the inducer salicylate (8).

TodX

As previously described, the *tod* operon consists of several genes that together are responsible for the degradation of toluene. When the sequence of this operon was examined and further sequenced, several new genes were discovered upstream of the cassette described. Directly upstream of *todF* is the gene *todX*. Then, in front of the *todX*, is a gene called *todR* that exhibits some homology to the *nahR* gene previously described. *TodR* was determined to have a negligible role in *tod* gene expression. Additionally, *todS* and *todT* were identified. *TodS* is suggested to be a sensor cytoplasmic protein. *TodT* has been suggested to be

the transmitter protein for *tod* gene regulation and when coupled with the TodS, can control *tod* gene expression. TodS and T have their own promoter while the other structural genes have a separate promoter.

TodX has been shown to possibly encode for three protein sizes. The mature protein is suggested to be 49kDa while the precursor is 51kDa and a truncated version translated from an internal start site is 40kDa in size. Sequence analysis shows similarity between the *todX* sequence and the sequence for a gene called *fadL* found in *E. coli*. The *fadL* gene encodes an outer membrane protein involved in the uptake of long chain fatty acids. The gene product of *todX* is only produced when toluene is present and therefore toluene is the inducer of *todX*. This is confirmed by the finding that the *todX* gene product is not required for its own synthesis. It has been postulated that *todX* is involved in the regulation of toluene degradation perhaps as a membrane protein controlling the uptake of toluene into the cell. It has been suggested that the TodX protein could cross the outer membrane and deliver the toluene to the enzymes responsible for degradation. It could also be conceived that the *todX* could facilitate transport of the toluene to TodS and TodT regulatory proteins inside the cell. It is possible that *todX* did not originate in *Pseudomonas putida* F1 but was picked up as the organism evolved (10).

Tn5

Tn5 is a transposon that was used in this thesis to create the strain with *todX* independently expressed by *nahRG*. A transposon is a mobile genetic element that inserts in to the host's DNA. Plasmids are also used in cloning for moving specified genes. However, plasmids remain extrachromosomal and the genes contained on a plasmid are under independent control of replication. Plasmids have several disadvantages. The cloned fragment has to be short, limiting what genes can be brought to a different cell by size. Additionally, plasmids are kept in a variety of copy numbers in cells. The copy number can effect expression or apparent expression of the genes contained on the plasmids. Copy number is difficult to control in the cell. Finally, antibiotic resistance must be constantly maintained in the cell line to continue selective pressure. This eliminates cells that spontaneously loose the plasmid or have it altered in some way. Continued selection pressure is unfeasible for *in vivo* techniques or for strains with potential for eventual release. Transposons can carry up to 10kb inserts and so make them ideal for this thesis situation. Markers are used on the transposon that do not confer significant selection advantage. Tn5 specifically has the broadest host range (6).

Specifically, a mini-Tn5 transposon is used in this research. This is a fully artificial transposon that inserts randomly in the chromosome instead of at a specific location. The transposase gene is responsible for the transposon moving to and from the chromosome. In the mini-Tn5 constructs, this gene is lost as a result of transposition. Therefore, insertions are very stable and do not “jump” from their insertion sites at a high rate (4).

FadL

FadL is an outer membrane protein of *Escherichia coli* that is involved in the cell taking in long-chain fatty acids. It is most likely associated with the peptidoglycan and is also a receptor for T2 bacteriophage. This protein binds to these fatty acids and allows them to come across the outer membrane and enter the cell. The protein is also heat-modifiable and it is thought that this property may have something to do with fatty acid transport across the cell membrane. This characteristic is common to several other membrane proteins from other gram-negative strains of bacteria. FadL shares significant homology with the *H. influenzae* type b outer membrane protein P1 suggesting that these two proteins may share a similar role in transport of fatty acids across a membrane (13).

INTRODUCTION

It has been known for several years that organisms exist that can naturally degrade organic compounds. However, only recently have these organisms been used for bioremediation purposes. With genetic engineering, strains now exist that can serve in bioremediation as well as biosensing. By creating fusions with the *lux* genes, light can be used to assay the presence of these compounds. The use of these biosensing strains has been tested in the lab, and it is important to optimize these strains for short response time if the technology is to be applied to *in situ* sensing. The gene *todX* was thought to be a transport protein for toluene so it was hypothesized that independent expression of the *todX* would decrease the response time of the bioreporter strain *Pseudomonas putida* TVA8. The data accumulated in this research seems to suggest that by independently expressing the *todX*, response time is not reduced for any of the compounds tested. This suggests that *todX* is not a transport protein but is perhaps a porin or is some artifact of a previously required mechanism.

MATERIALS AND METHODS

Strain construction in *E.coli*:

All digestions were carried out according to the manufacture's specifications for the enzyme in use. The mini-transposon Tn5TcNX/T2 was cut with the restriction enzymes *NotI* and *XbaI*. The *nahR* gene was cut out of the plasmid pLJS (2) using *AvrII* and *NotI*. Both of the digests were heat inactivated according to manufacture's specifications and ligated using T4 DNA ligase (Promega). The resultant transposon was named pNTET. The ligation was electroporated into SV-17 (Applegate) competent *E.coli* cells using a BTEX Electro cell manipulator 600 as previously described. The cells were plated on LB media containing 14µg/ml tetracycline as the selective agent. Transformants were re-streaked and a Bio 101 Miniprep Express Boiling Prep protocol was followed for quick extraction of the plasmid DNA. The samples were digested and run on a 1% agarose gel. The clone yielding the correct banding pattern was grown up and a large-scale prep was performed. The DNA was purified using a cesium chloride gradient and ultracentrifugation. This DNA was then digested to confirm that pNTET was isolated. Once confirmed, it was digested with *NotI* and *XbaI*. The *todX* gene was digested from a TA clone using *NotI* and *XbaI*. Following heat inactivation, the digests were ligated, and using the same procedure, the construct pNTTOD was obtained.

Construction of TVA8-TodX strains:

The pNTTOD construct in *E.coli* was bi-parentally mated with *Pseudomonas putida* TVA8 (2) by placing 1 ml of the donor strain (pNTTOD) in Eppendorf tubes and adding 25, 50, 100, 200, 400 or 500 µl of the recipient (TVA8) to each tube. Centrifuging in an Eppendorf microfuge at 10,000rpm for 5 minutes then pelleted the cells. The supernatant was poured off and 1 ml of fresh LB was added to each tube and the cells resuspended. The spin was repeated and the cells were washed two more times with sterile LB. The last wash was poured off and the cells allowed to sit in the residual media overnight at room temperature. Then the cells were resuspended and plated on LB plates supplemented with 50µg/ml kanamycin and 14µg/ml tetracycline with dilutions. The resultant transconjugants contained the modified transposon pNTTOD and were named TVA8-TodX.

Growth curves:

Growth curves were done to confirm that the insertion of the transposon had not interrupted necessary metabolic genes thus compromising strain fitness. The colonies obtained from the mating were restreaked and subjected to toluene vapor by placing a small amount of liquid toluene in the top of an Eppendorf tube in the lid of the petri plate and letting the plates sit for 3-4 hours. After that time, the plates were observed in the dark for light. Those colonies that bioluminesced in the presence of toluene vapor were selected for further study. These were grown up on minimal salts media MSM (KH_2PO_4 -0.68g, K_2HPO_4 - 1.73g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.1g, NH_4NO_3 - 1.0g in 1 liter, pH 7.0) supplemented with 10 μl of trace elements per 100 ml and with 500ppm succinate as the sole carbon source. The clones that grew the best were selected for growth curves on toluene along with the parent strain TVA8. The growth curves were performed by growing up the strains on YEPG media (1g dextrose, 2g polypeptone, 0.2g yeast extract, 0.2g NH_4NO_3 in 1 liter, pH 7.0) supplemented with phosphate buffer (0.5M $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) and antibiotics overnight at 30°C. One ml of these cultures was used to inoculate flasks with MSM + trace elements. Toluene was supplied in the vapor phase with the use of vapor bobbles filled half way with liquid toluene. The flasks were shaken at room temperature and 1ml aliquots removed every 30 minutes. These aliquots were read on a Spectrophotometer at 546 nm. OD was plotted versus time and the most fit strains were picked. The strain designated number 16 was chosen for further study.

Growing cell assays:

Light production was assayed as a function of toluene concentration and inducer concentration using a modified growing cell assay procedure. Cultures of TVA8 and TVA8-TodX #16 were grown up overnight at 30°C on YEPG media plus phosphate buffer with antibiotic selection with and without salicylate at 10ppm. These cultures were transferred to fresh YEPG + PO_4 buffer, appropriate antibiotics and salicylate if appropriate and grown to an OD of 0.35 measured at 546 nm. Two ml of MSM + trace elements with and without salicylate was put into glass scintillation vials. 2 ml of the appropriate culture was then added. Assays were done in triplicate. Toluene saturated MSM was added to several concentrations as determined by Henry's Law and the saturation of liquid toluene. Other compounds were tested in the same way, modifying the Henry's Law equation and the amount of compound added to the sample. Vials were shaken in a 27°C dark room and light readings were taken every 30 minutes using an

Oriel photomultiplier and digital display. The assay was continued for 3-4 hours until the reaction became oxygen limited and the final OD's of each vial were taken to complete the assay.

For some trials, the growing cell assay was done in modified 250 ml glass Erlenmeyer flasks with spectrophotometer cuvettes fused to the sides. This allowed for light and OD measurements to be done simultaneously. The above procedure was modified to retain the same concentrations of toluene by increasing the amount of MSM and culture added to start the assay. OD readings were taken along with light readings every 30 minutes in a Spectrophotometer 20.

TodX primers

Primers were designed to amplify the *todX* gene. The forward primer was designed 5'-3' as ATG AAG ATT GCC AGC GTG CTG GCA C and the reverse primer was designed as TTA AAA AAT TTT TGC TAT AGG AAA C. These were synthesized on a Beckman Oligo 1000 DNA Synthesizer using 30 nmol columns. They were then removed from the column following manufacturer's protocol and dried in a Speed Vac overnight. They were then resuspended in 500 µl TE.

TodX probe

A ³²P-labeled gene probe was made for the *todX* gene using DNA extracted from the *E.coli* pNTTOD strain. ³²PdCTP was used in place of dCTP in the PCR reactions. The following cycle was performed using a Perkin Elmer Cetus DNA Thermal cycler: 94°C for 1 minute, 50°C for 30 seconds, 72°C for 2 minutes and 4°C hold for 30 cycles. The labelled probe was purified through a Stratagene push column with STE and placed at -20°C.

Total mRNA extractions

Total mRNA was extracted from TVA8, and TVA8-TodX #16 with and without salicylate. The cells were grown up as described in growing cell assays using 10 and 0 ppm toluene and 10 ppm salicylate. They were allowed to shake undisturbed for 3 hours in triplicate. After 3 hours, 1.5 ml of cells were collected. The bacterial protocol for total mRNA extraction from RNeasy Total RNA kit (Qiagen) was followed and the RNA stored at -80°C until needed.

Slot blot

The mRNA from TVA8 and TVA8-TodX #16 with and without salicylate was treated with DNase for 20 minutes at 37°C and then further purified as specified in the RNeasy Total RNA kit

protocol (Qiagen). The samples were then quantitated using the kit protocol. Dilutions were made to give 10 µg per sample. 50 µl formamide was added followed by 15 µl formaldehyde and 9 µl 20x SSC (3 M NaCl, 0.3M sodium citrate). The samples were incubated at 70°C for 15 minutes and placed on ice for 5 minutes. Standards were prepared from the original TA clone DNA of todX. 100µl of these were added to 400 µl of 0.5 M NaOH and boiled for 4 minutes followed by incubation on ice. The protocol for slot blots was followed according to Sambrook et al. (14). The membrane was prehybridized at 60°C overnight in prehybridization solution (0.5M NaH₂PO₄, 1mMdisodium EDTA, 7% SDS, 1L dH₂O, pH 7.2). The probe was added and let hybridize overnight at 60°C. The blot was removed and washed 3 times for 15 minutes each in 2xSSC, 0.1%SDS solution. The blot was allowed to dry and then sealed in a bag and exposed to STORM phosphorimaging screen overnight. Following exposure, the screen was developed on a STORM imager.

Membrane protein extractions

Membrane protein was extracted from SV-17 and the E.coli pNTTOD and subsequently from TVA8 and TVA8-TodX #16 with and without salicylate according to the protocol outlined by Wang et al. 1995. From overnight LB cultures, 100 ml of culture was spun down at 8000 rpm for 10 minutes in a Beckman centrifuge. The pellet was resuspended in 10 ml 50mM Tris, 120 mM NaCl pH 7.6 and centrifuged. The pellet was then resuspended in 500 µl 50 mM Tris-HCl pH 7.6 50 mM KCl and the samples sonicated. The tubes were centrifuged again in a microfuge at 4000rpm for 10 minutes and the supernatant was removed and centrifuged again. The supernatant was removed and centrifuged in a tabletop Beckman ultracentrifuge at 50000rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl 50mM Tris-HCl pH 7.6 50 mM KCl and stored at 4°C.

Acrylamide Gels

The isolated membrane protein was quantitated using a BioRad Bradford Kit and compared to a standard BSA curve. The samples were diluted with 2xSDS sample buffer to give 5 µg per lane. The protocol for acrylamide gel preparation was followed (14) and a 12% gel prepared, loaded and run at 100 volts. The staining procedure was followed and the gel imaged on an imaging system.

Mineralization

Mineralization was carried out in biometer flasks fitted with needles for removal of samples. *PpF1* and a *PpF1* mutant deleted for *todX* (Wang et al. 1995) were grown up on LB overnight at room temperature. One ml was centrifuged and washed once with MSM + trace elements. This was inoculated into fresh MSM + trace elements in flasks with toluene vapor bobbars and they were shaken overnight. Samples were done in triplicate. One ml of water was added to scintillation vials. Ten ml of 0.5 M NaOH was placed in the side arm portion of the flasks. The cultures were diluted to the same OD and 30 ml of each was added to each flask. Radioactive ^{14}C toluene was added to each flask and they were put shaking in a dark room at 27°C. Every hour 500 μl was removed and put in the scintillation vials with the 1ml of water. Scintillation cocktail was added and the vials were allowed to sit overnight before being counted in a Beckman Scintillation counter. Total counts per minute (CPM) were calculated using the reported value and multiplying by 20 for the first sample. The next sample number was multiplied by 19 and added to the previous reported value. This was repeated decreasing the multiplier by one and adding the previous readings.

RESULTS

A PCR was done using primers specific for *todX* in the correct orientation. The banding pattern in the gel confirms that in the TVA8-TodX#16 strain, the *todX* gene is inserted in the correct orientation in the genome of the organism. The banding pattern was compared to a control organism with *todX* inserted in the opposite orientation, TVA8-RevTodX #17 (Figure 5).

Membrane protein extractions were carried out and run on acrylamide gels. This was first done with the *E.coli* SV-17 strains with the *nahR-todX* insertion. Samples were processed with and without salicylate and banding patterns analyzed (Figure 6). There are several bands that appear in the lane of the sample without salicylate, appear brighter in the lane with salicylate and are absent from the lane with the *E.coli* SV-17 strain. These bands correspond to the values for the various natures of the TodX protein (10). The extractions were repeated on the *Pseudomonas* strains resulting in the same banding pattern (Figure 7).

After the construction of several clones of TVA8-TodX, growth curves were done comparing the strains of TVA8-TodX with the parent TVA8 with toluene as the sole carbon source in a saturating situation (Figure 1). The strain #16 was picked for further study because it performed equal or better than TVA8 in this growth curve.

A growing cell assay was performed to compare the light of the TVA8-TodX strain with the parent strain TVA8 with and without salicylate over a range of toluene concentrations: 10, 1, 0.1, 0 ppm toluene. When this assay was initially performed optical density (OD₅₄₆ nm) was taken simultaneously with light in side-arm flasks. This OD data was used to normalize the light data in the analysis (Figure 2ab). There was a 30-minute faster response in the induced TVA8-TodX when compared to TVA8 on salicylate. In the samples without salicylate, the faster response was not seen. Additionally, the TVA8-TodX reached a higher light value than TVA8 in the experiments with and without salicylate.

The inducer level that was used throughout this data was 10ppm salicylate. Higher concentrations of salicylate were tried to attempt to achieve maximum induction. However, the samples induced with 10ppm salicylate performed similar to higher concentrations of salicylate. Additionally, all subsequent growing cell assays were done in scintillation vials as described in Materials and Methods.

A growing cell assay was done using TCE to confirm that the *todX* machinery was not increasing the amount of TCE available for induction (Figure 3). The light response was the same for the TVA8 strain

as for the TodX line. The growing cell assay procedure was repeated for a number of different compounds: ethylbenzene, benzene, and phenol (Table 2). In all of these cases, the faster response time was not observed when comparing the TVA8 samples with the TodX line with and without salicylate. However, a higher maximum light level was obtained in all cases by the TodX strain. The growing cell assay was repeated on a wider range of toluene concentrations (Figure 4). In this assay, the faster light response was not observed although the TodX line still attained higher maximum light levels.

Mineralization assays were done on *PpF1* and a deletion mutant of *PpF1*, *PpF1(todR::Kn, ΔtodX)* obtained from Wang et al. (10). Total counts per minute were calculated from reading taken every hour by multiplying the first time point by 20, multiplying the second time point by 19 and adding in the first, etc. for all time points measured. The data clearly shows a faster initial use of toluene by the *PpF1* strain as compared to the *ΔtodX* mutant strain (Figure 8). The triplicates were tightly clustered at first and became more diffuse as the experiment progressed.

Finally, slot blots using mRNA isolated from induced and uninduced TVA8 and TVA8-TodX #16 were run using *todX* as a probe (Figure 9). The marker hybridized very brightly and over-powers the first lane but it can be seen that the only signal is in the slots run with 10 ppm toluene. The samples were done in triplicate. When the lanes with and without salicylate are compared, it appears that there is a slight difference in the amount of *todX* mRNA in the slots grown with salicylate when compared to those without for TVA8 as well as TVA8-TodX #16. When the slots with TVA8 are compared to those with TVA8-TodX #16, it appears that there is more message in the TVA8 lane than the TVA8-TodX lane.

DISCUSSION

Through the independent expression of the gene *todX* in the toluene operon of *Pseudomonas putida* F1, it was hypothesized that response time of the bioluminescent reporter TVA8 could be reduced. A strain was constructed containing a *nahR-todX* fusion in a mini-Tn5 transposon in the reporter strain TVA8. The growth curve data suggest that insertion of this transposon did not affect any of the metabolic genes necessary for the cell to grow on and utilize toluene as the sole source of carbon.

The growing cell assay data initially suggested a faster response time was occurring with induction of the TodX strain. However, when the experiment was repeated without using OD to normalize the data, the faster response time was not observed. This could be due to variations in the experimental procedures, inaccuracies of the measuring devices, or perhaps the response time is so close that it can only be resolved when normalized with OD. However, throughout the growing cell assay data, the TVA8-TodX strain attained higher light levels on all compounds tested. Comparison of TVA8 and TVA8-TodX on TCE confirm that the *tod* machinery is not taking in TCE at a greater rate in the TodX line.

Both the PCR and membrane protein extractions confirm that the *todX* gene is present in the chromosome of TVA8 and it is being independently expressed by the *nahR*-salicylate induction system. The mRNA slot blot data suggests that there is a higher level of *todX* mRNA that is in the induced cell. However, it looks as if the level of mRNA is higher in the TVA8 samples when compared to the TVA8-TodX samples. This experiment needs to be repeated perhaps using smaller amounts of standards or loading two separate slot blots to avoid the standard washing out the sample lanes. Mineralization data shows that having the *todX* gene in the cell increases the initial rate at which it metabolizes toluene.

It has been proposed that TodX is an outer membrane transport protein for toluene. This data suggests that independently expressing this gene in a cell does not increase the rate at which toluene enters the cell. The data suggests that by using salicylate, the protein can be produced in greater amounts in a cell, but that this production of TodX does not affect the uptake rate of toluene as measured by bioluminescence. It is possible that the rate at which toluene enters the cell is not the rate-limiting step in this reaction. By bringing more toluene into the cell initially, there may be some other step that is necessary before induction of the *tod* operon can occur and the toluene is degraded.

It is probable that the TodX protein is a porin and allows unrestricted influx and efflux of toluene into and out of the cell. It is also possible that the protein is an artifact of a previously required mechanism even though it appears that its presence allows the cell to take in toluene and utilize it at a faster rate.

FUTURE WORK

There is much to be done in this area to help solidify our understanding of this system. More growing cell assays need to be done to confirm that data that I collected. Additionally, a better method for growing cell assays needs to be developed to allow for simultaneous OD and light readings in a closed environment. This may involve microtiter plate 96 well readers but with some modification to prevent escape of the organic to the air. Further mineralization assays need to be done possibly with simultaneous protein extractions to correlate the faster mineralization with more TodX protein in the cell. Additional work needs to be done to assess the nature of the TodX protein in the cell membrane. There is much that can be elucidated from the toluene degradation system and I hope that my work will provide stepping-stones for further study in this area.

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Table 1

Plasmid or strain	Relevant genotype or characteristics	Source
Plasmids/transposons		
Tn5TcNX/T2	mini-Tn5 transposon; Tc ^r	Applegate
pLJS	pBluescript without MCS with added <i>XbaI</i> , <i>NheI</i> , <i>AvrII</i> and <i>SpeI</i> ; Ap ^r	2
pNTET	mini-Tn5 with <i>nahR</i> ; Tc ^r	This work
pNTTOD	mini-Tn5 with <i>nahR</i> and <i>todX</i> ; Tc ^r	This work
Strains		
SV-17	Electrocompetent <i>E.coli</i>	Applegate
<i>Pseudomonas putida</i> F1	contains <i>tod</i> cassette	Applegate
TVA8	<i>P.putida</i> F1 containing mini-Tn5Kmtod-lux insertion in the chromosome; Km ^r	2
TVA8-TodX #16	TVA8 with mini-Tn5TcnahR-todX insertion in the chromosome; Km ^r , Tc ^r	This work
PpF1(<i>todR</i> ::Km, Δ <i>todX</i>)	<i>P.putida</i> F1 with Km insertion in <i>todR</i> and large deletion in <i>todX</i>	10

Growth of TVA8-TodX strains on toluene as the sole source of carbon.

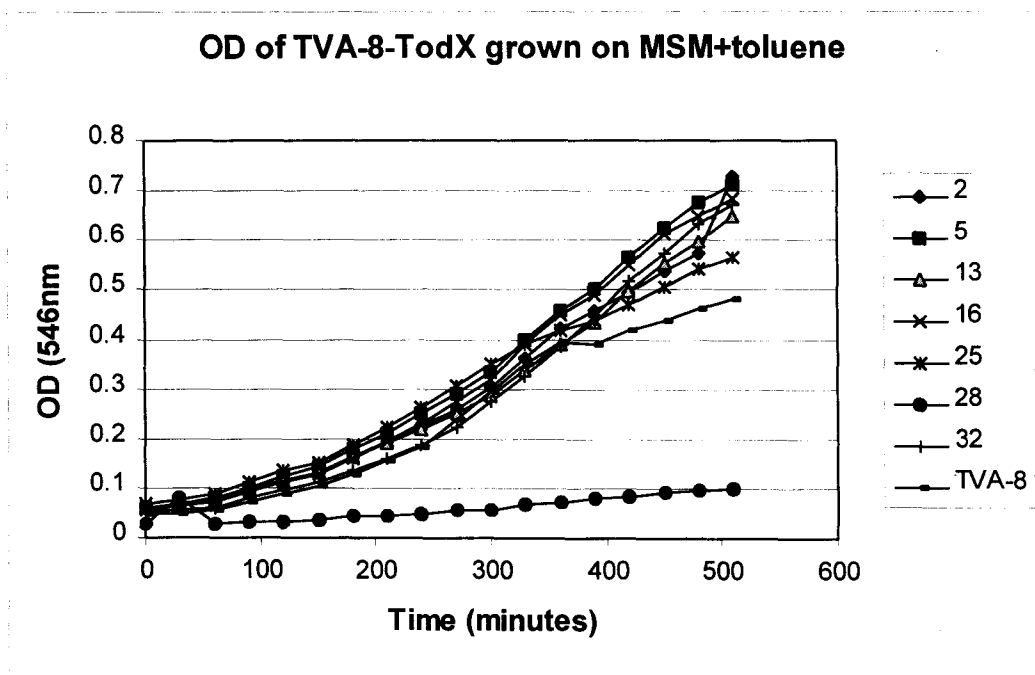


Figure 3a.
Bioluminescence of TVA8 under GCA conditions in scintillation vials with varying concentrations of TCE over time.

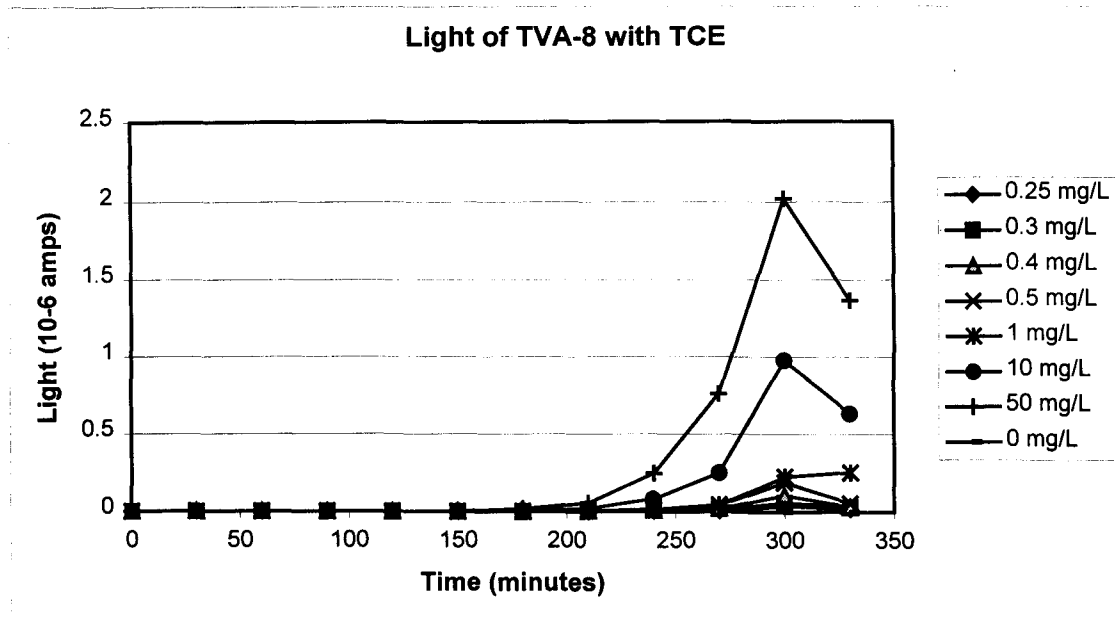


Figure 3b.
Bioluminescence of TVA8-TodX #16 under GCA conditions in scintillation vials with varying concentrations of TCE over time.

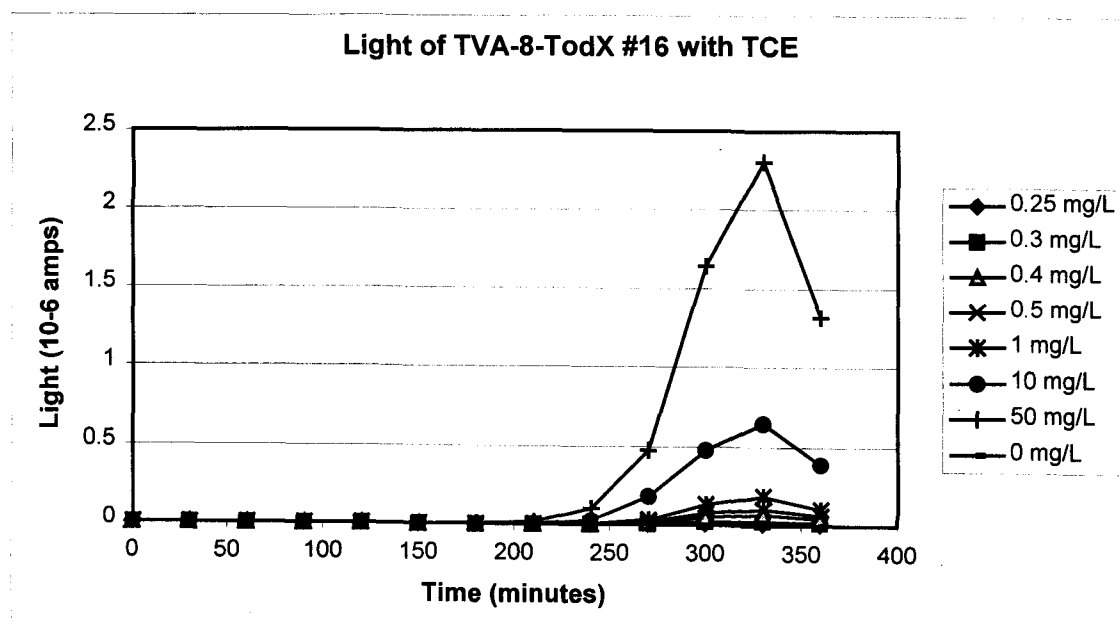


Table 2

All treatments reported are for 10ppm of the compound except * which is for 1ppm. Max light/OD values were determined by averaging triplicate values and then dividing by the average of the final OD.

Treatment/Strain	Response Time (minutes)	Total Exposure Time (minutes)	Maximum Light Level/OD (nA/OD)
<u>Benzene</u> TVA8 +	120	240	32.2
TVA8 -	120	240	13.6
TVA8-TodX #16 +	120	270	57.9
TVA8-TodX #16 -	180	270	38.7
<u>Phenol</u> TVA8 +	270	270	0.22
TVA8 -	150	240	3.74
TVA8-TodX #16 +	180	180	0.14
TVA8-TodX #16 -	180	180	0.12
* <u>Ethylbenzene</u> TVA8 +	240	300	4.98
TVA8 -	180	300	2.08
TVA8-TodX #16 +	210	300	2.84
TVA8-TodX #16	210	300	1.01
<u>Toluene</u> TVA8 +	120	300	289.8
TVA8 -	90	300	287.7
TVA8-TodX #16 +	120	300	551.7
TVA8-TodX #16	120	300	461.2
<u>TCE</u> TVA8 -	120	330	301.5
TVA8-TodX #16 -	180	360	309.1

Figure 4a

Bioluminescence of TVA8 and TVA8-TodX #16 under GCA conditions in scintillation vials with varying concentrations of toluene over time in the presence of salicylate.

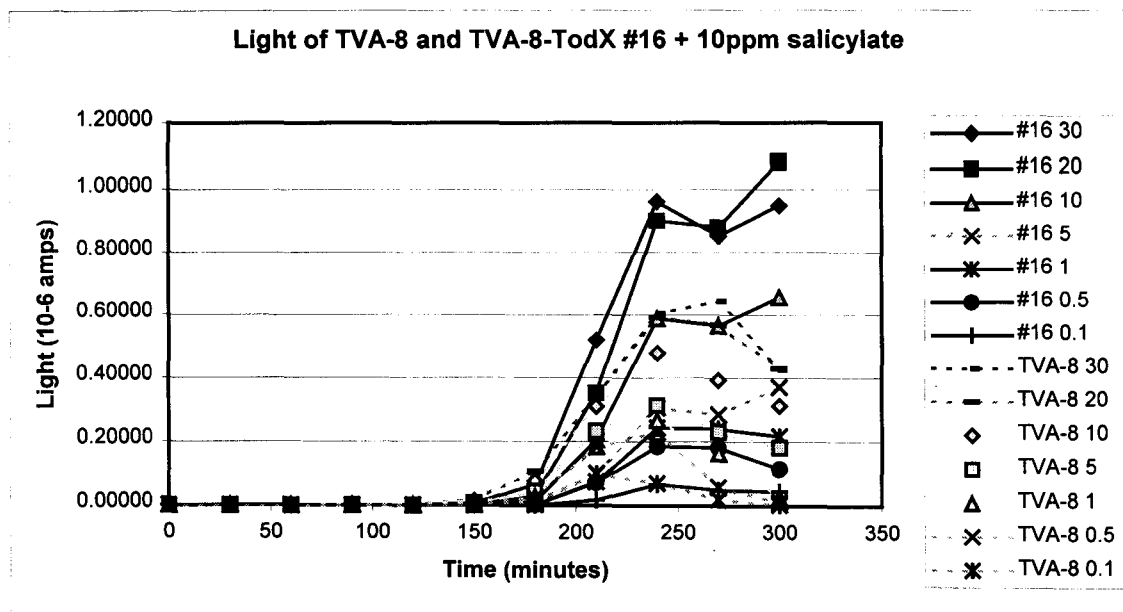


Figure 4b

Bioluminescence of TVA8 and TVA8-TodX #16 under GCA conditions in scintillation vials with varying concentrations of toluene over time in the absence of salicylate.

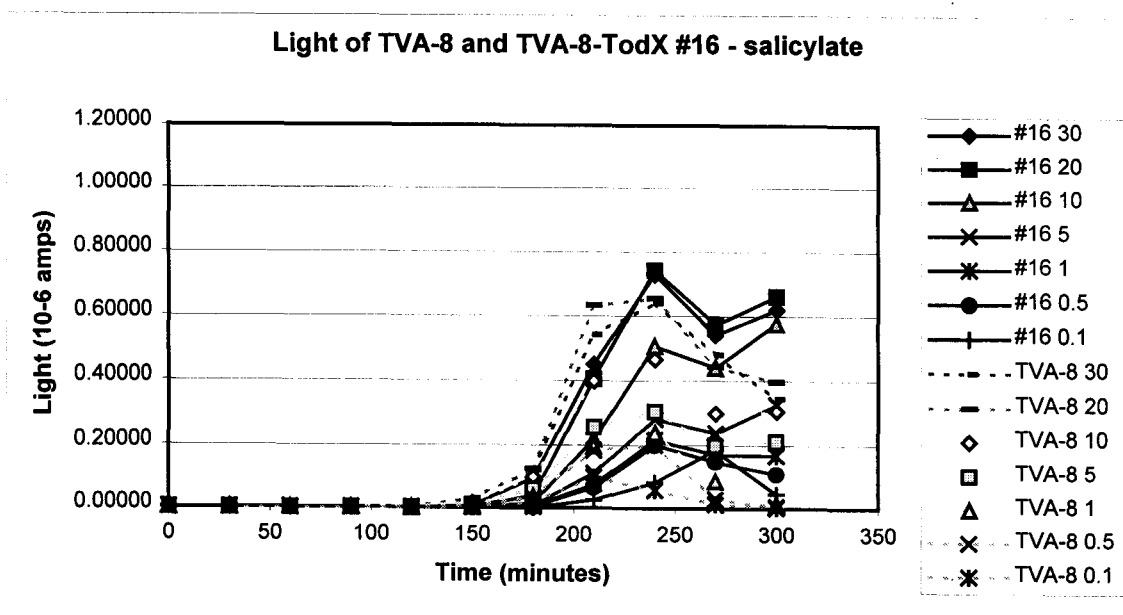


Figure 5
PCR of TVA8-TodX #16 DNA using *todX* primers confirming correct orientation and presence of *todX* gene as shown in lane 4.

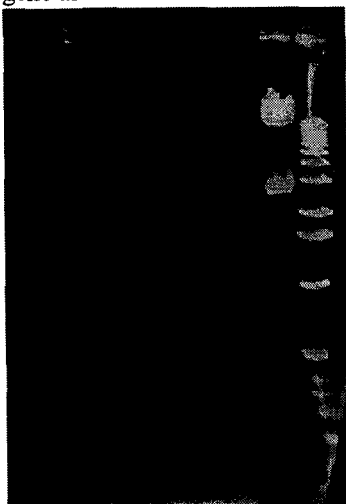


Figure 6
Membrane protein gel of *E. coli* SV-17 strains of pNTTOD with and without salicylate. Arrows indicate possible locations of *todX* protein.

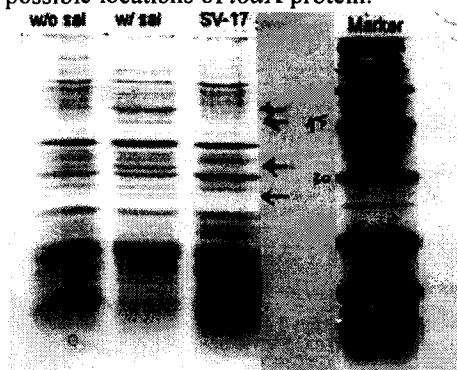


Figure 7
Membrane protein gel of *P. putida* strains of TVA8 and TVA8-TodX #16 with and without salicylate.

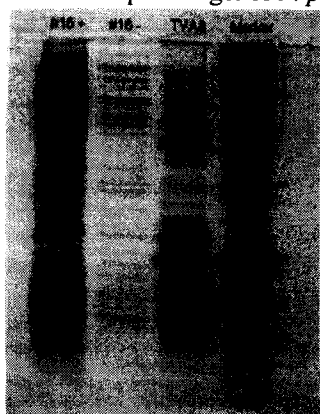


Figure 8

Total counts per minute obtained by mineralization experiment using *Pp* F1 and *Pp*F1 (*todR*::Kn, Δ *todX*) on 14 C labeled toluene over time.

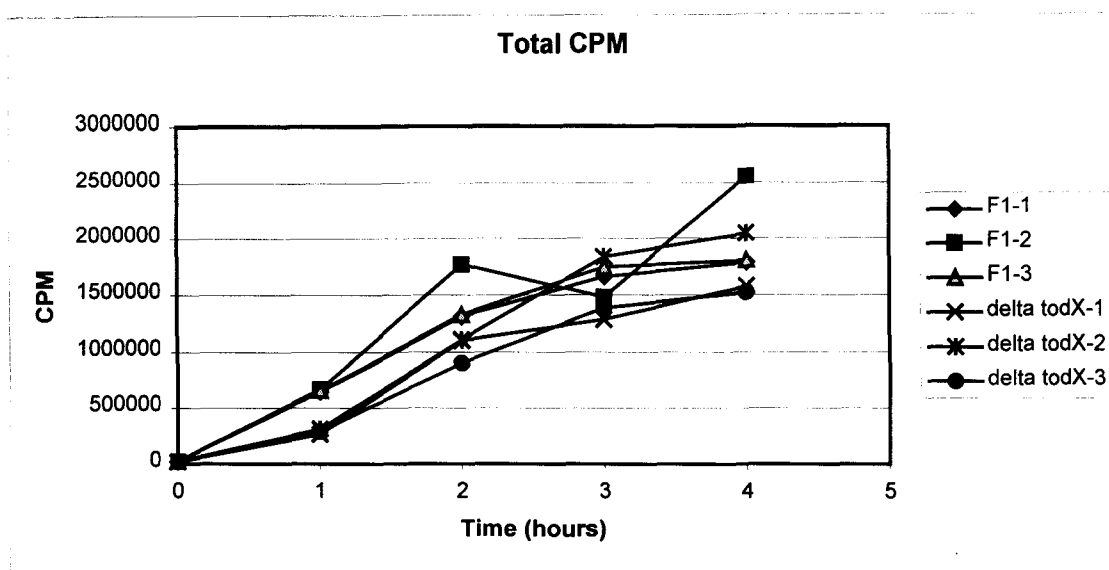


Figure 9

Slot Blot of mRNA probed with *todX*. Lane 1 is the standard. Lane 2 is TVA8 with salicylate at 10ppm toluene (first three) and 0ppm toluene (last three). Lane 3 is TVA8 without salicylate at 10ppm toluene (first three) and 0ppm toluene (last three). Lane 4 is TVA8-TodX #16 with salicylate at 10ppm toluene (first three) and 0ppm toluene (last three). Lane 5 is TVA8-TodX #16 without salicylate at 10ppm toluene (first three) and 0ppm toluene (last three).

