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REGULATED BIOLUMINESCENCE AS A TOOL FOR BIOREMEDIATION PROCESS MONITORING AND CONTROL OF BACTERIAL CULTURES

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ABSTRACT

An effective on-line monitoring technique for toxic waste bioremediation using bioluminescent microorganisms has demonstrated great potential for the description and optimization of biological processes. The lux genes of the bacterium Vibrio fischeri are used by this species to produce visible light. The lux genes can be genetically fused to the control region of a catabolic gene, with the result that bioluminescence is produced whenever the catabolic gene is induced. Thus the detection of light from a sample (monoculture, consortium, or bioreactor) indicates that genetic expression from a specific gene is occurring. We have used this technique to monitor biodegradation of specific contaminants from waste sites. For these studies, fusions between the lux genes and the operons for naphthalene (nah) and toluene/xylene (xyl) degradation were constructed. Strains carrying one of these fusions respond sensitively and specifically to target substrates. Bioluminescence from these cultures can be rapidly measured in a non-destructive and non-invasive manner. The potential for this technique in this and other biological systems is discussed.

INTRODUCTION

The development of bioreporter genes is one of the reasons for the rapid advances in the fields of genetics and physiology. A bioreporter gene is associated with a convenient assay, and can substitute for another gene that can not be assayed. The more useful bioreporters are associated with a biochemical assay that has any or all of the following characteristics: the test should be rapid and uncomplicated, inexpensive, reliable, sensitive, and should be free of any background signal. The best bioreporters will incorporate many of these characteristics, although all are lacking in some important facet.

The bioreporter that is used most frequently is the lacZ gene. This gene encodes the enzyme beta-galactosidase, which converts the sugar lactose into glucose and galactose. Its utility as a bioreporter arises from the use of synthetic substrates which produce easily measured colored products after enzymatic action of beta-galactosidase (5). These simple assays have been used in countless experiments to describe genetic expression; however, there are important limitations. Some of the reagents are expensive and the test, although fairly rapid, requires the destruction of part of the sample. Bioreporters which overcome these limitations are often needed.

A bioreporter that would avoid these drawbacks could rely on the measurement of a physical parameter, such as light. Light is produced in a biological reaction called bioluminescence by the marine microorganism, Vibrio fischeri, which lives symbiotically with certain species of deepsea fish. The intricacies of this symbiotic relationship are not well understood, but the genes for this trait (lux) have been successfully cloned (2). Extensive genetic analysis of the lux genes has resulted in the use of a subclone (luxCDABE) to produce light in other bacterial

species. The luxAB genes encode a heterodimeric luciferase enzyme that converts an aldehyde group to a carboxyl group on a suitable substrate. This reaction generates visible light as a byproduct. The luxCDE genes convert the carboxyl group back to the aldehyde, so that a pool of substrate is available for continuous light production. The former reaction is dependent on the presence of molecular oxygen, and so only aerobic reactions can be monitored using the lux genes as a bioreporter (4).

In a typical genetic construction the lux genes are fused to the control region (promoter) of the target gene. All of the regulatory genes necessary for the correct functioning of the target gene must also be supplied. The induction conditions for the gene under study will also induce the lux genes, and bioluminescence will result. If conditions are present that are known to cause induction, but no light production occurs, it may be assumed that some inhibitory condition or compound is also present in the reaction milieu. It is thus possible to use a lux fusion to study optimal expression conditions of a particular gene as well as to find appropriate conditions of expression in environmental samples. Often these fusions are located on plasmids - small, circular DNA sequences - that are easily introduced into bacterial species.

The efficacy of this technique in describing appropriate expression conditions, and the use of those conditions with environmental samples, is demonstrated here in a study of the degradation of hazardous waste compounds. The genes responsible for naphthalene catabolism (nah) and for toluene/xylene catabolism (xyl) from the soil bacterium Pseudomonas putida were fused to the lux genes to create sensitive and specific bioreporters. The success of this technique in defining induction conditions is evident in the correlation of waste degradation with periods of maximum light production. This technology should be more generally applicable to other aerobic bacterial processes. It is expected that increased efficiency of bioremediation as well as other processes will result from an analysis of bioluminescent reporter induction.

MATERIALS AND METHODS

Bacterial strains and plasmids. All genetic manipulations were performed in an Escherichia coli DH5 strain. Plasmid constructions were introduced into Pseudomonas putida PB2440 by conjugation. The plasmid pUTK9 (1) contains a nah-lux fusion that was constructed from the promoter region of the NAH7 naphthalene catabolic plasmid and the lux cloning vector pUCD615. The plasmid pUTK24 (Burlage et al., manuscript in preparation) contains a xyl-lux fusion constructed using the promoter region from the TOL toluene/xylene catabolic plasmid and pUCD615 (Figure 1).

Light detection. Light was detected and quantified using an Oriel photomultiplier model 77761 with a liquid light pipe and a collimating beam probe. This apparatus reports light as amperes of induced current, and is usually reported as nanoamps. The photomultiplier probe was kept in a light-tight chamber to reduce incident light.

Media and reagents. All reagents were greater than 99% pure. Naphthalene was added in a crystalline form. Toluene was added as a liquid at a final concentration of 0.015 mM. Relevant incubation conditions for experiments are given in the figure legends. Media in these experiments and protocols for genetic manipulations have been described (7).

RESULTS AND DISCUSSION

Genetic constructions. The essential characteristic of all the bioreporters described here is that they are the product of recombinant DNA manipulations. This is possible because the lux genes have been isolated on a convenient plasmid cloning vector called pUCD615 (6). This plasmid contains the luxCDABE structural genes, but does not carry a promoter (control) region that is essential for the expression of lux. Therefore a promoter region must be introduced into an appropriate position near the lux genes. All the techniques involved in this work are standard in the field of molecular biology, and are fairly inexpensive to perform or to purchase.

An example of one of the bioreporter constructions is presented in Figure 1. This is the cloning scheme for the production of plasmid pUTK24, a bioreporter of toluene and xylene presence. The TOL plasmid actually has the genes for the degradation of these compounds, and the regulatory genes xylS and xylR. We utilized a

small fragment of this large plasmid that contains the promoter of the catabolic genes. The new construction, pUTK24, was initially propagated in an E. coli strain, and then moved to a Pseudomonas strain. The new strain, RB1401, also contains the intact TOL plasmid, which provides the two regulatory genes and which allows the strain to degrade the contaminants. Thus this strain can both degrade toluene and report on its presence simultaneously.

It is entirely possible to make a vast number of these constructions, testing for myriad substances or conditions. Generally speaking, it is only necessary to obtain the promoter region of interest (often these can be isolated experimentally), the appropriate host regulatory genes (xylS and xylR in the above example) and the inducer substrate (toluene or xylene above). Other examples will be mentioned in the Discussion section, as well as possible uses.

Induction of bioluminescence. Figure 2 illustrates the generation of visible light by one of these bioreporters after induction with a specific substrate (1). The Pseudomonas strain RB1351 used here contains the nah-lux fusion plasmid pUTK9 and the intact NAH7 plasmid. The strain is able to both degrade and report on the presence of naphthalene. Light is measured in amperes as outlined in the Methods section. Within only a few minutes after naphthalene crystals were added to the lid of the plate, the light production from RB1351 has increased, and a few minutes later light production has reached a maximum value. It remains at this plateau as long as naphthalene crystals were observed (in this case, more than 16 hours). The response of the bioreporter is rapid to a specific inducer molecule, and can be measured in real time due to the on-line characteristic of the assay.

In liquid cultures the induced RB1351 strain gave a result that was different in significant details (1). Although the naphthalene was added while the culture was growing rapidly, the light production did not occur until the growth rate slowed down. It was later shown that naphthalene degradation was always correlated with light production, and that this catabolic system was under a growth-rate regulation. This was an unexpected finding, and was significant because it uncovered an important facet for the optimization of bioremediation in complex systems.

The sensitivity of these reporter strains has also been examined. This is important because contaminants of soil and groundwater are often present in low yet biologically significant amounts. For the nah-lux system the lower limit of detection is at least 45 part-per-billion (ppb) and has been reported as low as 0.1 ppb.

Bioluminescent bioreporters for environmental samples. Both nah-lux and xyl-lux plasmids were used in a recent study of the presence of specific contaminants in soil and water samples from sites near a fuel oil storage facility (Heitzer et al., manuscript in preparation). Typical results are shown in Figure 3, which describes data collected using the xyl-lux plasmid. Two representative soil samples are presented, TP01-08 and TP04-65. TP04-65 clearly shows induction of light, while TP01-08 demonstrates no light production. The former sample came from a site that was obviously contaminated with fuel oil, while the latter came from a relatively clean site. These results show that the contaminated site must have toluene or xylene as a fraction of the total contamination, since the reporter is specific for these compounds. It also demonstrates that this contaminant is **bioavailable** to the reporter strain. This is a very important quality for in situ work because the compounds must not only be present in the soil for bioremediation to have an effect, but those compounds must also be taken up by the bacterial cells and induce the appropriate catabolic genes.

It is evident from this experiment that bioreporters may be useful for a variety of aerobic processes besides bioremediation. The bioreactor can contain a consortium or a pure culture, the feedstock can be constant or variable. The bacterial strain that is actually performing the enzymatic activity is also reporting on its progress. In the case cited above, the light output continues until the toluene substrate is exhausted i.e. until it falls below a level sufficient for induction. This information could be very useful in designing a bioreactor system or optimizing the parameters of a particular reaction. The utility of this technique for complex bioreactor was recently described in an examination of bioreporter response to a variable feed stream (3).

Projects in development. Several other lux constructions have been created in this laboratory. A fusion to the genes for mercury reduction (mer) has produced a bioreporter of heavy metal contamination. Other constructions include constitutive light producers and bioreporters of stress conditions. Plans have also been made to mutagenize the lux genes in an attempt to create bioluminescence with different colors (wavelengths). These could be used to report on several genes in the same strain or in the same consortium.

The versatility of lux bioreporter strains makes them ideal for a variety of purposes. Experiments are in progress to optimize expression from the xyl-lux fusion in a bioreactor. The goal of this research is not only to optimize degradation of toluene and xylene, but also to learn about the physiology of the Pseudomonas bacterium and eventually to study the complex microbial interactions in a mixed bacterial culture. The knowledge gained from this series of studies may eventually expand the scope of microbial products and processes.

An unusual assimilation of microbial genetics, light detection apparatus and integrated computer technology will be used to control and modify bioreactor conditions. In this scheme, the bioluminescence from a reactor vessel will be detected by a photomultiplier which is monitored by a computer. If the light production falls below a certain threshold value the conditions are assumed to be no longer optimal, and a computer subprogram will be activated to adjust the process conditions. A series of interfaces with diagnostic equipment will allow the computer to determine whether key operating parameters are within normal ranges. When a non-optimal condition is discovered the conditions are appropriately modified. An appropriate length of time is allowed to pass for recovery of the bioluminescence, and the light is again sampled. If the output is normal the computer resumes normal sampling. If light output is still below threshold the diagnostic subprogram searches for other parameters. Certain key components of this system are already functional. When all units are in place it is believed that an efficient bioprocess control will be achieved.

SUMMARY

Our initial experiments were designed to determine the suitability of lux gene bioreporters for use as indicators of genetic expression under defined conditions. The acceptance of a new bioreporter by the scientific community is difficult, since the new bioreporter must offer advantages that are unavailable with other common systems. The lux system supplies many advantages that make it attractive for broad use, both in academia and in industry.

The specificity of the bioreporter for one or a few inducer substrates is an advantage inherent in using a genetic fusion as an indicator. Many such bioreporter strains can be constructed, not only for bioremediation of hazardous waste, but for any aerobic process. This might include industrial production of valuable recombinant proteins, antibiotics, biomass, or other products. Applications are only limited by worker availability and the description of suitable promoters.

The sensitivity of the lux system is also a great asset. It is possible to accurately measure light at extremely low intensities, and this means that very few cells can be detected. This makes the lux system valuable for bioreactor studies in which the bioluminescent strain may be present in low numbers, yet still be an important component of the consortium. This also means that in situ work might eventually be possible, so that these bioreporters might be portable sensors of environmental conditions.

Other important characteristics of this system include the speed of the assay. The results presented here were obtained on a real-time basis, allowing instant analysis of perturbations to the system. The ease with which the assay was performed allowed a many measurements to be taken cheaply, where other bioreporters would require selection of discrete timepoints as representative of the culture response. In addition, none of the sample was sacrificed for the assay, and the reactor was not disturbed during the procedure.

The experiments in progress should lead to new strategies for designing effective biological processes and for engineering of reactor systems. It is anticipated that further refinements will make the lux system more versatile, more sensitive and powerful, and more popular with the business and academic communities.

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FIGURE LEGENDS

Figure 1. Construction of a lux fusion plasmid. The lux plasmid vector pUCD615 is opened by the restriction enzyme EcoRI. The promoter (P) region of the TOL plasmid is isolated using EcoRI. The promoter fragment and the cut plasmid are combined and fused together using the ligase enzyme, creating the plasmid pUTK24. When this plasmid is introduced into a Pseudomonas strain, the strain becomes a bioreporter of toluene and xylene bioavailability. xyl -genes for toluene, xylene catabolism; lux - genes for bioluminescence.

Figure 2. Bioluminescence is induced by the presence of naphthalene. A nah-lux bioreporter strain was grown on a plate of LB agar until mature colonies formed. The low constitutive expression of light can be seen between 0 and 0.35 hours. Immediately after 0.35 hours naphthalene crystals were added to the lid of the plate. Light is measured by a photomultiplier and reported in nanoamps as described in the text.

Figure 3. The xyl-lux bioreporter strain is used to detect contaminants in soil samples. One gram soil samples were suspended in 4 ml of minimal medium in a small vial. The bioreporter strain was added at a concentration of 10^8 bacteria per ml. The positive control contained added toluene at a concentration of 0.015 mM; the negative control contained an uncontaminated soil sample. Light is reported in nanoamps.

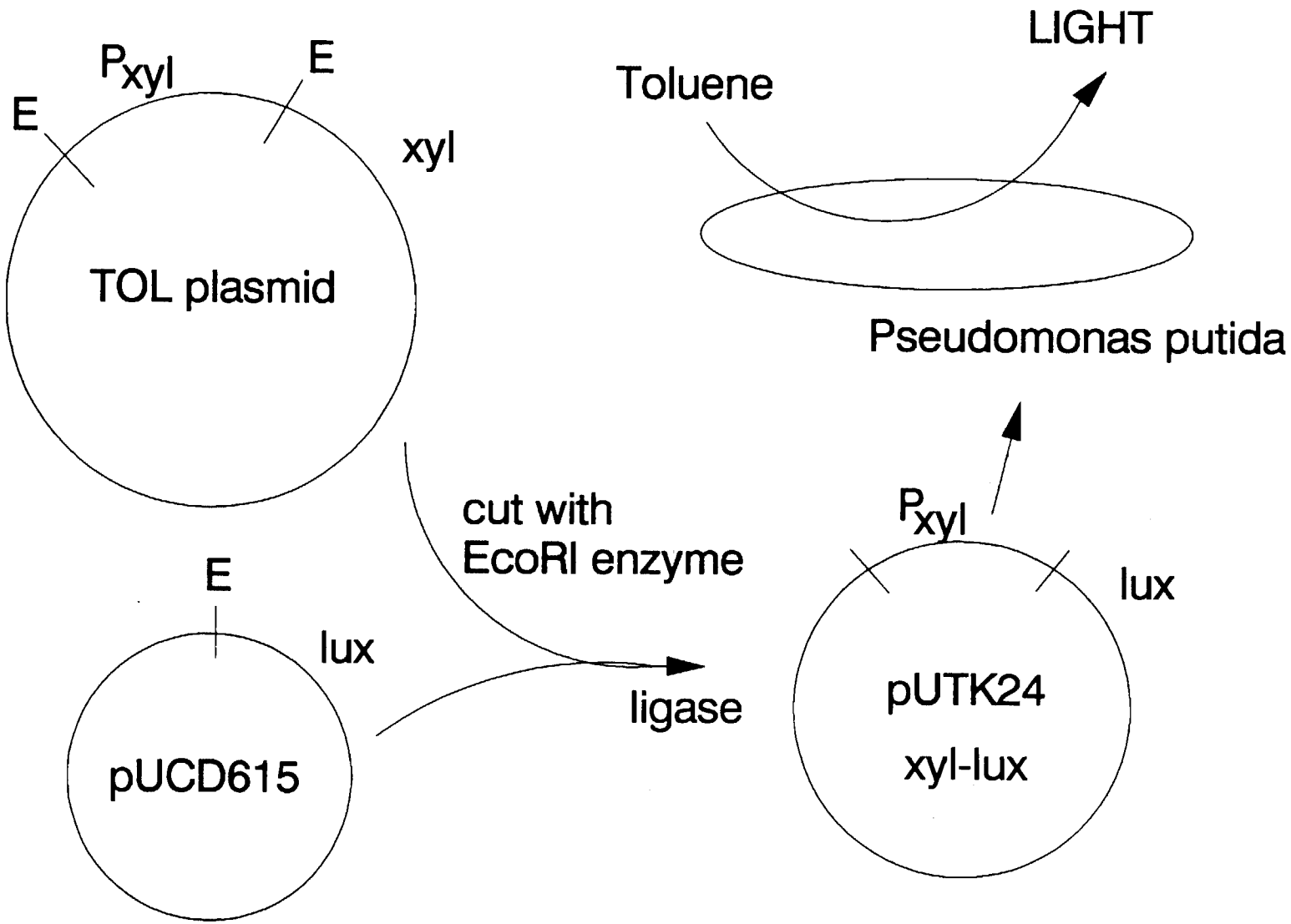
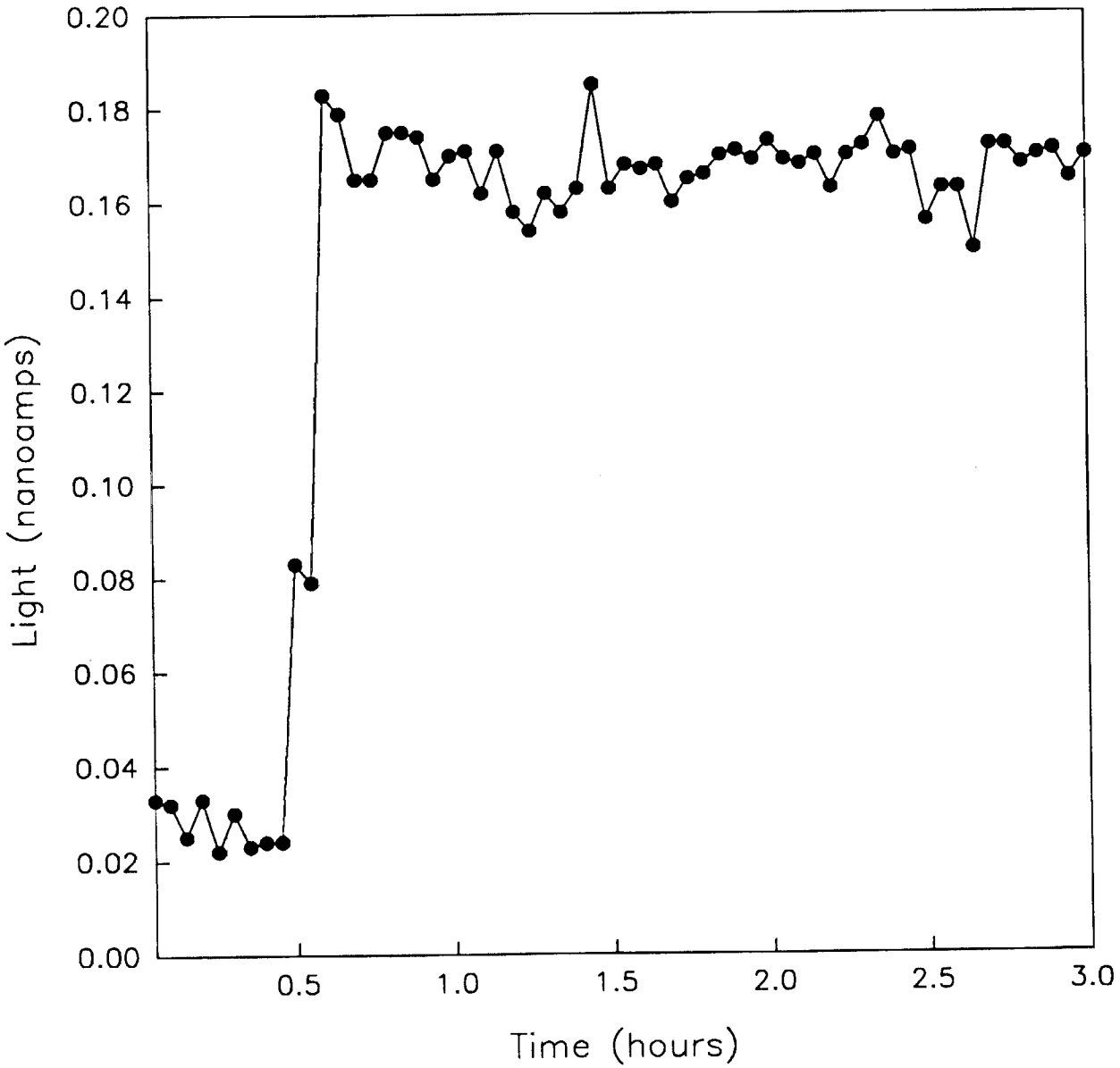
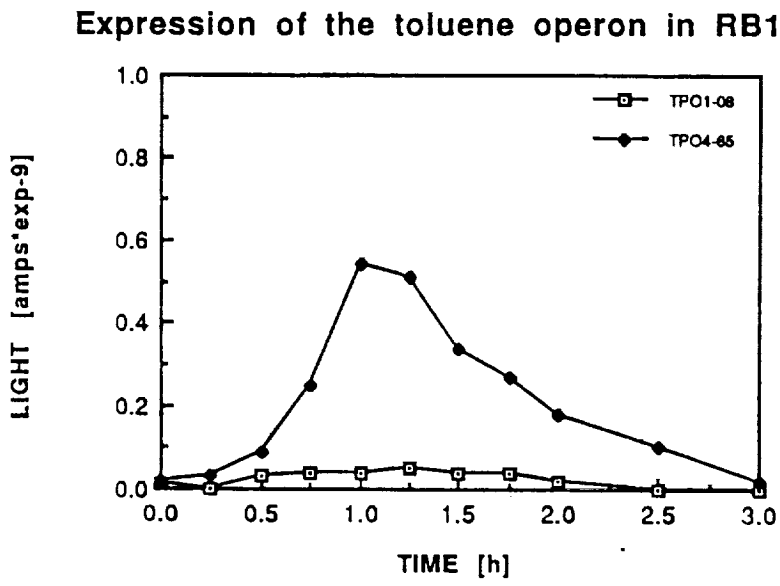
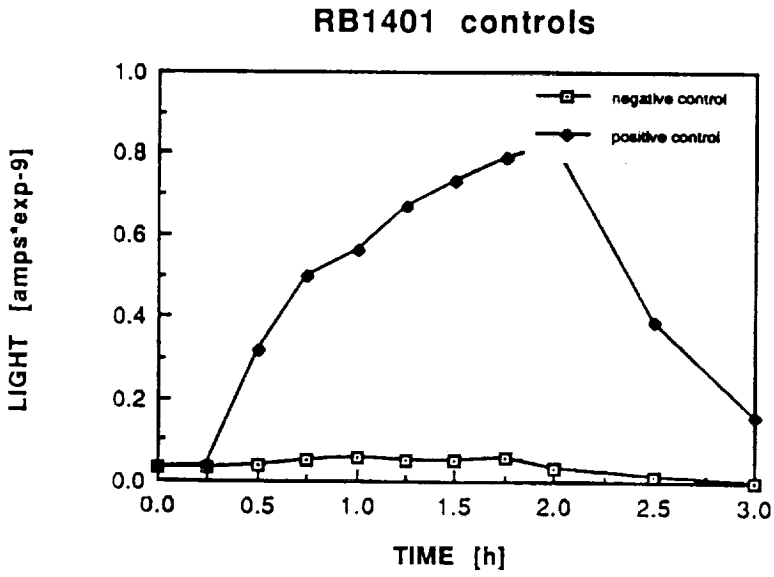


FIGURE 1 - lux plasmid construction

FIGURE 2 – Bioluminescence induced by naphthalene




Expression of the toluene operon in RB1401
LIGHT [amps*exp-9]
TIME [h]
TPO1-08
TPO4-65
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0


RB1401 controls
LIGHT [amps*exp-9]
TIME [h]
negative control
positive control
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0