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CHARACTERIZATION AND USE OF GENETICALLY-PROGRAMMED RESPONSES TO CHLORDANE, DINOSEB, BROMACIL, AND ARSENIC OXYANIONS

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ABSTRACT

Soil and aquatic ecosystems are constantly being exposed to chemical contaminants from industrial, agricultural, and municipal sources. For example, pesticides and herbicides are used on a world-wide scale to control a wide variety of harmful insects and competing weeds, to increase the production of food and fiber, and to facilitate modern agricultural production methods. Although these chemicals are designed to be selectively toxic towards target organisms, this is not absolute, and in most cases pesticides and herbicides are toxic toward non-target organisms, including humans. To identify genes which are transcriptionally regulated by cellular exposure to environmental pollutants, such as pesticides and herbicides, a 3000 *Esherichia coli* single-copy *luxAB* gene fusion library was previously screened in the presence of a pesticide or two herbicides (Costanzo, 1995). Five different clones whose luminescence is induced by either the herbicides, dinoseb and bromacil, or the pesticide, chlordane, were identified (Costanzo, 1995). Following partial characterization of the pesticide- and herbicide-responsive genes, luminescence experiments were repeated using new lots of bromacil, chlordane, and dinoseb. Despite numerous attempts to reproduce the dose-dependent light emissions, the original patterns of luminescence could not be duplicated. Experiments were therefore performed to determine the basis for the observed fluctuations in luciferase expression. Although difficulties were experienced when screening in the presence of chemically complex organic compounds, bacterial systems, such as the *E. coli luxAB* gene fusion clones, represent effective tools for studying the effects of toxic substances. Several common approaches are currently used to monitor and control environmental contamination. Direct analytical methods allow the determination of specific contaminant concentrations while biological toxicity assays measure harmful effects on living organisms, such as fish or *Daphnia*

spp. In recent years, microbial bioassays have gained popularity because they are sensitive, rapid, and cost-effective methods for measuring toxicity. The possibility of utilizing one *E. coli* chromosomal *luxAB* gene fusion clone, the *arsB::luxAB* clone (Cai and DuBow, 1996), to determine the presence of arsenic in a water effluent (Goodfellow effluent, Quebec) was investigated. The *arsB::luxAB* gene fusion clone detected trace amounts of arsenic-containing compounds in the water sample and may therefore be used as an environmental biosensor for arsenic detection.

ABRÉGÉ

Les écosystèmes terrestres et aquatiques sont constamment exposés à des contaminants chimiques de provenances industrielle, agricole et municipale. Des pesticides et des herbicides, par exemple, sont utilisés à l'échelle mondiale pour contrôler grands nombres d'insectes néfastes et de mauvaises herbes, pour augmenter la production de nourriture et de fibre, et pour faciliter les méthodes modernes de production agricole. Malgré le fait que ces produits chimiques soient dirigés specifiquement contre des organismes cibles, cela n'est pas absolu; dans la plupart des cas, les pesticides et les herbicides sont toxiques pour des organismes non-cibles dont les humains. Afin d'identifier des gènes qui sont réglés, au niveau de la transcription, par l'exposition cellulaire à des polluants environnementaux tels des pesticides et des herbicides, une librairie de fusions géniques uniques de *luxAB* dans *E. coli* fut mise en présence d'un pesticide et de deux herbicides (Costanzo, 1995). Cinq clones différents furent identifiés, dont la luminescence est induite par les herbicides dinoseb et bromacil, ou par le pesticide chlordane (Costanzo, 1995). Suivant la characterization partielle des ces genes sensibles aux herbicides et au pesticide, les expériences de luminescence furent répétées en utilisant de nouveaux lots de bromacil, chlordane et dinoseb. Malgré maintes tentatives de reproduire les émissions lumineuses montrant une dépendance de dose, les modèles de luminescence originaux ne purent pas être reproduits. Des expériences furent donc effectuées afin de déterminer les causes des fluctuations observées dans l'expression de la luciférase. Bien que nous avons eu des problèmes en utilisant des composés organiques complexes, des systèmes bactériens, tels que les clones de fusions géniques *luxAB* d' *E. coli*, représentent des outils efficaces pour étudier les effets de substances toxiques. Plusieurs approches communes sont présentement utilisées pour évaluer et contrôler la contamination

environnementale. Les méthodes directes d'analyse permettent de déterminer spécifiquement la concentration d'un agent polluant, tandis que les analyses de toxicité biologique measurent les effets néfastes sur des organismes vivants, tels les poissons ou *Daphnia spp.* Dans les dernières années, les "bio-analyses" microbiennes ont gagné de la popularité car elles sont des méthodes permettant de mesurer la toxicité de manière sensible, rapide et non-dispendieuse. Nous avons examiné la possibilité d'utiliser un clone d'*Escherichia coli* ayant une fusion chromosomale du gène *luxAB*, le clone *arsB::luxAB* (Cai and DuBow, 1996), pour déterminer la présence d'arsenic dans un effluant (effluant Goodfellow, Québec). Le clone *arsB::luxAB* détecta dans l'échantillon des traces de composés contenant de l'arsenic et pourrait donc être utilisé comme "biosensor" environnemental pour la détection d'arsenic.

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LIST OF ABBREVIATIONS

A	adenine
C	cytosine
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
DTT	dithiothreitol
G	guanosine
g	gram
IS	insertion sequence
kb	kilobase-pair
kDa	kilodalton
μg	microgram
μl	microliter
μM	millimolar
mg	milligram
ml	milliliter
mM	millimolar
ORF	open reading frame
PPM	parts per million
PPB	parts per billion

^{R}	resistance/resistant
RLU	relative light unit
T	thymidine
Tn	transposon
v/v	volume per volume
w/v	weight per volume

CHAPTER I

Literature Review

1.0. INTRODUCTION

The environment is constantly being exposed to a wide variety of contaminants from industrial, agricultural, and municipal sources. Consequently, many countries are now facing serious ecological and toxicological problems resulting from the release of toxic effluents into soil and aquatic ecosystems (Coleman and Qureshi, 1985; Bitton and Dutka,1986). Important goals in addressing these problems are the detection and elimination of pollutants which include living organisms, such as bacterial and parasitic pathogens, toxic organic chemicals, such as aromatics and hydrocarbons, inorganic chemicals, which include toxic heavy metal compounds, and chemical mutagens. Pollution caused by the release of these toxic pollutants has received a great deal of attention due to a high demand for chemicals, as well as the problem of ensuring the safety of food and water (Codina *et al.*, 1993). Once released into ecosystems, effects of these chemicals may be observed up to several seasonal cycles, after which they can become biologically unavailable through incorporation into organic and inorganic particles in sediments (Peterson *et al.*, 1996). These biological and chemical pollutants must therefore be continuously monitored and their levels controlled in order to conserve and protect ecosystems, wildlife, and human welfare.

Concentrations of chemical contaminants in soil and aquatic environments can be assessed via numerous methods. Detection of environmental pollutants has traditionally relied on highly sensitive and specific analytical chemistry methods (Van Dyk *et al.*, 1994). Older, conventional methods include atomic absorption spectroscopy (AAS) and chromatographic analysis tools, such as high performance liquid chromatography (HPLC) and gas chromatography (GC), which measure specific chemical components in water samples (Gu *et al.*, 1996). Fiber-optic research has also expanded in this area (Coulet and Blum, 1992; Blum *et al.*,

1993). For example, one assay has been developed to measure benzo(a)pyrene concentrations based on the interaction of benzo(a)pyrene with antibody-coated optical fibers. Antibody-bound benzo(a)pyrene is detected by measuring laser-induced fluorescence (Tromberg *et al.* 1988; Vo-Dinh *et al.,* 1987). Continued research in this area will undoubtedly provide biosensors for the detection of a wide array of other chemical compounds. Although effective, the analytical methods described above may have several shortcomings. First, they can be costly and labour-intensive (Kahru *et al.,* 1996). Second, they may not be able to distinguish pollutants that are available to biological systems from those that exist in the environment as inert, unavailable forms (Van Dyk *et al.,* 1994). This is a particular concern with respect to toxic metals. Bioavailability is therefore a critical issue in determining metal toxicity (Van Dyk *et al.,* 1994). Finally, analytical methods may fail to account for possible synergistic or antagonistic effects on biological organisms caused by chemicals present in complex mixtures (Kahru *et al.,* 1996).

As a result of these shortcomings, a trend has developed toward the use of living organisms to screen for toxic substances. The deleterious effects of toxic contaminants released into the environment have been assessed via acute and chronic toxicity tests, using mostly fish and invertebrate species (Peltier and Weber, 1985). However, these assays may be extremely labour-intensive and expensive. Therefore, toxicologists and environmental scientists have shifted their focus to short-term toxicity assays. The remainder of this chapter will explore the biological principles and applications currently in use to confront the problems of environmental toxicity and pollution. The development of short-term biological toxicity assays and biosensors will be discussed, along with the potential for these biosensors to complement conventional analytical monitoring methods.

2.0. SHORT-TERM TOXICITY ASSAYS

Short-term toxicity assays are based on microbial characteristics such as enzyme activity and biosynthesis, bioluminescence, motility, growth viability, ATP production, oxygen uptake, nitrification, and heat production. Therefore, by exploiting these characteristics, bacteria may be employed as environmental biosensors (Bitton and Koopman, 1992). A variety of short-term toxicity assays exist and have been utilized in the measurement of toxicity, genotoxicity, bioavailability, and biodegradability of numerous environmental pollutants.

2.1. *Enzymatic Assays*

Several microbial biosensors have been developed based on relationships between bacterial populations, present in soil or aquatic environments, and the effects of toxic substances on enzyme activity. Almost two decades ago, environmental scientists hypothesized that microbial enzyme activity could be a powerful indicator of the adverse effects caused by toxic environmental contaminants (Burns *et al.,* 1978). Environmental samples containing a particular contaminant can now be assayed for specific enzyme activities, based on colourimetric or luminescent tests (Bitton and Koopman, 1986). The effect of the toxic contaminant on bacterial enzyme activity can then be quantified using spectrophotometers, microtitre plate readers, fluorometers, or liquid scintillation counters. The degree of inhibition of enzyme activity, in the presence of the pollutant, can then be correlated the toxicity of the contaminant (Bitton and Koopman, 1992). Since the initial discovery of this relationship, many bacterial enzymes have been tested for their potential as environmental biosensors (Christensen *et al.,* 1982; Bitton and Koopman, 1986; Obst *et al.,* 1988).

Dehydrogenases are commonly used for this purpose in toxicity assays. Lehnard (1963) first proposed that dehydrogenase activity could be a

useful indicator of the toxic effects of inorganic chemicals, such as mercury, silver, and chromium, and organic chemicals, such as formaldehyde and phenol. Dehydrogenase activity was later found to correlate well with microbial flora present in aquatic ecosystems (Dermer *et al.,* 1980). Dehydrogenase enzyme inhibition assays involve the use of specific oxidoreduction dyes, such as triphenyl tetrazolium chloride (TTC), which become reduced in the presence of the dehydrogenase enzyme (Bitton and Koopman, 1986). In the case of TTC, dehydrogenase catalyzes a reduction reaction which results in the transformation of TTC, which is colourless, to triphenyl formazan, a red insoluble precipitate (Altman, 1976). Hence, a toxic contaminant present in an environmental sample will lead to inhibition of bacterial dehydrogenase activity, resulting in decreased reduction of TTC, and decreased formation of the red precipitate. Toxicity is then assessed by the degree of colour change observed (Bitton and Koopman, 1986). Toxicity tests based on the inhibition of enzyme activity, other than dehydrogenases, have also been investigated and these include ATPases (Riedel and Christensen, 1979), esterases (Guilbault and Kramer, 1964; Holland *et al.*, 1967), and phosphatases (Tyler, 1976).

Inhibition of bacterial enzyme biosynthesis, may also be representative of environmental toxicity (Cenci *et al.,* 1985; Reinhartz *et al.,* 1987). For example, β-galactosidase is an enzyme responsible for the breakdown of lactose. In *Escherichia coli*, the β-galactosidase enzyme is encoded by the *lacZ* gene. The *lacZ* gene belongs to a cluster of genes, collectively referred to as the *lac* operon (Jacob and Monod, 1961). The *lac* operon encodes proteins involved in the transport of lactose into the cell, regulation of the operon itself, and biosynthesis of the β-galactosidase enzyme (Jacob and Monod, 1961). *De novo* biosynthesis of β-galactosidase in *Escherichia coli* has been shown to be more sensitive to toxic chemical exposure than enzyme activity (Dutton *et al.,* 1988; Reinhartz *et al.,* 1987). Based on this discovery, a commercial toxicity assay, known as the Toxi-Chromotest®, was developed

(Reinhartz *et al.,* 1987). This assay exploits the fact that toxic chemicals exert an inhibitory effect on *de novo* biosynthesis of the β-galactosidase enzyme (Reinhartz *et al.,* 1987). *E. coli* β-galactosidase, in addition to lactose, also breaks down synthetic lactose analogues (Miller, 1992). Breakdown of these compounds by β-galactosidase causes a colour change when *E. coli* is grown in media containing the lactose analogue (Miller, 1992). Hence, the toxicity of environmental pollutants may be assessed by the absence of colour change, which is indicative of enzyme biosynthesis inhibition (Reinhartz *et al.,* 1987).

The effect of toxic chemicals on the *de novo* biosynthesis of other inducible enzymes, such as *E. coli* tryptophanase and *Bacillus licheniformis* α-glucosidase, has also been explored (Dutton *et al.,* 1990). For example, biosynthesis of α-glucosidase in *B. licheniformis* has been shown to be very sensitive to environmental contamination, especially by hydrophobic compounds and detergents (Dutton *et al., 1990*). Furthermore, *B. licheniformis* α-glucosidase synthesis is more sensitive to hydrophobic compounds than *E. coli* tryptophanase. This may be explained by the lack of an outer membrane in gram positive bacteria (Dutton *et al.,* 1990). Unlike the *Bacillus spp.*, gram negative bacteria, such as *E. coli*, have an outer membrane which inhibits hydrophobic compounds from entering the bacterial cell (Neidhart *et al.,* 1990).

2.2. *ATP-based Assays*

Early work by Brezonik and Patterson (1972) introduced the use of ATP production in the screening of contaminants in activated sludge systems. Further studies have confirmed the utility of ATP-based toxicity assays for water and waste water (Parker and Pribyl, 1984). A more recent approach is the ATP-TOX® assay developed by Xu and Dutka (1987). This test is based on both growth inhibition, via ATP measurement of bacteria, and luciferase activity. Inhibition of this enzyme is determined by adding a standard ATP

solution, as an enzyme substrate, followed by the measurement of light emission. Similar assays have also been based on the inhibition of ATP production in algae. One example is the Algae-Tox® assay which measures the inhibition of ATP production in *Selenastrum capricornutum* (Blaise *et al.*, 1986).

2.3. *Growth Inhibition Assays*

Analysis of growth rates of pure or mixed bacterial cultures has also proven useful in the detection of environmental contaminants (Alsop *et al,.* 1980; Trevors, 1986). These assays determine toxicity by the ability of environmental pollutants to inhibit bacterial growth. Inhibition of bacterial growth is detected simply by measuring changes in absorbance of the bacterial cultures, which is indicative of bacterial cell densities. Decreases in cell density therefore suggest increased toxicity by contaminated samples (Alsop *et al,.* 1980; Trevors, 1986).

2.4. *Ecological Effect Assays*

Cycling of nutrients by microorganisms in aquatic and soil environments may also be adversely affected by toxic chemicals. Carbon, nitrogen, phosphorus, and sulfur cycling are carried out by various groups of microorganisms and these processes may be subjected to inhibition by toxic chemicals (Bitton *et al.,* 1989). The carbon cycle may be affected by toxic chemicals and toxicity can be determined through measurement of microbial respiration. Several methods are currently available to measure respiration and these include oxygen electrodes, manometers, and electrolytic respirometers (King and Dutka, 1986). Furthermore, some of these assays are capable of measuring the inhibition of biodegradation of specific organic compounds, such as cellulose (Martin *et al.,* 1982; Wainwright, 1978).

Nitrogen fixation, ammonification, nitrification, and denitrification are all processes involved in the nitrogen cycle. Among these processes, nitrification is probably the most sensitive to the effects of toxic chemicals and environmental pollutants (Bitton and Koopman, 1992). Nitrification is carried out by two groups of chemoautotrophic bacteria; *Nitrosomonas spp.* oxidize ammonium to nitrite and *Nitrobacter spp.*, sometimes referred to as nitrifiers, convert nitrite to nitrate (Bitton and Koopman, 1992). A test based on the inhibition of respiration of *Nitrosomonas spp.* has been developed and may be used for rapid measurement of waste water toxicity (Alleman, 1988). Another test, based on the reduction of nitrite in *Nitrobacter spp.* in the presence of waste water, has also been developed and has been shown to be sensitive to the action of pesticides, heavy metals, and industrial effluents (Carlisle and Trevors, 1986; Bewley and Stotzky, 1983; Williamson and Johnson, 1981).

Anaerobic microorganisms, particularly methanogens, have also been shown to be very sensitive to environmental contamination. Owen *et al.* (1979) initially developed the Anaerobic Toxicity Assay (ATA) to determine the impact of toxic chemicals on gas production using easily degradable substrates, such as acetate or propionate. This assay has been used for testing the effect of industrial chemicals on methanogenesis (Benjamin *et al.*, 1984).

2.5. *Commercially Available Short-term Toxicity Assays*

Several short-term assays are currently commercially available. These toxicity tests include MetPad™, PolyTox, the ECHA Biocide Monitor, Microtox®, Biotox®, and Lumistox® (Table 1). MetPad® is designed for the detection of heavy metal toxicity. The test is based on inhibition of enzyme activity in a mutant strain of *E. coli* caused by bioavailable heavy metals in aqueous samples (Bitton *et al.*, 1992). A variation on this assay is the MetPlate® assay which is performed in micro-titer plates (Bitton *et al.*, 1994).

The PolyTox assay utilizes a mixture of bacterial cultures isolated from waste water. The assay is based on the reduction of respiratory activity of rehydrated bacterial cultures in the presence of contaminants (Elnabarawy *et al.,* 1988). The ECHA Biocide Monitor is a dipstick test for monitoring toxicity. It incorporates a test microorganism, present on the dipstick, and an oxidoreduction dye, tetrazolium salt, which is used to indicate the growth of the test bacterium. Toxicity detection is based on the inability of the bacteria to grow in the presence of contaminants (Dutka and Gorrie, 1989).

Numerous bacterial biosensors, based on bacterial characteristics other than enzyme inhibition and biosynthesis, have also been developed for assessing the toxicity of environmental samples. For example, bioluminescent organisms are widely distributed in nature and comprise a remarkably diverse set of species. Bacteria, fungi, fish, insects, shrimp, and squid all have identifiable light-emitting species (Meighen, 1991). Luminous bacteria are the most abundant and widely distributed of the light-emitting organisms and they are found in marine, freshwater, and terrestrial environments (Meighen, 1991). Bioluminescence in marine bacteria is driven by specific enzymes in a branched pathway of the electron transport system (Hastings *et al.,* 1985). It has long been known that toxic chemicals can adversely affect the light output of these bacteria and may therefore be used as an indicator of cell viability (Bulich, 1979). Various monitoring procedures have therefore been developed for the assessment of chemical pollutants on luminescent freshwater and marine biota (Bulich 1979; Bulich 1986). The most widely used assay, Microtox®, incorporates freeze-dried cultures of the constitutively bioluminescent bacterium, *Photobacterium phosphoreum* (Bulich, 1979; Bulich 1986). In the Microtox® assay, *P. phosphoreum* cells are added to a water sample, its luminescence is measured, and these numbers are compared to the light output of a control sample of untreated water (Bulich, 1979; 1986). This assay has been adopted by many environmental

laboratories and agencies and is extensively used in the assessment of sewage effluents, complex industrial wastes, fossil fuel process water, sediment extracts, sanitary landfills, and hazardous waste leachates (Munkittrick *et al.,* 1991).

Several other bacterial toxicity assays, based on the inhibition of light emission in bioluminescent bacteria, include BioTox® (Kahru, 1993) and Lumistox® (Fernandez et al., 1995). These toxicity assays, which also incorporate freeze-dried cultures of *P. phosporeum*, are variations of the Microtox® assay. Moreover, these toxicity assays have been used extensively in field experiments. For example, Lumistox® was used to evaluate the water quality of the Tormas river in Spain (Fernandez *et al.,* 1995). The BioTox® assay was also used by Kahru *et al.* (1996) to determine the toxicity of numerous pesticides. The use of Microtox® for continuous on-line monitoring of river water entering a water treatment plant in France was also investigated (Levi *et al.,* 1989). In this study, samples were monitored continuously and a computer triggered an alarm when samples were found to be toxic (Levi *et al.,* 1989). These detection systems, along with on-line monitoring of waste water and drinking water treatment plants, have proven to be extremely useful (Bitton and Koopman, 1992).

Table 1: Commercially Available Short-term Toxicity Assays

Assay	Toxicity Measured	Reference
MetPad™	heavy metal toxicity	Bitton *et al.*, 1992
MetPlate®	heavy metal toxicity	Bitton *et al.*, 1994
PolyTox	overall toxicity	Elnabarawy *et al.*, 1988
ECHA Biocide Monitor	overall toxicity	Dutka & Gorrie, 1989
Microtox®	overall toxicity	Bulich, 1979
Biotox®	overall toxicity	Kahru, 1993
Lumistox®	overall toxicity	Fernandez *et al.*, 1985
SOS Chromotest	mutagenicity	Quillardet *et al.*, 1982
Toxi-Chromotest®	mutagenicity	Reinhartz *et al.*, 1987
λ Inductest	mutagenicity	Moreau *et al.*, 1976

2.6. *The Future of Short-term Toxicity Assays*

Unlike conventional methods of pollution detection, the microbial biosensor assays described above are extremely cost-efficient. They also provide rapid responses to toxicants and require only modest laboratory equipment and space (Bitton and Koopman, 1992). A major disadvantage of these assays is that they only measure overall toxicity or genotoxicity to living organisms. To circumvent this problem, gene fusion biosensors, which detect environmentally-relevant concentrations and bioavailability of specific environmental pollutants, have been developed (Bitton and Koopman, 1992).

3.0. GENE FUSION BIOSENSORS

3.1. *Genetically-programmed Responses as Tools for Creating Biosensors*

The genome of all cells is programmed to respond to changes in the environment. These responses occur whether the changes are beneficial, as in the presence of a nutrient, or harmful, as in the presence of a toxic agent, and usually involve the turning on (or off) of selected genes. For example, *Escherichia coli* can express new and specific proteins when exposed to environmental stress. These proteins may be involved in detoxifying harmful compounds or they may be involved in the activation or repression of reactions which counteract or prevent further damage to the cell. One example of a cellular response to stress is the expression of a set of proteins when a growing cell is rapidly shifted to a higher temperature, termed the "heat shock" response. When the growth temperature of *E. coli* is rapidly shifted from 28°C to 42°C, at least seventeen new proteins are expressed. This is due to the activation of an alternate sigma factor, sigma-32, which replaces the normal sigma factor, sigma-70, on RNA polymerase. Sigma-32 alters the specificity of promoter recognition by RNA polymerase, resulting in the expression of a new set of proteins, the heat shock proteins (Neidhardt and VanBogelen, 1987). Many laboratories have used these types of genetically-programmed responses to determine the presence of toxic chemicals in soil and aquatic ecosystems.

3.2. *Reporter Genes for Biosensor Applications*

"Reporter genes" encode gene products (i.e. enzymes) which are easily measurable and are not normally expressed in the cells under study. Reporter genes are void of any regulatory or promoter elements and are therefore used to "report" or examine the expression of normal cellular genes. Ideally, reporter genes should detect environmentally-relevant concentrations of contaminants and these measurements should be proportional to gene expression. A reporter

gene that has been extensively used for these purposes is the gene encoding luciferase. For example, luciferase is the enzyme expressed by *P. phosphoreum* in the Microtox® assay (Bulich, 1979; 1986). Moreover, luciferase from the marine bacterium, *Vibrio harveyi,* has also been utilized (Meighen, 1991). Luciferase is a mixed-function oxidase which catalyzes a reaction requiring an aldehyde and a diflavin mononucleotide in the presence of oxygen and results in the production of a carboxylic acid, a flavin mononucleotide, a water molecule, and a photon of light at 490 nm (Meighen, 1991). The light produced from this reaction can be easily measured with photographic film, a scintillation counter, a luminometer, or the naked eye. Luciferase genes, when coupled to the expression of individual genes, can therefore be used as reporter genes to convert cellular gene expression into easily assayable light emission. Furthermore, measurement of luciferase expression is ideal because it is a more sensitive indicator of gene expression than most other reporter enzymes (Meighen, 1991).

One of the first reporter gene fusion assays exploited knowledge of the "SOS response" of *E. coli* (Quillardet *et al.,* 1982). The SOS response occurs when *E. coli* is exposed to mutagens (e.g. UV light) and results in the expression of proteins that repair DNA damage caused by mutagenic agents Under normal conditions, *E. coli* DNA repair enzymes are not expressed because their promoters are blocked by the LexA repressor. In the presence of excess single-stranded DNA (caused by DNA damage and/or stalled replication forks), the RecA protein, a protease, becomes activated and cleaves LexA, rendering it incapable of binding and repressing transcription. RNA polymerase is thus able to transcribe genes encoding repair enzymes, ultimately resulting in their expression and repair of the damaged DNA (Walker, 1987). The SOS Chromotest represents one of the first examples of a bacterial gene fusion biosensor for the detection of mutagens. In the SOS Chromotest, *sulA*, a gene whose expression is induced during the SOS response, is fused to the *lacZ*

gene such that expression of the *lacZ* gene and its gene product, β-galactosidase, is now under control of the bacterial SOS response. Upon exposure to a mutagen, β-galactosidase is therefore expressed and can be measured using colourimetric substrates (Quillardet *et al.*, 1982).

Similar techniques have been adopted by other laboratories (Guzzo and DuBow, 1991; Van Dyk *et al.*, 1994; Heitzer *et al.*, 1994). Through the creation of reporter gene fusions, investigators have succeeded in exploiting the genetically-programmed responses of bacteria to toxic environmental pollutants.

3.3. *Bacteriophages as Biosensors*

A series of assays have been developed to assess the mutagenic potential of chemicals. One example is the λ Inductest which involves the use of bacterial prophages. Lysogenic *E. coli*, containing a DNA damage-inducible λ prophage, are grown in the presence of a particular contaminant. If DNA damage occurs, the SOS response for DNA repair is triggered and the prophage lytic cycle is induced. Plaque formation therefore indicates the presence of mutagens in the sample (Moreau *et al.*, 1976). A similar technique, which combines bacteriophage-induction and luciferase gene fusions, has also been developed (Maillard *et al.*, 1996). In this assay, bacteriophages contain a promoterless *luxAB* reporter gene fused randomly to different locations in the phage genome. The recombinant phages are then used to lysogenize specific bacterial species, and one lysogen is chosen which exhibits increased luminescence in the presence of mutagens. This bacterial lysogen can therefore be grown in the presence of a contaminant. If the contaminant is mutagenic, induction of the prophage lytic cycle is triggered, the luciferase genes are expressed, and light is emitted, thus providing for sensitive detection of mutagens (Maillard *et al.*,1996).

Similar luminescent bacteriophages have also been designed to detect food-borne pathogenic organisms (Loessner *et al.*, 1996; Bloom *et al.*, 1993;

Jacobs *et al.,* 1993). Many conventional tests are currently available to monitor microbial contamination. However, due to the slow growth of many pathogenic microbes, conventional methodology does not allow for rapid identification of such contaminants, which is necessary for the prevention of disease and maintenance of food supplies. Luciferase is therefore being used to develop biosensors which detect these pathogens more sensitively and expediently. In these assays, bacteriophages contain the luciferase-encoding *lux* (prokaryotic origin) or *luc* (eukaryotic origin) genes. This bacterial detection system was first described in 1987, when Ulitzur and Kuhn cloned the bacterial luciferase (*lux*) genes into the bacteriophage lambda genome (Ulitzur and Kuhn, 1987). More recently, two groups have used this system to identify drug resistant strains of *Mycobacterium tuberculosis* or *Listeria* spp. from human sputum and contaminated food, respectively (Jacobs *et. al.* 1993; Bloom *et al.,* 1993; Loessner *et al.,* 1996). These species-specific, recombinant *lux*- or *luc*-containing phages work by infecting specific bacterial pathogens present in food or human sputum. Upon analysis via luminometry, the presence of viable pathogens is detected through infected phage-based luciferase expression and consequent increases in light emission. This system is extremely sensitive, rapid, and relatively inexpensive. Efforts are now under way to create and optimize luminescent bacteriophages for the detection of both common and disease causing bacteria in water (DuBow, 1993).

3.4. *Heat Shock Gene Fusions*

The "heat shock" response, a process that takes place in all organisms, is a response to abrupt increases in temperature whereby synthesis of large numbers of proteins is induced (Van Dyk *et al.,* 1994). In particular, the heat shock response is thought to allow cells to respond to protein unfolding (Craig *et al.,* 1991; LaRossa and Van Dyk, 1991). Proteins produced during the heat shock response include Hsp60 and Hsp70, which are molecular chaperones

having important cellular functions in protein folding and renaturation during both steady-state growth as well as during heat shock (Morimoto *et al.,* 1990). In *E. coli*, Hsp60 and Hsp70 are encoded by the *groEL* and *dnaK* heat shock-inducible genes, respectively. Another important heat-shock inducible gene, *grpE*, encodes an essential protein known to interact with the *dnaK* and *groEL* gene products (Ang and Georgopoulos, 1989; Langer *et al.,* 1992; Liberek *et al.,* 1991).

In addition to heat shock, several other stress conditions have also been shown to induce the synthesis of heat shock proteins in many organisms. Viral infection, a change from anaerobic to aerobic conditions, the presence of abnormal proteins, and exposure to various chemicals including ethanol, 2,4-dinitrophenol, sodium azide, hydrogen peroxide, heavy metals, and amino acid analogues are all known inducers of the heat shock response (Welch, 1990). In *E. coli*, a subset of proteins induced by heat shock can also be induced by starvation conditions or by the presence of toxic chemicals (Van Dyk *et al.,* 1994). Induction of the heat shock response is therefore thought to play an important role for survival in the presence of environmental contaminants (Van Dyk *et al.,* 1994). Consequently, investigators have proposed that monitoring of the heat shock response may also be a sensitive method for detecting environmental contaminants (Van Dyk *et al.,* 1994). To address the utility of the heat shock response for pollutant detection, Van Dyk *et al.* (1994) fused two known heat shock promoter elements, *dnaK* and *grpE,* to the promoterless luciferase reporter gene operon, *luxCDABE,* of the marine bacterium, *Vibrio fisheri*. These gene fusions were then cloned into multi-copy plasmids and transformed into specific *E. coli* bacterial strains. Metals, solvents, crop protection chemicals, and other organic molecules were found to rapidly induce light emission from *E. coli* strains containing these plasmid-borne fusions (Van Dyk *et al.,* 1994).

An important consideration when utilizing bacterial biosensors in toxicity assays is the permeability of bacterial cells to environmental contaminants. For example, the cell wall of gram negative bacteria is extremely complex, comprised of an outer membrane, a layer of peptidoglycan, and a cytoplasmic membrane. Moreover, this cellular envelope is rich in protein, phospholipids, and lipopolysaccharides (Neidhardt *et al.,* 1990). Due to its composition, the outer membrane of gram negative bacteria serves as an impermeable barrier preventing the escape of cellular components, such as enzymes. More importantly, however, with respect to biosensor applications, the outer membrane also serves as a strong barrier to numerous chemicals, including many large, hydrophobic compounds which could potentially damage the bacterial cell membrane (Neidhardt *et al.,*1990). Conversely, smaller, hydrophilic molecules can permeate the bacterial cell wall via specific protein channels, known as porins. Although the permeability of the outer membrane of gram negative bacteria to antibiotics has been well studied (Nikaido and Vaara, 1985), less is known about the effect of this permeability on the sensitivity of bacteria in short-term toxicity assays (Bitton *et al.,* 1988). Therefore, the chemical nature of the toxic compound may play an important role in determining the efficacy of the microbial biosensor. Since induction of the heat shock response most likely requires intracellular localization of toxic compounds, investigators also introduced a mutation at the *E. coli tolC* locus, which encodes a minor outer membrane protein (Van Dyk *et al.,* 1994). The new phenotype was shown to enhance detection of toxic hydrophobic molecules, such as pentachlorophenol, through alteration of the route of access across the outer membrane (Van Dyk *et al.,* 1994). Induction of stress promoters that respond to a wide variety of hostile environments therefore represent useful biosensors to detect the presence of numerous environmental pollutants (Van Dyk *et al.,* 1994).

3.5. *Luminescence-based Mercury Biosensors*

Selifonova *et al.* (1993) have constructed gene fusion biosensors for the detection of mercury II compounds. These biosensors were constructed by cloning the mercury resistance operon (including the regulatory elements) of transposon *21* (Tn*21 mer*) upstream of the promoterless *lux* operon (*luxCDABE*) from *Vibrio fisheri*. Plasmids harbouring these *mer-lux* genes fusions were then transformed into a particular strain of *E. coli* (Selifonova *et al.*,1993). The *mer* operon is the most well understood genetic system for bacterial detoxification of a heavy metal, and much of this knowledge was based on studies of Tn*21 mer* (Barrineau *et al.*, 1984). Tn*21 mer* is a narrow-spectrum mercury operon specifying resistance to and reduction of inorganic mercury, but not of organomercury compounds. It consists of six functional genes, *merRTPCAD*, and is tightly regulated by Hg(II) (Selifonova *et al.*,1993). Furthermore, the sensitivity of the *mer-lux* biosensors was increased through introduction of the *merTPC* transport functions of the *mer* operon (Selifonova *et al.*,1993). In doing so, the investigators demonstrated that the mercury biosensors could detect Hg(II) in natural waters at the 0.5 to 1000 nM concentration range. At the 0.5 to 50 nM concentration range in defined medium, the biosensors responded to Hg(II) in a linear manner. Moreover, these ranges of concentrations encompass levels found in natural waters, as well as those commonly encountered in contaminated environments (Selifonova *et al.*,1993). Selifonova *et al.* (1993) have therefore demonstrated that the mercury biosensors provide a simple bioassay for the semi-quantitative detection of Hg(II) in contaminated waters and industrial waste waters (Selifonova *et al.*,1993).

3.6. *Luminescence-Based Naphthalene Biosensor*

Burlage *et al.* (1990) and King *et al.* (1990) have genetically engineered biosensors for monitoring the bioavailability and catabolism of naphthalene

and its degradation intermediate, salicylate. The bacterium utilized, *Pseudomonas flourescens* HK44, carries a plasmid, pUT21, which encodes a naphthalene degradation gene and a bioluminescent reporter gene (Burlage *et al.,* 1990; King *et al.,* 1990). Plasmid pUT21 contains a transcriptional fusion between the *luxCDABE* operon from *Vibrio fisheri* and the *nahG* gene of the naphthalene/salicylate degradation operon. Exposure of *P. flourescens* HK44 to both naphthalene and salicylate resulted in increased gene expression and consequently, increased bioluminescence (Burlage *et al.,* 1990; King *et al.,* 1990). Investigators subsequently illustrated a linear relationship between the naphthalene/salicylate concentration and overall bioluminescence. This assay therefore represents a specific, quantitative bioassay for the detection of these pollutants (Burlage *et al.,* 1990; King *et al.,* 1990).

Heitzer *et al.* (1994) also recently developed an optical whole-cell assay, incorporating the naphthalene biosensor, for the on-line monitoring of naphthalene and salicylate in waste water effluents. The luminescent *P. flourescens* HK44 biosensor was immobilized onto the surface of an optical light guide using a translucent matrix, strontium alginate. The biosensor probe was then inserted into a measurement cell which simultaneously received the waste water effluent (Heitzer *et al.,* 1994). Exposure to both naphthalene and salicylate resulted in rapid increases in bioluminescence. The bacterial biosensor was then tested using contaminated environmental samples (Heitzer *et al.,* 1994). Bioluminescent responses were obtained upon exposure to either an aqueous solution of JP-4 jet fuel or an aqueous leachate from a gas plant, which were known to contain naphthalene (Heitzer *et al.,* 1994).

3.7. *Luminescence-based Biosensor for the Detection of Benzene Derivatives*

A gene fusion biosensor has also been developed for the detection of benzene derivatives (Kobatake *et al.,* 1995). This biosensor was constructed through fusion of firefly luciferase (*luc*) to specific genes of the TOL plasmid (Kobatake *et al.,* 1995). The TOL plasmid of *Pseudomonas putida* encodes a series of enzymes involved in the degradation of benzene and its derivatives. Expression of these enzymes is controlled by the XylR and XylS regulatory proteins, whose promoters are activated in the presence of aromatic compounds (Kobatake *et al.,* 1995). The gene encoding firefly luciferase was inserted under the control of the *xylS* promoter, and the resulting gene fusion plasmid, pTSN316, was transformed into a strain of *E. coli* (Kobatake *et al.,* 1995). Expression of luciferase in this biosensor was induced in the presence of aromatic compounds, the lower detection limit for m-xylene being 5 μM (Kobatake *et al.,* 1995). This biosensor therefore offers a rapid and sensitive detection method for some potentially harmful by-products of industrial pollutants (Kobatake *et al.,* 1995).

3.8. *Luminescence-based Heavy Metal Biosensor*

Guzzo and DuBow (1991) constructed a library of over 3000 gene *E. coli* clones, each of which contained a single insertion of the promoterless luciferase genes, *luxAB*, from the marine bacterium, *Vibrio harveyi*. Upon screening this collection of clones with a variety of different compounds, a clone was isolated whose luminescence is induced in the presence of various heavy metals, namely, aluminum, copper, iron, and nickel (Guzzo *et al.,* 1991). Analysis of this clone revealed that this heavy metal-inducible gene was *fliC,* a gene involved in flagella biosynthesis (Guzzo *et al.,* 1991). Hence, this *luxAB-fliC* gene fusion clone may potentially be used as an environmental biosensor for the detection of bioavailable heavy metals.

3.9. *Luminescence-based Arsenic Biosensor*

A gene fusion biosensor was also constructed for the detection of arsenic- and antimony-containing compounds present in the environment (Diorio *et al.,* 1995; Cai and DuBow, 1996). Unlike the plasmid-encoded gene fusions, this biosensor consisted of the *luxAB* genes, derived from *Vibrio harveyi*, fused to the chromosomally-located *arsB gene* of *E. coli* (Cai and DuBow, 1996). The *arsB* gene belongs to an operon responsible for arsenic detoxification (Diorio *et al.,* 1995). This gene encodes a membrane-localized arsenite ion-specific export pump (Diorio *et al.,* 1995; Cai and DuBow, 1996).

3.10. *Additional Luminescence-based Biosensors*

Several other luminescence-based biosensors have been developed for the detection of various heavy metals and organic compounds such as copper, cadmium, cobalt, chromium, zinc, nickel, tributyl tin, and dimethylsulfoxide (Table 2) (Collard *et al.,* 1994; Corbisier *et al.,* 1993; Rouch *et al.,* 1995; Guzzo and DuBow, 1994a; Briscoe *et al.,* 1996).

4.0. THE FUTURE OF GENE FUSION BIOSENSORS

The development of gene fusion biosensors represents an exciting alternative to toxicity testing. Although biosensors are still in the early stages of development, reporter bacteriophages and luciferase gene fusion biosensors allow investigators to determine both the bioavailability and the physiological effects caused by exposure to environmentally-relevant concentrations of specific contaminants. One problem that has still not been overcome by these microbial bioassays is that no single microbial bioassay can detect all categories of environmental toxicants with equal sensitivity (Bitton and Koopman, 1992). A "battery of tests" approach, incorporating several microbial assays, has therefore been recommended (Blaise *et al.*,1991).

Table 2: Heavy Metal-responsive Luciferase Gene Fusions.

Biosensor	Gene Fusion	Compound Detected	Reference
Heat Shock	*dnaK*, *grpE* genes from *E. coli*	metals, solvents, pesticides, and other organic molecules	Van Dyk *et al.*, 1994
Mercury	*merT, merTCP, merTPCAD* genes from transposon Tn*21*	Hg^{2+}	Selifonova *et al.*, 1993
Naphthalene	*nahG* gene from *P. fluorescens*	naphthalene/salicylate	Heitzer *et al.*, 1994
Benzene derivatives	*xylS* gene from *P. putida*	aromatic compounds e.g. m-xylene	Kobatake *et al.*, 1995
Copper	*pcoE* gene from plasmid pRJ1004 of *E. coli*	Cu^{2+}	Rouch *et al.*, 1995
Cadmium	*cadA* gene from *S. aureus*	Cd^{2+}	Corbisier *et al.*, 1993
Cobalt	*cnr* and *czc* operons from *A. eutrophus*	Co^{2+}	Collard *et al.*, 1994
Zinc	*czc* operon from *A. eutrophus*	Zn^{2+}	Collard *et al.*, 1994
Arsenic	*arsB* gene from *E. coli*	As^{3+} and As^{5+}	Diorio *et al.*, 1995
Heavy metals	*fliC* gene from *E. coli*	Al^{3+}, Cu^{2+}, Ni^{2+}, Fe^{3+}	Guzzo *et al.*, 1991
Nickel	*celF* gene from *E. coli*	Ni^{2+}	Guzzo & DuBow, 1994

OUTLINE OF THE THESIS

All living cells have evolved the capacity to adapt or cope with environmental stress. This ability to adapt to new environments may be attributed to alterations in gene expression occurring within the cell, such as the induction or repression of one or several genes. Through use of a 3000 *Escherichia coli luxAB* gene fusion library, our laboratory has identified several genetically-programmed responses to toxic inorganic compounds (Guzzo *et al.,* 1991; Guzzo and DuBow, 1994a; Briscoe *et al.,* 1996). As part of my undergraduate research project, genetically-programmed responses to toxic organic compounds, such as pesticides and herbicides, were identified (Costanzo, 1995). However, despite numerous attempts, dose-dependent expression of luciferase and subsequent light emissions, observed in the original study (Costanzo, 1995), could not be reproduced. Experiments were therefore designed to determine the basis of these inconsistencies.

The development of bacterial gene fusion clones represents a powerful tool for monitoring toxic environmental substances. Environmental toxicity tests, employing microorganisms, have gained much popularity due to their ability to provide data rapidly and sensitively, as well as their cost-effectiveness. We therefore investigated the possibility of utilizing a well characterized arsenic-responsive *Escherichia coli* gene fusion clone as an environmental biosensor.

This thesis reports the results obtained while conducting this research.

Chapter II includes a synopsis of studies conducted as an undergraduate where genetically-programmed responses in *E. coli* to the herbicides, bromacil and dinoseb, and the pesticide, chlordane, were identified. The remainder of the chapter reports experiments performed to determine the basis for variability in luciferase expression and light emission.

Chapter III describes the use of a previously characterized *E. coli* clone, the *arsB*::*luxAB* chromosomal gene fusion clone, as a biosensor for the detection of arsenic-containing compounds present in an aquatic sample taken from the St. Lawrence River, Québec.

The experimental procedures utilized throughout these analyses are reported in their respective chapters.

Chapter IV summarizes the results obtained. Concluding remarks and directions for future research are also discussed.

CHAPTER II

Characterization of Genetically-programmed Respones in *Escherichia coli* to Chlordane, Dinoseb, and Bromacil

INTRODUCTION

Pesticides and herbicides have been developed to control a wide variety of harmful insects and competing weeds, respectively. By the very nature of their use in weed and pest control, they are common contaminants of food, water, and domestic products. Although pesticides and herbicides are designed to be selectively toxic towards target organisms, this is not absolute, and pesticides and herbicides can be toxic, to varying extents, towards non-target organisms (Hodgson and Levi, 1987; McEwen and Stephenson, 1979). In previous experiments, the 3000 clone *luxAB* (single-copy) gene fusion library (Guzzo and DuBow, 1991) was screened separately in the presence of each of two herbicide mixtures, bromacil and dinoseb, and one pesticide mixture, chlordane (Costanzo, 1995).

Bromacil works by interfering with photosynthesis (Gosselin, 1984; Meister, 1992) and is commonly used for brush control on non-cropland areas. It is also used to selectively control weeds in citrus and pineapple crops (Menzie, 1974). Once released into the environment, bromacil binds loosely to soil particles, remains water soluble, and has a very long half-life. Moreover, bromacil readily leaches through soil, eventually contaminating ground water (Cohen, 1984). Dinoseb is a phenolic herbicide used in soybean, vegetable, and fruit crops for the selective control of grass and broadleaf weeds (Cohen, 1984). Evidence has been found which suggests that dinoseb binds to certain organic and clay soils (Cohen, 1984). Dinoseb is also very stable in surface water and has been found in streams at concentrations of approximately five parts per billion (PPB) (Cohen, 1984).

Bromacil and dinoseb are both highly toxic to mammals, birds, and fish, and prolonged exposure may lead to inhibition of cellular division (Sarkar *et al.*, 1993; Verschueren, 1983). Extensive exposure to bromacil or dinoseb has been shown to have teratogenic effects, causing developmental abnormalities in rat fetuses (Walker and Keith, 1991). Bromacil and dinoseb are also

considered possible human carcinogens (Hall *et al.,* 1978). Gastroenteritis, swollen lymph nodes, bleeding heart, and abnormal adrenal glands were observed in sheep following long-term exposure to bromacil (Gosselin, 1984). Chronic exposure to elevated concentrations of dinoseb has been found to interfere with cellular metabolism and ATP production. This interference is the basis for most toxic effects related to the compound (Hall, 1978; Walker and Keith, 1991).

Another widely used pesticide, chlordane, is an organochlorine insecticide used for the control of ant, grasshopper, subterranean termites, and other insects (Verschueren, 1983). In soils, chlordane is very persistent, having a half-life of approximately four years (Agency for Toxic Substances and Disease Registry, 1989). However, chlordane has also been detected in surface waters, suspended solids, sediments, drinking water, sewage sludge, and urban run-off (U.S. E.P.A., 1990; Toxnet, 1995).

Chlordane is extremely toxic to birds, fresh water invertebrates, and bacteria (Meister, 1992). It exerts a wide array of toxicological effects with respect to chronic exposure . It is extremely lipophilic and can therefore accumulate in body fat and lipid-containing organs such as adipose tissue, kidneys, muscle tissue, liver, and brain (Hardell *et al.,* 1996; Hayes and Laws, 1990). Extensive mammalian exposure may cause serious chronic and cumulative toxicity, including central nervous system disorders, liver and kidney damage, and blood diseases (Hartely and Kidd, 1983). Excessive exposure to chlordane has also been shown to cause severe alterations in the human immune system including aberrant peripheral T and B cell regulation and autoimmune activation (McConnachie and Zahalsky, 1992; Theus *et al.,* 1992). Finally, chlordane has also been shown to induce single-stranded breaks in DNA (Venkat *et al.,* 1995).

Considering the universal exposure to toxic chemicals such as pesticides and herbicides, very little is known regarding the biological

responses to environmental stress caused by low or intermediate levels of exposure. The genome of all cells is programmed to respond to changes in the environment. These responses occur whether the changes are beneficial, as in the presence of a nutrient, or harmful, as in the presence of a toxic agent, and usually involve the turning on (or off) of selected genes. Many genes in *Escherichia coli* whose products are involved in the metabolism of toxic agents are currently unknown. To identify genes that are transcriptionally regulated by cellular exposure to environmental pollutants, a "library" was constructed in which chromosomal genes in *E. coli* were fused to a transcriptional "reporter gene". Promoterless reporter gene fusions can facilitate the measurement of transcription and can be used *in vivo* to study transcriptional control and to elucidate gene function (Guzzo and DuBow, 1991). The promoterless reporter gene employed in the construction of this library was the *luxAB* operon isolated from the marine bacterium, *Vibrio harveyi*. The *luxAB* operon is very useful because it encodes the enzyme luciferase which catalyzes a quantifiable luminescent reaction. Furthermore, transcription of a gene, fused to the *luxAB* reporter gene, is proportional to the amount of light produced (Meighen, 1991).

To create chromosomal *luxAB* gene fusions, the reporter gene was inserted between the two inverted repeats (IS*50*L and IS*50*R) of a modified Tn*5* transposon. This transposable element was altered such that its left inverted repeat consisted of only the outer 23 base pairs. This modification was made to ensure that the left end of the transposable element did not contain any transcriptional start or stop sequences. As a result, expression of the integrated *luxAB* operon, when fused to a chromosomal gene, was now under the control of an upstream promoter. A tetracycline resistance gene (tet^R) was also placed between the inverted repeats of the transposable element, serving as a selectable marker. The Tn*5*-*luxAB* element was then inserted into a ColE1-based plasmid and named pFUSLUX (Guzzo and

DuBow, 1991). ColE1 plasmids contain an origin of replication which, when introduced within a bacterial host, allows the plasmid to replicate autonomously. ColE1 replicons initiate replication with an RNA primer, called RNA 2. To isolate and select *E. coli* clones containing the Tn*5-luxAB* transposable element inserted randomly, and in single copy, into their genome, plasmid pFUSLUX was transformed into an *E. coli* strain which contained the ampicillin resistance-encoding p15A-based plasmid, pTF421 (Guzzo and DuBow, 1991). Plasmid pTF421 overproduces RNA1. Since RNA1 is complementary to RNA2, it hybridizes to RNA2 and inhibits the initiation of ColE1 replication (Fitzwater *et al.,* 1984). Thus, replication of pFUSLUX is inhibited upon transformation into a strain containing pTF421. Theoretically, only clones containing the Tn*5-luxAB* element transposed from pFUSLUX randomly into the *E. coli* chromosome will grow after transformation and selection on the basis of tetracycline resistance (*tet* ^{R}), from the Tn*5-luxAB* insert, and ampicillin resistance (*amp* ^{R}), from plasmid pTF421.

Through use of this 3000 clone *luxAB* (single-copy) library, our laboratory has successfully identified genes in *E. coli* which are induced in the presence of toxic metals (Guzzo and DuBow, 1994a; Guzzo and DuBow, 1994b; Cai and DuBow, 1996; Briscoe *et al.,* 1996). My undergraduate research project involved screening the *E. coli luxAB* gene fusion library in the presence of complex, organic chemicals, namely, the herbicides, bromacil and dinoseb, and the pesticide, chlordane (Costanzo, 1995). Three concentrations (0 μg/ml, 1 μg/ml, and 10 μg/ml) of each herbicide or pesticide were used in the initial screening of the *E. coli* gene fusion library to identify genes whose level of transcription (i.e. luciferase expression) was altered by these organic chemicals. Five different clones were identified whose luminescence was induced by either one of the herbicides or by the pesticide in a dose-dependent manner (Figure 1). Two gene fusion clones, LF20206 and LF20207, were found whose luminescence increased with augmenting

concentrations of chlordane. These clones were designated CHL1 and CHL2, respectively. Two additional gene fusion clones, LF20204 and LF20205, showed a dose-dependent increase in luminescence following exposure to dinoseb. These clones were designated DIN1 and DIN2, respectively. The remaining *E. coli* gene fusion clone, LF20208, designated BRO, also showed concentration-dependent increases in light emission following exposure to bromacil (Costanzo, 1995).

Following identification and isolation of these pesticide- and herbicide-responsive clones, experiments were performed to determine the Tn*5-luxAB* copy number within the genomes of these luminescent clones. Southern blotting analysis of the *E. coli* strains BRO (LF20208), CHL1 (LF20206), CHL2 (LF20207), DIN1 (LF20205), and DIN2 (LF20206) revealed that the Tn*5-luxAB* element was present in single copy (Figure 2A and 2B) (Costanzo, 1995). In all cases, a single band was observed in the samples digested with the restriction endonuclease, *Sal*I, and two bands where observed upon *Eco*RI digestion (Figure 2A). Of the two bands observed following *Eco*RI hydrolysis, one band was constant in size for each clone, representing the single internal *Eco*RI fragment located within the Tn*5-luxAB* element (Figure 2A and 2B). The second band varied in size, representing the DNA segment spanning the *Eco*RI site in the *luxAB* genes to the next *Eco*RI site located in the chromosome (Figure 2A and 2B). Faint bands observed at approximately 7 kilobase (Kb) represent non-specific cross-hybridization of the non-radioactively labeled *luxAB* probe to the multi-copy plasmid, pTF421, harboured by the *E. coli* clones (Guzzo and DuBow, 1991) (Figure 2A) (Costanzo, 1995).

Subsequent experiments involved cloning the pesticide- and herbicide-responsive genes (Figure 3) (Costanzo, 1995). The upstream regions of the *chl1* and *din1* gene fusions were cloned using the *tet* ^{R} gene in the Tn*5-luxAB* cassette as a selectable marker. Cloning was performed by enzymatically

digesting genomic DNA from strains LF20206 and LF20204 with *Hin*dIII and ligating the DNA to a suitable plasmid vector, such as plasmid pUC119 (Vieira and Messing, 1982), hydrolyzed with the same restriction endonuclease (Figure 3). Since no *Hin*dIII restriction sites exist in either the *tet* ^{R} or *luxAB* genes , the only clones which survived selection were those which contained the intact *tet*^{R} gene, the *luxAB* genes, the truncated IS*50*L, and the chromosomal sequence located between IS*50*L and the adjacent chromosomal *Hin*dIII site (Figure 3). Plasmids pCHL1 and pDIN1 were obtained using this procedure (Costanzo, 1995). Following restriction mapping, the DNA fragments adjacent to the *luxAB* genes in plasmid pDIN1 was determined to be approximately 1.35 Kb. Conversely, plasmid pCHL1 was found to contain approximately 2.4 Kb of chromosomal DNA. The upstream region of the *chl2* gene fusion was also cloned in one step (Costanzo, 1995). In this case, chromosomal DNA was hydrolyzed with the restriction endonuclease, *Sal*I, and the *luxAB* genes were used as a selectable marker. Since there are no *Sal*I restriction sites in the *luxAB* genes, desired clones contained the *luxAB* genes, the truncated IS*50*L element, and the adjacent chromosomal *Sal*I restriction site. Plasmid pCHL2, containing approximately 5.5 Kb of chromosomal DNA, was obtained using this procedure. Similar attempts to clone the second dinoseb-responsive gene, *din2*, and the bromacil-responsive gene, *bro*, were unsuccessful (Costanzo, 1995).

To precisely identify the pesticide- and herbicide-responsive genes, the junctions between IS*50*L and the sites of the Tn*5-luxAB* insertions were sequenced from plasmids pCHL1, pCHL2, and pDIN1. The sequences were then analyzed by computer homology searches against the GENBANK database. It was determined that the Tn*5-luxAB* element of the CHL1 clone (LF20206) was inserted at 76.9 minutes on the *E. coli* chromosome (Figure 4A). This site of insertion corresponds to a previously sequenced region

containing a putative open reading frame whose gene product has yet to be elucidated (Sophia *et al.,* 1994). The nucleotide and amino acid sequences of this uncharacterized protein are illustrated in Figure 5. Computer analysis also revealed that this unknown open reading frame may encode a protein consisting of 130 amino acids. This putative protein is hydrophobic in nature and has a predicted isoelectric point of 10.9. Similar to LF20206, the Tn*5-luxAB* element of the CHL2 clone (LF20207) also randomly inserted within a previously sequenced open reading frame, encoding an uncharacterized protein (Sophia *et al.,* 1994). This site of insertion was located at 78.8 minutes on the *E. coli* chromosome (Figure 4B). Finally, the DIN1 clone (LF20204) contained the Tn*5-luxAB* element inserted at 35.3 minutes on the *E. coli* chromosome (Figure 4C). This insertion site lies within a region downstream of the *cspB* gene, which encodes a major cold shock protein (Lee *et al.,* 1994). Since this chromosomal region contains no open reading frames, it is likely that transcription of the promoterless *luxAB* genes is controlled by the regulatory region of the *cspB* gene (Costanzo, 1995).

The work presented in this chapter focuses on attempts to reproduce the dose-dependent light emission in the presence of chlordane, bromacil, and dinoseb. Analyses were performed to determine the nature of the experimental inconsistencies observed when luminescence assays were repeated under varying conditions. Experiments are presented which rule out a genetic basis for the lack of reproducibility of the luminescence assays. Based on experiments presented in this chapter, hypotheses concerning the complex chemical nature of the compounds tested, the sensitivity of the reporter gene used in the assays, and the limitations of the *E. coli* gene fusion library are discussed.

MATERIALS AND METHODS

1.0. Bacterial Strains and Growth Media

Table 1 describes bacterial strains and plasmids used. Media used in the luminescence assays include Luria Bertani Broth (LB) [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto yeast extract, 2 N NaOH], M9 Minimal Media [0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl] (Miller, 1992), and TCMG media [1% (w/v) BBL trypticase, 0.5% (w/v) NaCl, 1 mM $MgSO_4$] (Baker *et al.,* 1983). Antibiotics were used at the following concentrations: ampicillin (0.04 mg/ml) and tetracycline (0.01 mg/ml).

2.0. Assay for Light Production

This technique is taken from Guzzo and DuBow (1994a). Briefly, 1-cm^2 patches of cells were grown overnight on petri dishes containing 12.5 ml LB agar [LB broth, 1.5% (w/v) agar], M9 agar [M9 minimal media, 1.5% (w/v) agar] or TCMG agar [TCMG broth, 1.5% (w/v) agar] along with 0, 1, or 10 μg/ml (final concentration) (unless otherwise specified) of bromacil, chlordane, or dinoseb (Accustandard, New Haven, USA). *E. coli* strain LF20208 was grown in the presence of bromacil; *E. coli* strains LF20206 and LF20207 were grown in the presence of chlordane; and *E. coli* strains LF20204 and LF20205 were grown in the presence of dinoseb. Plates were then placed upside down and exposed to Agfa Curix RP-1 X-ray film at 23°C (unless otherwise specified) after the addition of 100 μl decyl aldehyde (Aldrich, Milwaukee, USA) to the covers of the petri dishes. X-ray films were developed at various times. Upon developing, light emission was quantified by scanning the X-ray film on a flatbed scanner linked to a phosphorimager SF apparatus (Molecular Dynamics, Sunnyvale, USA). Light emission from each patch of cells was then analyzed using the ImageQuant software program (Molecular Dynamics, Sunnyvale, USA).

3.0. Isolation of Total Cellular DNA

Ten ml of cells (*E. coli* strains LF20204, LF20206, and LF20207) were grown for 18 hours in the presence of tetracycline and ampicillin. The cells were then pelleted by centrifugation (10 minutes, 4°C, 5000 xg) and resuspended in 1.4 ml 10X TE buffer [100 mM Tris-HCl (pH 8.0), 10 mM EDTA]. Sodium dodecyl sulfate [10% (w/v)] and RNase A [1 mg/ml in 10 mM Tris-HCl (pH 7.6)] were added to a final concentration of 0.53% (w/v) and 0.21 mg/ml, respectively, and the mixture was incubated at 37°C for 2 hours. Pronase [20 mg/ml in 10 mM Tris-HCl (pH 7.6)] was then added to a final concentration of 1.9 mg/ml and the mixture was incubated at 37°C for 2 hours. The DNA was then purified by performing 3 phenol extractions [phenol saturated in 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and 4 ether extractions and precipitated with 70% ethanol. Flocculent DNA fibers were immediately isolated with a micropipet, dried by vacuum dessication, and resuspended in 50 μl 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

4.0. Conditions for Enzymatic Hydrolysis of DNA

Total cellular DNA was subjected to hydrolysis with 3 units per μg DNA of restriction endonuclease, in Digestion Buffer [6.5 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 75 mM NaCl, 12 mM β–mercaptoethanol, 0.25 mg/ml BSA] for 4 hours at 37°C and subsequently inactivated at 65°C for 10 minutes. All enzymes used in this study were purchased from Pharmacia (Baie d'Urfe, Canada).

5.0. Southern Blotting and Hybridization of Probe

The *luxAB* hybridization probe was obtained by hydrolysis of plasmid pFUSLUX with *Bam*HI. The 3.2 kilobase (Kb) *luxAB* genes were isolated from a 0.75% horizontal agarose gel and purified using the Gene Clean II kit (Bio 101, Vista, USA). Approximately 200 ng *luxAB* DNA was uniformly labeled

with [α-^{32}P] dGTP or dCTP (3000 Ci/mmole, Amersham) using oligonucleotide primers from the Regional DNA Synthesis Laboratory (Calgary, Canada), according to the random priming method (Sambrook *et al.*, 1989). For the Southern blot, 10 μg of total cellular DNA was isolated (*see* Isolation of Total Cellular DNA) and hydrolyzed with the restriction endonucleases, *Sal*I or *Eco*RI. The DNA was subjected to electrophoresis through a 0.75% agarose gel at 20 volts for approximately 16 hours and transferred to a Hybond-N membrane (Amersham Ltd., Oakville, Canada). Approximately 1 x 10^8 to 1 x 10^9 cpm/μg of the labeled *luxAB* probe was added to the membrane. Pretreatment of the membrane, hybridization with the iabeled probe, and washing were all performed according to Sambrook *et al.* (1989).

6.0. P1 Transductions

This protocol is taken from Miller (1992).

6.1. Preparation of P1*vir* Lysate

Saturated cultures of *E. coli* strains LF20204, LF20206, and LF20207 were diluted 1:50 into LB containing 5mM $CaCl_2$ and then incubated at 37°C for 1 hour. The bacterial cells (10^8 cells) were then absorbed with 10^7 P1*vir* bacteriophage for 20 minutes at 37°C. Molten R-Top agar [1% (w/v) bacto tryptone, 0.1% (w/v) bacto yeast extract, 0.8% (w/v) NaCl, 0.8% (w/v) agar, 2 mM $CaCl_2$, 0.5% (w/v) glucose] was added to the P1*vir*-absorbed cells and this mixture was then plated on R-media [differs from R-Top agar because media used for R-plates contains 1.2% (w/v) agar rather than 0.8% (w/v) agar]. The plates were then incubated at 37°C for 8 hours. The soft R-Top agar layer was then scraped off the plates and transferred to a centrifuge tube. The R-plates were subsequently washed with 1 ml of LB which was also transferred to the centrifuge tube. Five drops of chioroform were then added to the tubes containing the R-Top agar and LB wash. This mixture was

subjected to centrifugation (10 minutes, 4°C, 5000 xg) to remove cell debris. The resulting supernatant, containing the P1*vir* phage, was subsequently used in the transduction experiment.

6.2. Transduction with P1*vir* Lysate

A fresh 5 ml saturated culture of *E. coli* MG1655 was pelleted by centrifugation (10 minutes, 4°C, 5000 xg) and resuspended in an equal volume of MC buffer (0.1 M $MgSO_4$, 5 mM $CaCl_2$). MG1655 cells (10^8 cells) were then absorbed with 10^7 P1*vir* bacteriophage (0.1 ml lysate) for 20 minutes at 37°C. Sodium citrate (0.5 M, final concentration) was added to the cell-lysate mixtures and 0.1 ml samples were plated on LB [LB broth, 1.5% (w/v) agar] containing tetracycline (0.01 mg/ml).

RESULTS

Inconsistent Results Observed in Repeated Luminescence Assays

In previous experiments, five clones were identified and isolated from a *luxAB* gene fusion library which demonstrated dose-dependent increases in light emission following exposure to the herbicide mixtures, bromacil and dinoseb, or the pesticide mixture, chlordane (Figure 1) (Costanzo, 1995). Three different trials of luminescence assays yielded consistent dose-dependent increases (Costanzo, 1995).

Following cloning and partial characterization of the pesticide- and herbicide-responsive genes, luminescence assays were repeated using new, lots of chlordane, dinoseb, and bromacil. However, subsequent luminescence assays did not yield results consistent with those previously observed employing the original lots of chemicals. Figure 6 illustrates one of the numerous luminescence assays performed on solid LB agar media. It is evident that none of the original *E. coli* clones, DIN1 (LF20204), DIN2

(LF20205), CHL1 (LF20206), CHL2 (LF20207), or BRO (LF20208) demonstrated the previously observed, two- to five-fold, dose-dependent increases following exposure to the appropriate organic compound (compare Figure 1 and Figure 6). In fact, in the particular assay depicted in Figure 6, DIN1 (LF20204) and BRO (LF20208) exhibited a relatively constant level of light emission regardless of the amount of dinoseb or bromacil present in the media (Figure 6). Moreover, luciferase expression seemed to decrease in the DIN2 (LF20205), CHL1 (LF20206), and CHL2 (LF20207) clones with increasing concentrations of dinoseb and chlordane (Figure 6). It should be noted that the patterns of light emission varied with each different assay performed.

Luminescence assays were performed over thirty times (Table 2). Cells derived from 50% (v/v) glycerol or LB stab stocks (three to six months old) were assayed. Colonies which seemed to show dose-dependent increases in light emission were chosen and streaked onto fresh LB agar plates to ensure purity of the strains. Since all progeny cells were derived from the same parental clone, they should exhibit the same levels of luciferase expression and hence yield the same luminescence pattern when exposed to the appropriate compound. However, light emission patterns fluctuated with each cell generation. One of these luminescence assays was randomly chosen and analyzed quantitatively using a phosphorimager (Figures 7, 8, and 9). The DIN1 clone (LF20204) demonstrated a constant level of luciferase expression, emitting approximately 1.8 X 10^6 relative light units (RLUs) (Figure 7A), whereas the DIN2 clone (LF20205) showed a decrease in light emission in the presence of increasing concentrations of dinoseb (Figure 7B). Similar to the DIN2 clone, decreased luciferase expression and luminescence was also observed with the CHL1 (LF20206) and CHL2 (LF20207) clones for this particular trial (Figure 8A and 8B). Finally, the bromacil-responsive clone also exhibited an inconsistent pattern of light emission following exposure to

increasing concentrations of the herbicide (Figure 9). It should be noted that patterns of luminescence varied each time the experiments were repeated. For example, decreases in light emission were also observed between 0 and 1 μg/ml followed by slight increases in luminescence noted between 1 and 10 μg/ml (data not shown).

Luminescence assays described above were all performed exactly the same way as those used to originally identify and isolate the five clones. They were all performed using LB agar media, in the presence of 0, 1, and 10 μg/ml of bromacil, dinoseb, or chlordane. Furthermore, all clones were exposed to the appropriate pesticide or herbicide at room temperature (23°C). Luminescence assays were then performed under varying conditions. For example, although LB-agar is optimal for cell growth, it is a chemically-undefined mixture composed of complex components, such as whole cell yeast extracts. It was hypothesized that certain factors or ligands present in the media may bind the chemicals, rendering them inaccessible to the cells. Luminescence assays were therefore repeated incorporating defined media, such as TCMG (Baker *et al.,* 1983) and M9 minimal media (Miller, 1992). Furthermore, light emission assays were performed using more chemically pure Noble agar as opposed to technical grade agar. The effect of exposure temperature was also investigated. Luminescence assays were conducted where the clones were exposed to the pesticide or herbicides at 15°C, 23°C,or 37°C. The range of concentrations of chemicals was also tested. Cells were exposed to concentrations of bromacil, dinoseb, and chlordane ranging from 0 to 1 μg/ml as well as from 0 to 40 μg/ml. The inconsistent results obtained from the luminescence assay were unaffected by these changes (Table 2).

Since the two chlordane- and the first dinoseb-responsive genes had been previously cloned and partially characterized (Costanzo, 1995), future experiments focused on these three clones.

Determination of Whether Inconsistencies Observed in Luminescence Assays Are Attributable to Transposition or Genomic Rearrangements

It was hypothesized that the difficulties encountered in reproducing the original luminescence assays may be of a chemical or genetic nature. Over a year had passed since the original Southern blot experiment (Figure 2), which illustrated that our clones contained single-copy chromosomal insertions of the *luxAB* genes. Since the *Tn5*-luxAB element is functional in transposition, it is possible that secondary transposition occurred within the genomes of our clones, potentially complicating the luminescence readings. To determine whether a genetic phenomenon, such as secondary transposition of the *Tn5-luxAB* genes, or other genomic rearrangements, was responsible for the inconsistent light emissions, Southern blotting experiments were repeated. However, unlike the original analysis (Figure 2A), the *luxAB* probe used in this experiment was radioactively labeled.

Southern blot analysis of *E. coli* strains CHL1 (LF20206), CHL2 (LF20207), and DIN1 (LF20208) revealed that the Tn*5-luxAB* cassette was still present in single copy (Figure 10). In all cases, a single band, and two bands were observed in the samples hydrolyzed with *Sal*I and *Eco*RI, respectively (Figure 10A). Of the two bands observed following *Eco*RI hydrolysis, one band was constant in size for each clone, representing the single internal *Eco*RI fragment located within the Tn*5-luxAB* element (Figure 10A and 10B). The second band varied in size, representing the DNA segment spanning the *Eco*RI site in the *luxAB* genes to the next *Eco*RI site located in the chromosome (Figure 10A and 10B). More importantly, however, the migration patterns of the bands, to which the radiolabeled *luxAB* probe hybridized, were identical to the migration patterns observed in the original analysis (compare Figure 2A and Figure 10A) thus confirming, within a limit of detection of 100 base pairs, that transposition of the *luxAB* reporter gene or

other forms of genomic rearrangements had not occurred within the chromosomes of the chlordane- and dinoseb-responsive clones.

P1 Transductions

The 3000 clone *luxAB* gene fusion library was originally constructed using *E. coli* DH1. *E. coli* DH1 has a *recA*$^-$ *genotype*, thus making these cells more susceptible to spontaneous mutagenesis as a result of their inability to perform in recombinational DNA repair or to express genes under the control of the "SOS" regulon (Friedberg *et al.*, 1995). Hence, it was possible that our original clones had accumulated random point mutations within the pesticide- or herbicide-responsive promoters or within genes encoding proteins involved in the regulation of these promoters thereby altering luciferase expression. Since the Tn*5-luxAB* transposon was shown to be intact (Figure 10), it was possible to transduce the reporter gene element into a wild-type, *recA*$^+$ *E. coli* strain to determine whether the observed inconsistent light emissions were due to spontaneous mutations in the pesticide- or herbicide-responsive genes. P1 transduction experiments were therefore performed using bacteriophage P1*vir* to infect the parental *E. coli* strain (*E. coli* DH1) of the CHL1, CHL2, and DIN1 clones. The resulting lysates were then used to infect a wild-type *E. coli* strain, *E. coli* MG1655. *E. coli* MG1655 transductants, containing the Tn*5-luxAB* element inserted upstream of the chlordane- or dinoseb-responsive genes, were selected on the basis of tetracycline resistance. The resulting transductants (LF20209, LF20210, and LF20211) were then screened for dose-dependent increases in light emission following exposure to dinoseb or chlordane. Dose-dependent increases in light emission from these transductants were not observed (Figures 11, 12, and 13). The dinoseb transductant, LF20209, containing the *luxAB* reporter downstream of the dinoseb-responsive gene, exhibited inconsistent fluctuations in light emission following exposure to the herbicide (Figure 11). The two chlordane

transductants, LF20210 and LF20211, demonstrated a slight decrease in luminescence and a relatively constant level of light emission, respectively (Figure 11). Luminescence assays utilizing these P1*vir* transductants were repeated numerous times and the results were analyzed via densitometry using a phosphorimager (Figures 12 and 13). In one particular assay, the dinoseb transductant (LF20209) showed a slight decrease in luminescence. In the absence of dinoseb, 1.5 x 10^6 RLUs were emitted from the colonies (Figure 12). However, in the presence of 10 μg/ml dinoseb, light emission decreased to less than 1.0 x 10^6 RLUs (Figure 12). The first chlordane transductant (LF20210) showed a drastic decrease in light emission, falling from 2.2 x 10^6 RLUs in the absence of chlordane to 0.8 x 10^6 RLUs in the presence of 1 μg/ml chlordane (Figure 13A). Finally, the amount of light emitted from the second chlordane transductant (LF20211) remained relatively constant in the presence of 0, 1, and 10 μg/ml chlordane (Figure 13B). Since the *luxAB* reporter genes were transduced into a wild-type strain (*E. coli* MG1655), these results suggest that the inconsistencies observed in the luminescence assays may not be attributable to spontaneous mutations in the host pesticide- or herbicide-responsive genes.

DISCUSSION

Previous studies (Costanzo, 1995) involved identification of five clones containing the *luxAB* genes fused to chromosomal genes in *E. coli.* Transcription of these genes was induced in the presence of the herbicides, bromacil and dinoseb, or the pesticide, chlordane. Two clones, CHL1 (LF20206) and CHL2 (LF20207), exhibited increased luminescence in the presence of chlordane (Figure 1). Two clones, DIN1 (LF20204) and DIN2 (LF20205), demonstrated increased light emission in the presence of dinoseb (Figure 1) and a final clone, BRO (LF20208), was induced following exposure to bromacil (Figure 1). Light emission increased two- to five-fold in all cases

(Costanzo, 1995). Southern blotting analysis confirmed that the these five clones contained a single copy of *luxAB* genes inserted randomly within the host chromosome (Figure 2A and 2B).

Following initial characterization of the pesticide- and herbicide-responsive genes, numerous attempts were made to reproduce the dose-dependent luminescence assays originally observed. It should be noted that these luminescence assays were performed using new lots of compounds, different from those used to isolate the five responsive clones in the original assays. Despite repeated attempts, the dose-dependent results could not be reproduced with any consistency (Figures 6, 7, 8, and 9). Colonies which showed a dose-dependent increase in luminescence, following exposure to either bromacil, chlordane, or dinoseb, were isolated and grown on fresh media to ensure genetic purity. However, subsequent assays, employing these progeny cells, yielded fluctuating light emissions in the presence of 0, 1, and 10 μg/ml of pesticide or herbicide. These results were surprising since the progeny cells were genetically identical to the parental colony which exhibited dose-dependent light emission and should therefore exhibit similar patterns of luciferase expression. Furthermore, inconsistencies and fluctuations were obtained regardless of the experimental conditions. For example, changes in the type of media used, temperature, or concentration range of the compounds did not to affect the outcome of the luminescence assays (Table 2). It was therefore hypothesized that one or more genetic alterations, having accumulated within the pesticide- and herbicide-responsive clones over the course of a year, may be responsible for the lack of reproducibility of the once consistent light emission experiments. Another explanation for the observed fluctuations and variable light emissions may lie within the complex nature and composition of the pesticides and herbicides themselves.

Experiments were therefore designed to determine whether the cause of the experimental inconsistencies was genetic in nature. The 3000 clone

luciferase gene fusion library was constructed by inserting the *luxAB* reporter genes between the inverted repeats of a modified Tn*5* transposon (Guzzo and DuBow, 1991). Tn*5* is a composite transposon in which antibiotic resistance genes are flanked by two nearly identical insertion sequences, IS*50*R and IS*50*L (Reznikoff, 1993). The transposable element used in the construction of the library was altered such that its left inverted repeat (IS*50*L) was truncated to consist of only the outer 23 base pairs. This modification was made to ensure that the left end of the transposable element did not contain any transcriptional start or stop sequences (Guzzo and DuBow, 1991). However, the right inverted repeat (IS*50*R) is a fully functional transposable element, encoding an enzyme, known as TnP (Reznikoff, 1993). TnP is a transposase which catalyzes transposition of the Tn*5* or IS*50* sequences from which it is encoded (Reznikoff, 1993). In addition to transposition, transposable elements, such as Tn*5*, are associated with other types of genetic rearrangements such as deletions, inversions, and chromosomal fusions (Reznikoff, 1993).

Southern blotting experiments (Figure 10) were therefore performed to determine whether the variability of luciferase expression and light emission could be attributed to secondary transposition events or to other forms of genomic rearrangements having occurred within the responsive clones. However, hybridization of the *luxAB* probe to genomic DNA isolated from *E. coli* strains CHL1 (LF20206), CHL2 (LF20207), and DIN1 (LF20204) revealed that the Tn*5*-*luxAB* element was still present in single copy (Figure 10A and 10B). In addition, based on extensive similarities observed in band migration patterns between the Southern blots, it was concluded that transposition or other forms of genomic rearrangements had not occurred within the genomes the CHL1 (LF20206), CHL2 (LF20207), and DIN1 (LF20204) clones (compare Figure 2A and 10A).

To further investigate the possibility of genetic alterations within the chromosomes of our clones, P1*vir* transduction experiments were performed. In addition to employing a modified Tn*5* transposon, the 3000 clone luciferase library was constructed using *E. coli* strain DH1. *E. coli* DH1 contains a *recA*$^-$ genotype. The product of the *recA* gene plays a central role in cellular responses to DNA damage (Walker, 1995). The RecA protein is involved in repair of daughter-strand gaps generated when cells attempt to replicate a damaged DNA template. It is also implicated in the repair of double-stranded breaks along with translesion replicative bypass (Friedberg *et al.*, 1995). As a result of this vital and integral role in DNA repair, DH1 cells which do not produce a functional RecA protein may be more susceptible to spontaneous mutagenesis. Hence, it was postulated that our original pesticide- and herbicide-responsive clones may have accumulated random point mutations within the responsive promoters or within genes encoding proteins involved in the regulation of these promoters. Such a phenomenon may explain the erratic patterns of luciferase expression and light emission. To test this hypothesis, the Tn*5*-*luxAB* element was transduced from the DH1 parental strain to a wild-type, *recA*$^+$ *E. coli* strain, *E.coli* MG1655. Following transduction, luminescence experiments were repeated. However, luciferase expression remained inconsistent and light emissions varied with each trial of the assay (Figures 11, 12, and 13). It was therefore concluded that fluctuations in light emissions following exposure of the clones to the appropriate compound, were not attributable to mutagenesis. The data obtained from the Southern blot analysis and the P1*vir* transduction experiments ruled out the possibilities of transposition, genetic rearrangements, or accumulation of spontaneous point mutations. These results suggest that the inconsistency and variability of luciferase expression and light emission, following exposure to increasing concentrations of either bromacil, chlordane, or dinoseb, may not be of a genetic nature.

Another possible explanation may be based on the chemical nature of the media used. The majority of luminescence assays were performed in LB-agar which is a complex, nutrient-rich media. Due to its undefined nature, it was postulated that various unknown components, present in LB-agar, could act as ligands and bind the pesticides and herbicides rendering them inaccessible to the cells. To test this hypothesis, other types of media, such as TCMG (Baker *et al.,* 1983) and M9 minimal media (Miller, 1992), were used in the luminescence assays. Furthermore, technical grade agar, normally used in the preparation of solid media, was substituted with a more pure form of agar, Noble agar (Table 2). The use of minimal media and Noble agar enabled the performance of the luminescence assay under more pure, chemically-defined parameters. However, use of these minimal media did not restore the consistent, dose-dependent light emissions originally observed.

The variability in luminescence may be also attributed to the complex nature and formulations of the pesticides and herbicide mixtures themselves. For example, technical grade chlordane is composed of up to fifty different chemicals including various chlorinated hydrocarbons and their by-products (Hartley and Kidd, 1983). These individual components are purchased separately and subsequently mixed by the supplier. According to the supplier, the lots of individual chemical components vary constantly and are commonly contaminated with impurities (personal communication, Accustandard). Moreover, the exact amounts and the ratios of the components which make up these pesticides and herbicides are not well-defined (Hartley and Kidd, 1983). Hence, it is possible that due to variations in chemical production, the luminescence assays performed with the initial lots of chemicals yielded consistent, reproducible results whereas subsequent assays, using new lots of compounds, were not reproducible.

The varying composition of these chemicals may also have affected their ability to permeate the bacterial cell membrane. Gram negative bacteria,

such as *E. coli*, possess an extremely complex cell wall comprising an outer membrane, a layer of peptidoglycan, and a cytoplasmic membrane. As a result, the cell wall acts as an effective barrier against large hydrophobic molecules, such as bromacil, chlordane, and dinoseb (Neidhardt *et al.*, 1990). Therefore, it may be possible to introduce mutations into our clones, to destabilize the cell wall and hence, increase permeability. For example, VanDyk *et al.* (1994) created luminescent biosensors using a *tolC*$^-$ bacterial mutant. The *tolC* gene encodes an outer membrane protein which serves as an ion transport channel (Neidhardt *et al.*, 1990). Such mutations therefore increase bacterial cell wall permeability allowing organic compounds, such as pesticides and herbicides, greater accessibility to the cell, ultimately allowing them to interact with specific genes or proteins located within the bacterial host.

Another explanation for the variability observed in the luminescence assays may involve the *luxAB* genes themselves. Luciferase activity is easily assayable and extremely sensitive. The *luxAB* reporter gene system has been successfully used by our laboratory to identify toxic metal-inducible genes (Guzzo *et al.*, 1991; Guzzo and DuBow 1994a; Cai and DuBow, 1996). Moreover, the next chapter of this thesis will illustrate the effectiveness of the *luxAB* reporter gene system to detect arsenic-containing compounds in environmental samples. However, the compounds used in these studies were all inorganic, pure chemicals. Due to the extreme sensitivity of the luciferase enzyme and the different variables involved in the bioluminesence pathway, it is possible that reproducibility of the two- to five-fold increase in light emission, observed following exposure to bromacil, chlordane, or dinoseb may have been affected by various factors associated with the bioluminescent reaction.

Luciferase is a heterodimeric, mixed-function oxidase which catalyzes the following quantifiable bioluminescence reaction (Meighen, 1991):

$$FMNH_2 + RCHO + O_2 \Leftrightarrow FMN + H_2O + RCOOH + h\nu_{(490\ nm)}$$

This light emitting reaction involves an intracellular, luciferase-catalyzed oxidation of the reduced flavin mononucleotide ($FMNH_2$) and a long-chained aliphatic aldehyde by molecular oxygen (Stewart and Williams, 1992). From a biochemical viewpoint, bacterial bioluminescence requires oxygen, a source of energy, a luciferase enzyme, and a long chain fatty aldehyde (Stewart and Williams, 1992). The $FMNH_2$ required for the luminescence reaction is supplied by the bacterial cell, whereas the long-chain aldehyde may be added exogenously. The above reaction is highly specific for $FMNH_2$ and modification of the flavin ring or removal of the phosphate group decreases luciferase activity significantly (Meighen, 1991). Since the reaction catalyzed by luciferase consists of numerous variables such as oxygen, $FMNH_2$, and aldehyde, great care must be taken to ensure that these do not become rate-limiting (Slauch and Silhavy, 1991). It is possible that the new lots of chemicals, in addition to the component(s) responsible for the two- to five-fold increase in light emission, may have also contained different ratios of other components along with unidentified impurities. The presence of these other compounds may have elicited an inhibitory effect on luciferase expression and the bioluminescence reaction. These impurities may have altered oxygen or $FMNH_2$ levels in the cell or affected the reducing potential of the five clones. The occurrence of such phenomena may explain the variability observed in the luminescence assays. Future experiments may therefore include conducting luminescence assays following exposure of the clones to the individual, pure components which make up bromacil, chlordane, and dinoseb to attempt to determine which component(s) is responsible for the increases in light emission.

As a result of the sensitivity and the variables involved in luciferase expression and the bioluminescence pathway, future experiments should also focus on fusing the regulatory regions of our pesticide- and herbicide-

responsive clones to other reporter genes, such as the *lacZ* gene. The *lacZ* gene, encoding β-galactosidase, has been widely used in genetic fusion studies. Although not as sensitive as *luxAB*, the *lac* operon is one of the most understood reporter systems in which numerous genetic and biochemical aspects have been characterized and defined (Silhavy and Beckwith, 1985). Furthermore, since lactose, the substrate of the system, is a sugar which can be utilized by *E. coli* as a sole carbon source, there are several indicator media available for detecting β-galactosidase sugar metabolism (Silhavy and Beckwith, 1985). Furthermore, the compound o-nitrophenyl-β-d-galactosidase (ONPG) is also hydrolyzed by β-galactosidase, yielding o-nitrophenol, which is yellow in solution (Miller, 1992). Miller (1992) described an assay for β-galactosidase activity using this compound. This assay has been standardized such that *lacZ* reporter gene expression can be measured in defined units (Miller Units) (Miller, 1992). Construction of *lacZ* fusion clones which respond to bromacil, chlordane, and dinoseb may therefore be necessary to minimize *luxAB* artifacts (Higgins *et al.,* 1994). Other reporter genes which may be utilized in the study of our pesticide- and herbicide-responsive genes include the *galK* gene, which encodes galactokinase, the *cat* gene, encoding chloramphenicol acetyl transferase, and the green fluorescent protein (GFP) isolated from yeast (Slauch and Silhavy, 1991).

It should be noted that other laboratories have successfully used the *luxAB* reporter system to identify genes whose levels of transcription are altered by exposure to organic compounds (VanDyk *et al.,* 1994; Kobatake *et al.,* 1995; Burlage *et al.,* 1990; King *et al.,* 1990). These compounds, which include benzene, naphthalene, and toluene, are common organic solvents, easily obtainable in large quantities. Moreover, in these studies, the *luxAB* gene fusions were cloned onto multi-copy number plasmids within the host cell. Therefore, future experiments may also include cloning the pesticide- and herbicide-responsive gene fusions onto multi-copy number plasmids prior to

conducting luminescence assays in the presence of bromacil, chlordane, and dinoseb. Once these possibilities have been investigated and consistent, reproducible gene expression is observed upon exposure to the appropriate pesticide or herbicide, experiments should be designed to further characterize these genes and their products. Such experiments may provide valuable insight into the molecular mechanisms of toxicity of these pesticides and herbicides. Finally, following characterization, assays such as those described in the next chapter of this thesis, should be developed to test the potential applications of these gene fusions as environmental biosensors.

TABLE 1. Bacterial Strains and Plasmids

Strains/Plasmids	Characteristics	Reference
E.coli MG1655	F^-, λ^-	Jensen, 1993
E. coli NM522	[(*supE thi*)(*lac-proAB*)*hsd5* (r_{k-1},m_{k-}) (F'*proAB* *lac*^{q}Z)M15]	Gough and Murray, 1983
E. coli DH1	[F^- *recA1 gyrA96 thi* *hsdR17* (r_{k-1}, m_{k+}) *supE44* *relA1*]	Hanahan, 1983
E.coli LF20102	DH1 strain containing the *amp* ^{R} ColE1 RNA1 over-producing plasmid pTF421	Guzzo and DuBow, 1991
E. coli LF20204 (DIN1)	LF20102, Tn*5-luxAB::din1*	Costanzo, 1995
E. coli LF20205 (DIN2)	LF20102, Tn*5-luxAB::din2*	Costanzo, 1995
E. coli LF20206 (CHL1)	LF20102, Tn*5-luxAB*::*chl1*	Costanzo, 1995
E. coli LF20207 (CHL2)	LF20102, Tn*5-luxAB*::*chl2*	Costanzo, 1995
E. coli LF20208 (BRO)	LF20102, Tn*5-luxAB*::*bro*	Costanzo, 1995
E. coli LF20209	MG1655 x LF20204 (P1)	this study
E. coli LF20210	MG1655 x LF20206 (P1)	this study
E. coli LF20211	MG1655 x LF20207 (P1)	this study
pTF421	pACYC177 based plasmid, *amp* ^{R}, over-produces RNA1	Fitzwater *et al.*, 1984
pFUSLUX	ColE1based plasmid, *tet* ^{R} *luxAB* from *Vibrio harveyi*	Guzzo and Dubow, 1991

Table 2: Types of Luminescence Assays Performed

Clones	Media	Temperature	Concentration range	No. Times Performed	Luminescence Pattern	Date
CHL1, CHL2	LB-agar[a]	23°C	0, 1, 10 µg/ml	3	dose-dependent increase	09/95
DIN1, DIN2	LB-agar	23°C	0, 1, 10 µg/ml	3	dose-dependent increase	09/95
BRO	LB-agar	23°C	0, 1, 10 µg/ml	3	dose-dependent increase	09/95
CHL1, CHL2	LB-agar LB-noble[b] TCMG[c]-agar TCMG-noble M9[d]-agar M9-noble	23°C 15°C 37°C	0, 1, 10 µg/ml 0, 0.001, 0.005, 0.01 µg/ml	>30	fluctuations	09/96-08/97
DIN1, DIN2	LB-agar LB-noble TCMG-agar TCMG-noble M9-agar M9-noble	23°C 15°C 37°C	0, 1, 10 µg/ml 0, 0.1, 1, 2, 5, 5, 10, 20 µg/ml	>30	fluctuations	09/96-08/97
BRO	LB-agar LB-noble TCMG-agar TCMG-noble M9-agar M9-noble	23°C 15°C 37°C	0, 1, 10 µg/ml 0, 0.1, 1, 2, 5, 5, 10, 20 µg/ml	>30	fluctuations	09/96-08/97

[a]: purchased from Anachemia (Montréal, Canada)

[b]: purchased from Difco (Montréal, Canada)

[c]: see Materials and Methods

[d]: see Materials and Methods

Figure 1. Light emission of 1-cm^2 patches of strains LF20204 (DIN1), LF20205 (DIN2), LF20206 (CHL1), LF20207 (CHL2), and LF20208 (BRO), grown in the presence of 0, 1, and 10 ppm (μg/ml) of the herbicides, bromacil and dinoseb, and the pesticide, chlordane. See Materials and Methods for experimental details. Taken from Costanzo, 1995.

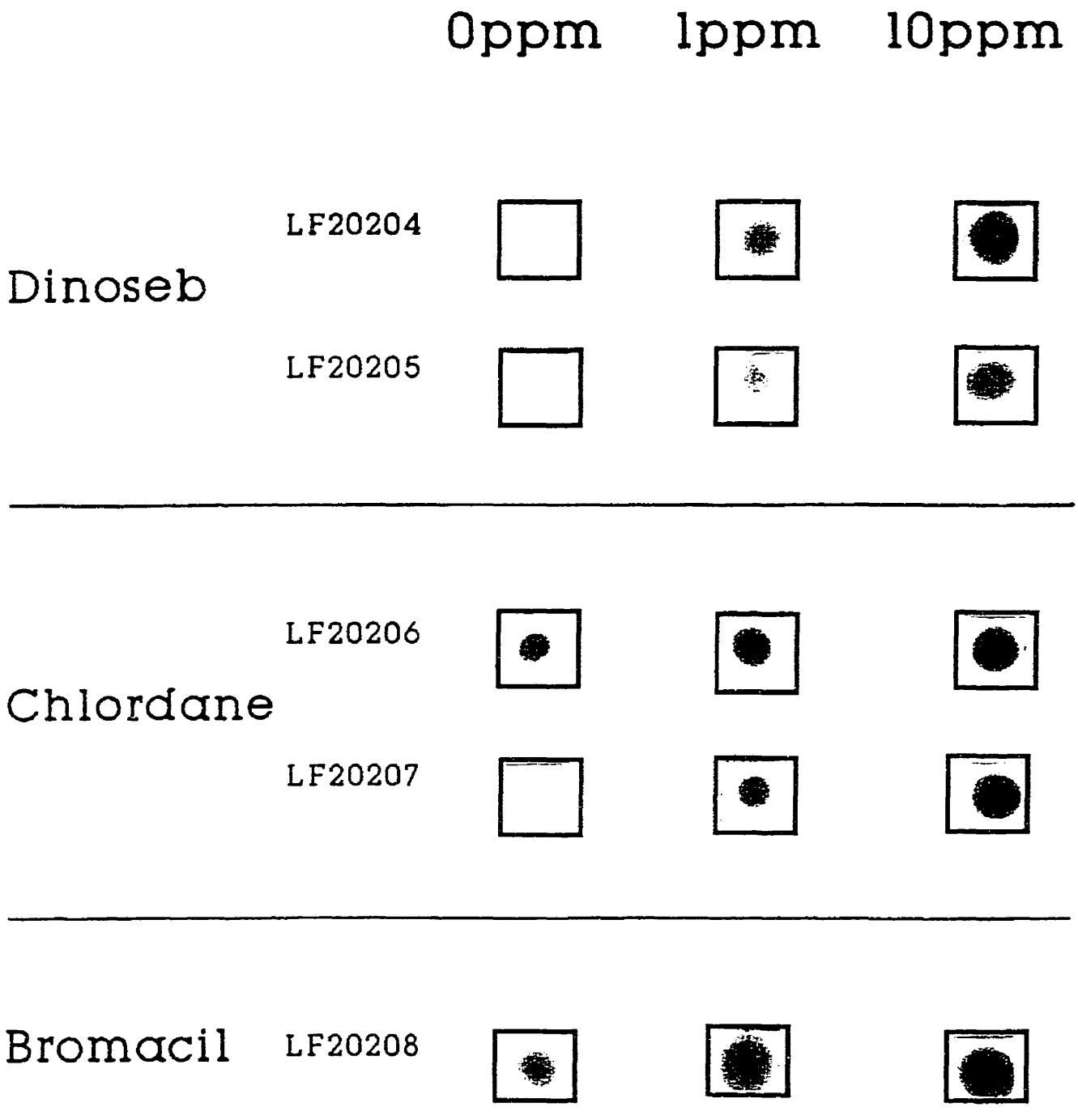
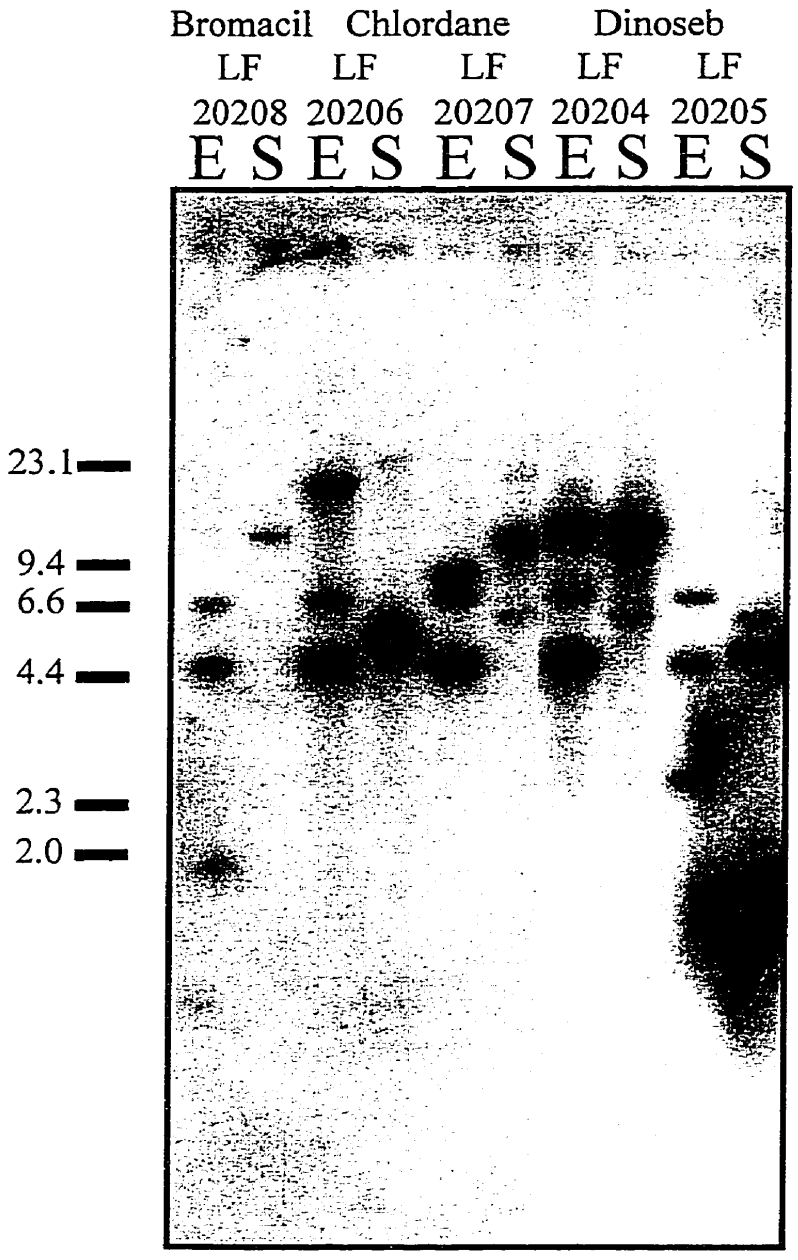

0ppm
1ppm
10ppm
Dinoseb
LF20204
LF20205
Chlordane
LF20206
LF20207
Bromacil
LF20208

Figure 2. (A) Southern blot analysis of *Eco*RI- and *Sal*I-cleaved cellular DNA from clones LF20208 (BRO), LF20206 (CHL1), LF20207 (CHL2), LF20204 (DIN1) and LF20205 (DIN2). Sizes in kb of marker DNAs are indicated at the left. Restriction enzymes used are abbreviated as follows: *Eco*RI (E), and *Sal*I (S). See Materials and Methods for experimental details. (B) Schematic diagram of the Tn*5-luxAB* element inserted within the host chromosome. *Eco*RI and *Sal*I sites are indicated. Adapted from Costanzo, 1995.

A)


Bromacil
Chlordane
Dinoseb
LF 20208
LF 20206
LF 20207
LF 20204
LF 20205
E S E S E S E S E S
23.1
9.4
6.6
4.4
2.3
2.0

B)

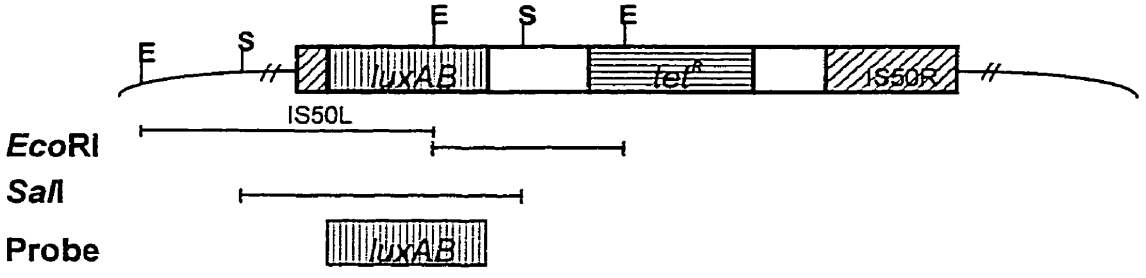
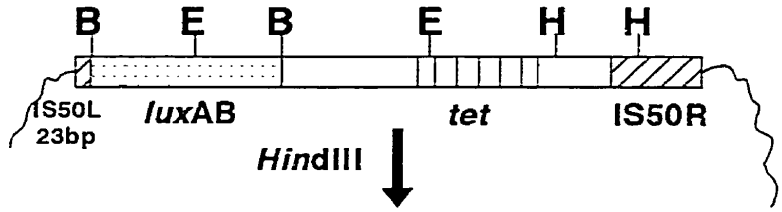
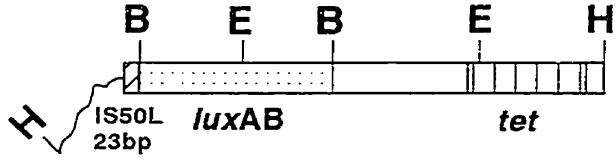

E
S
E
S
E
IUXAB
tet
IS50R
IS50L
EcoRI
SalI
Probe
IUXAB

Figure 3. Schematic diagram for the cloning and sequencing of the herbicide- and pesticide-responsive genes. Restriction enzyme sites are abbreviated as follows: *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). See Materials and Methods for experimental details. Taken from Costanzo, 1995.

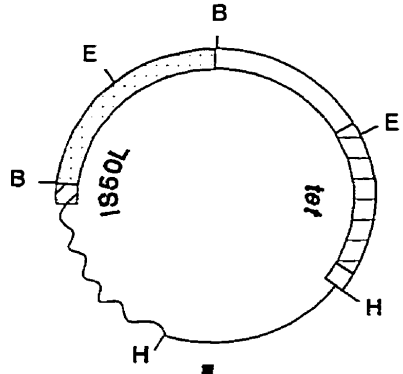


*Hin*dIII



ligate to pUC119/*Hin*dIII

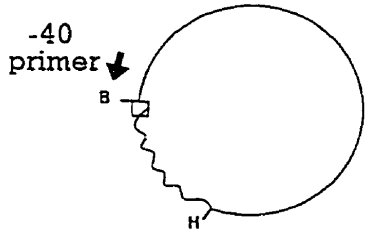
Select for *tet*^{r}



Isolate *Bam*HI/*Hin*dIII fragment clone into pUC119/*Bam*HI-*Hin*dIII

Isolate ssDNA

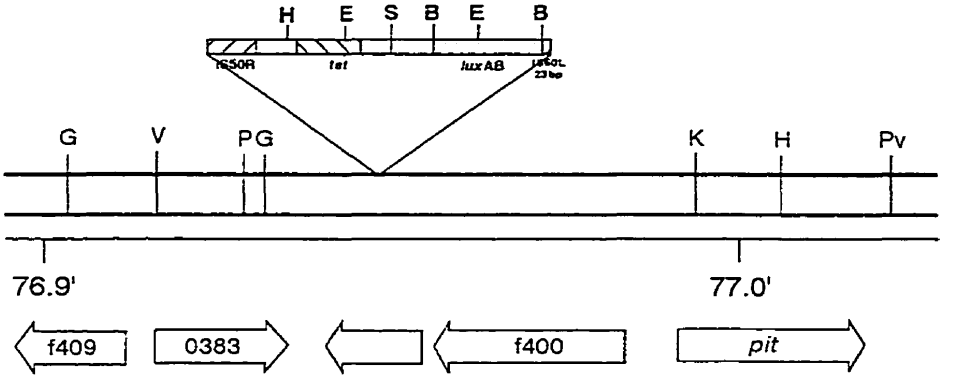
Sequence using primer hybridizing to pUC119



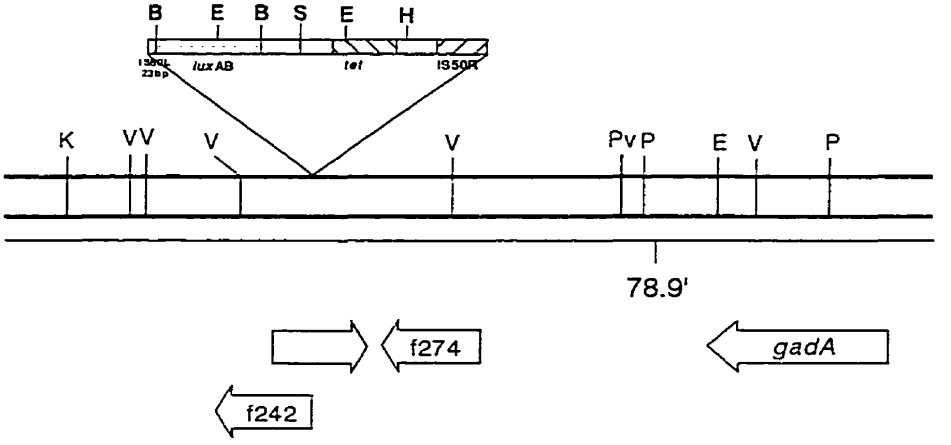
Determine region (and DNA sequence) of *E. coli* chromosome

Figure 4. Maps illustrating Tn*5-luxAB* insertion sites in relation to the published restriction map of *E. coli* (Rudd, 1992) for clones (A) CHL1 (LF20206), (B) CHL2 (LF20207) and (C) DIN1 (LF20204). Numbers below maps indicate minutes on the *E. coli* chromosome. Unshaded Arrows indicate proximal identified genes in their study and their orientations. Shaded arrows represent genes characterized in this study and their orientation. Restriction enzyme sites are abbreviated as follows: *Bam*HI (B), *Eco*RI (E), *Sal*I (S), *Hin*dIII (H), *Bgl*I (G), *Eco*RV (V), *Pst*I (P), *Kpn*I (K), and *Pvu*II (Pv). Taken from Costanzo, 1995.

A)


H E S B E B
IS50R
tet
luxAB
IS50L 23bp
G V PG K H Pv
76.9'
77.0'
f409
0383
f400
pit

B)


B E B S E H
IS50L 23bp
luxAB
tet
IS50R
K VV V V Pv P E V P
78.9'
f274
gadA
f242

C)

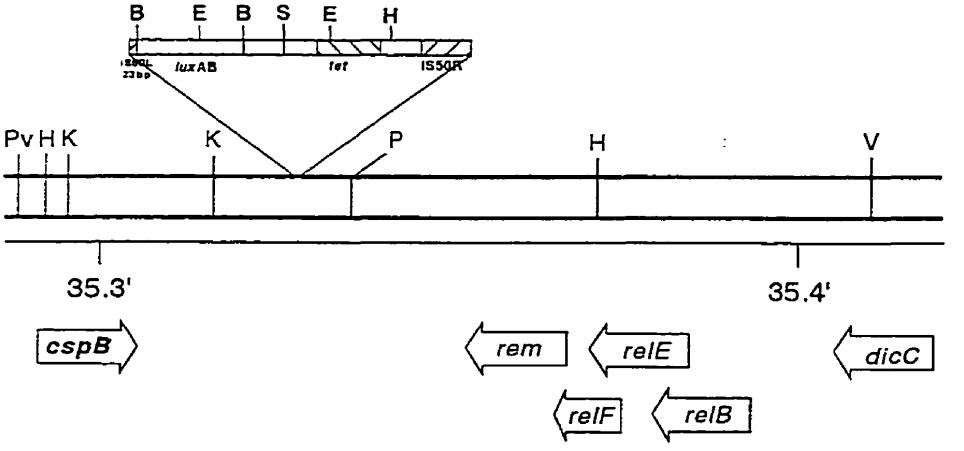

B E B S E H
IS50L 23bp
luxAB
tet
IS50R
Pv H K K P H V
35.3'
35.4'
cspB
rem
relE
dicC
relF
relB

Figure 5. Nucleotide sequence of the region adjacent to the Tn*5-luxAB* insertion of clone CHL1 (LF20206). Amino acid sequence of the putative protein encoded within this region is illustrated below the nucleotide sequence. **Tn*5*** indicates the exact site of insertion Translation begins at an ATG codon and terminates at a TAA codon. -35 and -10 promoter consensus sequences are underlined and typed in bold face. Taken from Costanzo, 1995.

```
  1  TGA CCT ATT TCC TAA AGA TGA AAA TTA TTA GAG TCG CAT TAA AAA TGA GCT AAT

 55  TTT GAT AGT GGT TAT CTT GTG ATT ATT TTC TAA TGA GCC CGT GAA CTG AAA CCC
        -35                                    -10

109  TCC ATG CTT AAT ATA AGG TGG ATG GAA AGG TGA TTG AAA ACT CAC TCA GTG GCC

163  AGA TCT TTA TGA AAT ATG AAC AGG TTG AAA AAA ACA GTA ACT TTG CTG TTT TTT
  1                  Met Asn Arg Leu Lys Lys Thr Val Thr Leu Leu Phe Phe

217  ATA CTT AAC CAC TAT GCA TTA ATG CTG CGT TAT TTC ATG ATG CCT AAG AAA AAC
 14  Ile Leu Asn His Tyr Ala Leu Met Leu Arg Tyr Phe Met Met Pro Lys Lys Asn

271  CAG CGT TAC GCA AAT GGT CAA CGC TGG TTT TAT CCG GTA CGT TGC AAT TAT TTT
 32  Gln Arg Tyr Ala Asn Gly Gln Arg Trp Phe Tyr Pro Val Arg Cys Asn Tyr Phe

325  TTA GCA GAA CCC GCT TCT AAT ATT GAA ACG ATT GAG AAC AAC GTA AAG CAT ACC
 50  Leu Ala Glu Pro Ala Ser Asn Ile Glu Thr Ile Glu Asn Asn Val Lys His Thr

            Tn5
                V
379  GCT CCC AAA CCG ACC AGA ACT CGC GAA GGA ATA AAA TAT CCC ATG TCG GTC TGC
 68  Ala Pro Lys Pro Thr Arg Thr Arg Glu Gly Ile Lys Tyr Pro Met Ser Val Cys

433  GCC ATT TCC GCA AGA AAC GAT GCC AGG AAA AGG CAA AAC AGG CAG GTG AAG ACG
 86  Ala Ile Ser Ala Arg Asn Asp Ala Arg Lys Arg Gln Asn Arg Gln Val Lys Thr

487  GGA ATC ATC GGT ATT CTG TTG GCT AAC GAA CAG GTA CGT CTC CAT ACC AGT GCC
104  Gly Ile Ile Gly Ile Leu Leu Ala Asn Glu Gln Val Arg Leu His Thr Ser Ala

541  AGT AGC CAG ACT TTT GAG AAT ATG CTG TAA CAG ATC ATT CCG AGG CAA ATA AGA
122  Ser Ser Gln Thr Phe Glu Asn Met Leu ***

595  ATA ATG CCG GGA GCC AGT CGG GCG CTT GCA TCG GAA CTG ACT AAG ACG TAT ATC

649  CCC TGC AGT ACC GTG ATT GAG CCG AGA AAA ATA ACC CAA TAA CAC CAC AGC CAG

703  TGT TCT TTA GTT GAA AAC GTA TTA CGT
```

Figure 6. Light emissions of 1-cm^2 patches of strains LF20204 (DIN1), LF20205 (DIN2), LF20206 (CHL1), LF20207 (CHL2), and LF20208 (BRO), grown in the presence of 0, 1, and 10 PPM (mg/ml) of the herbicides, bromacil and dinoseb, and the pesticide, chlordane. See Materials and Methods for experimental details.

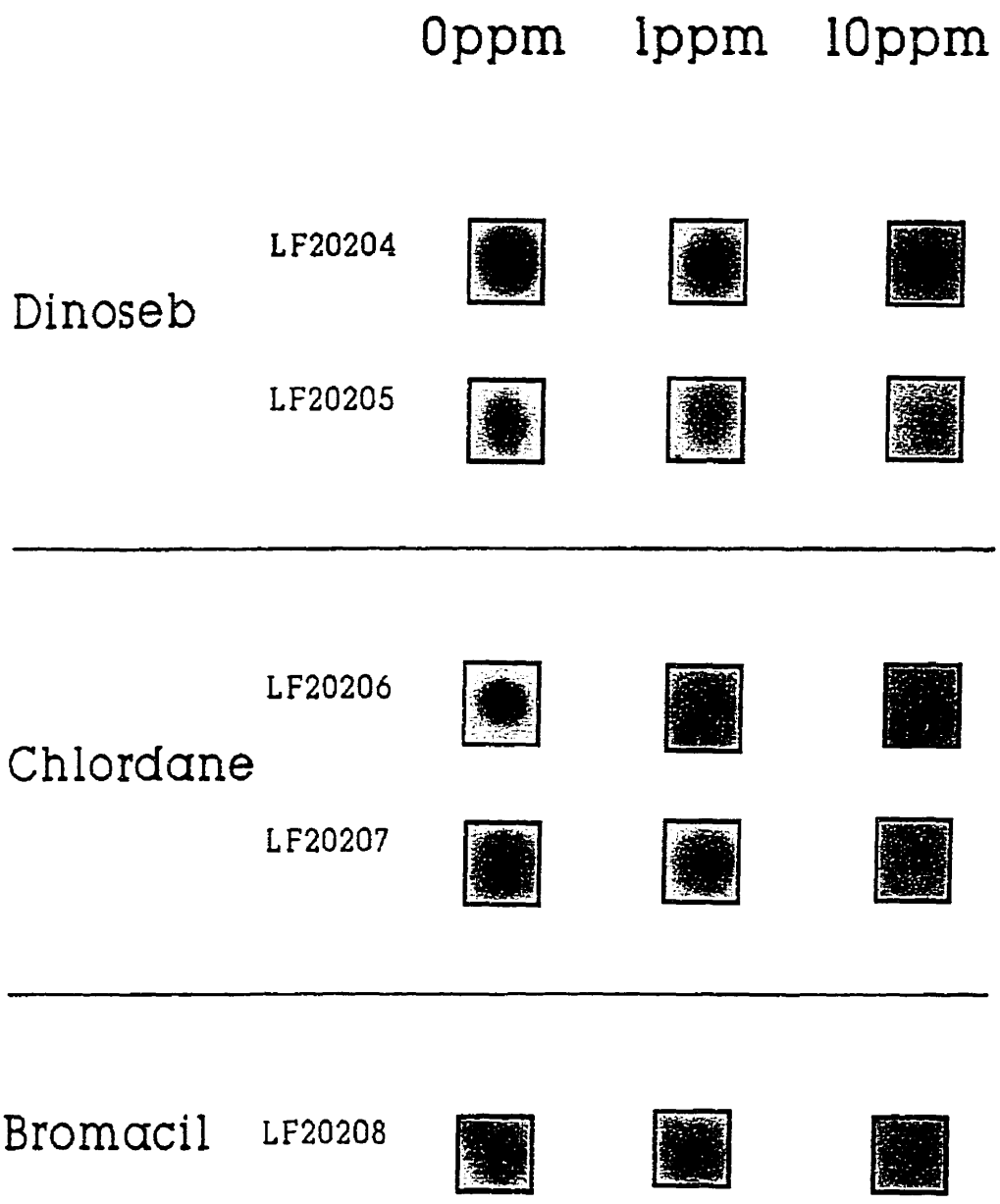
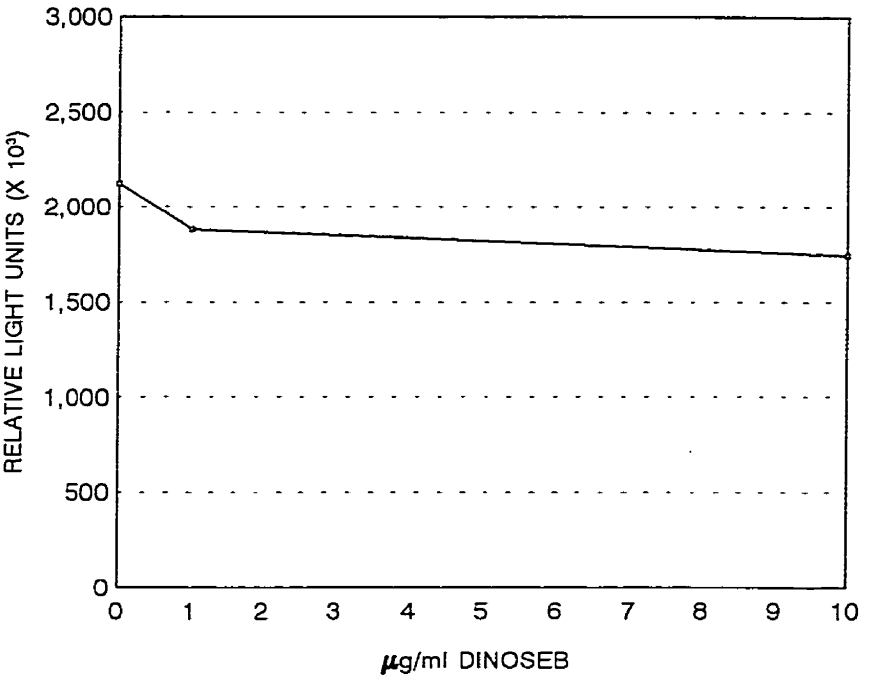

0ppm
1ppm
10ppm
LF20204
Dinoseb
LF20205
LF20206
Chlordane
LF20207
Bromacil
LF20208

Figure 7. Densitometric analysis of luminescence emitted by strains (A) DIN1 and (B) DIN2 grown in the presence of 0, 1, and 10 PPM (mg/ml) dinoseb.

A)


3,000
2,500
2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml DINOSEB

B)

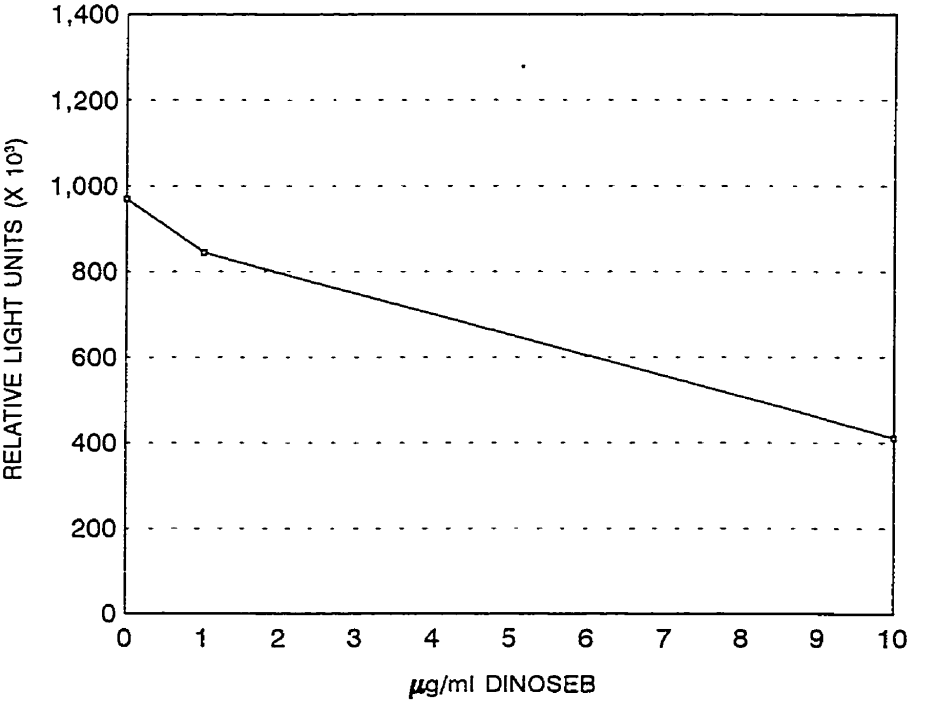
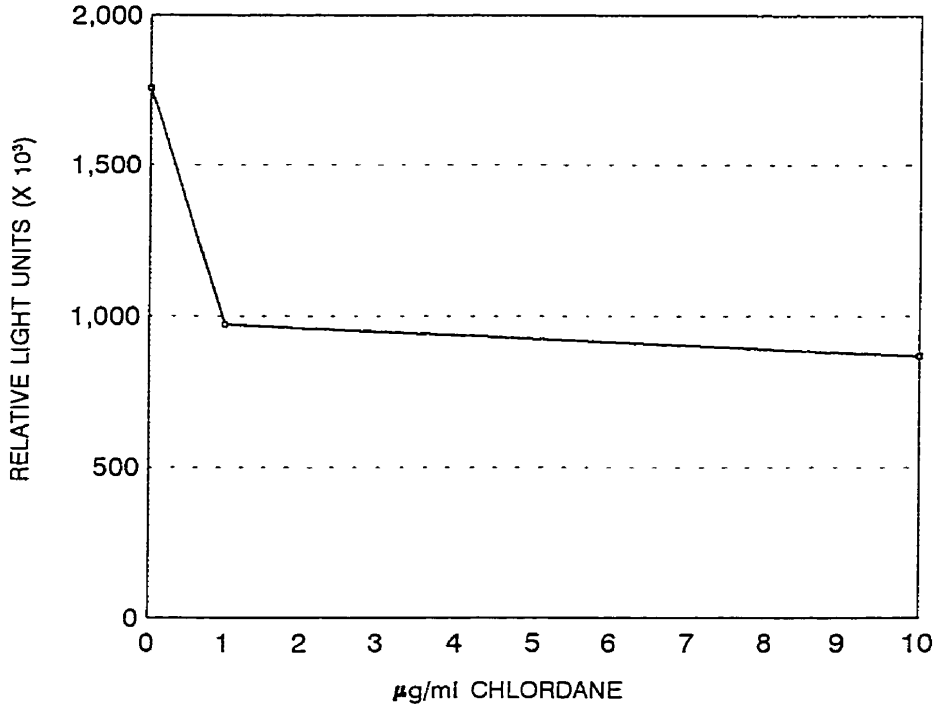

1,400
1,200
1,000
800
600
400
200
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml DINOSEB

Figure 8. Densitometric analysis of luminescence emitted by strains (A) CHL1 and (B) CHL2 grown in the presence of 0, 1, and 10 PPM (mg/ml) chlordane.

A)


2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml CHLORDANE

B)

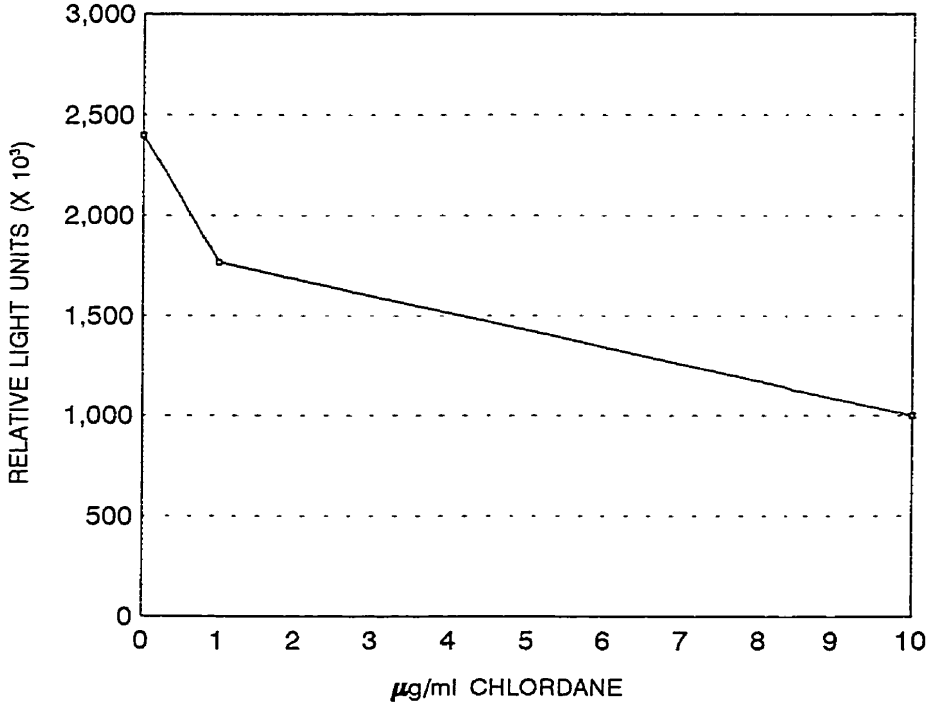

3,000
2,500
2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml CHLORDANE

Figure 9. Densitometric analysis of luminescence emitted by strain BRO grown in the presence of 0, 1, and 10 PPM (mg/ml) bromacil.

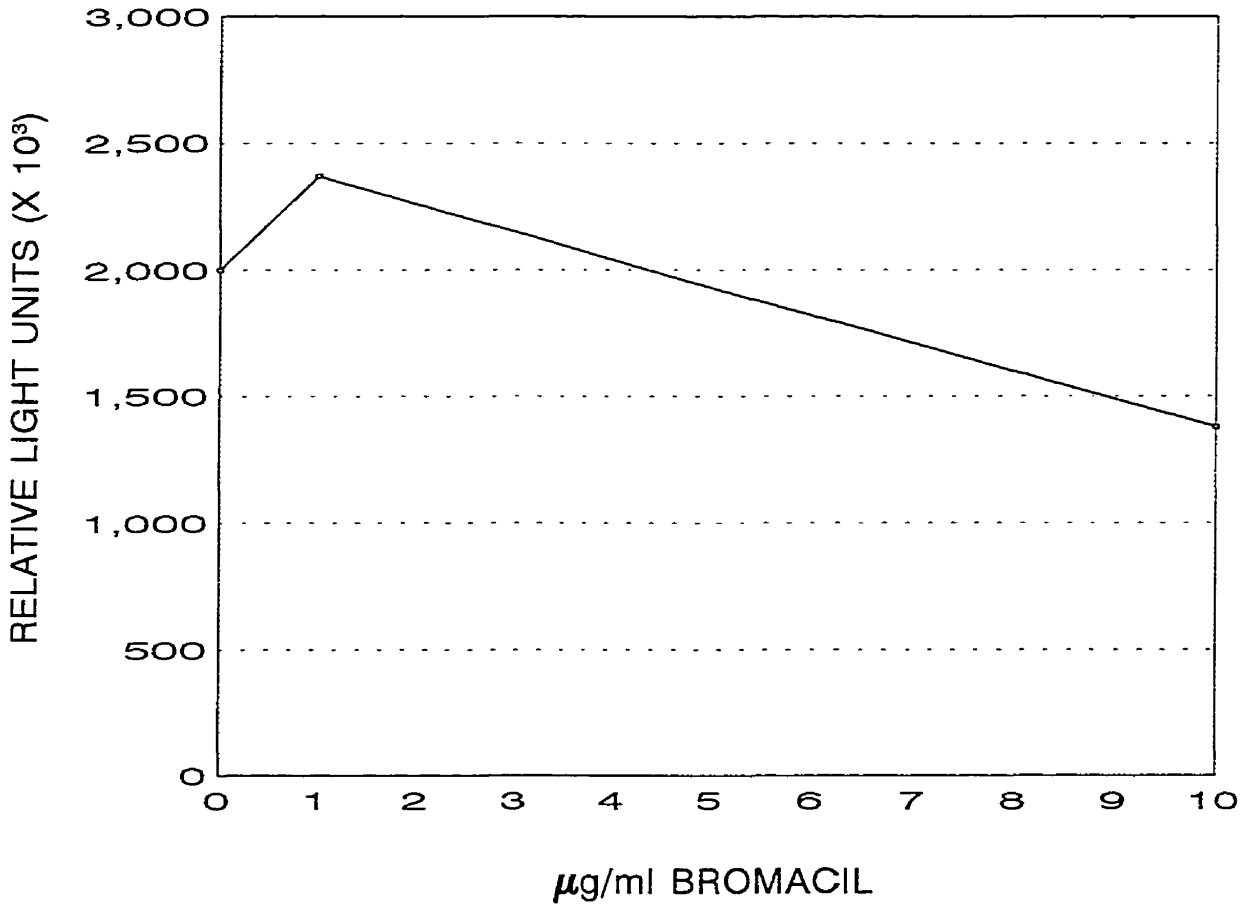
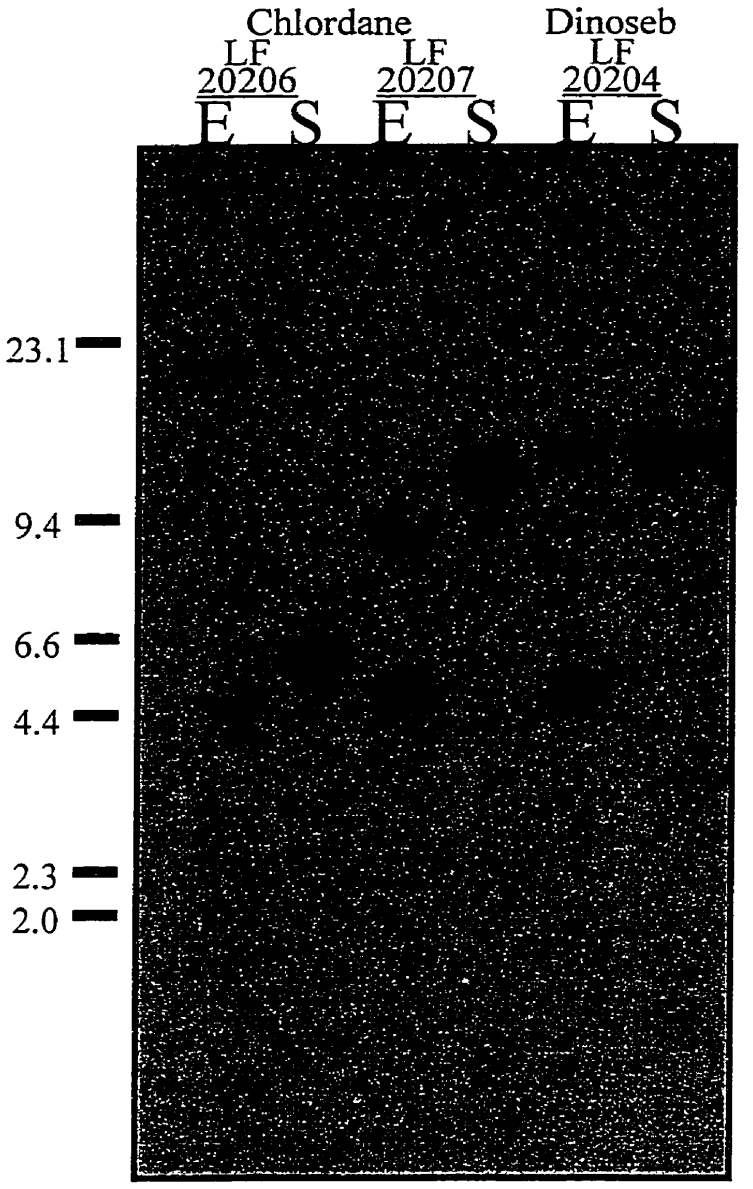

3,000
2,500
2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0
1
2
3
4
5
6
7
8
9
10
µg/ml BROMACIL

Figure 10. (A) Southern blot analysis of *Eco*RI- and *Sal*I-cleaved cellular DNA from clones CHL1 (LF20206), CHL2 (LF20207), and DIN1 (LF20204). Sizes in Kb of marker DNAs are indicated at the left. Restriction enzymes used are abbreviated as follows: (E) *Eco*RI, and (S) *Sal*I. See Materials and Methods for experimental details. (B) Schematic diagram of the Tn*5-luxAB* element inserted within the host chromosome. *Eco*RI and *Sal*I sites are indicated

A)


Chlordane
Dinoseb
LF 20206
LF 20207
LF 20204
E S E S E S
23.1
9.4
6.6
4.4
2.3
2.0

B)

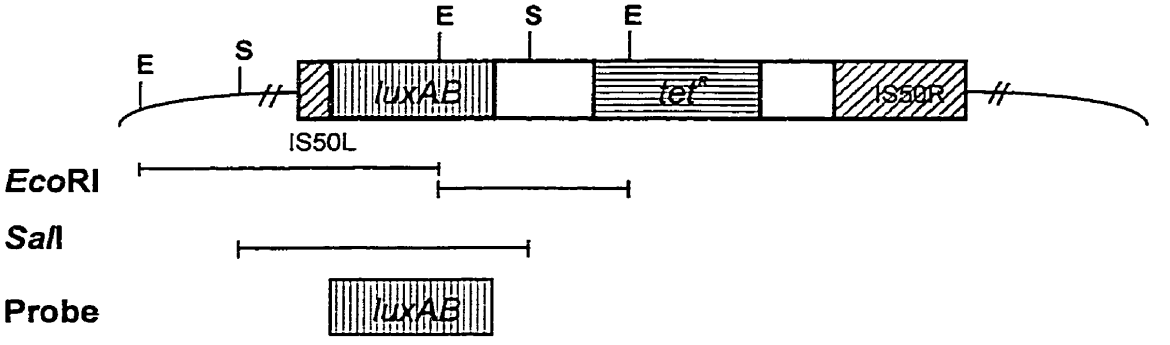

E
S
E
S
E
luxAB
tet
IS50R
IS50L
EcoRI
SalI
Probe
luxAB

Figure 11. Light emissions of 1-cm^2 patches of MG1655 transductant strains LF20209 (DIN1), LF20210 (CHL1), and LF20211 (CHL2), grown in the presence of 0, 1, and 10 PPM (mg/ml) of the herbicide, dinoseb, or the pesticide, chlordane. See Materials and Methods for experimental details.

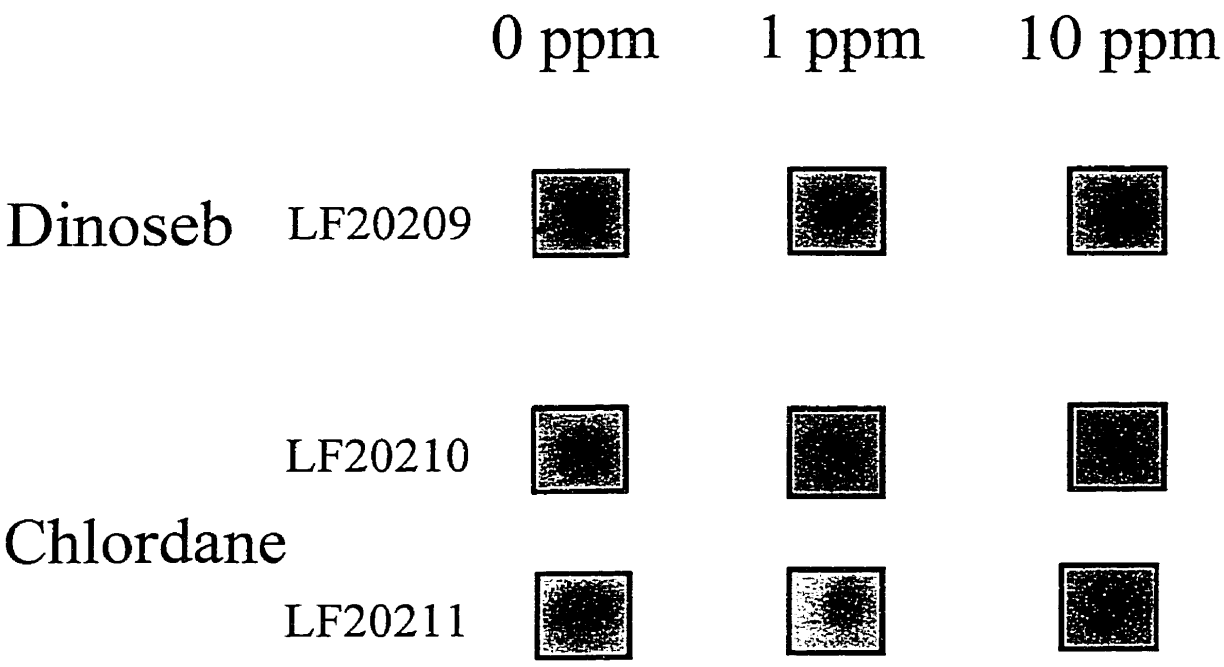

0 ppm
1 ppm
10 ppm
Dinoseb
LF20209
LF20210
Chlordane
LF20211

Figure 12. Densitometric analysis of luminescence emitted by the MG1655 transductant strain, DIN1 (LF20209) grown in the presence of 0, 1, and 10 PPM (mg/ml) dinoseb.

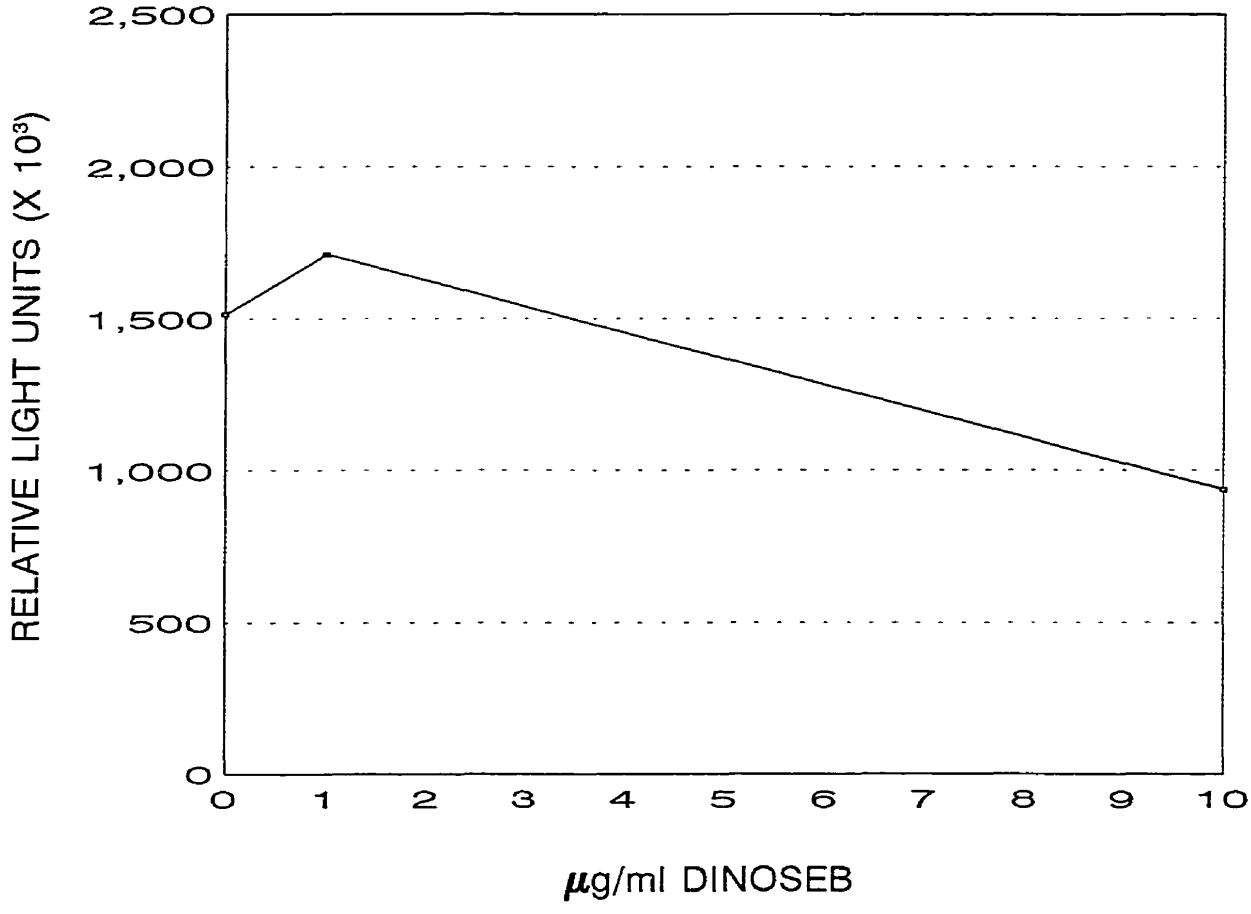
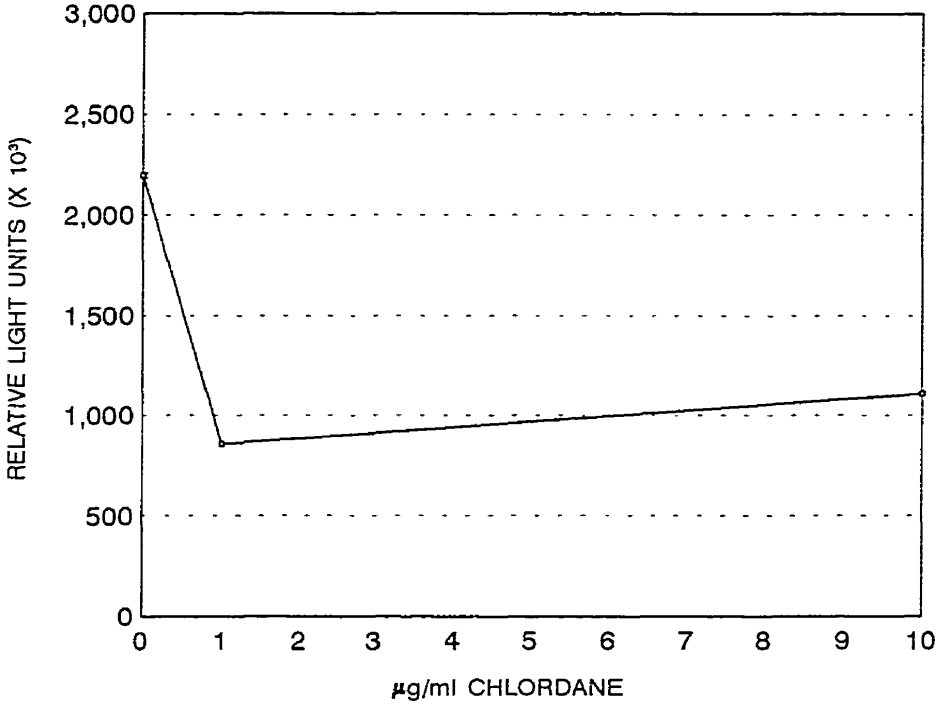
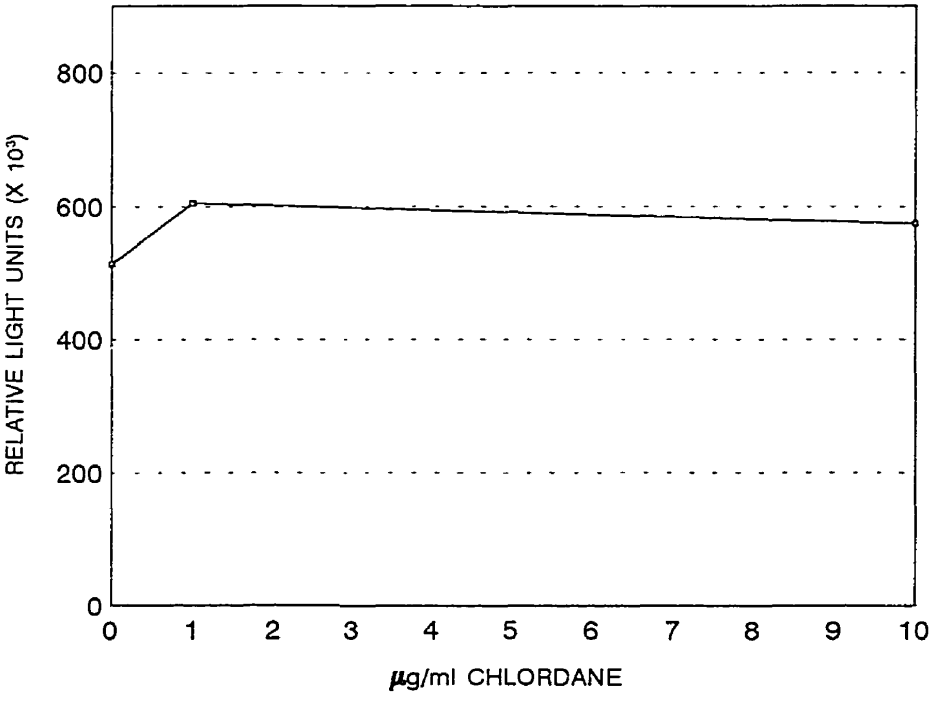

2,500
2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0
1
2
3
4
5
6
7
8
9
10
µg/ml DINOSEB

Figure 13. Densitometric analysis of luminescence emitted by MG1655 transductant strains (A) CHL1 (LF20210) and (B) CHL2 (LF20211) grown in the presence of 0, 1, and 10 PPM (mg/ml) chlordane.

A)


3,000
2,500
2,000
1,500
1,000
500
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml CHLORDANE

B)


800
600
400
200
0
RELATIVE LIGHT UNITS (X 10³)
0 1 2 3 4 5 6 7 8 9 10
µg/ml CHLORDANE

CHAPTER III

Use of an Arsenic Oxyanion-responsive *Escherichia coli* Luciferase Clone for the Detection of Arsenic Compounds in an Environmental Aquatic Sample.

INTRODUCTION

Arsenic is a ubiquitous element with metalloid properties. In water, arsenic is usually found in its trivalent (arsenite) or pentavalent (arsenate) form (Andrae, 1977). The toxic properties of these arsenic oxyanions have been well-documented. These include acute, subacute, and chronic effects involving the respiratory, gastrointestinal, cardiovascular, nervous, and haematopoietic systems (Done and Peart, 1971; Woods and Fowler, 1977; Hine *et al.,* 1977). Adverse health effects of arsenic may therefore be local or systemic. Acute and subacute effects caused by the ingestion of inorganic arsenic compounds, mainly arsenic (III) oxide, are also well-documented in the literature. The major lesion is profound gastrointestinal damage (Hine *et al.,* 1977). Other acute symptoms and signs include muscular cramps, facial oedema, and cardiac abnormalities (Done and Peart, 1971). Chronic effects, although not as well studied, include local damage to the respiratory system, skin, liver, cardiovascular and nervous systems upon inhalation and/or ingestion of arsenic compounds (Hameda and Horiguchi, 1976). There is also sufficient evidence to associate exposure to inorganic arsenic with carcinomas of the lung and skin (Blot and Fraumeni, 1972; Friberg and Cederlof; 1978; Jackson and Grarnge, 1975; Lee and Frauneni, 1969; Mabuchi *et al.,* 1979; Sommers and McManus, 1953; Reymann *et al.,* 1978). Given the devastating effects of these inorganic arsenic oxyanions, the sensitive and rapid detection of arsenic in the environment is essential.

In 1973, Hedges and Braumberg (1973) found that gram positive and gram negative bacteria both have plasmid-mediated resistance to arsenic and antimony. It was later discovered that this resistance was due to a cluster of genes, termed the *ars* operon. The plasmid-encoded *ars* operon is made up of five open reading frames, designated *arsRDABC*. This operon is transcribed as a single transcriptional unit initiated at a site just upstream of *arsR*. ArsR is

a *trans*-acting repressor which controls basal level of expression of the operon (SanFrancisco *et al.*, 1990; Wu and Rosen, 1991; Wu and Rosen, 1993a). ArsD is a negative regulator of the operon (Wu and Rosen, 1993b). ArsA encodes an arsenite- and antimonite-inducible ATPase (Rosen *et al.*, 1988; Mei-Hsu *et al.*, 1991). ArsB encodes a membrane pump for efflux of arsenite and antimonite ions (Wu and Rosen, 1992; Dey and Rosen, 1995). Finally, ArsC encodes an arsenate reductase (Rosen *et al.*, 1991; Gladysheva *et al.*, 1994). Several groups have recently found a chromosomally-located *ars* operon homologue in *Escherichia coli* and other gram negative bacterial species (Sophia *et al.*, 1994; Carlin *et al.*, 1995; Diorio *et al.*, 1995). This finding established a potential evolutionary relatedness between the plasmid-encoded and the chromosomal *ars* operons in bacteria, the only difference being that the *E. coli* chromosomal *ars* operon comprises three open reading frames which share high homology with the *arsR*, *arsB*, and *arsC* genes. Finally, expression of this operon in *E. coli* has been shown to be induced by arsenic and antimonite oxyanions and the inducibility of arsenical compounds appears to reflect their relative toxicity (Diorio *et al.*, 1995; Cai and DuBow, 1996).

In this study, we therefore examined whether the *arsB*::*luxAB* clone, described by Cai and DuBow (1996), could be used in the monitoring of aquatic samples for arsenical compounds. These results were also compared with results from ß-galactosidase assays in which an *arsB*::*lacZ* fusion clone was used to monitor the same contaminated samples. Finally, results obtained from these biosensor experiments were compared to data from chemical analyses performed by Environment Canada.

MATERIALS AND METHODS

1.0. Bacterial Strains and Growth Media

Table 1 describes bacterial strains and plasmids used. Media used in the luminescence assays include Luria Bertani Broth (LB) [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto yeast extract, 2 N NaOH] (unless otherwise specified). Antibiotics were used at the following concentrations: ampicillin (0.04 mg/ml) and tetracycline (0.01 mg/ml).

2.0. Liquid Luminescence Assay

Escherichia coli strains, LF20012, MG1655, and 40 were grown at 37°C overnight in 10 ml of Luria Bertani (LB) broth A solution of 2X LB broth [2% (w/v) tryptone, 2% (w/v) NaCl, 1% (w/v) Bacto-yeast extract] was prepared. The overnight *E. coli* cultures were diluted 20-fold into the autoclaved solution of 2X LB broth containing tetracyline. The bacterial cultures were then divided into 5 flasks and grown for 1 h at 37°C to ensure logarithmic growth of the culture (A_{600}=0.2-0.3). Cells were then exposed to equal volumes of either a 0.1 μg/ml (PPM) arsenite solution (American Chemical Ltd., Montréal, Canada), a 1.0 μg/ml (PPM) arsenate solution (Fisher Scientific, Ltd., Montréal, Canada), an equal volume of filtered and unfiltered arsenic-contaminated water (Goodfellow Effluent, Environment Canada), or an equal volume of sterile delH_2O. Samples of contaminated water effluent were collected by Environment Canada from the St. Lawrence River, Québec (Goodfellow Effluent). Samples of contaminated water were filtered using 0.45μm nitrocellulose membranes. Samples contained tetracycline at a final concentration of 0.01 mg/ml. All samples were then incubated for 1.5 h at 37°C. Following incubation, samples were diluted in LB broth to a final A_{600} of 0.05 and then aliquoted into luminometer cuvets to be measured in a Tropix Optocomp I luminometer (MGM Instruments, Hamden, Conn.). Total relative

light units (RLUs) were determined for 10-s intervals upon injection of 100 μl of dodecyl aldehyde (diluted 1:100 LB broth) (Aldrich, Milwaukee, USA). Luciferase activity is expressed as the number of photons emitted per second per A_{600} unit.

3.0. β-galactosidase Assay

E. coli strains LF20001, MG1655 and 40 were grown at 32°C overnight in LB broth in the presence of ampicillin (0.04 mg/ml). Eighty μl of the overnight cultures were then added to 5 ml fresh medium containing either 0.1 μg/ml (PPM) arsenite (American Chemicals Ltd., Montréal, Canada), 1.0 μg/ml (PPM) arsenate (Fisher Scientific Ltd., Montréal, Canada), an equal volume of filtered and unfiltered arsenic-contaminated water (Goodfellow Effluent, Environment Canada), or an equal volume of sterile delH_2O. Cells were then grown at 32°C until they reached early exponential phase (2-5 X 10^8 cells/ml). The cultures were then cooled on ice for 20 min. The cell density was recorded (A_{600}). An aliquot of 0.2 ml of each culture was immediately added to 1.8 ml Z buffer [60 mM $Na_2HPO_4 \bullet 7\ H_2O$, 40 mM $NaH_2PO_4 \bullet H_2O$, 10 mM KCl, 1 mM $MgSO_4 \bullet 7\ H_2O$, 50 mM β-mercaptoethanol]. Eighty μl of chloroform and 40 μl of a 0.1% sodium dodecyl sulfate (SDS) solution were added to each assay mixture. Tubes were vortexed for 10 seconds and placed in a 28°C water bath for 5 min. The reaction was started by adding 0.4 ml ONPG [4 mg/ml in 100 mM phosphate buffer]. After sufficient yellow colour had developed, the time of the reaction was recorded. The reaction was stopped by adding 1 ml of a 1 M Na_2CO_3 solution. A control solution containing 0.2 ml ONPG and 1.8 ml Z buffer was also incubated along with the basal-level cultures. The optical density was recorded (A_{420}). Light scattering was corrected for by obtaining the absorbance at another wavelength (A_{550}) where there is no contribution from o-nitrophenol. β-galactosidase enzyme units were calculated as follows: $1000X(OD_{420} - 1.75XOD_{550}) / t\ X\ v\ X\ OD_{600}$ (Miller, 1992).

RESULTS

Luminescence Assays With Filtered and Unfiltered Arsenic-Contaminated Water Samples

The biosensor potential of a previously constructed *arsB*::*luxAB E. coli* clone (Diorio *et al.,* 1995; Cai and DuBow, 1996) was tested for its ability to detect the presence of arsenic compounds in a contaminated water sample (Goodfellow Effluent, Environment Canada). Luminescence assays were performed using three *E. coli* strains, *E. coli* 40 (Δ*lac*), *E. coli* MG1655 (wt), and the *arsB*::*luxAB E. coli* fusion clone, LF20012. Light emitted from the three strains was measured following their exposure to sterile delH_2O, a 0.1 PPM arsenite solution, a 1.0 PPM arsenate solution, an unfiltered sample of the arsenic-contaminated water effluent, and a filtered sample derived from the same arsenic-contaminated effluent.

E. coli LF20012, containing the *arsB*::*luxAB* chromosomal fusion, exhibited a 17-fold increase in light emission, following a 1.5 hr exposure to the 0.1 PPM arsenite solution and to the 1.0 PPM arsenate solution (Figure 1). Increases in light emission were also observed following exposure of strain LF20012 to the filtered and unfiltered arsenic-contaminated water effluents. Exposure to the unfiltered water sample resulted in a 3-fold increase in luminescence, while exposure to the filtered water sample resulted in a 4-fold increase in light emitted from the *E. coli* LF20012 culture (Figure 1). Only basal levels of light emission were observed when the *E. coli luxAB* fusion clone was grown in the presence of sterile delH_2O (Figure 1). Unlike *E .coli* strain LF20012, *E. coli* 40 and MG1655 did not exhibit any significant increases in luminescence (data not shown).

β-galactosidase Assays With Filtered and Unfiltered Arsenic-Contaminated Water Samples

β-galactosidase assays were performed using three *E. coli* strains, *E. coli* 40, *E. coli* MG1655, and the *arsB*::*lacZ E. coli* fusion clone, LF20001. β-galactosidase activity was measured following exposure to sterile delH_2O, a 0.1 PPM arsenite solution, a 1.0 PPM arsenate solution, an unfiltered sample of the arsenic-contaminated water effluent, and a filtered sample derived from the same arsenic-contaminated effluent. MG1655 exhibited basal level β-galactosidase activity. Furthermore, this level of activity was not affected by the presence of the arsenite, arsenate, or the arsenic-contaminated water effluent samples (Figure 2). *E. coli* 40, a Δ*lac* strain, did not exhibit any β-galactosidase activity (Figure 2). Unlike *E. coli* MG1655 and *E. coli* 40, *E. coli* LF20001 exhibited a 34-fold increase in β-galactosidase activity following exposure to the 0.1 PPM arsenite solution and a 31-fold increase in the presence of the 1.0 PPM arsenate solution (Figure 2). Exposure to the filtered arsenic-contaminated water sample resulted in a 5-fold increase in β-galactosidase activity of the *E. coli lacZ*::*arsB* fusion clone (Figure 2). The unfiltered water sample also stimulated a 5-fold increase in β-galactosidase (Figure 2). Finally, sterile delH_2O did not stimulate any β-galactosidase activity in the *arsB*::*lacZ* fusion clone (Figure 2).

DISCUSSION

In this study, we examined the potential of the *Escherichia coli arsB*::*luxAB* clone (LF20012) described by Cai and DuBow (1996), and the *Escherichia coli arsB*::*lacZ* clone (LF20001) described by Diorio *et al.* (1995), to detect the presence of arsenical compounds in aquatic samples. The results obtained from these experiments were then compared with chemical data obtained from Environment Canada.

In the absence of any arsenic-containing solutions, the *arsB::luxAB* clone exhibited basal levels (less than 1 X 10^6 RLUs) of light emission (Figure 1). In previous experiments, it was shown that concentrations of 0.1 PPM arsenite and 1.0 PPM arsenate maximally induced the expression of the *arsB::luxAB* fusion and thus, these concentrations were used as positive controls (Cai and DuBow, 1996). As expected, dramatic increases in light emission (approximately 1.7 X 10^7 RLUs) were observed following exposure of the *arsB::luxAB* clone to the arsenite and arsenate solutions (Figure 1). Following exposure of the *arsB::luxAB* clone to filtered and unfiltered samples of the arsenic-containing water effluent, approximately 3.6 X 10^6 and 4 X10^6 RLUs were emitted, respectively (Figure 1). Chemical analyses performed by Environment Canada revealed that the unfiltered water effluent contained a total arsenic concentration of 0.28 PPM (data not shown). The species and nature of arsenic present in the water sample has not yet been determined. However, it was previously shown that in oxygenated water, arsenic is prevalent in its pentavalent, arsenate form (Cullen and Reimer, 1989). If this is the case, the results obtained from the luminescence assays described here correlate well with the available chemical data. (Figure 1). However, further chemical analyses, determining the exact nature and species of the arsenic present in the effluent, is necessary to make any firm conclusions.

To confirm the results from the luminescence assays and to eliminate the possibility that luminescence was due to a *luxAB*-specific phenomenon, β-galactosidase assays were also performed. In these experiments, the *E. coli arsB::lacZ* clone (Diorio *et al.*, 1995) was exposed to the same arsenic-containing water effluents described above. In the absence of any arsenic-containing solutions, the *arsB::lacZ* clone exhibited only basal levels (0.5 Miller Units) of β-galactosidase expression (Figure 2). However, in the presence of 0.1 PPM arsenite and 1.0 PPM arsenate, dramatic increases of approximately 34 and 31 Units of β-galactosidase were observed, respectively

(Figure 2). Expression of the *arsB::lacZ* clone was also induced upon exposure to the filtered and unfiltered arsenic-containing water effluents, where approximately 5 Units of β-galactosidase activity were observed (Figure 2). The results obtained from the β-galactosidase assay therefore correlate well with the results obtained from the luminescence assay. It was concluded that induction of the *arsB* gene by arsenic present in the water effluent is not a reporter gene-specific phenomenon. These experiments were repeated using the parental strain of the *arsB::lacZ* clone, *E. coli* 40, and in all cases, no β-galactosidase activity was observed.(Figure 2). However, these results were not surprising since *E. coli* 40 has a Δ*lac* genotype (Bukhari and Metlay, 1973) and thus cannot produce the β-galactosidase enzyme. When the experiment was performed using a wild-type *E. coli* strain, MG1655 (Jensen, 1993), basal levels of β-galactosidase activity (2-3 Units) were observed (Figure 2). These clones therefore represent a potential means for rapid, effective, and inexpensive detection of arsenic-containing compounds present in aquatic ecosystems. With further development, these clones may be used to complement traditional analytical methods of toxicity testing by taking the bioavailability of arsenic contaminants into account.

Table 1: Bacterial Strains

Strain	Characteristics	Reference
E. coli MG1655	F^-, λ^-	Jensen, 1993
E. coli 40	F^-, *Δpro-lac, rpsL, trp*	Bukhari and Metlay, 1973
E. coli LF20012	*arsB::luxAB* in E. coli 40	Cai and DuBow, 1996
E. coli LF20001	*arsB::lacZ* in E. coli 40	Diorio et al., 1995

Figure 1. Luciferase bioassay 1.5 h following induction of the *arsB::luxAB* clone with (■) one volume of sterile delH$_2$0, (▣) one volume of 0.1 ppm arsenite solution, (□) one volume of 1.0 ppm arsenate solution, (▣) one volume of filtered Goodfellow effluent, (▣) one volume of unfiltered GoodFellow effluent. Luciferase assays were performed in triplicate and the average was plotted. In all cases, the standard deviation was less than 5%. Refer to Materials and Methods for experimental procedures.

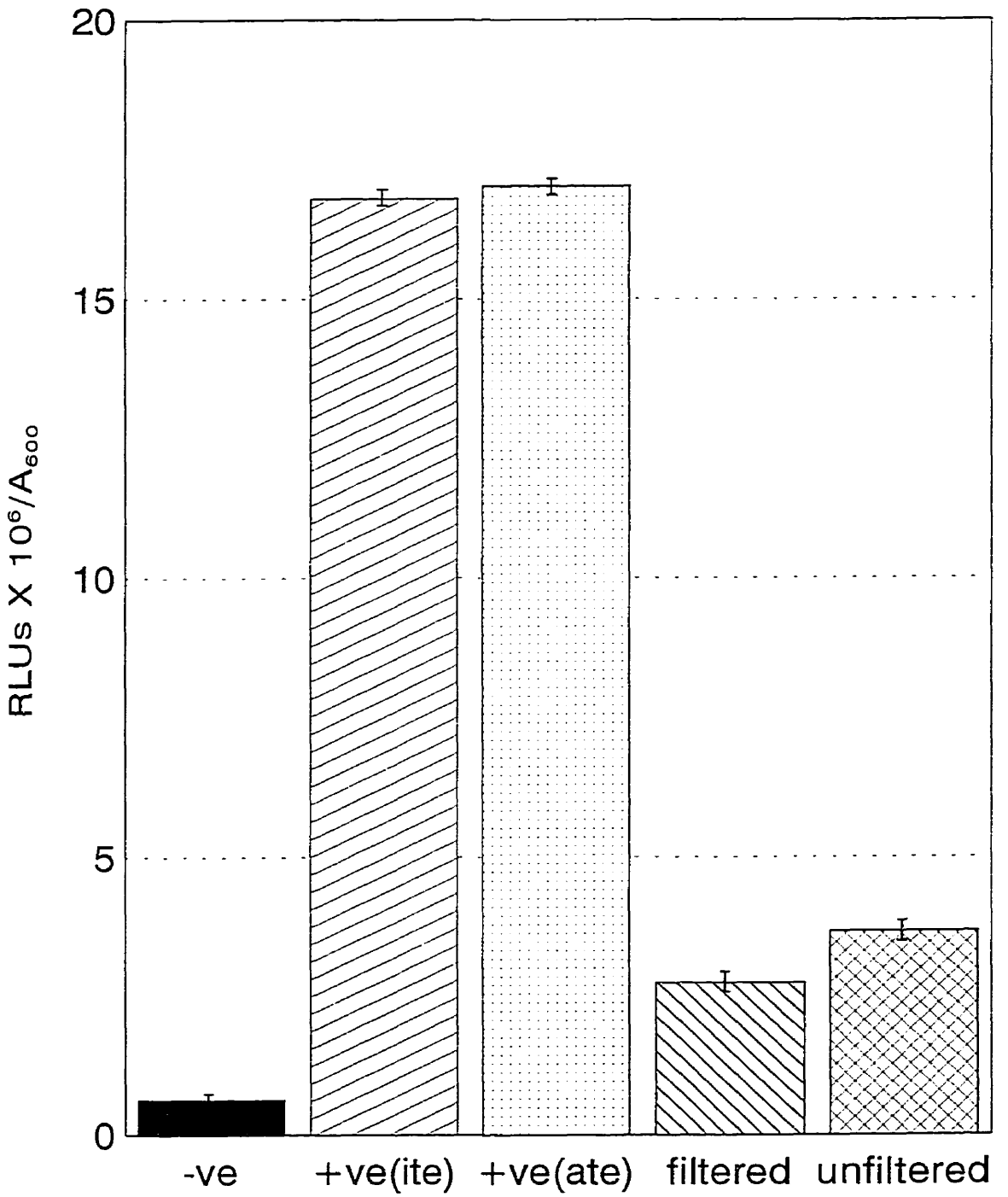
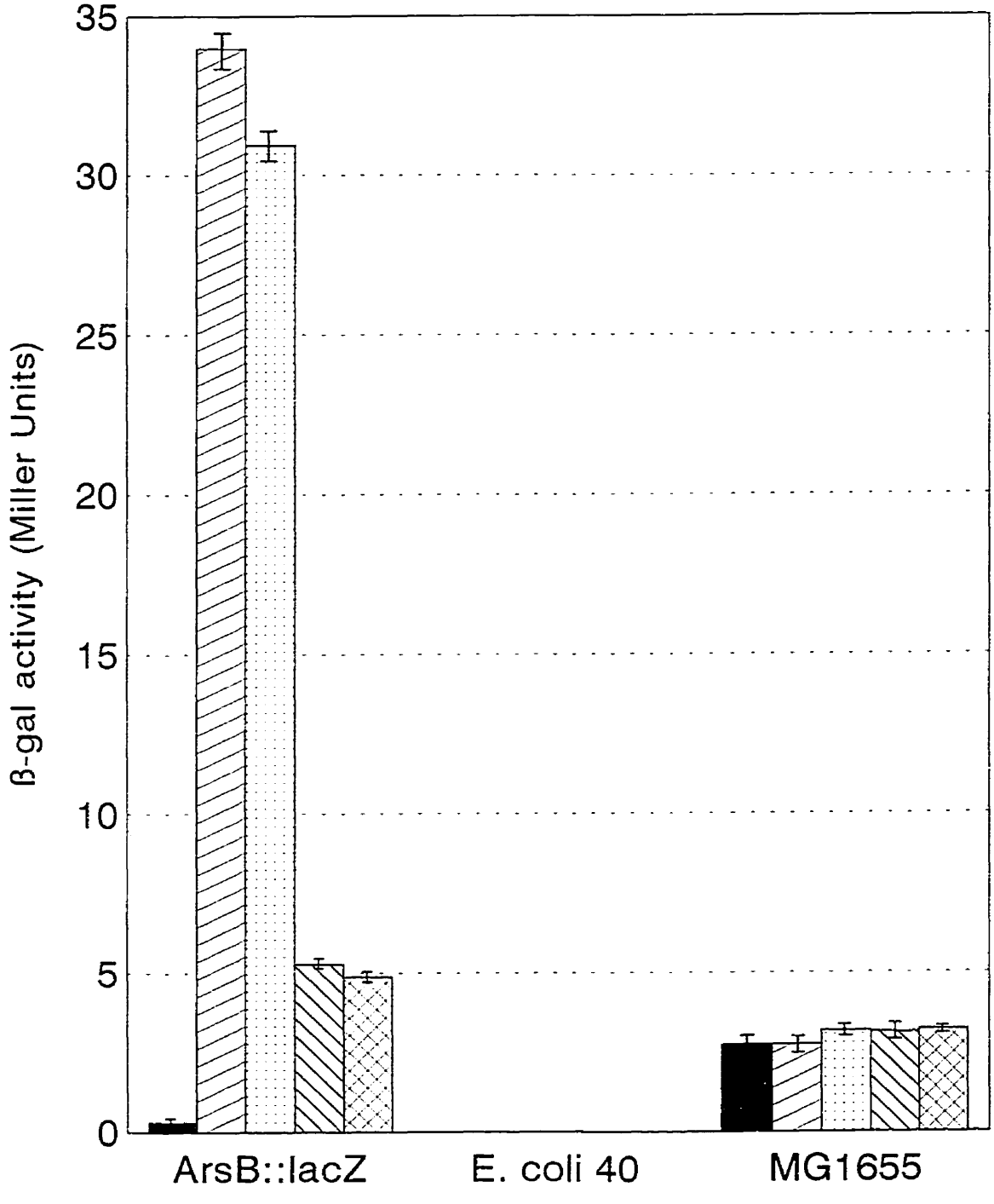

20
15
10
5
0
RLUs X $10^6/A_{600}$
-ve
+ve(ite)
+ve(ate)
filtered
unfiltered

Figure 2: β-galactosidase bioassay 1.5 h following induction of the *arsB::lacZ* clone with (■) one volume of sterile delH$_2$0, (▨) one volume of 0.1 ppm arsenite solution, (□) one volume of 1.0 ppm arsenate solution, (▨) one volume of filtered Goodfellow effluent, (▨) one volume of unfiltered GoodFellow effluent. β-galactosidase assays were performed in triplicate and the average was plotted. Error bars depict the calculated standard deviation. In all cases, the standard deviation was less than 5%. Refer to MATERIALS and METHODS for experimental procedures.


35
30
25
20
15
10
5
0
β-gal activity (Miller Units)
ArsB::lacZ
E. coli 40
MG1655

CHAPTER IV

Conclusions and Future Directions

Our environment is constantly being exposed to a myriad of chemicals, such as pesticides and herbicides. However, little is known about their biological effects on cells. These effects may involve the induction of gene expression producing proteins which alter the cell's physiology, allowing it to cope with the pesticide- or herbicide-induced stress. The first part of the thesis therefore focused on the characterization of genes in *Escherichia coli* whose expression was altered upon cellular exposure to the herbicides, bromacil and dinoseb, and to the pesticide, chlordane. To identify these genetically-programmed responses, a modified Tn*5-luxAB* *E. coli* gene fusion library (Guzzo and DuBow, 1991) was screened in the presence of increasing concentrations of bromacil, chlordane, and dinoseb (Costanzo, 1995). Using this 3000 clone library, our laboratory has identified several genetically-programmed responses to various inorganic, toxic, metal compounds. (Guzzo *et al.,* 1991; Guzzo and DuBow, 1994a). However, the library had never been screened for the presence of *E. coli* clones which respond to chemically complex, organic compounds such as pesticides and herbicides.

Five distinct clones which exhibited increases in light emission following exposure to either bromacil, dinoseb, or chlordane were isolated from this library (Costanzo, 1995). Characterization of these pesticide- and herbicide-responsive genes involved cloning, mapping, and sequencing. Following partial characterization of these genes, luminescence experiments were repeated. However, unlike the dose-dependent increases in light emission originally observed, luminescence assays performed with new lots of compounds were inconsistent yielding erratic patterns of light emissions. Thus, experiments were performed to determine the cause of these inconsistencies. Southern blotting analyses and P1*vir* transduction experiments were performed to determine if a genetic phenomenon was responsible for fluctuations in luciferase expression and luminescence patterns. From these experiments, it was concluded that inconsistent light emissions were not caused by secondary transposition of the

Tn*5-luxAB* element or genomic rearrangements or by the accumulation of point mutations within the chromosomes of the responsive clones. Furthermore, luminescence assays were performed under varying conditions to determine the effects of media and temperature on luciferase expression. Based on results presented in chapter two, it was hypothesized that the complex nature of the compounds tested coupled with the extreme sensitivity of the bioluminescence pathway may have been responsible for the variability observed in luciferase expression and luminescence.

Future experiments to further characterize the pesticide- and herbicide-responsive genes should involve performing luminescence assays following exposure of the clones to the pure, individual components comprising the complex pesticides and herbicides. Moreover, additional experiments should incorporate the use of other well characterized, more defined reporter genes, such as *lacZ*, to minimize *luxAB* artifacts. Following characterization of the luminescence reactions, future studies may involve over-expression of the proteins encoded by the two chlordane-responsive, putative open reading frames. Additional experiments should also be designed to study the regulatory cascade involved in *cspB* induction. These experiments may ultimately elucidate the functions and relationships of these genes to the pesticide, chlordane, and the herbicide, dinoseb. Following characterization of these genes and their corresponding gene products, assays, such as those described in chapter three, should also be developed to test the potential of these luminescent clones as biosensors to detect the presence of chlordane and dinoseb in the environment.

With increased world-wide industrialization and a concomitant higher demand for chemicals, both developed and developing countries are facing increasing ecological and toxicological problems from the release of toxic contaminants into the environment (Bitton and Koopman, 1986). In response to these expanding stresses, a multitude of biological assays have been

developed to assess the impact of toxic chemicals on our ecosystems. The long-term effects of chemicals discharged into aquatic environments are now being realized and research efforts are now being directed to short-term toxicity tests which can alert monitoring agencies of the potential dangers associated with toxic chemical release (Bitton and Koopman, 1986). The second part of the thesis therefore focused on determining whether a well characterized arsenic/antimony-responsive *E. coli arsB::luxAB* fusion clone, LF20012 (Cai and DuBow, 1996), could be used as a biosensor to detect arsenic compounds present in an aquatic sample obtained from the St Lawrence River, Québec (Goodfellow Effluent, Environment Canada).

Liquid luminescence assays were performed in which the arsenic-responsive *E. coli* clone was exposed to an arsenic-contaminated water sample, as well as several control samples, containing known concentrations of arsenite or arsenate. A three- to four-fold increase in light emission was detected following exposure of the clone to either filtered or unfiltered water effluents. If the arsenic present in the water effluent is predominantly in its pentavalent form (Cullen and Reimer, 1989), then luminescence results obtained from our assay correspond well with chemical data obtained from Environment Canada. However, further investigations and chemical analyses are necessary to determine the exact nature and species of arsenic present in the water effluent. Similar experiments were performed in which β-galactosidase activity was measured in an *E. coli arsB::lacZ* fusion clone, LF20001 (Diorio *et al.*, 1995), following exposure to the same arsenic-contaminated water effluent and control samples. Results obtained from the β-galactosidase assay confirmed that the light emission observed in the presence of the arsenic-contaminating water effluent was not due to a reporter gene-specific phenomenon.

The results obtained from these experiments suggest that metal-responsive *E. coli* clones, identified and isolated in our laboratory, have the

potential to be exploited as biosensors. Furthermore, the application of these biosensors in the environment may complement existing bioassays by detecting the presence of intermediate, biologically-relevant concentrations of environmental contaminants, while at the same time taking the bioavailability of these contaminants into account.

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IMAGE EVALUATION
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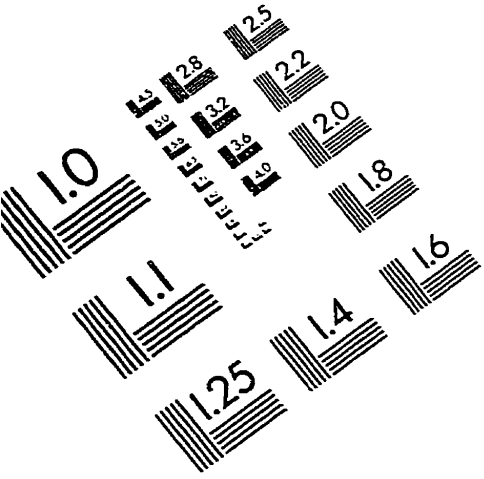

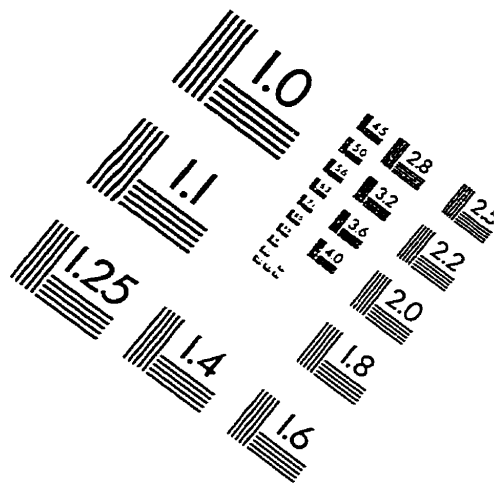

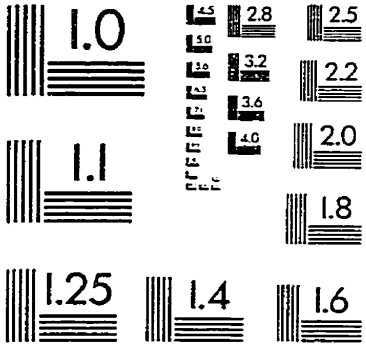

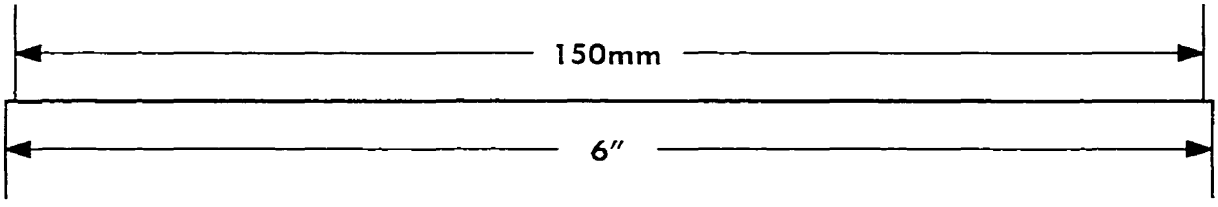

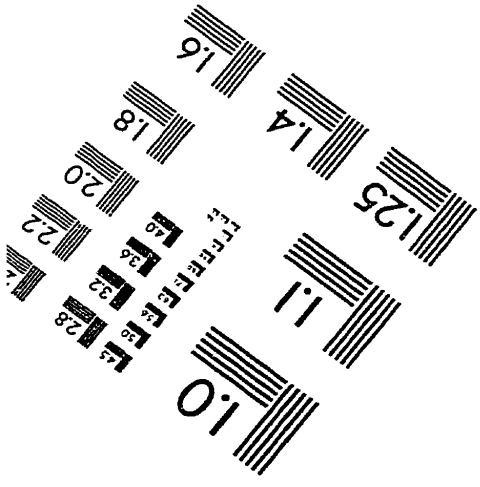

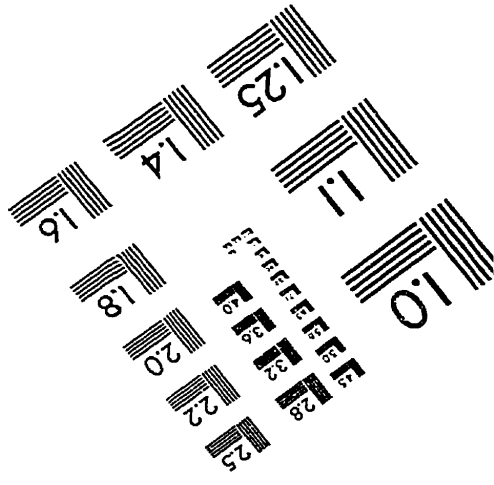

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