DEVELOPMENT AND CHARACTERIZATION OF A *recA*-BASED BIOSENSOR FOR DNA-DAMAGING AGENTS

By

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CHAPTER I

STATEMENT OF THE PROBLEM

Studies of ecosystems and the physiological states of microbial communities have long relied on destructive and invasive methods. These methods yield results that are dependent on a delayed, remote examination of the communities. The need for real-time, *in situ* monitoring devices in order to gain more accurate insights into the functioning of natural microbial communities is great and requires the development of new techniques (Miller and Poindexter, 1994). A whole-cell biosensor that can be incorporated into a microbial community would be extremely useful in real-time sensing of community health before, during, and after bioremediation.

Fusions with reporter genes (i.e. *lacZ, catE*) have been extensively used to quantify the level of gene expression. While these methods are very useful in the laboratory, they are of limited use in environmental studies because of the invasive nature of the tests and for their inability to allow real time, *in situ* analysis of community response.

The study described here was carried out in order to construct a transcriptional fusion of the *Pseudomonas aeruginosa recA* promoter to the bioluminescence (*lux*) genes from *Vibrio fisheri*. *P. aeruginosa recA*

offers an ideal candidate for a stress indicator in a environmental setting. In this study I fused the *recA* promoter to a promoterless *lux* cassette and I demonstrate the utility of using the strain carrying the *recA-lux* fusion as a whole cell biosensor for DNA-damaging environmental stresses such as UV radiation and chemical pollutants.

This construct allows monitoring of the levels of DNA-damaging stress to which an environmental microbial community is subjected by measuring the levels of induction of *recA* gene activity of cells carrying the fusion. This bioluminescent reporter offers great advantages for *in situ* monitoring. The production of light by this genetic construction is dependent on the *recA* gene promoter. Light production can be measured without subjecting the cells to destruction. This reporter system offers real-time analysis and sensitive measurements.

CHAPTER II

LITERATURE REVIEW

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative , rod-shaped heterotrophic microorganism. *P. aeruginosa* is aerobic, non-fermentative, and varies in length from 2-4 μm. It is motile and produces a slime layer at all times. In certain environments, such as the lungs of cystic fibrosis patients, *P. aeruginosa* turns mucoid by producing the exopolysaccharide, alginate. *P. aeruginosa* is ubiquitous in nature owing its success in colonizing numerous environments to its biphasic growth pattern. Under certain environmental conditions, *P. aeruginosa* is highly motile and can swim from one site to the other, while in other habitats it uses exopolymers, such as alginate, to adhere to surfaces and multiply as a sedentary organism. The latter mode of growth is preferred by *P. aeruginosa* in nature and is manifested by the formation of biofilms (Costerton *et al.*, 1987). In nature, biofilms are initiated by mucoid *P. aeruginosa* upon adherence of cells to a solid surface. *P. aeruginosa* cells engulf themselves in secreted exopolymers (primarily alginate) (Gacesa,1998). As a biofilm matrix, alginate was shown to be important as a virulent factor in complications of cystic fibrosis (Govan and Harris,

1986) and other diseases such as urinary-tract infections (Nickel *et al.*, 1985). Alginate has also been shown to play a major role in biofouling of man-made materials (Christensen and Characklis, 1990). In addition, alginate endows *P. aeruginosa* with protection against antibiotics (Hodges and Gordon, 1991).

Many plasmids have been isolated from *P. aeruginosa.* They belong to a variety of incompatibility groups. In general, they are highly stable and very difficult to cure from cells (Doggett, 1979).

P. aeruginosa is pervasive in nature it is found in the rhizosphere, water and soil (Galli *et al.*, 1992). This ubiquitous nature makes *P. aeruginosa* an environmentally important organism that is appropriate to use in the development of biosensors for microbial communities in diverse environments.

Biofilms

Costerton *et al.* (1995) stated:

> Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media.

In the environment, bacteria can generally be found in three states: (i) When nutrients are available, biofilms are established and cells grow in communities. (ii) Cells can be planktonic, which allows colonization of new habitats, such cells usually originate from other biofilms. (iii) When

nutrient conditions are unfavorable, cells reduce in size and enter a dormant state known as starvation survival. This state is reversible upon change in nutrient availability.

Biofilms were first described by Zobell and Anderson (1936) and since then, they have been recognized as an important and ubiquitous growth phase of bacteria in aquatic environments. Studies on *P. aeruginosa* have shown that cells in biofilms are phenotypically distinct from planktonic cells. Adhesion of *P. aeruginosa* cells to surfaces triggers the upregulation of genes (i.e. *algC* and *algD*) that control the synthesis of alginate (Hoyle *et al.*, 1993). Secretion of alginate is crucial for the formation of the biofilm, because it allows concentration of nutrients and adhesion of other bacteria from diverse species.

Biofilm *P. aeruginosa* owe their phenotypic transformation to a sigma factor that controls numerous genes involved in the formation of the biofilm (Martin *et al.*, 1994). This factor is similar to the sigma factor that controls sporulation in gram-positive bacteria and the one which controls the rough-smooth transformation in gram-negative bacteria (Costerton *et al.*, 1995). Biofilms have been shown to release planktonic cells at a steady rate which is important for the survival of the organism because it allows survival and colonization of new habitats. The separation of cells from biofilms is also under physiological control (Applegate and Bryers, 1991).

The structure of biofilms is very complex. After adhesion, synthesis of exopolysacharride is coupled to cell division. This leads to the formation of microcolonies (Costerton *et al.*, 1995). A microcolony, as the basic unit

of the biofilm, can either contain a single species or a mixture of species that cooperate at the metabolic level. In either case, microcolonies are believed to possess a certain level of homeostasis. A mixed community in a biofilm may contain primary producers such as photosynthetic cells and heterotrophs that thrive by scavenging biomass of dead cells. Networks of water channels have been found in biofilms, these channels are responsible for the exchange of nutrients and metabolites with the bulk fluid (Costerton *et al.*, 1995). In conclusion, a biofilm is a complex mode of growth that is favored by bacteria because it endows a certain level of homeostasis and protection from harsh environments or harmful agents.

The RecA protein and its functions

The *Escherichia coli* RecA is a multi-functional molecule that plays key roles in a number of different processes : Homologous recombination, DNA repair, SOS network regulation, SOS mutagenesis, and other processes. The *E. coli* RecA protein has a molecular weight of 37,800.

Mutation in the *recA* locus confers recombination deficiency to *E. coli* (Willetts, 1969). In homologous recombination, RecA catalyses post-replicational strand exchange between homologous DNA molecules (Cox *et al.*, 1982).

In DNA repair, the homologous recombination functions of the RecA protein are useful in restoring a damaged DNA molecule (e.g. chromosome) by exchanging homologous strands with an undamaged DNA molecule (Howard-Flanders and Theriot, 1966).

The RecA protein has several important regulatory functions.

When DNA is damaged, RecA is activated and acts as a coprotease by stimulating the autoproteolytic cleavage or LexA. LexA is a repressor that acts on a number (up to 20) of unlinked genes, including *recA* and *lexA*, collectively known as the SOS Network (Little and Mount, 1982). Cleavage of the LexA protein results in expression of all of the SOS genes. Among these genes are *uvrA*, *uvrB* , and *uvrC* which are the major components of the Nucleotide Excision Repair Pathway. Their gene products excise the damaged DNA region allowing for resynthesis of DNA and thus repairing the molecule (Miller and Kokjohn, 1990).

In *E. coli*, RecA is also involved in regulation of SOS Mutagenesis. When activated, RecA derepresses expression of umuC and *umuD*, members of the SOS network. While UmuC and UmuD are mutagenesis proteins, UmuD must be post-translationaly modified to be active. The coprotease activity of RecA stimulate the autoproteolytic cleavage of UmuD, in a fashion similar to LexA cleavage producing a functional UmuD' protein. UmuD' and UmuC form a complex that allow the DNA polymerase III to bypass damaged DNA (Shinagawa *et al.*, 1988; Rajagopalan *et al.*, 1992). The $UmuCD_2$' complex is also thought to require the direct contribution of the RecA protein to carry out this function (Sweasy *et al.,* 1990). It is proposed that RecA interacts directly with the $UmuCD_2$' complex to rescue polymerases stalled at DNA lesions (Bates and Bridges, 1991). There is evidence that RecA is involved in a number of other processes such as induction of induced stable DNA replication (iSDR) (Magee and Kogoma, 1990), constitutive stable DNA

replication (Cao and Kogoma, 1993) and chromosome partitioning (Allman *et al.*, 1991).

The coprotease function of RecA is also necessary for the induction of various prophages. RecA stimulates autoproteolytic cleavage of the *E. coli* phage lambda cI repressor (Quillardet *et al.*, 1982). Temperate phages such as *P. aeruginosa* phage D3 have also been shown to respond to the RecA protein. RecA activation is used by the phage as a monitor of the level of DNA damage in the host (Miller and Kokjohn, 1987).

Eighty-eight *recA* genes from prokaryotes have been cloned and sequenced to date (Grubert *et al.*, 1998). RecA is highly conserved and it is believed to be universally found in the genomes of eubacteria (Miller, 1992). Expression of the *recA* gene is inducible by a number of DNA-damaging agents such as solar ultraviolet radiation and chemicals such as mitomycin C (Miller and Kokjohn,1990).

P. aeruginosa RecA

Wild-type *P. aeruginosa* are more sensitive to ultraviolet radiation than are wild type *E. coli* (Kokjohn and Miller, 1985). This finding is interesting because *P. aeruginosa* is autochthonous in soil and aquatic habitats. Microbial communities in these environments are constantly exposed to DNA-damaging agents such as UV and chemical agents that necessitate repair systems for survival (Friedberg *et al.*, 1995). *E. coli,* on the other hand, exists primarily in the intestinal tract of mammals. This may seem to protect it from environmental insults, however, the intestinal tract is an environment rich in free radicals and thus is constantly

exposed to oxidative damage.

Kokjohn and Miller (1985) have cloned the *P. aeruginosa recA* gene. The RecA protein of *P. aeruginosa* has a predicted molecular weight of 36,877 and shows a 71% identity with the amino acid sequence *E.coli* RecA protein (Sano and Kageyama, 1987). The *P. aeruginosa recA* gene complements defects in homologous recombination in *E. coli recA* mutants and restores post-replication recombinational repair as well as inducible repair after UV damage in these cells (Kokjohn and Miller, 1985). *P. aeruginosa* has been shown to be inducible by DNA-damaging agents (Miller and Kokjohn, 1988). While *P. aeruginosa* has been shown to have an SOS-like response (Warner-Bartnicki and Miller, 1992) this species lacks Weigle reactivation and Weigle mutagenesis after exposure to UVC (Simonson *et al.* 1990) or quinolone (Benbrook and Miller, 1986). This suggests that this species lack umuDC-like genes (Simonson *et al.*, 1990). When *P. aeruginosa recA* gene is introduced into an *recA*-deficient mutant of *E. coli,* UV-inducible mutagenesis and repair are complemented. In addition, a *lexA* analog has been isolated from *P. aeruginosa* (Garriga *et al.*, 1992) which indicates that a RecA-LexA regulatory network is possible in this organism. R2, an IncP9 plasmid, that is specific for *P. aeruginosa* has been shown to allow expression of Weigle reactivation and UV-induced mutagenesis in $RecA^+$ *P. aeruginosa* cells (Simonson *et al.*,1990). Two other plasmids, pKM101 and R46 which belong to incompatibility group N and carry *umuDC* analogs,*mucAB* , have also been shown to confer DNA-damage-inducible UV resistance and mutagenesis activities to *P. aeruginosa* (Kokjohn and

Miller, 1994). Starvation, dessication, oxidative damage or any stituation where DNA replication is blocked seem to induce *recA* expression. The block in the fork results in single-stranded DNA, which is belived to be the signal that activates the RecA protein.

Bioluminescence

The use of bioluminescence genes as reporters of gene expression became possible with the cloning of the *lux* operon from various organisms. Bioluminescent bacteria are very diverse. Most of them, however, belong to one of three genera : *Vibrio, Photobacterium* or *Xenorhabdus*. They are widely distributed in nature and can be found in terrestrial, freshwater, and, more commonly, in marine environments. luminescent bacteria can be free living or in symbiosis with other animals . Two of the most studied luminous bacteria are *Vibrio fisheri* and *Vibrio harveyi* which are free living marine organisms.

The *lux* systems from *V. fisheri* and *V. harveyi* have been cloned and expressed in *E. coli* (Baldwin *et al.*, 1984; Engerbrecht et al., 1983). There are five structural genes in the *lux* operon (*luxCDABE*). *luxA* and *lux* B code for the two subunits of the luciferase, α and ß respectively. *luxC*, *luxD,* and *luxE* code for the reductase, the transferase, and the synthetase polypeptides respectively. The LuxCDE proteins form the complex fatty acid reductase (Meighen and Dunlap, 1993). The five structural genes needed to make bioluminescence are organized in one operon in many luminous bacteria.

In *V. fisheri* , there are two regulatory genes *luxI* and *luxR*

upstream of *luxC*. *luxI* is a member of the same operon that contains the structural genes, and it encodes a polypeptide responsible for the synthesis of an autoinducer (N-[3-oxohexanoyl] homoserine lactone) (Engerbrecht and Silverman, 1984). *luxR* is located upstream of *luxI* but is transcribed in the opposite direction. It is thought to encode a receptor protein for the autoinducer (Meighen and Dunlap, 1993). LuxR binds the autoinducer to stimulate the expression of the operon containing the structural genes and *luxI* (Meighen, 1991)

The *V. fisheri* luciferase is a heterodimer of the two subunits α (41 KDa) and β (37 kDa). It is stable at temperatures of 30°C and lower. It starts loosing activity rapidly at 37° C (Stewart and Williams, 1992). The luciferase oxidizes $FMNH_2$ (reduced flavin mononucleotide) and a long-chain fatty acid aldehyde in the presence of oxygen and emits 490 nm light. The resulting products of the reaction are FMN, a long-chain fatty acid and water (Meighen, 1991) (Figure 1)

The long-chain fatty acid is synthesized by the fatty acid reductase to allow for recycling of the aldehyde and thus, continuous bioluminescence. The reductase is a multi-enzyme composed of an ATP-dependent synthetase subunit (42 kDa), an NADPH-dependent reductase subunit (54 kDa) and an acyltransferase subunit (33 kDa) (Rodrigues *et al.*, 1983). The reductase complex is formed by four subunits of each component mentioned above (Meighen and Dunlap, 1993). The reductase oxidizes NADPH (nicotinamide adenine dinucleotide phosphate) using ATP to turn the long-chain fatty acid into the corresponding aldehyde

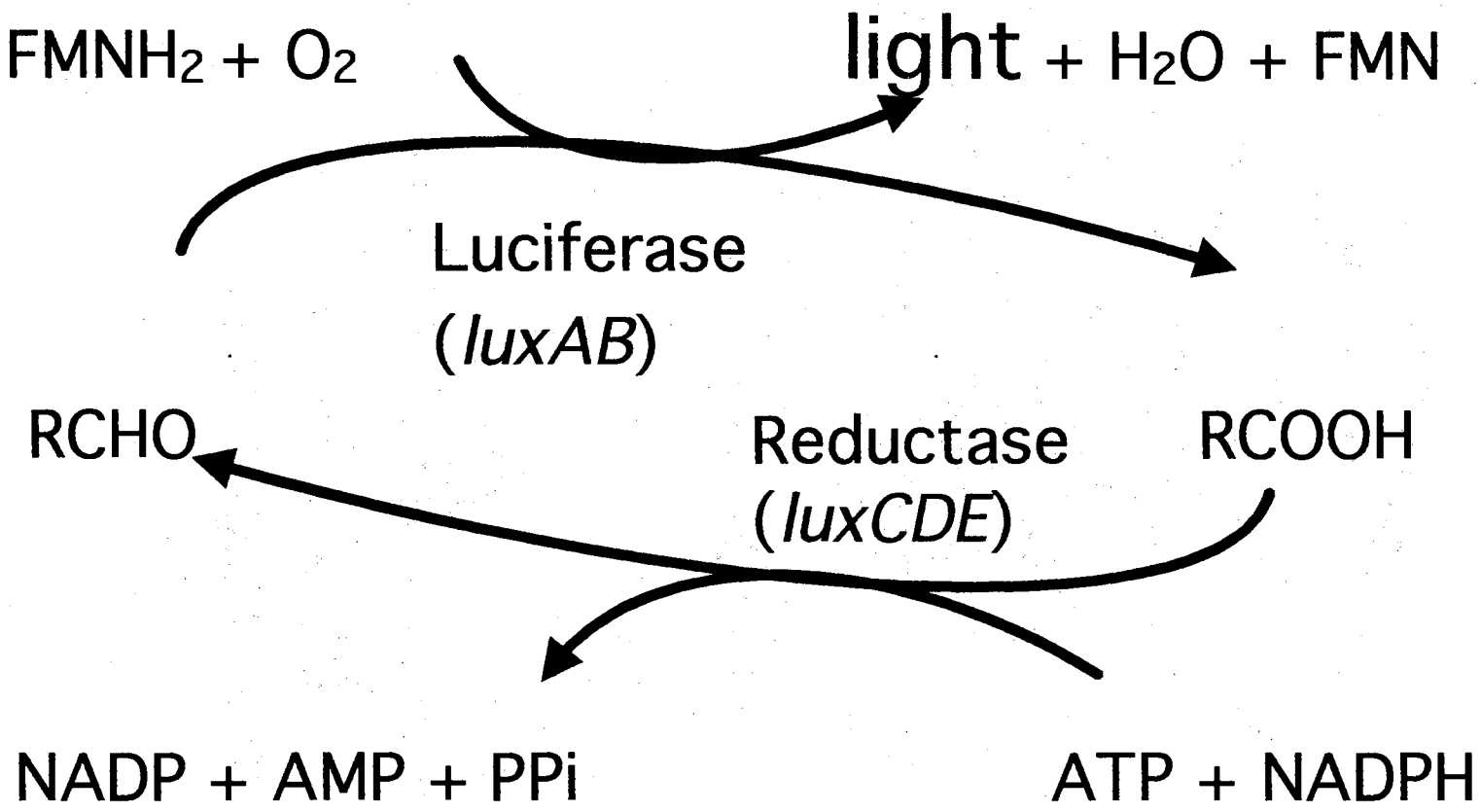


Figure 1. Bioluminescence reactions.

(Meighen, 1991)

The *lux* genes have been introduced into a number of non-luminescent bacteria. When only *luxAB* are introduced into a new host, aldehyde must be added to replenish the supply of the luciferase substrate. The five structural genes, however, can be introduced into a non-luminescent bacterium such as *P. aeruginosa* as a single operon (*luxCDABE*) to eliminate the need to add external aldehyde. All the other reactants (e.g. $FMNH_2$, NADPH, ATP, etc) are abundant in most organisms (Meighen and Dunlap, 1993). The *lux* operon (*luxCDABE*) is controlled by a single promoter. This promoter can be replaced with other regulatory sequences allowing the study of the regulation of different promoters, the response of bacteria to different conditions, or the recognition of the *luxCDABE* carriers in the environment. The *lux* operon has been used successfully as a reporter of gene expression to monitor naphthalene biodegradation and bioavailability (Burlage *et al.*, 1990).

DNA damage

DNA is a dynamic molecule that partakes in many chemical processes such as replication, transcription, and transposition. These processes are essential for cell function and division. Chemical changes in the bases of DNA can arise either spontaneously due to the instability of the chemical bonds in the molecule or as a result of errors in replication, recombination, or as a result of repair of damage to the molecule. Other changes in DNA arise from its interaction with chemical agents, DNA being a reactive molecule, or with radiation (i.e. ultraviolet light) since

DNA is a chromophore. As the carrier of the genetic information for most living organisms, DNA has to maintain a high level of integrity to ensure proper inheritance of traits. Living organisms have developed ways to repair DNA damage (see section on *recA).* If an alteration in the DNA escapes repair and is inherited, it is considered a mutation. Such mutations can be either beneficial or deleterious to the organism depending on its environment. Mutagenesis plays an crucial role in the diversity and evolution of organisms.

a) Effects of Ultraviolet radiation

Solar ultraviolet radiation (UVR: 100-400 nm) (Figure 2) is one of the most important environmental stresses to which microorganisms are exposed. UVR is mutagenic and even cytotoxic (Hanawalt and Haynes, 1965; Rosenstark, 1989). Historically, UV has been a model for studying DNA damage. Even though, UVR can damage most macromolecules in an organism, the most important target is DNA since it is the genetic material and exists in unique or very low copy number in cells.

UVR is significantly absorbed by the atmosphere, air is responsible for absorption of wavelengths below 200 nm, while stratospheric ozone absorbs wavelengths below 300 nm. With depletion of stratospheric ozone, our environment is being exposed to higher intensities and a broadened spectrum of UVR.

The UVR spectrum is divided into three wavelength ranges: UVA, UVB and UVC.Each of these classes has different effects on microorganisms (Friedberg *et al.*, 1996). Solar UVR that reach earth is

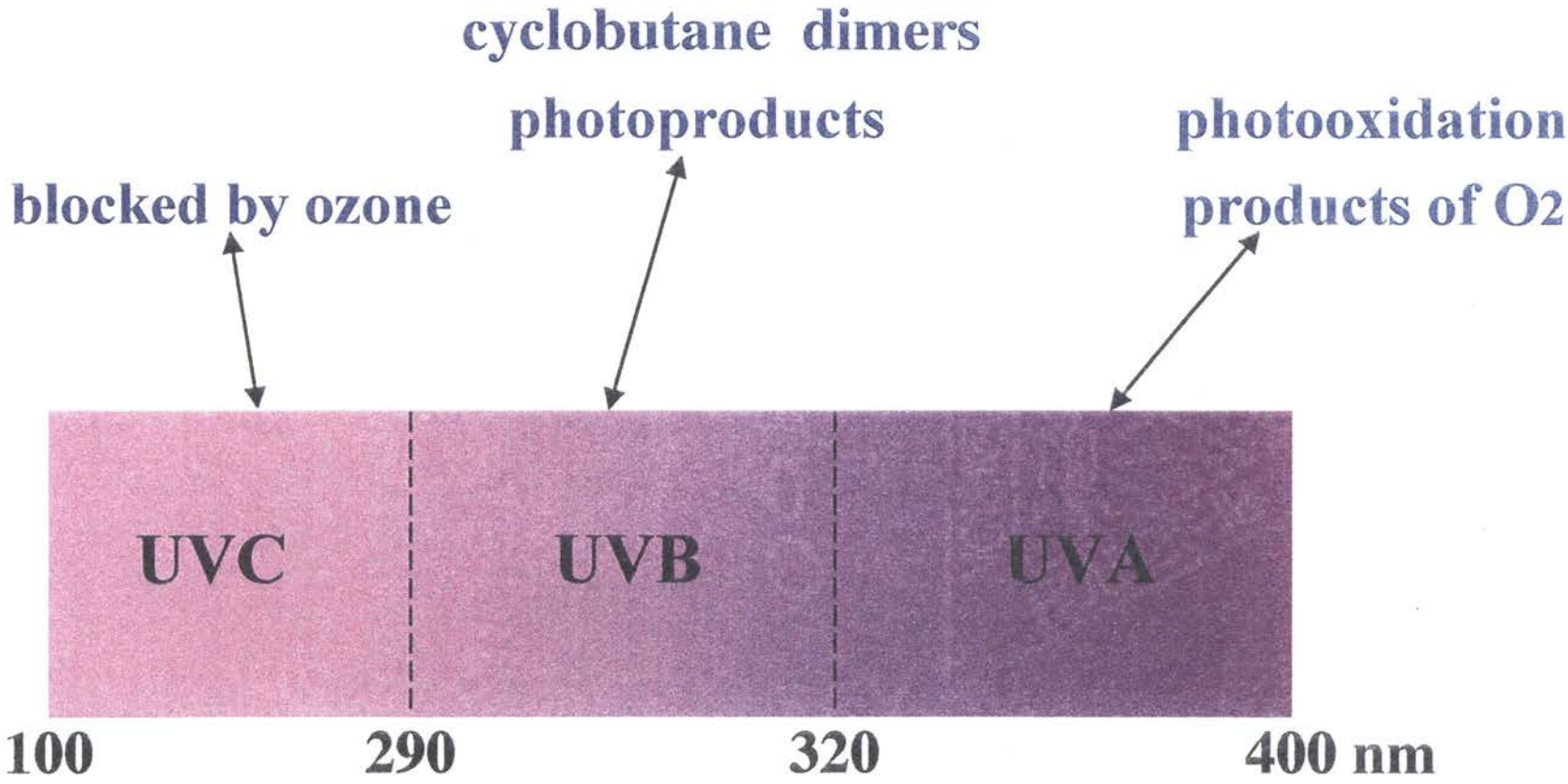


Figure 2 . Ultraviolet radiation spectrum.

composed mainly of UVA and UVB. UVA ranges from 320 nm to 400 nm. It is the least energetic class of UVR. Besides its implication in damaging proteins and membranes, UVA indirectly damages DNA by creatingreactive oxygen compounds (e.g. H_2O_2, O_2^-, etc.), by photooxidation of O_2, which cause single strand-breaks in DNA. The lethal action of UVA has been mainly associated with its interaction with absorbing molecules, and chromophores, such as, quinones, flavins, and steroids (World Health Organization, 1994).

UVB ranges from 290 nm to 320 nm and is more deleterious to DNA than UVA. Since UVB is absorbed by DNA, it directly alters nucleotides by creating cyclobutane pyrimidine dimers and photoproducts (e.g. pyrimidine - pyrimidone (6-4)). Cyclobutane dimers are due to the formation of a covalent four-membered ring between two adjacent pyrimidines (Hanawalt, 1975). (6-4) photoproducts are formed by the covalent binding of the sixth carbon of one pyrimidine to the fourth carbon of the adjacent pyrimidine. The (6-4) photoproducts are mainly thymine-thymine dimers, cytosine-cytosine dimers and rarely thymine-cytosine dimers. (6-4) cytosine-thymine are not seen in UVB irradiated DNA (Friedberg et al., 1995). The photoproducts cause major distortions in the double helix of DNA that inhibit DNA replication by blocking progress of DNA polymerase III. Depletion of the ozone layer due by pollution has resulted in higher levels of biologically effective UVB reaching the earth (World Health Organization, 1994).

Although blocked by the ozone layer, UVC (100 nm to 290 nm) is

potentially more damaging than UVB because it is absorbed by DNA very efficiently.

b) Chemical agents that damage DNA

Many chemicals are known to interact with DNA in a deleterious fashion. Research on the effects of certain chemicals on DNA has been fueled by the increasing awareness of environmental mutagens and carcinogens. DNA-damaging chemicals are classified according to their effects. Alkylating agents (i.e. methyl methane-sulfonate) are electrophiles that add an alkyl group (i.e. CH^{+}_{3}) at various atoms of the nucleotides. They are very reactive with the nitrogen and the oxygen atoms found on the rings all four DNA bases.

A second class of chemicals that damage DNA are cross-linking agents (i.e. nitrogen mustard), these chemicals are responsible for covalently binding bases one to another. The cross-links formed can be interstrand if the bases are situated on two opposite strands or intrastrand if the bases are on the same DNA strand.

Another kind of cross-links are caused by psoralens. Such cross-linking activity requires photoactivation of these by long-wavelength UV radiation. These compounds are typically planar three ring aromatic that cause interstrand cross-links which introduce helix distortions. Other chemicals can become damaging to DNA after metabolism and they are especially relevant to animals, base analogs have also been shown to be potentially damaging *in vitro* (Friedberg *et al.*, 1996).

Artificially immobilized cells

Immobilization of cells is the physical confinement of whole microorganisms in a solid matrix while retaining viability. The cell-loaded matrix is typically in contact with a liquid medium that allows feeding and possibly washing out of byproducts from the cells. (Klein and Wagner, 1983). Mattiason (1983) compared cell densities in reactors with immobilized cells and a liquid culture and discovered that a solid matrix allowed a much higher cell density. This feature is crucial for developing systems for bioconversion or treatment of pollutants because it offers greater activity of cells and thus greater yields. One of the most important advantages of the use of immobilized cell in bacterial studies, as compared to liquid cultures, is the possibility of repeated use of the cell matrix and the absence of loss of biomass due to wash out in a continuous flow situation.

Cells can be immobilized in three different manners:
1) Covalently linking the cells to a solid surface using a chemical crosslinker. 2) A porous carrier matrix is prepared first, the cells are then added and allowed to adsorb. 3) The most common method of immobilization is the physical entrapment of cells in a three dimensional polymer network (i.e. alginate). The cells are grown to the desired density then they are mixed with a solution of alginate. A divalent cation is then added to promote crosslinking of alginate which forms a solid mesh trapping the cells inside. Traditionally calcium chloride has been used as the crosslinking agent, strontium chloride use ,however, was shown to form a stronger matrix (King *et al.*, 1990). This method has been shown to

produce a good model for study of natural biofilms (Stewart *et al.*, 1998).

Biosensors

Biosensors are sensing elements that couple a biological component with a physical transducer (Figure 3). There are many types of biological components, such as, enzymes, immunoglobulins and whole cells. The biological element provides selectivity for the analyte being studied which is crucial when the analyte is present in a mixture. Biological elements offer high sensitivity compared to chemical sensors because of the catalytic properties of enzymes or the specificity of immunoglobulins. The transducer component of the biosensor generates a measurable signal. Signals can be electrical (i.e. voltage, current), or optical (i.e. light) (Wise and Najafi, 1991). The first type of biosensors were enzyme electrodes such as the one developed by Clark and Lyons (1962) to monitor the level of glucose in the body. They used the enzyme glucose oxidase which catalyses the oxidation of glucose to gluconic acid. Oxygen was used as the oxidizing agent and the consumption of oxygen was monitored through electrochemical reduction in a platinum electrode. Glucose levels were read as fluctuations in current.

Biosensors that use whole cells have also been used and offer many advantages. Whole-cell biosensors offer the same sensitivity and selectivity as enzymes. In addition they are easier to prepare and cheaper than isolated enzymes to use. Enzymes that are part of a whole cell are also more stable in natural environments than are their isolated counterparts. With the advent of genetic engineering, whole cell

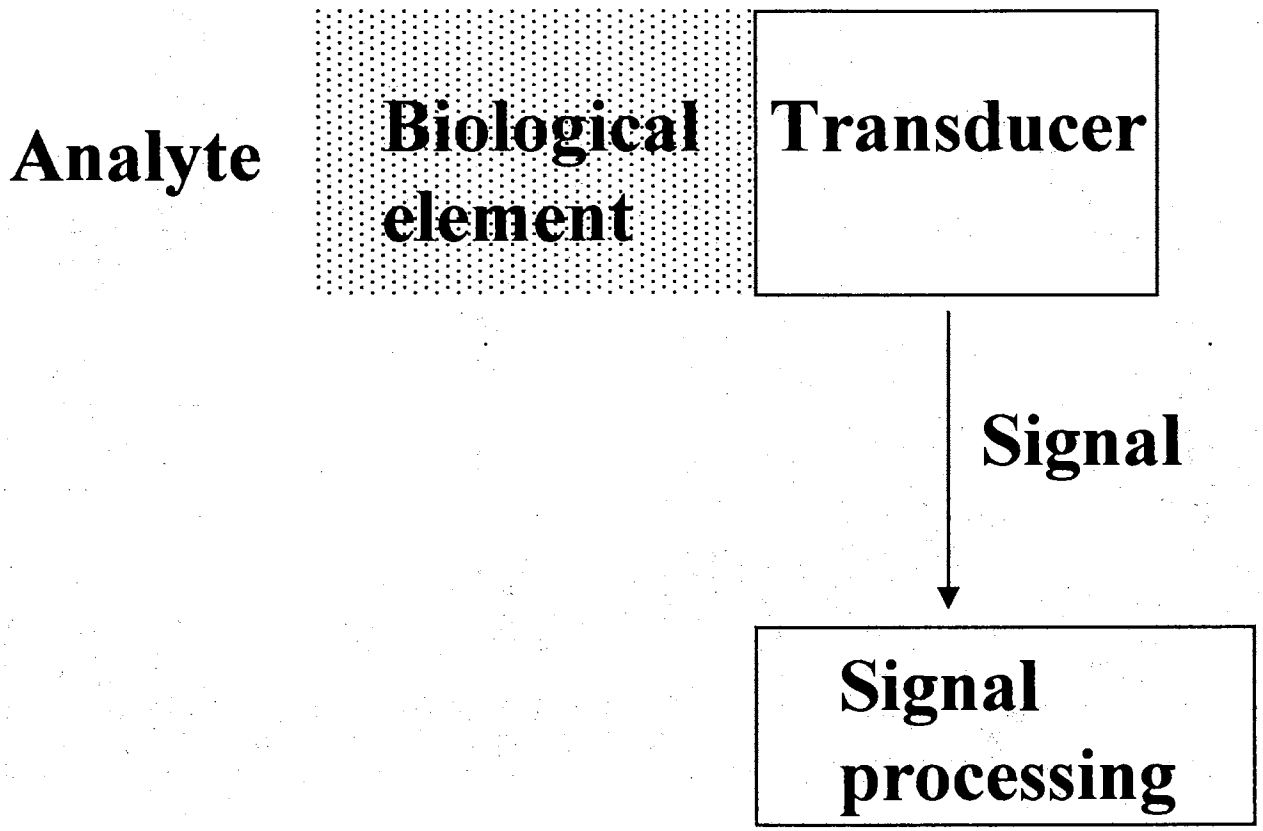


Figure 3. General scheme of biosensors

biosensors can be designed for a limitless number of analytes and conditions (Eggins, 1996). Historically, most biosensors that have been developed were geared for clinical use. Recently, however, environmental issues have prompted scientists to develop biosensors to monitor pollutants in water and soil. The use of plasmid-based fusions and whole-cell biosensors for such endeavors has been reported. Such biosensors are very useful in environmental settings because they can be integrated into a microbial community of interest. A *Pseudomonas fluorescens* biosensor was used for detection if naphthalene and its degradative intermediate, salicylate were degraded in the environment (King *et al.*, 1990). Another study reports the development an *E. coli* biosensor for detection of heat shock (Vollmer *et al.*,1997). All these biosensor used plasmid fusions of the gene of interest and a bioluminescence operon. Whole-cell biosensors are more suitable to environmental research than other kind of biosensors (i.e. enzyme-based biosensors) because they offer more comprehensive results such as bioavailability for the analyte, its effects on living organisms as well as concentration.

The biosensor used in this study was constructed by fusing the promoter of the *P. aeruginosa recA* (Kokjohn and Miller, 1985) gene to the *lux* operon from *V. fisheri* (Meighen,1991). *recA* expression which acts as an indicator of stress, controls the expression of the *lux* genes which produce the signal to be measured. The analyte that is measured by our biosensor is DNA damage, the biological component is RM4440, or more specifically *recA* and the signal produced is light (Figure 4).

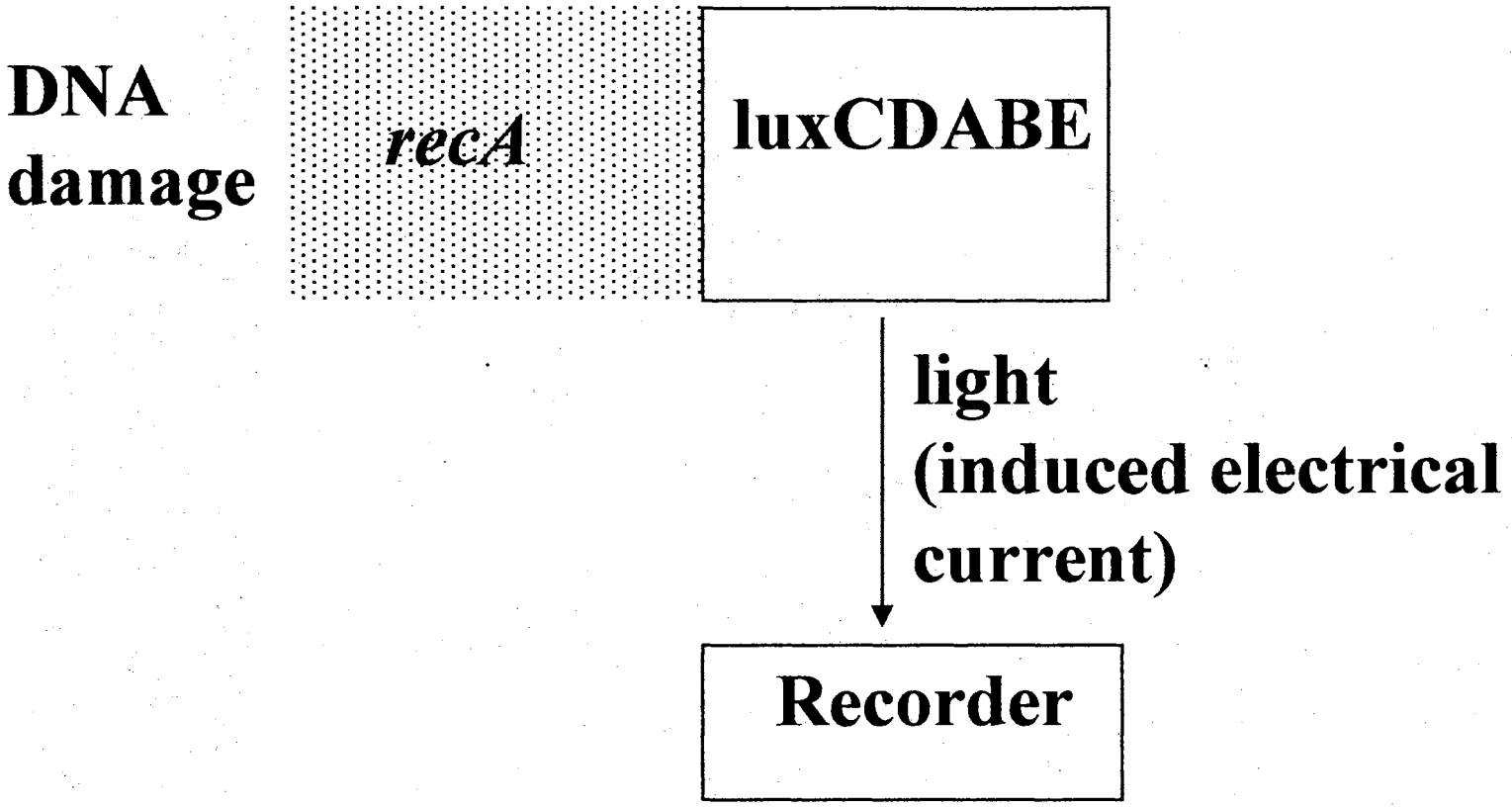


Figure 4. Diagram of RM4440 as a biosensor.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

All strains used in this study are described in Table 1. Plasmids are listed in Table 2. *P. aeruginosa* strain FRD1 was obtained from D. Ohman (Ohman and Charkrabarty, 1981) and was used throughout these studies. Original cloning was done in *E.coli* strain DH5α (Maniatis *et al.*, 1989) obtained from Promega (Madison, Wisconsin). The microorganisms RM4440 and RM4439 have been deposited in the American Type Culture Collection (ATCC). The collection numbers are ATCC 87704 and ATCC 87705 for RM4440 and RM4439 respectively.

Culture media and growth conditions

E. coli was grown in Luria Broth (LB) medium (Miller and Ku, 1978) at 37° C for all cloning purposes. *P. aeruginosa* was grown in Pseudomonas Isolation Agar (PIA) obtained from Difco Laboratories, Detroit Mi. For experiments where light production was measured, Light Measuring Medium (LMM) was used to culture the bacteria. It contained (in grams per liter): peptone, 20 g; $MgCl_2$, 1.4 g; potassium sulfate, 10 g; glycerol,

Table 1: Bacterial strains used in this study.

Strain or plasmid	Genotype	Source or Reference
E. coli		
JC11372	*recA56, argE3, leuB6, proA2, supE44*	(Kokjohn and Miller, 1985)
DH5α	*recA1, endA1, gyrA96, lacZ*	(Maniatis et al., 1982)
RM4221	*E. coli* B harboring pRK2013	(Figurski and Helinski, 1979)
RM4153	JC11372 harboring pKML2003	(Kokjohn and Miller, 1985)
RM4439	DH5α harboring pMOE15	This study
P. aeruginosa		
FRD1	prototrophic	(Ohman and Chakrabaty,1981)
RM4440	FRD1 harboring pMOE15	This study

Table 2: Plasmids used in this study

Plasmid	Relevant Characteristics[1]	Reference or Source
pUCD615	IncW, ori_{pBR322}, ori_{pSa}, promoterless *luxCDABE*, Ap^r, Km^r	(Rogowsky et al. 1987)
pRK2013	Helper plasmid for conjugal transfer, Km^r	(Figurski and Helinski,1979)
pKML2003	*P. aeruginosa recA* gene cloned on pBR322, Ap^r, Tet^r	(Kokjohn and Miller, 1987)
pMOE15	*P. aeruginosa recA* promoter fused to *luxCDABE*, Ap^r, Km^r	This study

[1]Phenotypic designations are as follows: Ap^r, ampicillin resistance in *E. coli*, carbenicillin resistance in *P.aeruginosa*.; Km^r, kanamycin resistance; Tet^r, tetracyclin resistance.

20 ml. The medium was adjusted to pH 7.0. This medium is rich and complex and it should provide ample nutrition for bioluminescence production. Plates were made by adding 13 g of bacteriological agar per liter of medium (US biological, Swampscott, MA) . For these experiments, *P. aeruginosa* was grown in LMM at room temperature in a Multigen Chemostat, purchased from New Brunswick Scientific CO, Inc. (Edison, NJ), with constant agitation and sterile aeration. Growth was monitored by viable counts and light scattering using a Klett-Summerson Colorimeter. $Klett_{660}$ units were read.

Antibiotics were added to the various media when appropriate for selection of plasmids. They were used at the following concentrations: ampicillin, 50 μg/ml; and kanamycin, 50μg/ml for *E. coli.* For *P. aeruginosa* carbenicillin was used in place of ampicillin at a concentration of 500 μg/ml of culture medium. Kanamycin was used at 500 μg/ml of medium for *P. aeruginosa.*

In the alginate matrix experiments, *Pseudomonas* minimal medium (PMM) was used as a nutrient source. PMM contains (in grams per liter): potassium phosphate, dibasic (K_2HPO_4), 7.0 g ; potassium phosphate, monobasic (KH_2PO_4), 3.0 g ; sodium citrate ($NaC_6H_5O_7.2H_2O$), 0.5 g ; magnesium sulfate, 7.0 g; hydrate ($MgSO_4.7H_2O$), 0.1 g; and ammonium sulfate ($(NH_3)2SO_4$),1. RM4440 was grown in one liter culture to mid-exponential growth phase in PMM at room temperature. The cells were pelleted and resuspended in half the volume of saline and used to prepare the alginate matrix.

Plasmid construction

The cloned *P. aeruginosa recA* gene was obtained on the plasmid pKML2003 (Kokjohn and Miller, 1987). A 1.7 Kb *Sal*I fragment that contains the *recA* promoter was isolated from pKML2003 and subcloned into the *Sal*I site of the dephosphorylated *lux* plasmid vector pUCD615 (Rogowsky *et al.*, 1987). Colonies were grown on ampicillin-kanamycin (each at a concentration of 50 μg/ml each) plates. Plasmid preparations were performed on transformants by alkaline lysis method (Maniatis *et al.*, 1989). Plasmids carrying an insert were subjected to restriction enzyme analysis to determine the orientation of the promoter. One transformant that contained the desired construct of the *recA* promoter fused to the *lux* cassette in the direction allowing expression of the *lux* genes through the *recA* promoter (pMOE15) was identified and used for subsequent studies.

DNA sequencing

Sequencing was done in the core facility at Oklahoma State University. Nucleotide sequences were determined using Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, FS (Perkin Elmer) in the automated sequencer 373 DNA sequencer (applied Biosystems). An oligonucleotide that is complementary to a region in the luxC gene was synthesized(Oklahoma State University core facility) and used as a primer to sequence towards the *recA* gene promoter upstream of *luxC* (Figure 5).

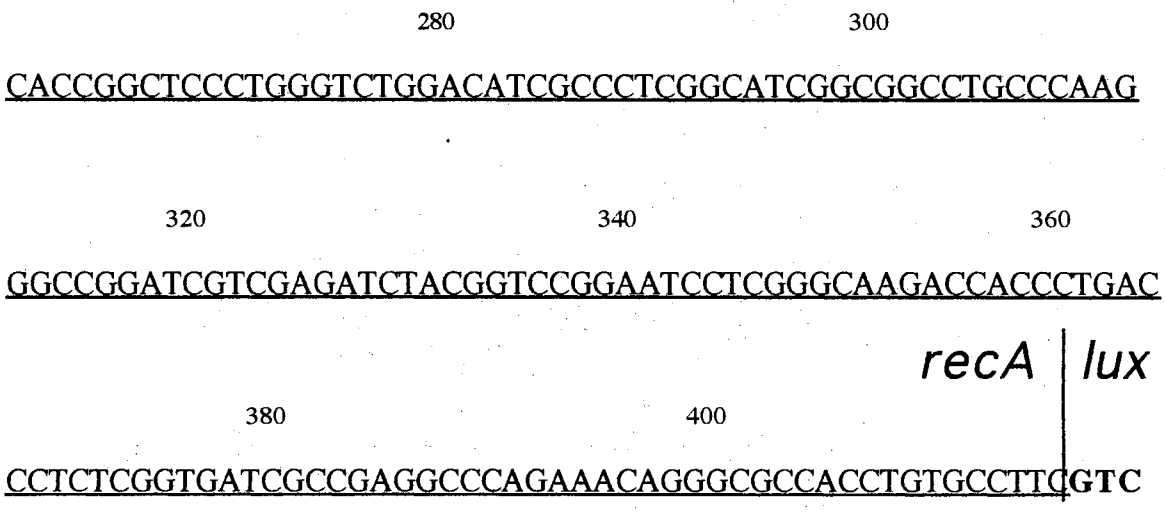


Figure 5 . Sequence of the *recA*-promoter-*lux* fusion junction at nucleotide 418 of the *P. aeruginosa recA* gene (underlined). The *SalI* site used for subcloning is shown (bold). The rest of the sequence is the *lux* cassette from pUCD615.

Triparental Mating

pMOE15 was mobilized from *E. coli* into wild-type *P. aeruginosa* by a modification of the triparental mating method of Ruvkun and Ausubel (1981), using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). *P. aeruginosa* strain FRD1 was grown at 43°C with low oxygenation to inactivate the restriction system (Rolfe and Holloway, 1968). The *E. coli* strain RM4439 was used as the donor of pMOE15 and RM4221 contained the helper plasmid. Cultures of these bacteria were grown to 40 $Klett_{660}$ units. One hundred microliters each of the recipient, donor, and helper cultures were mixed and filtered onto the surface of a 0.45 μm pore size cellulose nitrate filter. The filter was then placed on a PIA plate without selection and incubated at 37°C for 16 h. This surface mating mixture was then suspended in 1 ml of saline, and transconjugants were then selected by plating 50 μl of this suspension on PIA plates containing kanamycin (500 μg/ml) and carbenicillin (500 μg/ml).

Light measurements

A flexible liquid light guide (Oriel instruments, Stratford, CO) was used to transmit light from the sample into a photomultiplier tube Model 77340 (Oriel instruments, Stratford, CO) which produces an induced current. The photomultiplier was connected to a Photomultiplier (PMT) Readout Model 7070 (Oriel instruments, Stratford, CO) with a digital display to read the current in Amperes (A). The PMT readout was sensitive to 1 nA of induced current. The PMT readout was connected to a chart pen

recorder to allow continuous light reading.

UV exposure

UV was generated using one of three different lamps depending on the UV range desired. UVC (Wavelength of 100-290 nm) was generated using a germicidal bulb (Model XX-15F) emitting a peak wavelength of 256 nm and a fluence of 1,100 $\mu W/cm^2$ at a 15 cm distance. UVB (Wavelength of 290-320 nm) was generated using a lamp (Model XX-15F) with a peak emission of 302 nm and a fluence of 1,800 $\mu W/cm^2$ at a 15 cm distance. UVA (Wavelength of 320-400 nm) was generated using a lamp (Model XX-15F) with a peak emission of 365 nm and a fluence of 1,200 $\mu W/cm^2$ at a 15 cm distance. UV lamps were purchased from Spectronics Corporation (Westbury, New York). Doses of UV radiation were determined with a UVX radiometer (Ultra Violet Products, San Gabriel, CA) fitted with a sensor for UVC, UVB or UVA.

Alginate matrix

One volume of resuspended cells was mixed with two volumes of sterile low-viscosity sodium alginate (from Macrocystic pyrifera (kelp)) (3.5% weight/weight in 0.9% NaCl) and one-half volume of sterile glycerol. The mixture was then aliquoted in 15 ml volumes and stored at -70° C.

Sample preparation and exposure to UV

Fifteen milliliters of the alginate cell suspension was passed through a 20-

gauge needle using a syringe and dripped into a 0.1 M strontium chloride solution to form small beads of 4 mm in diameter. The mixture was stirred for 15 min. Alginate crosslinks upon mixing with strontium chloride immobilizing the cells within the matrix. The beads were then taken out of the cross-linking solution and placed in an electrophoresis tray. An electrophoresis apparatus (Biorad) was used as an incubation chamber. Incubation was started by passing PMM medium through a monolayer of beads at room temperature at a flow of 3.5 ml per minute (Figure 6). In different experiments, the alginate beads were exposed to increasing doses of ultraviolet radiation. In order to measure the maximum transmittance of the alginate beads, the fluences of UVC, UVB and UVA was measured using the proper UV radiometer directly and through a 4 mm slab of alginate. Percent transmittance was calculated by dividing the fluences through the alginate by the fluences measured directly and multiplying by one hundred (Figure 17).

Exposure of biofilm to chemical agents

100 ml PMM was prepared and the chemical to be tested was added to the appropriate final concentration. The beads were prepared as described above. The beads were then added to the PMM-chemical mixture and stirred for an hour. The beads were transferred to the electrophoresis chamber for incubation. PMM medium was used for maintenance and light production was monitored as previously described.

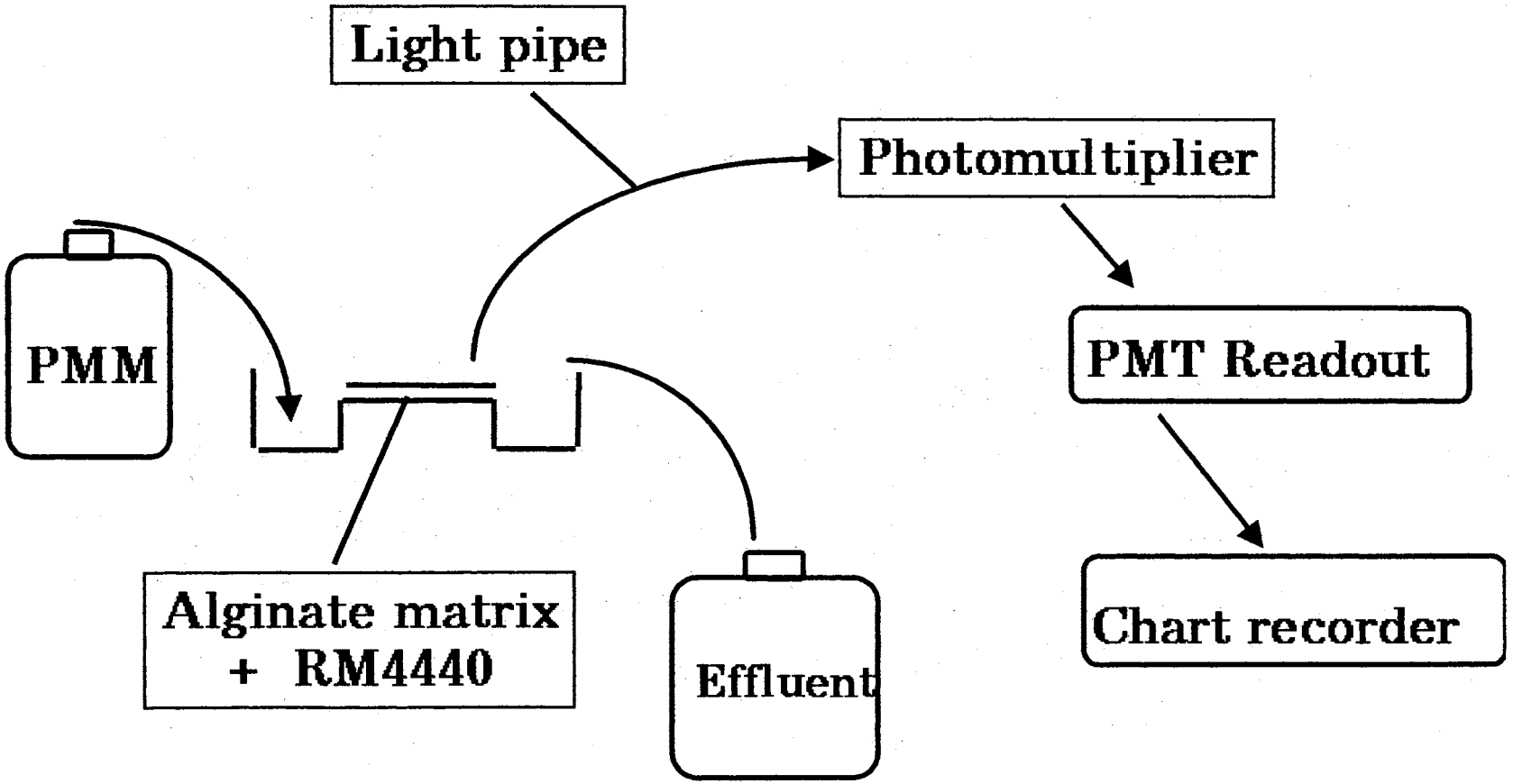


Figure 6. Schematic diagram of set-up of alginate matrix.

Viable-cell counts of immobilized cells

Viable cell counts were taken before and immediately after UV exposure of the cells trapped in the alginate beads. Before exposure to UV, five beads were removed and transferred to 1 ml of 50 mM $(NaPO_3)_n$. The beads were mixed until completely dissolved. Serial dilutions were made in saline and 50 µl were plated on LB. After exposure to UV, five beads were removed and the procedure was repeated. Plates were incubated at 37 °C for 24 hours and counted. Post UVC and UVB incubations were conducted in the dark to prevent light repair.

Electron Microscopy

Electron microscopy was done in the Oklahoma State veterinary medicine microscopy laboratory. Scanning electron microscopy was done on beads containing entrapped RM4440 cells before and after exposure to 10 J/m^2 of UVC. The electron microscope used was a JEOL 35 U. The beads were placed in a fixative solution (3% glutaraldehyde and 3% sucrose) for 3 hours. They were then washed with 0.2 M sodium cacodylate buffer (pH 7.4). Following refrigeration overnight, the beads were dehydrated in a graded concentration series of ethanol (50, 70, 90, 95, 100%). They were dried to the critical point required for electron microscopy and coated with a heavy metal (gold). An acceleration voltage of 25 KV was used .

CHAPTER IV

CONSTRUCTION AND CHARACTERIZATION OF RM4440

One of the important aspects of bioremediation is the need to assess damage in the environment. In order to meaningfully estimate that damage, an *in situ* method that is nondestructive, very sensitive and allows real-time analysis is needed. Bioluminescence is such a method, since it offers all the criteria mentioned above, and so can be used as a reporter of bacterial distress. The genes responsible for light production in *V. fisheri* have been cloned and characterized (Baldwin *et al.*, 1984). The five-gene operon can be expressed in a number of organisms (e.g. *E.coli* , *P. aeruginosa* etc) When the *lux* operon is fused to the promoter of a gene of interest, it acts as a transcriptional reporter that allows the monitoring of the expression of the particular gene.

In order to take advantage of this reporter operon in environmental studies, in this chapter, fusion of the promoter of *recA* gene to the luxCDABE operon is described. A microorganism carrying a *recA-lux* fusion can be introduced into a microbial community and act as a whole

cell biosensor for environmental stresses such as ultraviolet radiation. In *P. aeruginosa, recA* has been cloned and characterized, its induction has been shown to be mediated by DNA-damaging agents (Miller and Kokjohn, 1988). *recA* expression is, therefore, a reliable indicator of DNA-damage stress in *P. aeruginosa.*

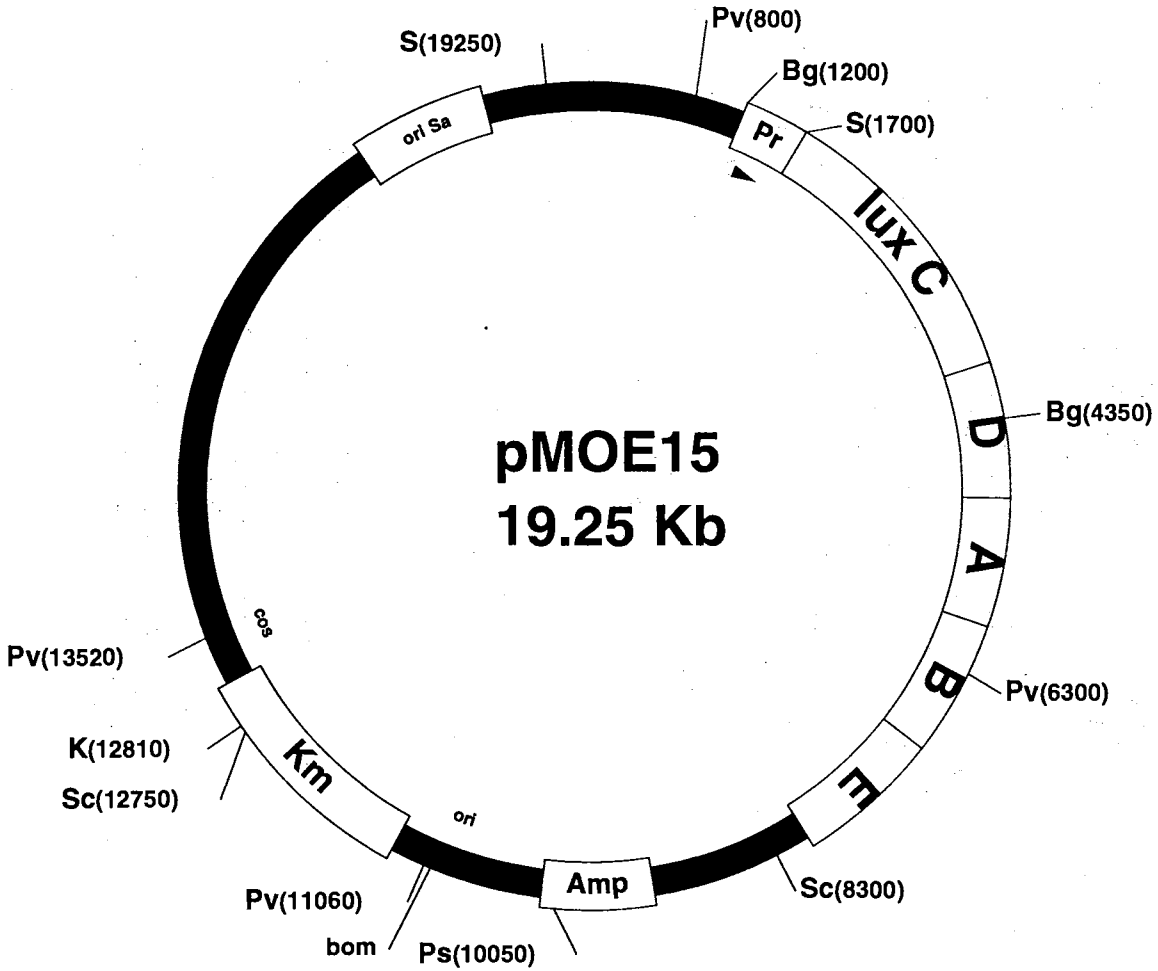
Construction of a *recA-luxCDABE* fusion

The *P. aeruginosa recA* promoter was subcloned into pUCD615 (Rogowsky *et al.* 1987). A 1.7 Kb *Sal*I fragment that contains the *recA* promoter was ligated to the dephosphorylated *Sal*I-digested*lux* plasmid vector pUCD615. The orientation of the promoter was confirmed by restriction enzyme analysis. The resulting construct, pMOE15 (Figure 7) was transferred to *P. aeruginosa* strain FRD1 by triparental mating. The exact location of the *recA-luxCDABE* fusions was identified by DNA sequence analysis (Figure 5). The strain showed a background level of luminescence which is consistent with the fact that *recA* is constitutively expressed (Figure 8).

Light production increased in response to UVC exposure

A 1 l batch culture of RM4440 was grown to mid-exponential phase. The cells were then collected by centrifugation at 8000 rpm for 10 min. The cell pellet was suspended in saline and exposed to various doses of UVC radiation (Range of time of exposure: 0-86 s). After exposure, the cells were collected and transferred to fresh LMM to continue light measurements. Viable counts were taken at 30 minutes intervals to allow

Figure 7. Restriction map of pMOE15. The region indicated by Pr is the *Pseudomonas aeruginosa recA* promoter from pKML2003. The promoter was subcloned into a SalI site in the multiple cloning site of pUCD615. There are several translational stop codons downstream of the *recA* promoter. The *lux* operon is under the control of the recA promoter. Bg, *Bgl* II; K, *Kpn* I; Ps, *Pst*I ; Pv, *PvuI* I; S, *sal* I; Sc, *sac* I; cos, packaging site of phage lambda; ori, origin of replication from pBR322; ori Sa origin of replication from plasmid *pSa; bom*, basis of mobilization.


pMOE15
19.25 Kb
S(19250)
Pv(800)
Bg(1200)
S(1700)
Pr
lux C
D
A
B
E
Bg(4350)
Pv(6300)
Sc(8300)
Amp
ori
Ps(10050)
bom
Pv(11060)
Km
cos
Pv(13520)
K(12810)
Sc(12750)
ori Sa

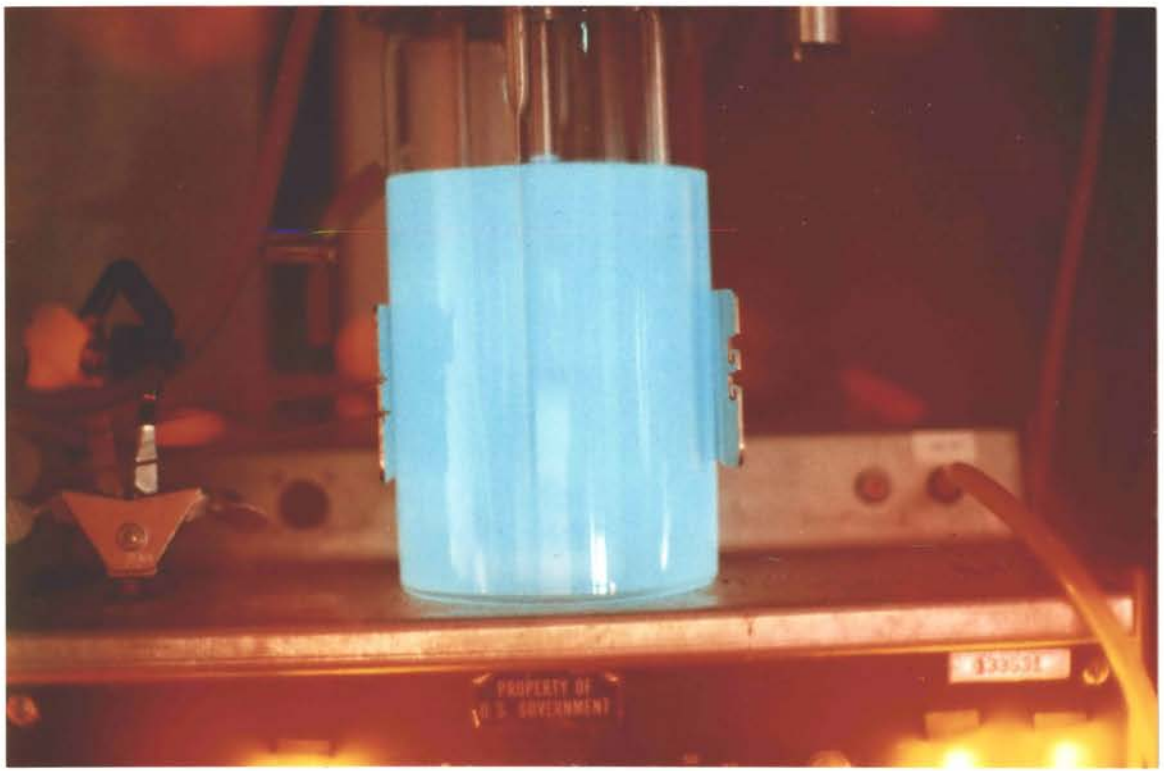


Figure 8. Photograph of batch culture of RM4440 taken in the dark (3 minutes exposure). Cells show background due to the relatively low constitutive expression of *recA*.

normalization of the light produced to cell concentration. All manipulations subsequent to UVC exposure were done under amber light (Kodak Wratten) to eliminate photoreactivation. Post-UVC incubations were carried out in the dark to avoid light repair. Viable counts were taken throughout the experiments, and light production was monitored continuously. A batch culture that was not exposed to UVC was used as a control.

The effect of UVC on growth was evident from the growth curves of the exposed cultures (Figure 9). After irradiation, exponential growth halted and the number of viable cells dropped. After a period of time during which repair of the DNA damage took place, exponential growth resumed. RM4440 showed a basal level of bioluminescence without exposure to UVC. This agreed with previous studies indicating that *recA* is constitutively expressed in bacterial cells. However, when RM4440 cultures were exposed to various doses of UVC, the intensity of bioluminescence produced by the population of cells increased to a maximum as early as 30 min following irradiation (Figure 10). Detection of increased *recA* expression by light production was found to be very sensitive compared to other methods (e.g., ß-galactosidase induction or Western analysis of RecA protein concentrations) used to monitor *recA* expression. With those methods detectable increases in *recA* gene expression are not observed until as much as 2 h after exposure of the cells to UV radiation (Miller and Kokjohn, 1988).

After UVC exposure, bioluminescence increased to a maximum and stayed constant for a period of 8 hours before returning to the pre-

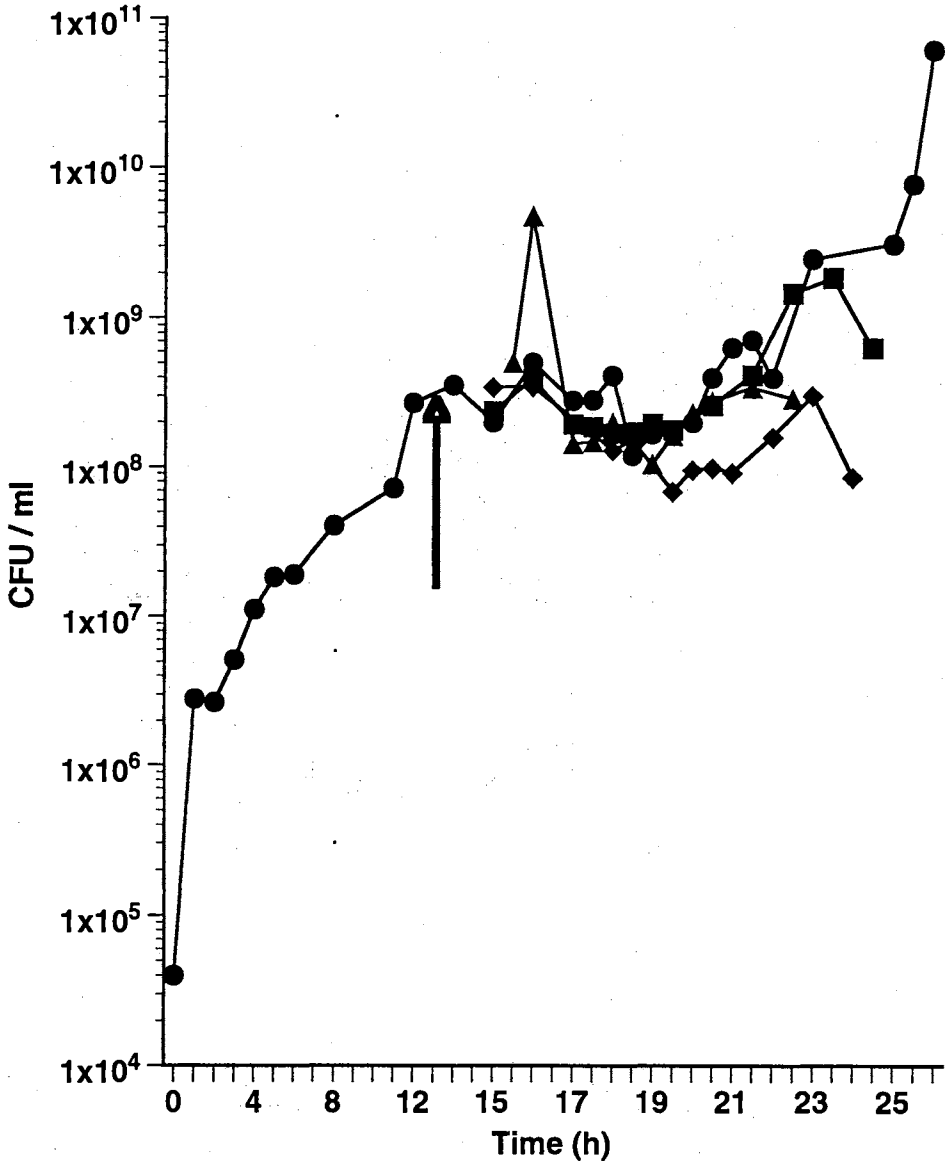


Figure 9. Growth curves of RM4440 irradiated with increasing doses of UVC; ■, 5 (J/m2), ●, 10 (J/m2), ▲, 15 (J/m2) and ◆, 20 (J/m2). The cells were grown as batch cultures and exposed to UVC at mid-log phase. Viable counts were taken at 30 minutes intervals. Arrow indicates time of exposure. All incubations were done at room temperature.

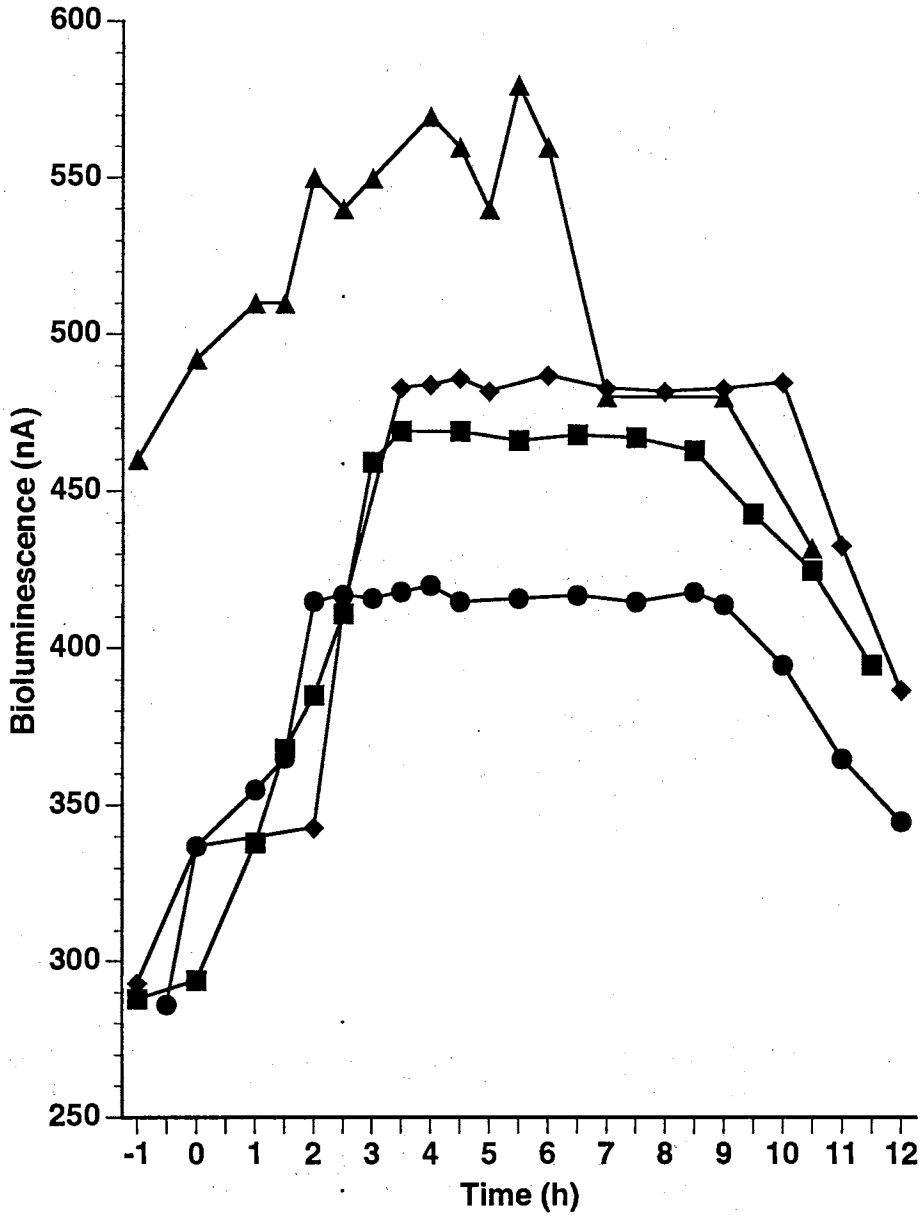


Figure 10. Light production RM4440 in response to increasing doses of UVC: ■, 5 (J/m2); ●, 10 (J/m2); ▲, 15 (J/m2); ◆, 20 (J/m2). The cells were grown as batch cultures and exposed to UVC at mid-exponential phase. All incubations were done at room temperature in the dark.

exposure level (Figure 10). The duration of the response is dose dependant. The higher the UVC dose the longer the response.

In order to account for cell death or growth, the concentration of viable cells (CFU/ml) were determined at various times through the experiment. The ratio of light production to CFU was determined in order to estimate the amount of light per cell and, thus, the amount of *recA-luxCDABE* gene expression per cell. The nA-to-CFU ratio was plotted against time of incubation (Figure 11). In the control culture which was not exposed to UV radiation, there was no increase in bioluminescence throughout the experiment. However, when cells were exposed to UVC , there was a peak in the increased light production from the culture after irradiation. The size of the bioluminescence response peak was found to be dose dependant (Figures 11 and 12). A dose of 5 J/m^2 produced the lowest response with an increase of 2.3 fold, while the 20 J/m^2 produced the greatest response with an increase of 4.78 fold. The elapsed time post irradiation before the peak of the light response was reached was also found to be dose-dependence characteristic (Figure 11 and 12) The lower the UVC dose, the shorter the time before the peak response. There was an increase of approximately 30 min in the maximum response for each 5 J/m^2 increase in the UVC dose.

Discussion

I have demonstrated in this study the development of a whole cell biosensor for DNA-damaging stresses such as UV radiation. The *P.aeruginosa recA* promoter was transcriptionaly fused to a promoterless *luxCDABE* cassette and introduced into a wild-type *P.aeruginosa*. This

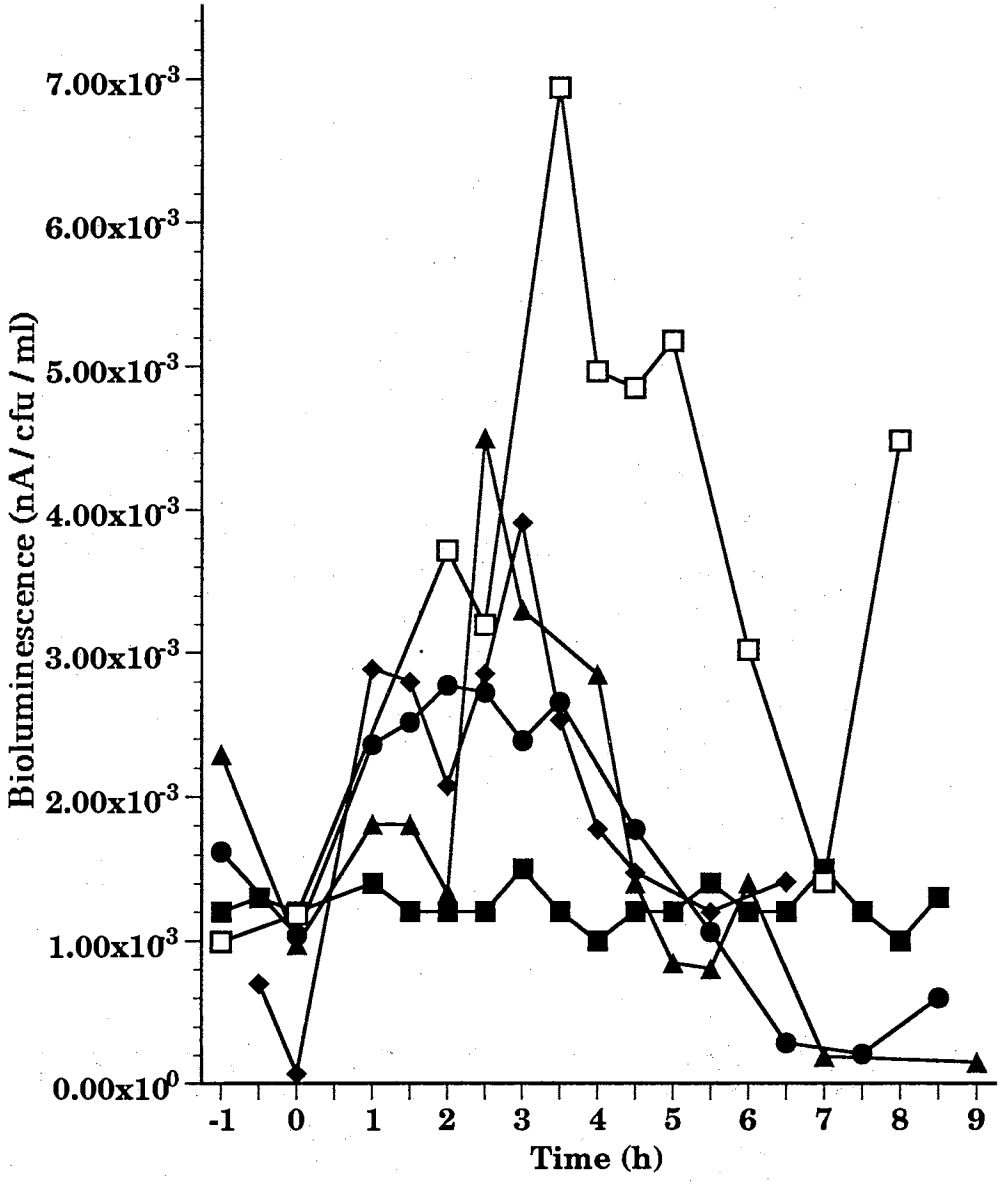


Figure 11. Normalized Response of RM4440 in response to induction by increasing UVC doses: ■, 0 (J/m2); ●, 5 (J/m2); ▲, 10 (J/m2); ◆, 15 (J/m2); □, 20 (J/m2). Viable counts were taken at 30 min intervals. Bioluminescence response is expressed in nA/CFU/ml.

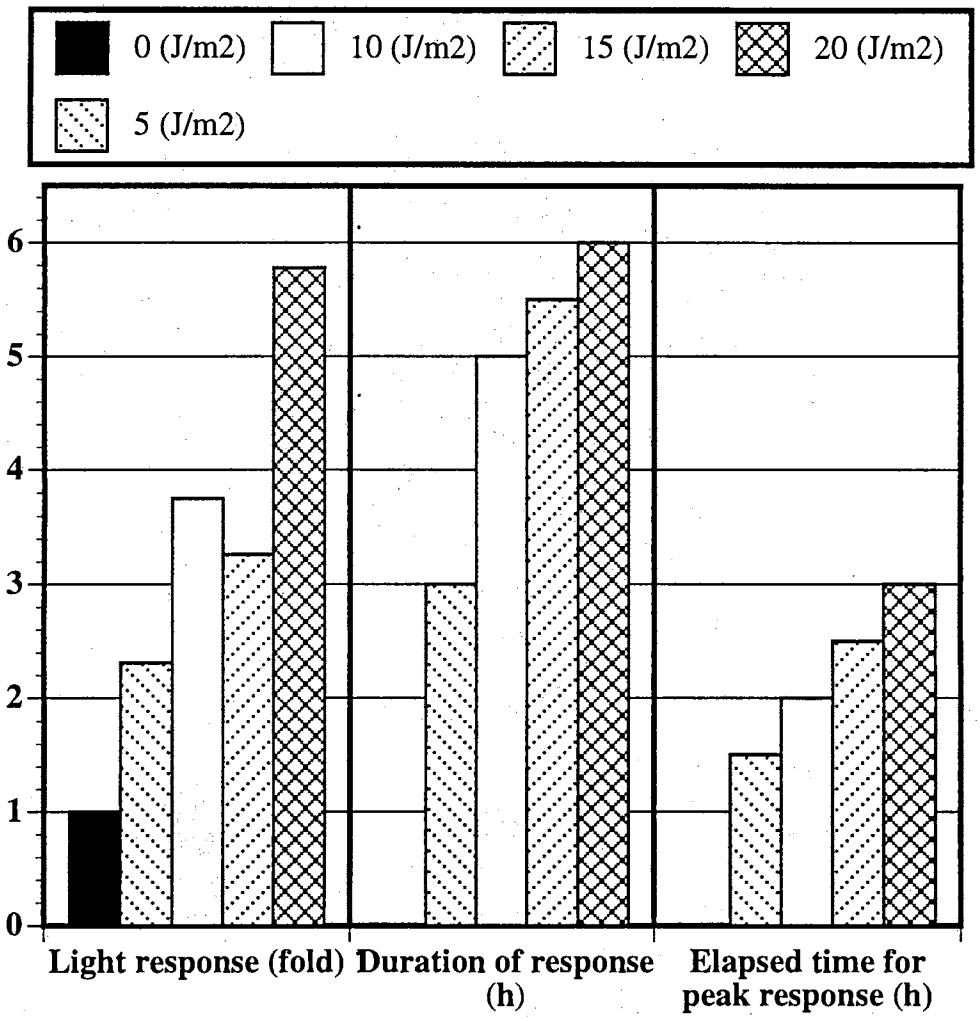


Figure 12. Comparative response of RM4440 to increasing doses of UVC. Light response is shown as a relative increase compared to baseline bioluminescence (no irradiation). Increases in bioluminescence was dose dependant

construct produces light in a dose-dependent response to exposure to UV light.

The response was rapid and directly proportional to UV dose with respect to various parameters including duration of population response, maximal levels of cellular response, and the elapsed time before the appearance of the maximal response. When cells were not exposed to UVC, specific light production (per cell) stayed leveled during the entire experiment. When cells were exposed to UVC, an increase in light production was observed indicating induction of increased expression of the *recA* gene in these cells. The maximal response was proportional to the UVC dose experienced, indicating that the level of induction of *recA* expression was proportional to the amount of DNA damage present in the cell. As the dose increased, the peak response was delayed for longer and longer periods of time. This observation suggests that increased DNA damage reduced the cells ability to initiate transcription (Witkin, 1969), thus increased time elapsed before expression of *recA* to repair the damage.

Our construct will allow us to follow the expression of *recA* in wild-type *P. aeruginosa* in a non-invasive manner. It has the potential to provide real-time analysis with high sensitivity and reproducibility. *P. aeruginosa* is a natural member of soil and freshwater bacterial communities. I believe that this construct can be introduced into various environmental situation to allow monitoring of *in situ* DNA-damaging stress. Constructs of this type will make it possible to gain insight into the state of natural environmental bacterial communities both in pristine

and polluted habitats. They may also be useful in monitoring the bioremediation of sites polluted with various DNA-damaging xenobiotics. They will allow monitoring of the actual state of the community *in situ* possibly without the need to disturb the habitat.

CHAPTER V

STUDY OF RM4440 IN A BIOFILM

In order for RM4440 to be useful as an environmental biosensor, it was of necessary to test RM4440 in conditions that resemble the natural habitat of *P. aeruginosa.* A large number of environmental microorganisms live in biofilms. These biofilms form an important niche that requires careful study. *P. aeruginosa*, in nature, prefers a sessile mode of growth and is in fact responsible for the development of biofilms through the excretion of exopolymers. In the previous chapter, development of RM4440 was described, and its response to UVC damage under batch culture conditions in a rich medium was studied. In this part of the study, RM4440 was studied under immobilization conditions in order to improve our understanding of how biofilm microbial communities cope with stress. These studies allowed us to test the usefulness of RM4440 as biosensor in a different environmental setting (i.e. a biofilm). RM4440 was immobilized in an alginate matrix to simulate conditions that might be found in a biofilm. Minimal medium was used to characterize the biosensor organism's response to increasing doses of UVC, UVB and UVA.

GROWTH CHARACTERISTICS OF RM4440

Growth curves of RM4440 and FRD1

In order to study the growth characteristics of the biosensor, growth curves were done on the host strain (FRD1) without the plasmid (Figure 13) and RM4440 which contains pMOE15 (Figure 14). Two 50 ml Pseudomonas Minimal Medium batch cultures were inoculated from an overnight culture. No carbon source was added to the medium (PMM). The flasks were incubated at room temperature with vigorous shaking. Klett-Summerson measurements were taken periodically to assess growth. Under these conditions, FRD1 had an average doubling time of G = 135 min. RM4440 had a slightly longer doubling time of G = 150 min. The growth rates for FRD1 and RM4440 were $\mu = 0.44\ h^{-1}$ and $\mu = 0.40\ h^{-1}$ respectively. The lower growth rate of RM4440 is probably due to the presence of the plasmid which adds more burden on the cell to replicate the extra DNA.

RM4440 uses alginate as a nutrient

To investigate whether RM4440 is able to use unlinked alginate as a carbon source another batch culture was monitored for growth. A 1 % solution alginate was added to PMM and the culture was incubated at room temperature with vigorous shaking. Klett-Summerson measurements were taken periodically. The experiment was done twice (Figure 15). The average doubling time of RM4440 was G = 60 min and the growth rate $\mu = 1.0\ h^{-1}$. The growth rate was two-fold faster in the presence of alginate than without which indicates that RM4440 uses

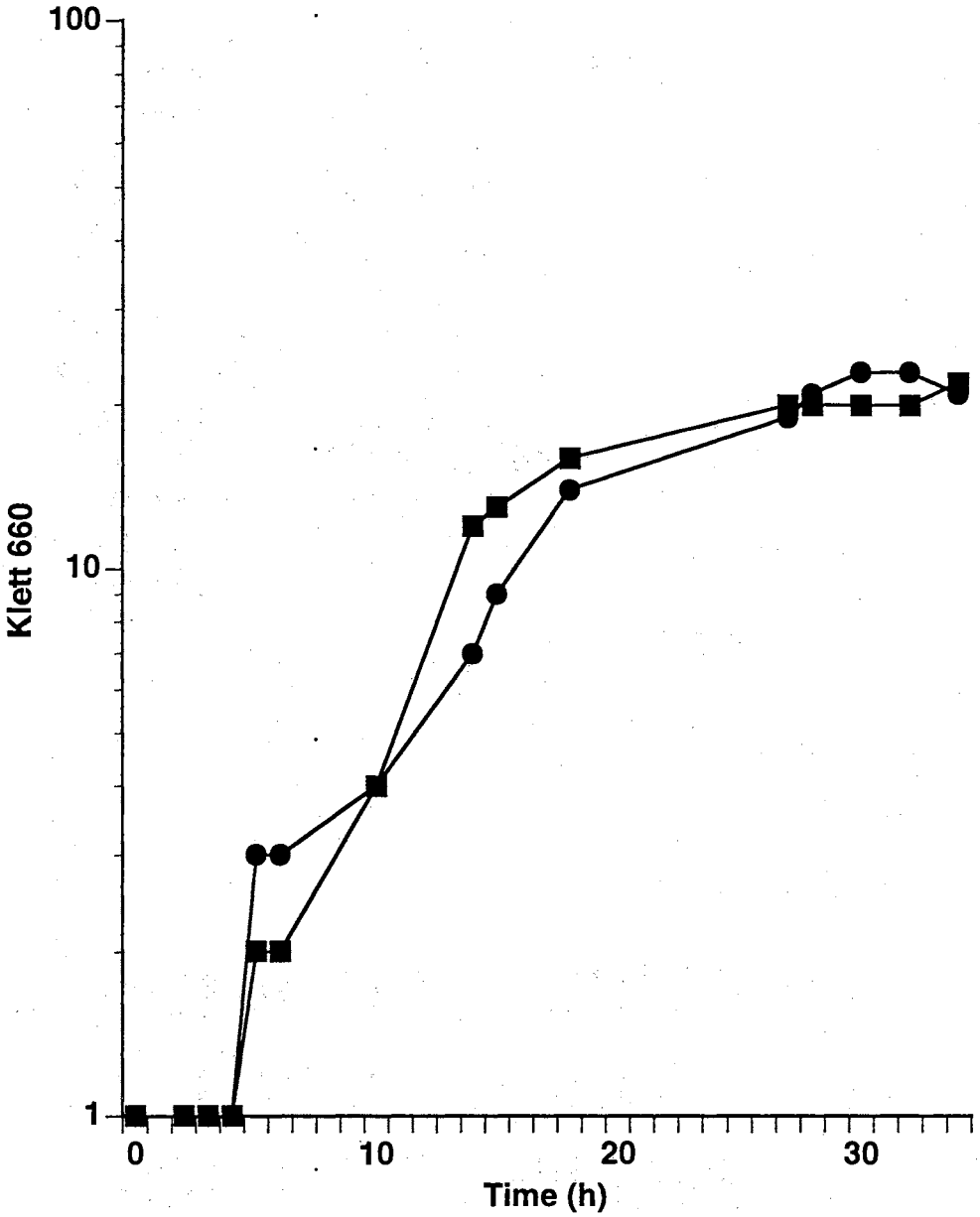


Figure 13. Growth curve of FRD1 in liquid PMM. The cultures were incubated at room temperature. The experiment was done twice.

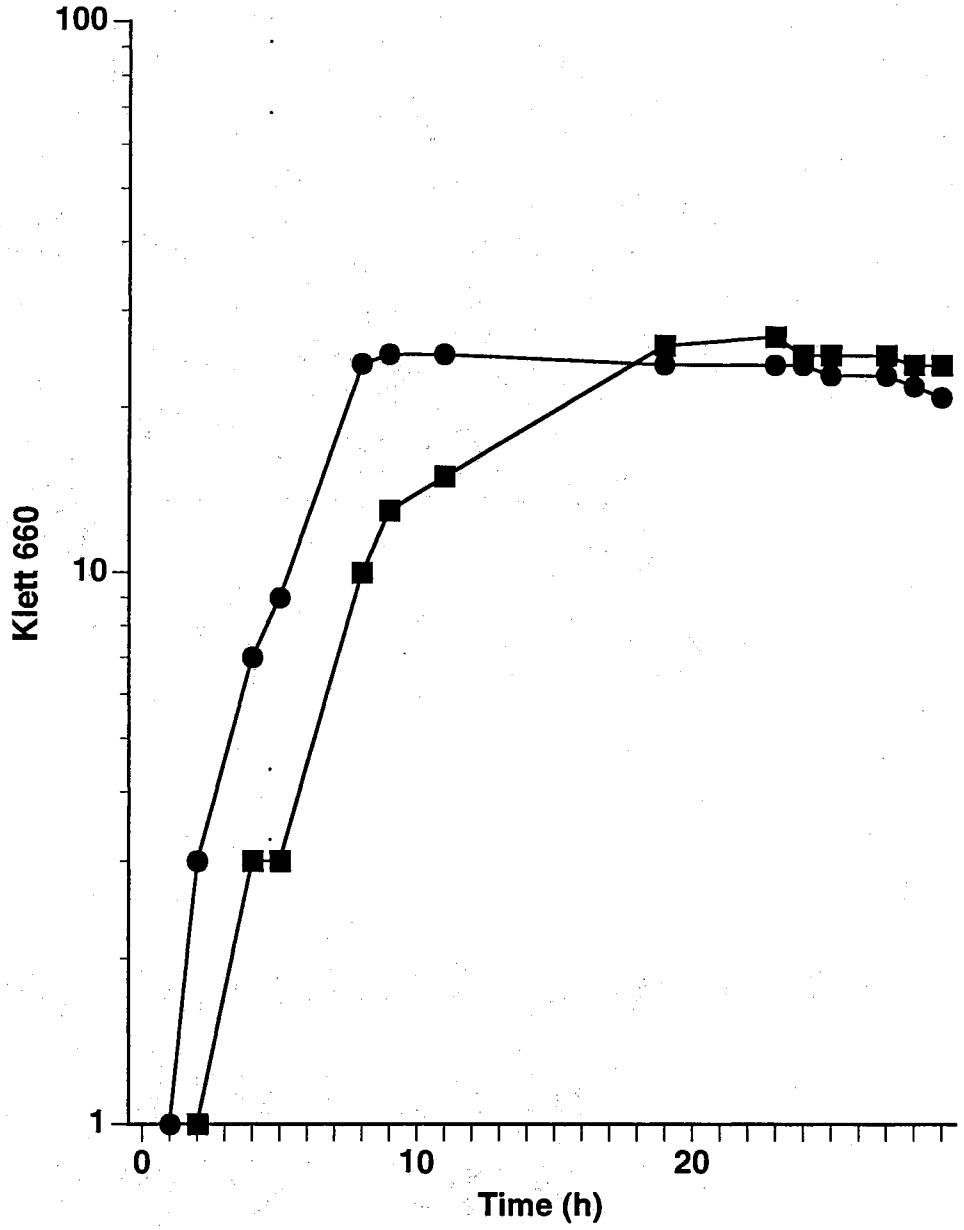


Figure 14. Growth curve of RM4440 in liquid PMM. The cultures were incubated at room temperature. The experiment was done twice. ■, Expt-1, ●, Expt-2;

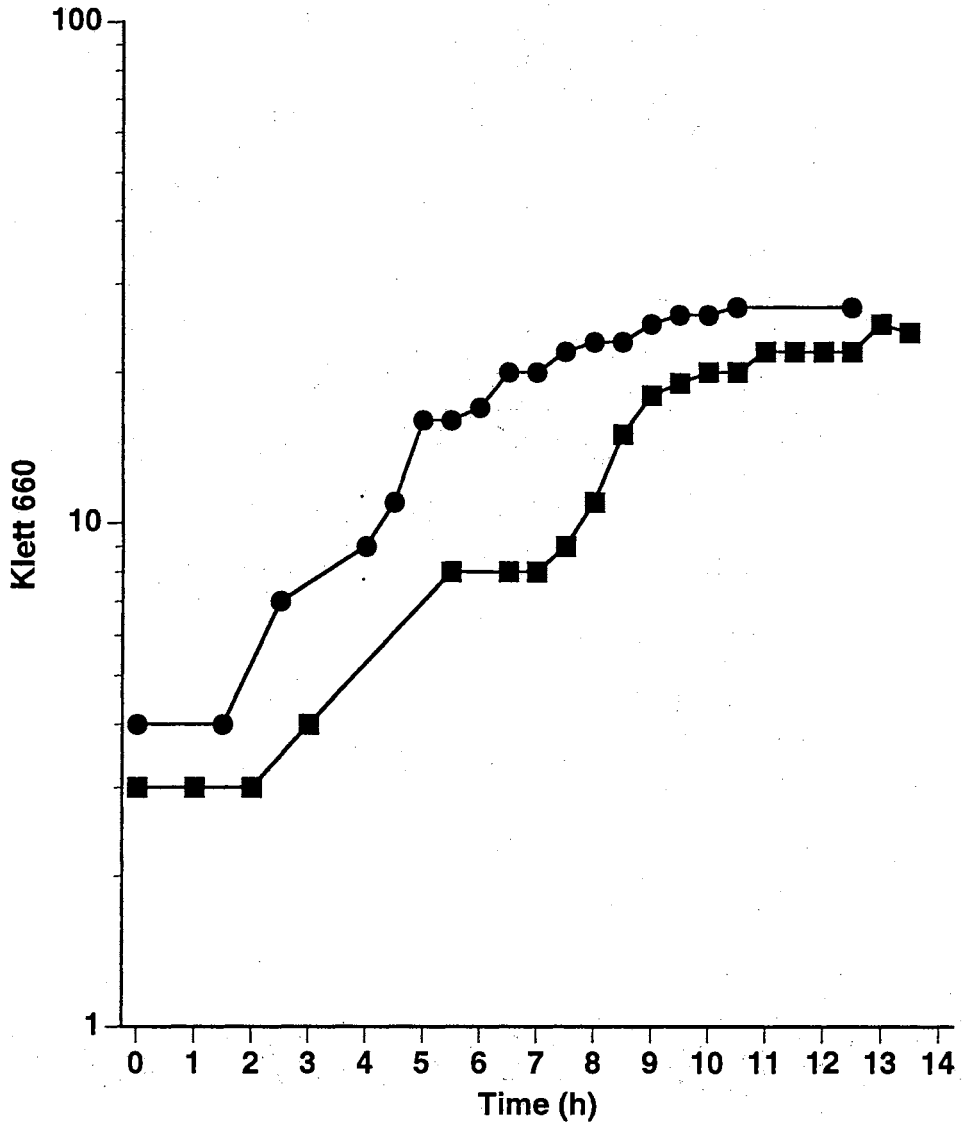


Figure 15. Growth curve of RM4440 in liquid PMM supplemented with 1% alginate. The cultures were incubated at room temperature. The experiment was done twice. ■, Expt-1, ●, Expt-2;

alginate as a carbon source under batch culture conditions.

Growth curve of RM4440 in the alginate matrix

When cells are immobilized in an alginate matrix the physical environment is different from batch cultures because the alginate is covalently crosslinked into a network. FRD1 carries the *algL* gene which encodes an alginate lyase. This lyase is believed to be important in the release of cells from the biofilm by breaking down the alginate network (Monday and Schiller 1996). This Lyase may also be used for release of alginate for consumption by cells.

Growth of RM4440 was analyzed under immobilization conditions. RM4440 was immobilized into 4 mm alginate beads. A monolayer of beads was fed continuously with *Pseudomonas* Minimal Medium at a flow rate of 3.5 ml/min. Samples for viable counts were taken every 2 hours by dissolving 5 beads and plating serial dilutions on LB plates. Growth in the matrix was monitored for a period of 30 h. Under these conditions, RM4440 showed a growth pattern similar to a batch culture (Figure 16). The exponential growth phase showed a doubling time of RM4440 was G = 168 min and the growth rate $\mu = 0.36\ h^{-1}$. A phase with a constant number of cells followed the exponential phase. The growth rate in the alginate matrix was faster than that of the batch culture with alginate because The immobilized cells were fed continuously with PMM while the batch culture has an exhaustible source of nutrients.

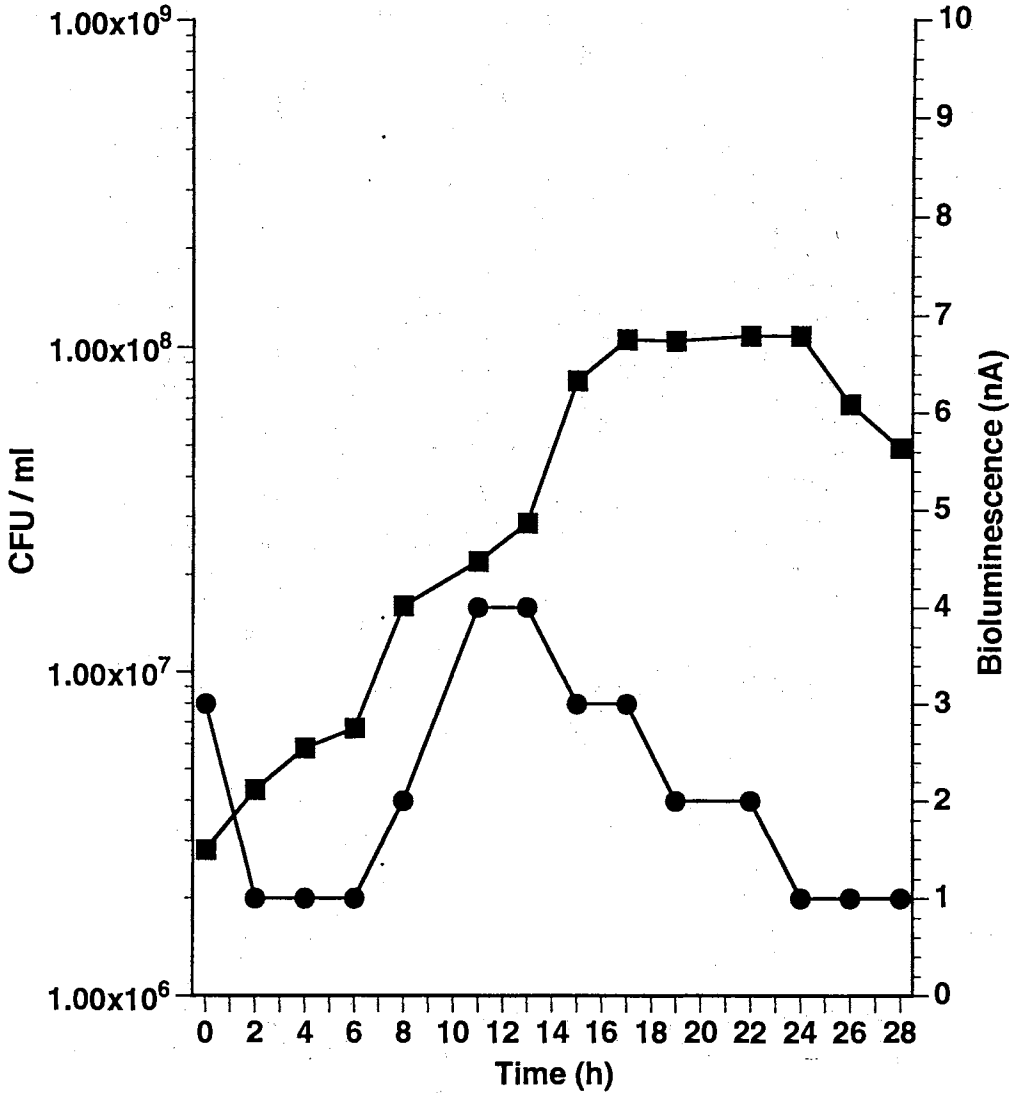


Figure16. Growth curve of RM4440 immobilized in the alginate matrix. The experiment was done at room temperature with PMM as the maintenance medium. Samples were taken every two hours. The cells were released from the matrix using sodium polyphosphate. Bioluminescence was continuously monitored. ■, CFU/ml; ●, bioluminescence(nA).

Alginate beads

RM4440 cells were physically entrapped in the beads of strontium alginate matrix. The beads were 4 mm in diameter. UV transmission of alginate was measured as described in materials and methods and was found to be 13 % of UVC, 31 % of UVB and 33 % of UVA radiation to which the biofilm was exposed (Figure 17). Scanning electron microscopy was done on the beads to observe the distribution and the effects of UVC stress (10 J/m^2) on the cells in the matrix (Figure 18). The cells on the surface of untreated beads were distributed individually and showed no obvious clumping. Most cells were partially buried in the matrix although the harsh treatment and washing of the beads prior to microscopy may have washed some cells out. The cells exposed to UVC were filamentous which indicated an inhibition of cell division similar to the SOS-associated filamentation observed in *E. coli* (Hill et al., 1997). This phenomenon insures the proper partitioning of genetic materials to daughter cells. In *E. coli*, two pathways coordinate DNA replication and cell division, a *sfi*-dependent and a *sfi*-independent pathway. In the *sfi*-dependent pathway, the gene *sfiA* is induced as part of the SOS response, and the protein SfiA binds FtsZ, an essential protein involved in septal biogenesis. This binding prevents septation and results in the formation of filaments (Higashitani et al., 1995). In *sfi*-independent filamentation, SfiA is not required, however DNA damage is necessary, as well as the derepression of the SOS regulon (Hill et al., 1997).

In *P. aeruginosa*, filamentation has been observed in response to various antibiotics (Chen *et al.*,1997; Elliot and Greenwood, 1984). To date,

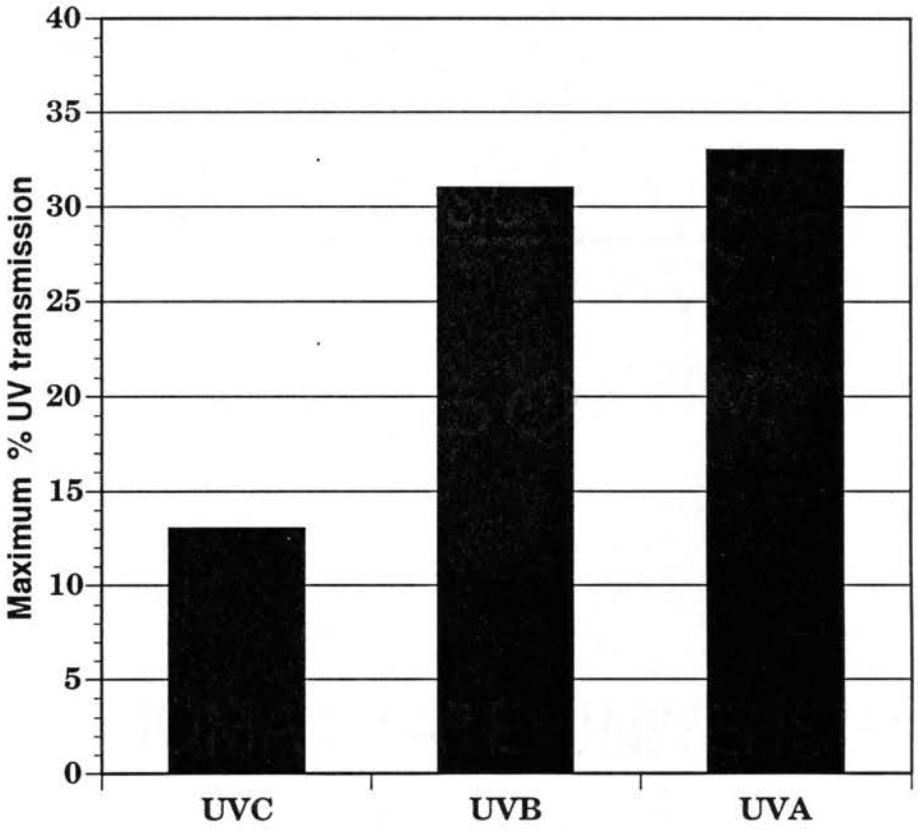


Figure 17. Maximum % UV transmittance by the alginate matrix. Fluences of UV were measured directly and through an 4 mm (diameter of bead) thick alginate layer that has been crosslinked.

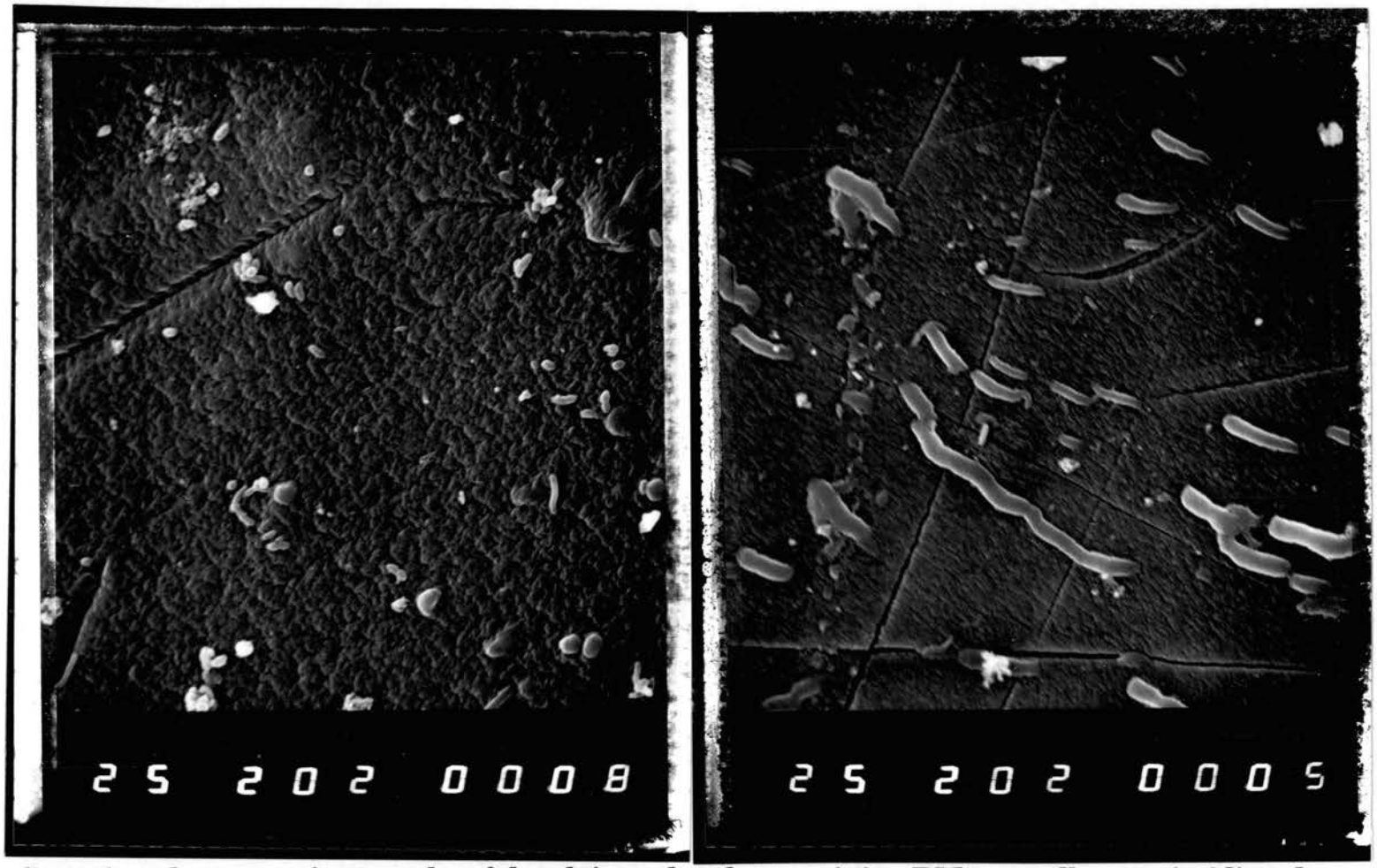


Figure 18. Scanning electrom micrographs of the alginate beads containing RM4440 cells. a. unirridiated control. b. exposure to 10 (J/m2) of UVC. Stressed cells show filamentation due to inhibition of cell division. Magnifivcation 2000x.

however, no *sfiA* analog has been isolated from *P. aeruginosa*. Thus, the mechanisms of filamentation remain unknown in this organism.

RM4440 responds to UVC stress

RM4440 cells were trapped in a cross-linked alginate matrix and exposed to increasing doses of UVC light. The dose of UVC radiation was delivered as a single pulse and visible (490 nm) *lux*-dependent light production was monitored continuously following exposure (Figure 19). Viable counts were taken before and after irradiation to account for cell death.

The bioluminescent response was normalized by dividing the amount of light by the number of cells after each UVC exposure. RM4440 showed a rapid and reproducible increase in bioluminescence in response to UVC stress. The response profile was similar for all doses, a lag period of 30 min followed the pulse of UVC before visible light production began to increase steadily (Figure 20). Peak bioluminescence was observed 4.5 h after irradiation for all doses except for the 12.5 J/m^2 dose for which it was 6.5 h. The response of RM4440 showed an overall dose-response profile consistent with results obtained previously in batch culture experiments (Elasri and Miller, 1998) (Figure 21). The UVC dose related to the response by linear regression equation of :

Bioluminescence response = $1.94\ 10^{-6}$ * (UVC dose) + $1.69\ 10^{-6}$

with a regression coefficient of $r^2 = 0.72$.

The survival rate of RM4440 in the matrix decreased as the dose of UVC increased (Figure 22), it was however higher than the survival rate

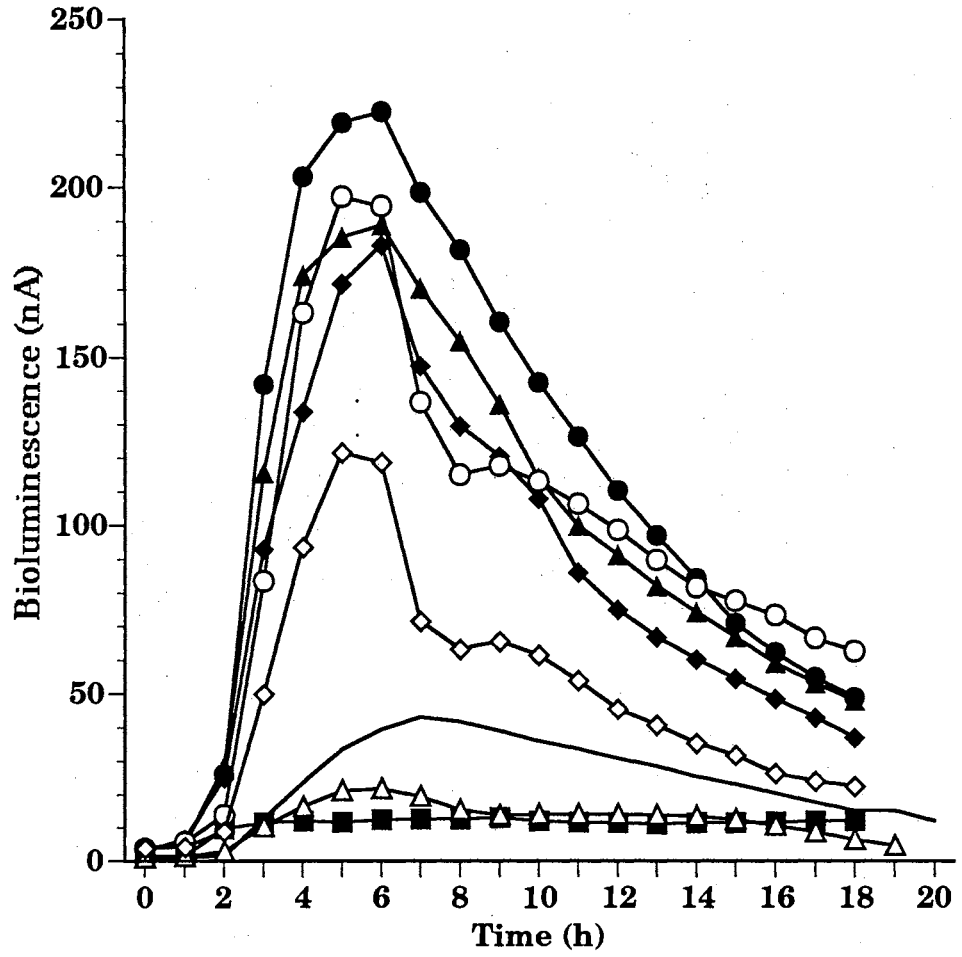


Figure 19 . Bioluminescence response of RM4440 to increasing doses of UVC (J/m2) in biofilm : ■, 0; ●, 2.5; ▲, 5; ◆, 10; □, 12.5; O, 15 ; △, 17.5; ◇ , 20. Each point represents the mean of three independent experiments.

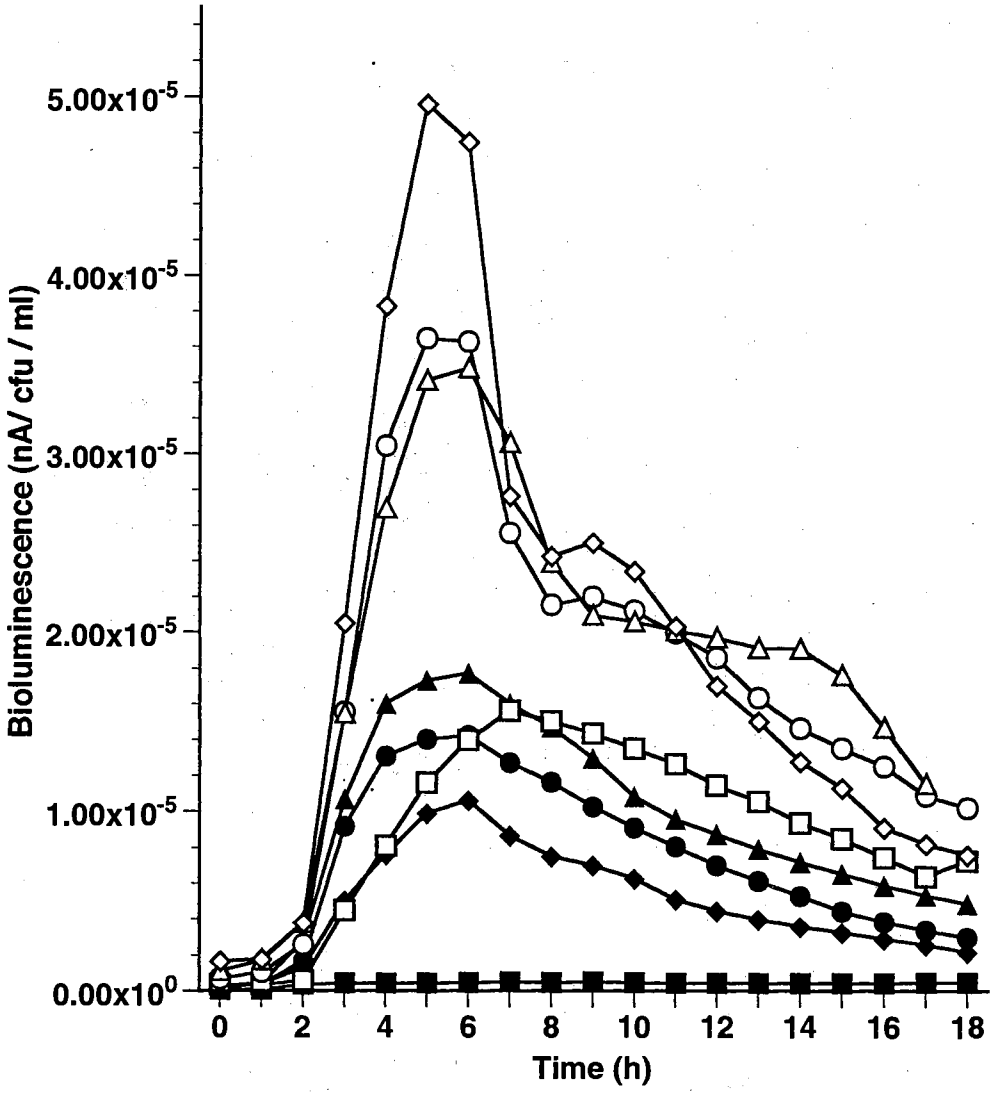


Figure 20 : Normalized bioluminescence response of RM4440 to increasing doses of UVC (J/m2) in biofilm : ■, 0; ●, 2.5; ▲, 5; ◆, 10; □, 12.5; O, 15 ; △, 17.5; ◇ , 20. The response was normalized by dividing the light produced by number viable cells in 1 ml of matrix (nA/CFU/ml). Each point represents the mean of three experiments.

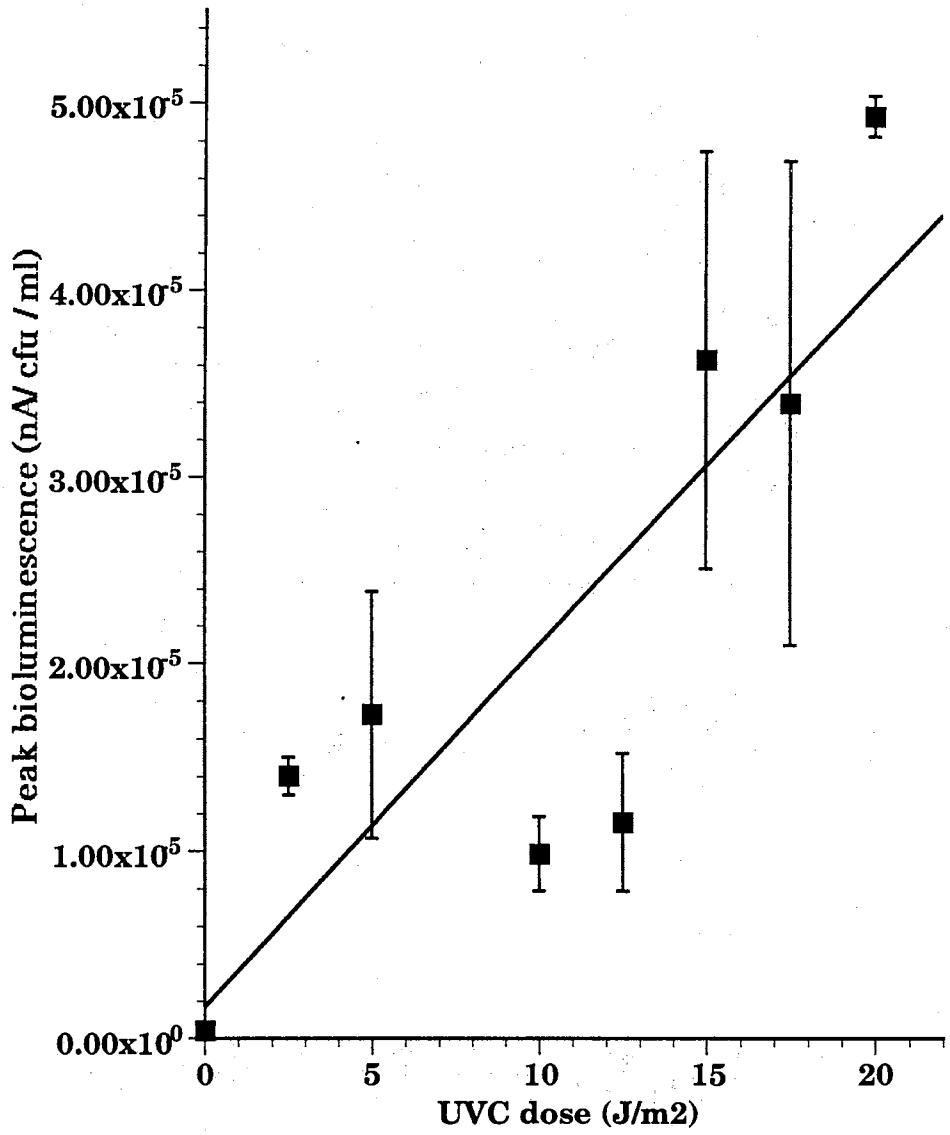


Figure 21. Peak bioluminescence after exposure of RM4440 biofilm to increasing doses of UVC. The peak response was 5 hours post irradiation for most doses. The data represent the mean of three independent experiments. (regression coefficient of r2 = 0.72)

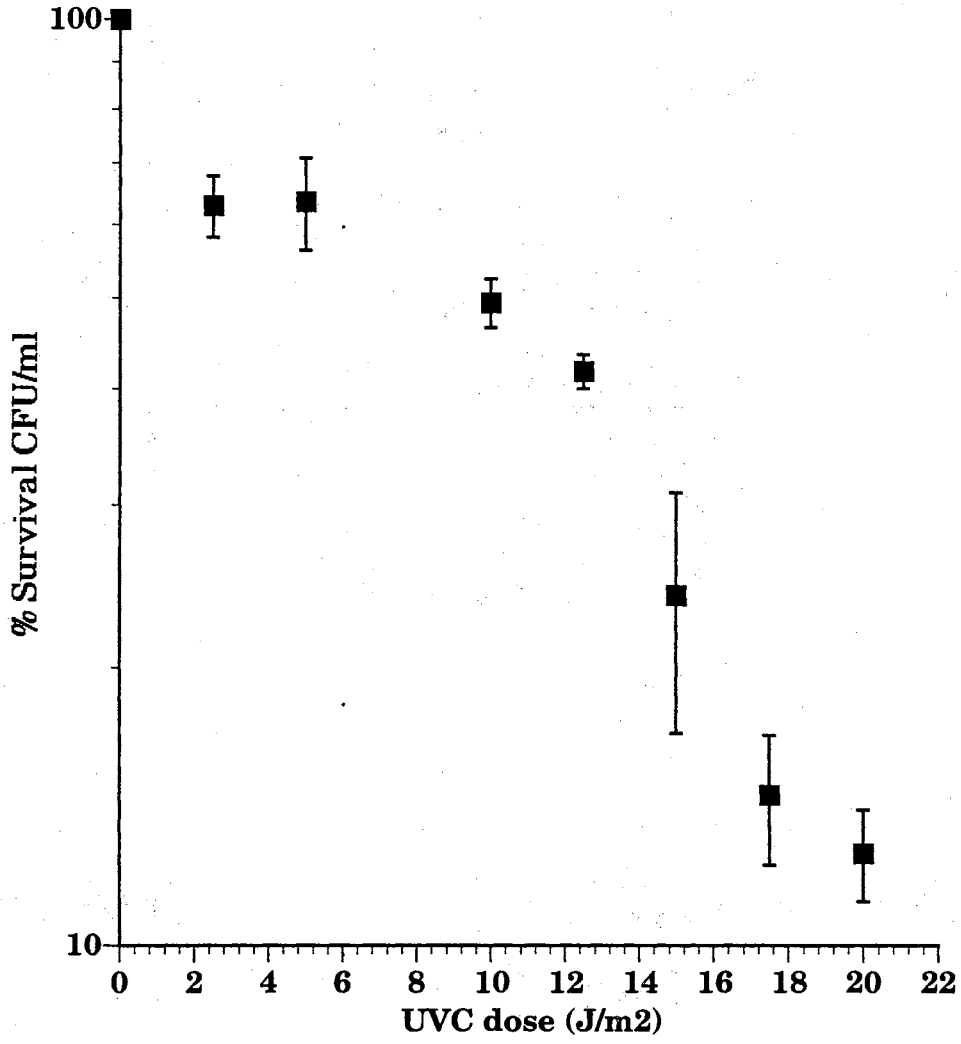


Figure 22: Survival rate of RM4440 under increasing doses of UVC. Data represents the mean of three independent experiments.

observed in rich liquid medium (Simonson et al., 1990). Although UVC is poorly transmitted by the matrix more than any other range of UV (13% maximum transmittance), it has shown the most bioluminescent response. This is probably due to the fact that UVC is still the most efficient range of UV at causing damage and thus inducing the SOS response. Although, the environmental relevance of UVC is limited, because of its germicidal effects, there is great interest by many industries in destruction of biofilms that are responsible for fouling of man-made materials. An understanding of the dynamics of how biofilm cells cope with UVC stress is critical.

RM4440 responds to UVB stress

RM4440 was exposed to increasing doses of UVB radiation in a fashion similar to the procedure used for UVC irradiation. In order for the study to be environmentally relevant, UVB doses were chosen based on average daily doses of UVB reaching the earth (Booth, 1997). Similarly to the results obtained following UVC irradiation, the response profile of the irradiated biofilms was marked by a lag period of 30 min and peak responses were observed 4.5 h post irradiation (Figure 23).

Dose dependance was not as apparent with UVB as it was with UVC (Figure 24). However, when peak responses (5 h for most doses) were plotted as a function of the UVB dose administered, there was a tendency for bioluminescence to increase as dose increased (Figure 25). The UVB dose related to the response by linear regression equation of :

Bioluminescence response = $1.33\ 10^{-7}$ * (UVB dose) + $2.44\ 10^{-6}$

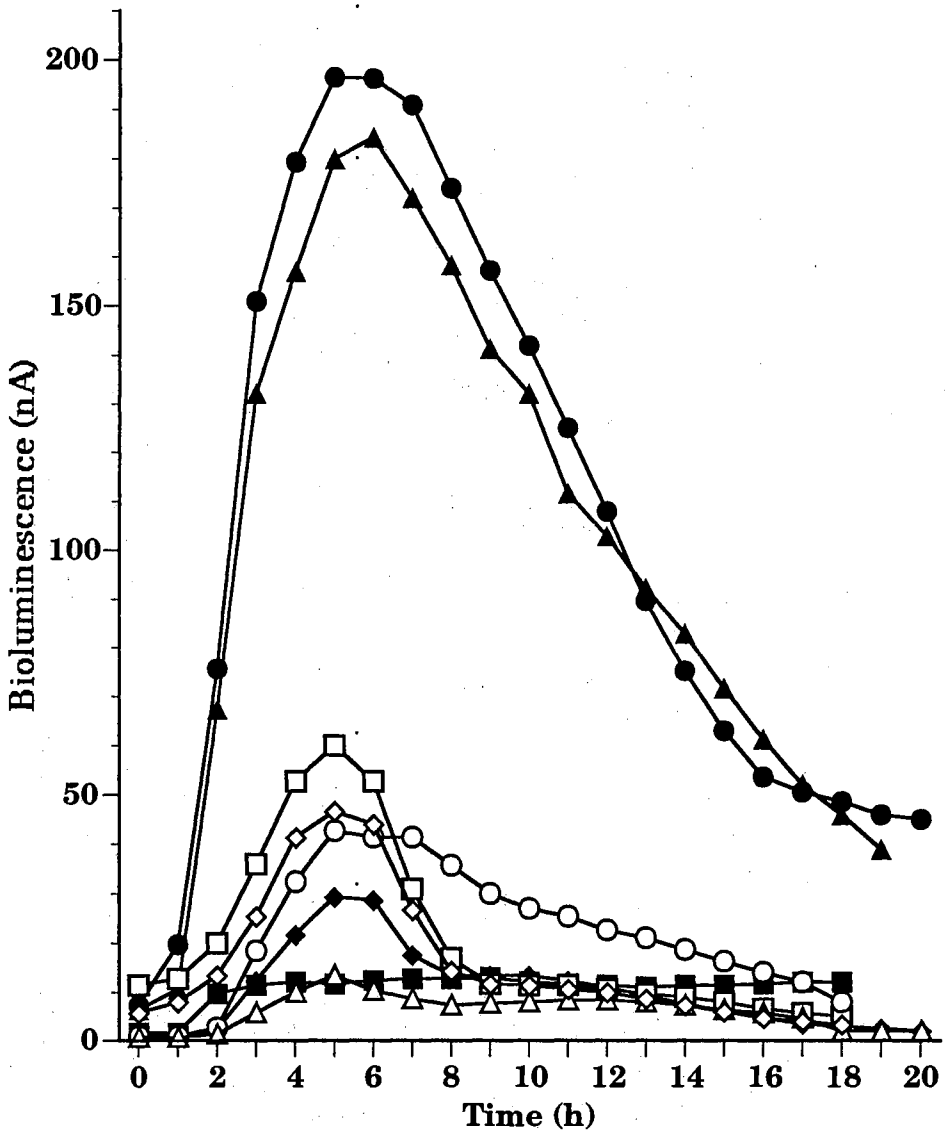


Figure 23. Bioluminescence response of RM4440 to increasing doses of UVB (J/m2)in biofilm : ■, 0; ●, 25; ▲, 50; ◆, 75; □, 100;O, 110;Δ, 120; ◇, 125. Each point represents the mean of three independent experiments.

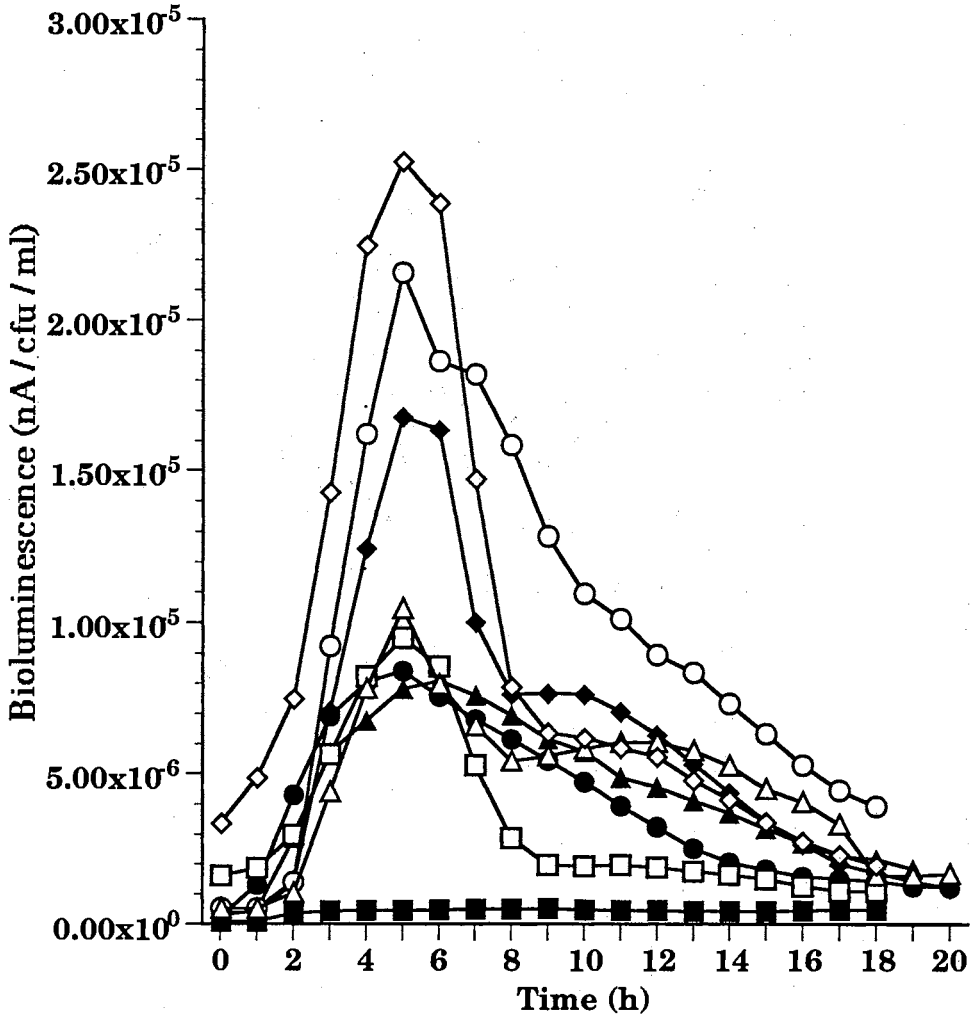


Figure 24. Normalized bioluminescence response of RM4440 to increasing doses of UVB (J/m2) in biofilm: ■, 0; ●, 25; ▲, 50; ◆, 75; □, 100;○, 110;△, 120; ◇, 125. The response was normalized by dividing the light produced by number viable cells in 1 ml of matrix (nA/CFU/ml). Each point represents the mean of three independent experiments.

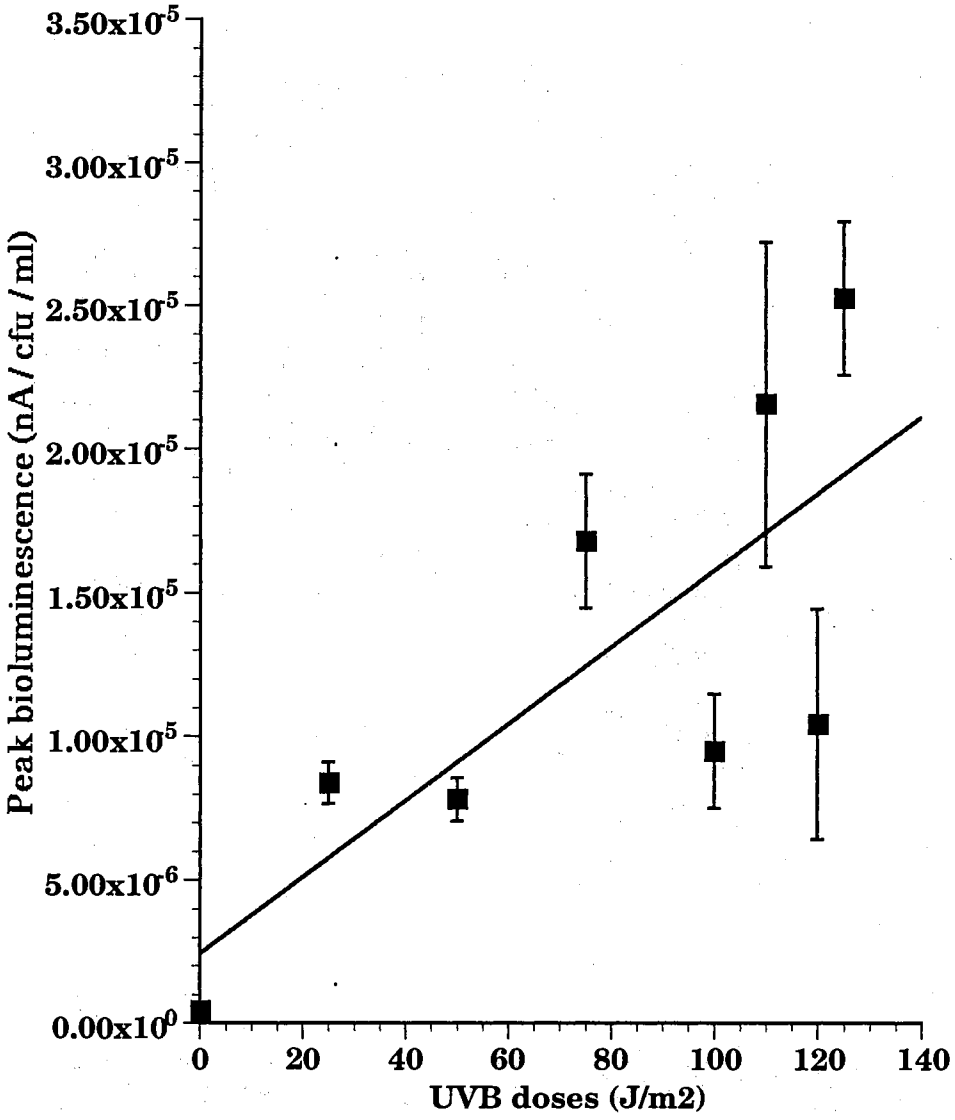


Figure 25. Peak bioluminescence after exposure of RM4440 to increasing doses of UVB in biofilm. The peak response was 5 hours post irradiation for most doses. The data represent the mean of three independent experiments. (regression coefficient of r2 = 0.58)

with a regression coefficient of $r^2 = 0.58$.

The survival rate of cells under various doses was monitored (Figure 26). Survival rate decreased with higher doses of UVB. The survival rate of cells in the biofilm was greater than that of free cells owing to the physical shielding of the alginate matrix. This is important in improving our understanding of the role of biofilms. In nature, it appears that sessile microbial communities are likely to surpass planktonic cells in coping with UV stress in environmental settings.

RM4440 responds to UVA stress

UVA doses of up to 20,000 J/m^2 were used to test the response of biofilms containing RM4440 to near UV light. Bioluminescence stayed at basal levels indicating little or no induction of *recA* expression. Doses used in this study were within the range of the average dose of UVA light that reaches the earth daily.

Effects of psoralen treatment on RM4440

In order to investigate the effects of UVA in the presence of a photosensitizer on microbial communities in biofilms, beads were stirred in 100 ml of PMM containing psoralen (final concentration of 10 mg/l) for 30 min and subsequently exposed to an hour of ambient light or a pulse of UVA (8000 J/m^2 and 16000 J/m^2). Psoralen was also added to the biofilm in the dark as a control. Treatment of RM4440 to psoralen and UVA resulted in increased bioluminescence (Figure 27 and 28). A loss in viability was also noted. This demonstrated that *recA* expression was induced in

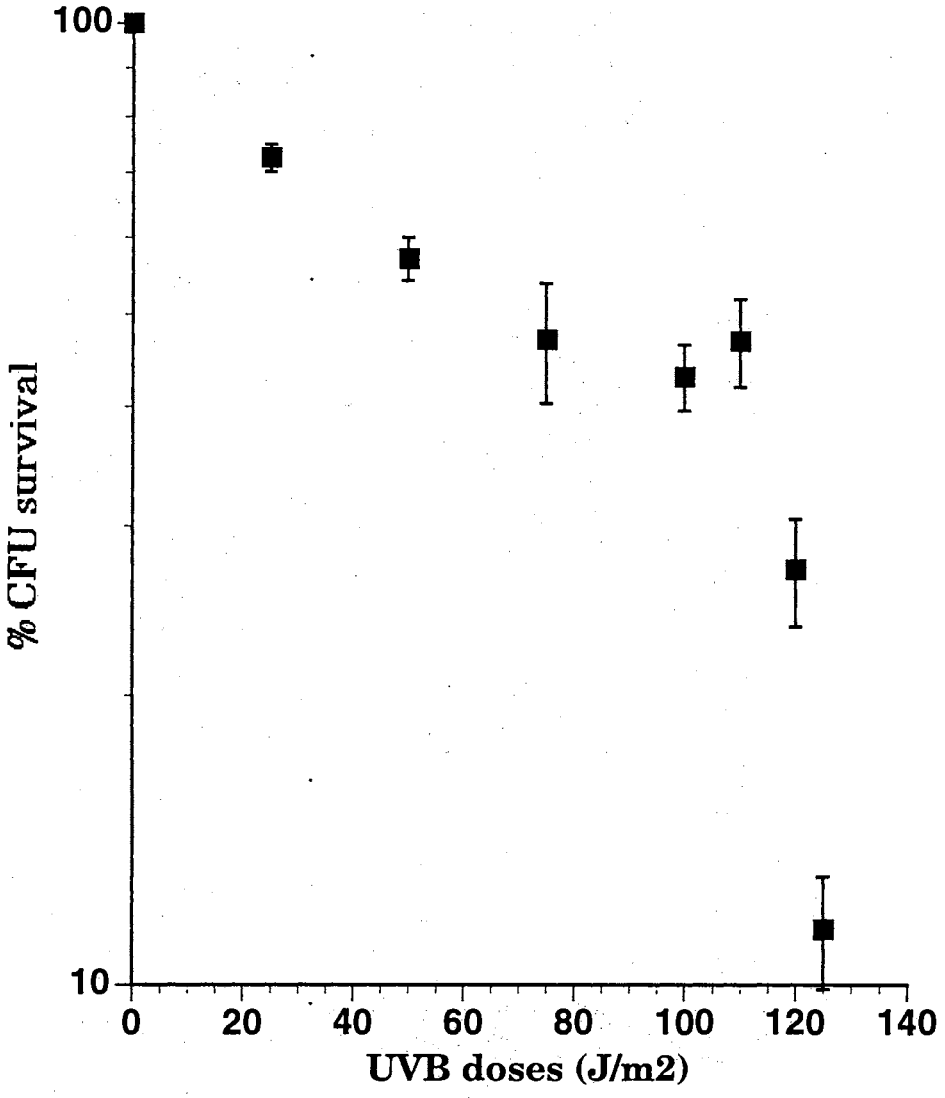


Figure 26. Survival rate of RM4440 under increasing doses of UVB. Data represents the mean of three independent experiments.

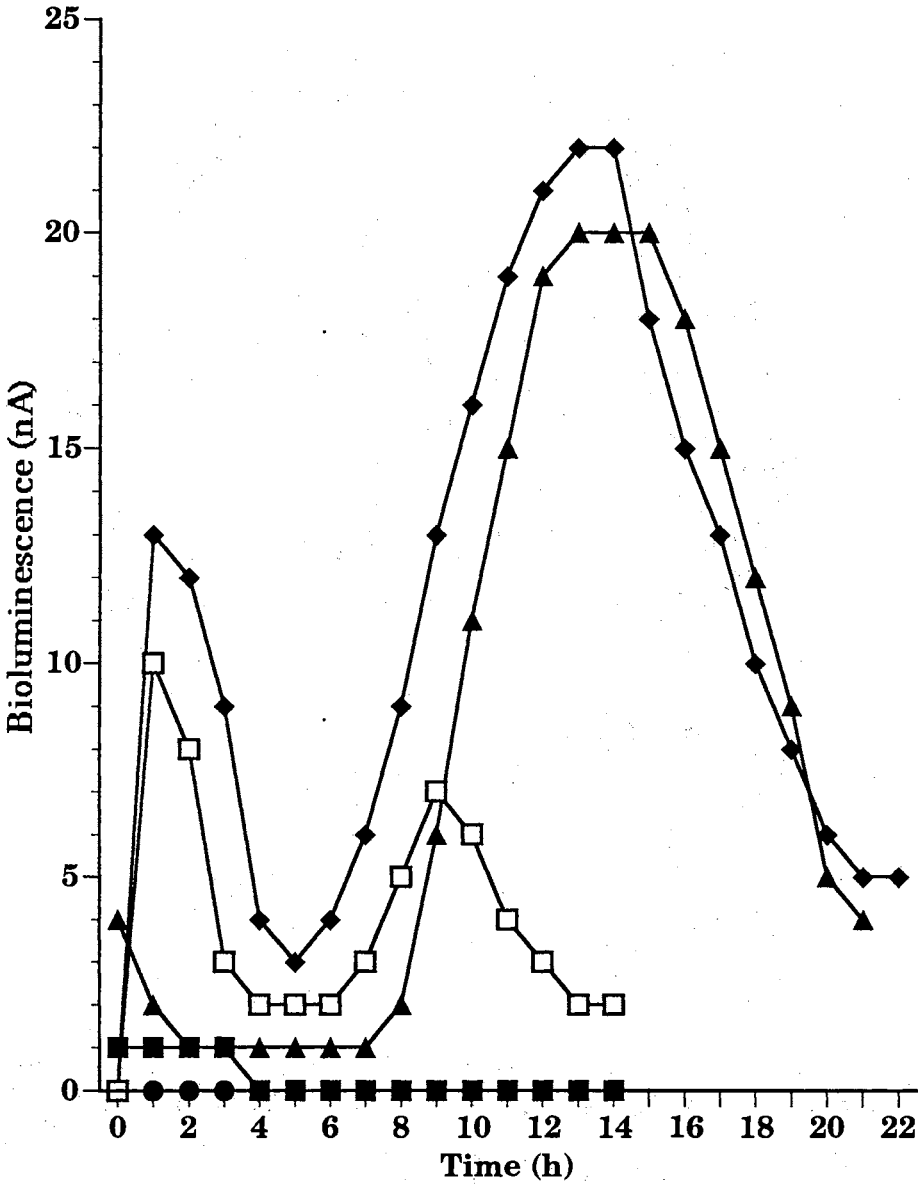


Figure 27. Bioluminescence response of RM4440 after exposure to UVA and psoralen. The biofilm beads were stirred in PMM containing psroralen. ■, UVA only; ●, Psoralen only; ▲, ambient light and psoralen; ◆, 8000 (J/m2) UVA and psoralen; □, 16000 (J/m2) UVA and psoralen.

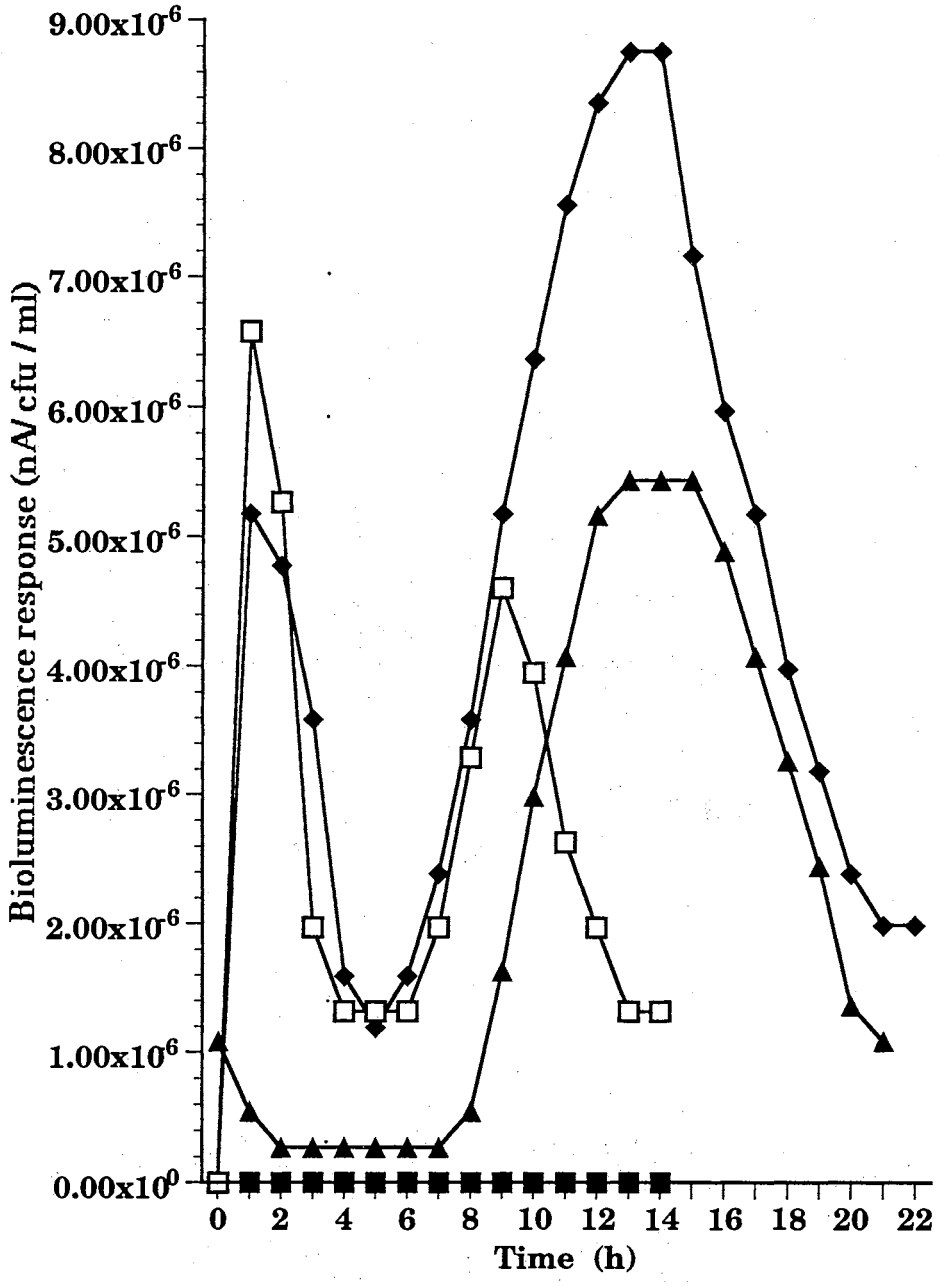


Figure 28 . Normalized bioluminescence response of RM4440 to UVA and psoralen after exposure to UVA and psoralen in biofilm. The biofilm beads were stirred in PMM containing psroralen for an hour. ■, UVA only; ●, Psoralen only; ▲, ambient light and psoralen; ◆, 8000 (J/m2) UVA and psoralen; □, 16000 (J/m2) UVA and psoralen.

response to covalent binding of psoralen to DNA upon UVA exposure. The psoralen-dark control did not show a bioluminescent response, it did however cause cell death. The profile of the response to psoralen and UVA was different from the responses to UVB and UVC. Bioluminescence showed two peaks, an early one that is reminiscent of the rapid response to UVB or UVC exposure and a delayed response that appeared several hours later. This profile may be due to an early phase of damage that is caused by DNA-bound psoralen which causes immediate injury. The SOS system is induced in response and some damage is repaired. The second phase of damage may be an effect of residual psoralen that is trapped in the matrix or of secondary damage to other macromolecules (i.e. proteins) which in turn interact with DNA and damage it (Averbeck, 1989). These results are consistent with previous studies showing the involvement of the SOS system in repair of psoralen damage (Bauluz et al., 1991; Sladek et al., 1989). When psoralen was added to the RM4440 biofilm in the dark, a genotoxic effect was observed. However there was no noticeable *recA* expression. Quinto et al. (1984) showed that in the dark, psoralen causes frameshift mutagenesis in bacterial cells , through non-covalent interaction with DNA . This type of mutagenesis may explain the genotoxic effects observed with RM4440 after the dark-psoralen treatment.

Environmental insights

I characterized the response of a *P. aeruginosa recA* promoter-*luxCDABE* fusion under conditions that more closely resemble the

natural setting of microorganisms found in soil and fresh water habitats. Minimal medium was used to maintain a low nutritional-content environment. I immobilized cells in an alginate matrix to simulate the way mucoid *P. aeruginosa* engulf themselves in secreted exopolymers (primarily alginate) when establishing a biofilm in nature. Biofilms have been shown to be the preferred mode of growth of *P. aeruginosa* in aquatic environments (Costerton et al., 1987). The alginate excreted by cells in their native habitat is believed to be used by cells for establishment of biofilms upon adhesion of cells to solid surfaces in nature (Costerton et al., 1987; Davies and Geesey, 1995; Gacesa, 1998). Alginate as a biofilm matrix has been shown to be important in complications of cystic fibrosis (10) and other diseases such as urinary tract infections (Nickel et al., 1985). Bacteria entrapped in alginate has also been shown to play a major role in fouling of man-made materials (Christensen and Characklis, 1990). In addition, alginate endows *P. aeruginosa* with protection against antibiotics (Hodges and Gordon, 1991). I discovered that the alginate biofilm transmits only small amount of UV radiation protecting the cells from exposure (13% of UVC, 31% of UVB and 33% of UVA). This suggests that the exopolymer may be a natural defense mechanism used to attenuate UV light exposure in nature. This attenuation was evident from the higher rate of survival of the immobilized cells that was observed when compared to experiments done in liquid cultures after exposure to various doses of UV (See chapter IV, and Kidambi et al., 1996).

The ability of RM4440 to monitor the effects of UVC and UVB on microorganisms in an artificial biofilm was demonstrated. The response

profiles for UVC and UVB were similar supporting the observation that they cause similar types of damage to cells. UVC is considered to be more efficient in damaging DNA and lower doses caused significant induction of *recA* expression. No bioluminescence response was observed with UVA alone when immobilized RM4440 were exposed. This may be due to the absorption of UVA by the alginate matrix. However, the presence of a diffusible, exogenous photosensitizer, caused indirect damage effects to RM4440 in the alginate biofilm following UVA exposure. UVA has been shown to induce *recA* expression in marine isolates (Booth, 1997) and in *P. aeruginosa* (Kidambi et al., 1996). In the Kidambi *et al.* study, *P. aeruginosa* was exposed to UVA in liquid media which may reflect on the increased UV sensitivity of planktonic cells in the environment. In this study, the organisms were sheltered by the matrix and thus the increased UV resistance. The presence of photosensitizers in the environment has the potential to render UVA a important stress even in biofilm communities. RM4440 has the potential to be used as a monitor for biological stress in various environments. As a biosensor it can be incorporated into sessile, as well as planktonic bacterial communities.

CHAPTER VI

RESPONSE OF RM4440 TO VARIOUS CHEMICALS

The need to monitor environmental pollutants that may be mutagenic, carcinogenic, or teratogenic has become increasingly important. Various techniques have been developed for this purpose. The most promising methods are based on microorganisms. Bacterial tests have been used to predict mutagenicity and carcinogenicity of chemical compounds. The most prominent ones are the Ames Test (Ames *et al.*, 1975) and the SOS Chromotest (Quillardet *et al.*, 1982). Bacteria-based tests are utilized because of the rapid growth of microorganisms in defined media and the low cost associated with them. The Ames Test, also known as, the Salmonella/Microsome assay is the most prominent of these tests. It uses a number of mutant- *Salmonella* strains and tests for their reversion from histidine auxotrophy to prototrophy. The SOS Chromotest is an *E. coli*-based test that relies on a fusion of the β-galactosidase gene to the *sfiA* gene promoter. *sfiA* is a member of the SOS Network, that is induced in response to various DNA-damaging stresses (Walker *et al.*, 1984). In order to test RM4440 effectiveness in detecting DNA damage caused by

agents other than UV, a battery of potential DNA-damaging chemicals were tested.

Testing of various chemicals on the RM4440 biofilm

RM4440 biofilms were exposed to one of a series of chemicals as described in chapter III. This set of chemicals was chosen for a pilot study to assess the response of sensor bacteria in a biofilm situation. Table 3 shows the response of this community to the different chemicals tested. A number of chemicals were effective in causing light production. The response to these chemicals was delayed when compared to UV responses. This may be due to the difference in the mechanisms of action of these two types of agents (i.e. radiation and chemicals). Both agents may cause damage either by direct interaction with DNA or through interaction with a secondary target that eventually causes DNA damage. Radiation hits the target DNA directly and the damage occurs instantly and thus the response is rapid. Chemicals on the other hand, have to cross two major barriers through diffusion. The first barrier is the biofilm matrix which may decrease the diffusion rate of chemicals due to its semi-solid structure. The second major barrier is the bacterial cell membrane. The cytoplasm may also slow down the rate of diffusion of chemicals. Response to chemicals was observed after up to 7 hours post exposure for some agents. This response is slow when compared to UV radiation which shows a response as early as 30 minutes post irradiation (See chapter V).

Methyl methane-sulfonate is a known strong inducer of the SOS response

Table 3. Response of RM4440 to various chemicals

Chemical tested	SOS induction[a]	Known SOS inducers[b]
Ethanol	_	_ (c)
Hydrogen peroxide	_	+ (d)
Cadmium chloride	_	_ (c)
Benzene	_	_ (c)
Hexachlorobenzene	_	_ (c)
Ether	_	_ (c)
Pentachlorobenzene	_	_ (c)
Phthalic acid	_	_ (c)
Arsenic acid	_	_ (c)
Methyl methane-sulfonate	+	+ (e)
Benz (a) anthracene	+	+ (f)
Benzo (b) fluoranthene	+	+ (f)
Dibenz (a,h) anthracene	+	+ (f)
Benzo (a) pyrene	+	+ (g)
Psoralen	_	NA (j)
Psoralen + UVA	+	+ (h)
Hexacholorobutadiene	+	NA (i)
Vinyl chloride	+	NA(j)

Table 3 (continued)

(a), data for SOS induction are (-) for light production of less than ten fold the baseline bioluminescence, (+) more than 10 fold baseline bioluminescence. (b), references of results reported in other studies: (c), Quillardet and Hofnung, 1993; (e), Quillardet and Hofnung, 1985; (d), Ptitsyn *et al.*, 1997; (f), Mersch-Sundermann et al., 1992; (g), Quillardet *et al.*, 1985; (h), Bauluz et al., 1991; (j), this study; NA, not appliquable.

(Quillardet and Hofnung, 1985). This chemical demonstrated a dose response relationship similar to the one observed with radiation (Figure 29 and 30). This study illustrates the potential for use of RM4440, not only as a detector, but also as a gauge of the amount of DNA-damaging chemical agents present *in situ*. A method for quantitative analysis of the health of a microbial community is very valuable in evaluating the effectiveness of bioremediation processes.

In general, the response of RM4440 is specific since there was no light production in response to exposure to chemicals that are known not to induce the SOS response (Table 3). Hydrogen peroxide, however, yielded a negative response although is known to induce the SOS network through oxidative damage. This may be due to two factors, first RM4440 is a *P. aeruginosa* which is catalase positive and second, the fact that the RM4440 assay was done in an alginate matrix which may diminish the oxidative effect of the highly reactive H_2O_2 molecule. Previous studies on the effects of H_2O_2 were done mainly with *E. coli* cells that were directly exposed to hydrogen peroxide in a planktonic environment (Ptitsyn *et al.*, 1997). This protective role of biofilms may extend to chemical exposure as well as, exposure to UV light.

Discussion

RM4440 was shown to be responsive to chemical assaults on DNA in a biofilm setting. The response was dose dependent and specific. This illustrate the usefulness of RM4440 as a tool for ecological studies in polluted ecosystems. An evaluation of the effects of various agents on an

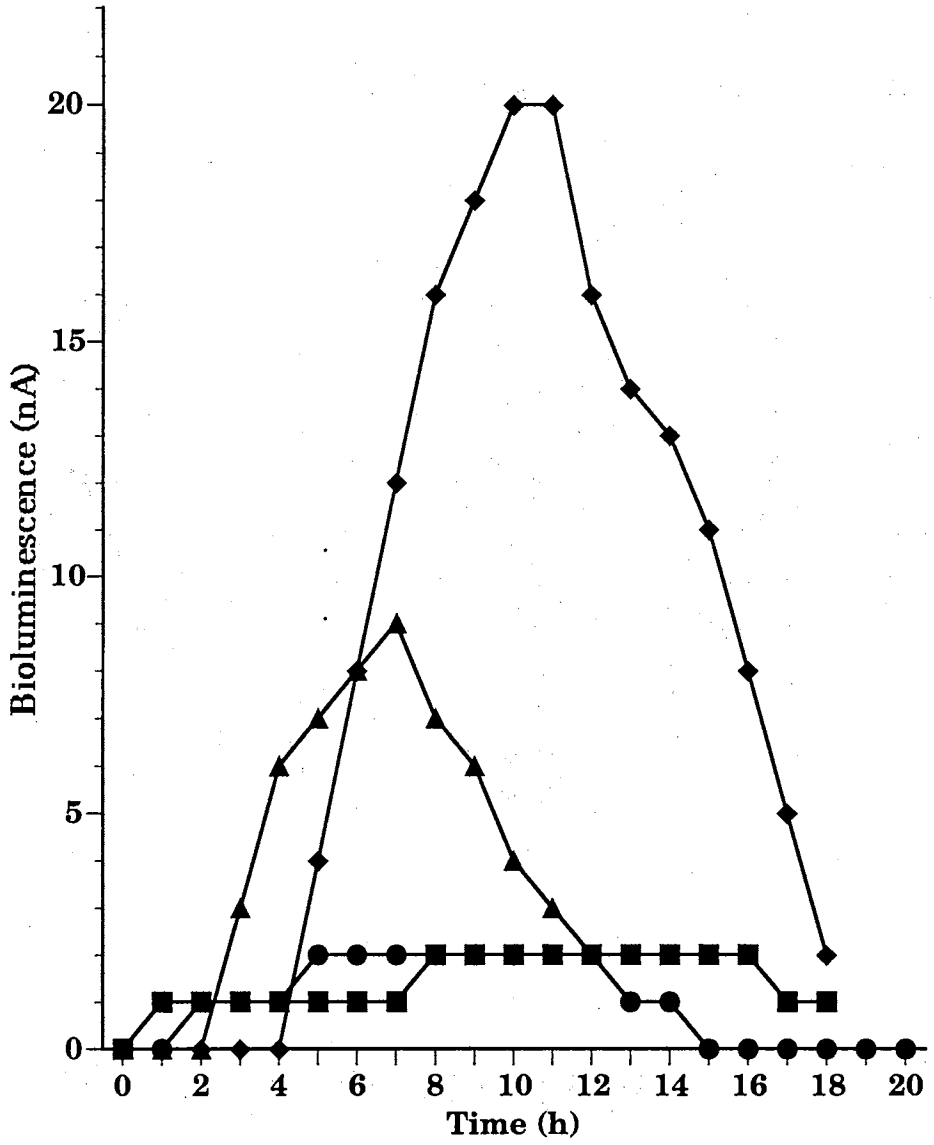


Figure 29. Response of RM4440 to increasing concentrations of methyl methane-sulfonate. ■, 0 %; ●, 0.02 %; ▲, 0.1 %; ◆, 0.2 %. The beads containing RM4440 were stirred in 100 PMM for 1 hour, before incubation.

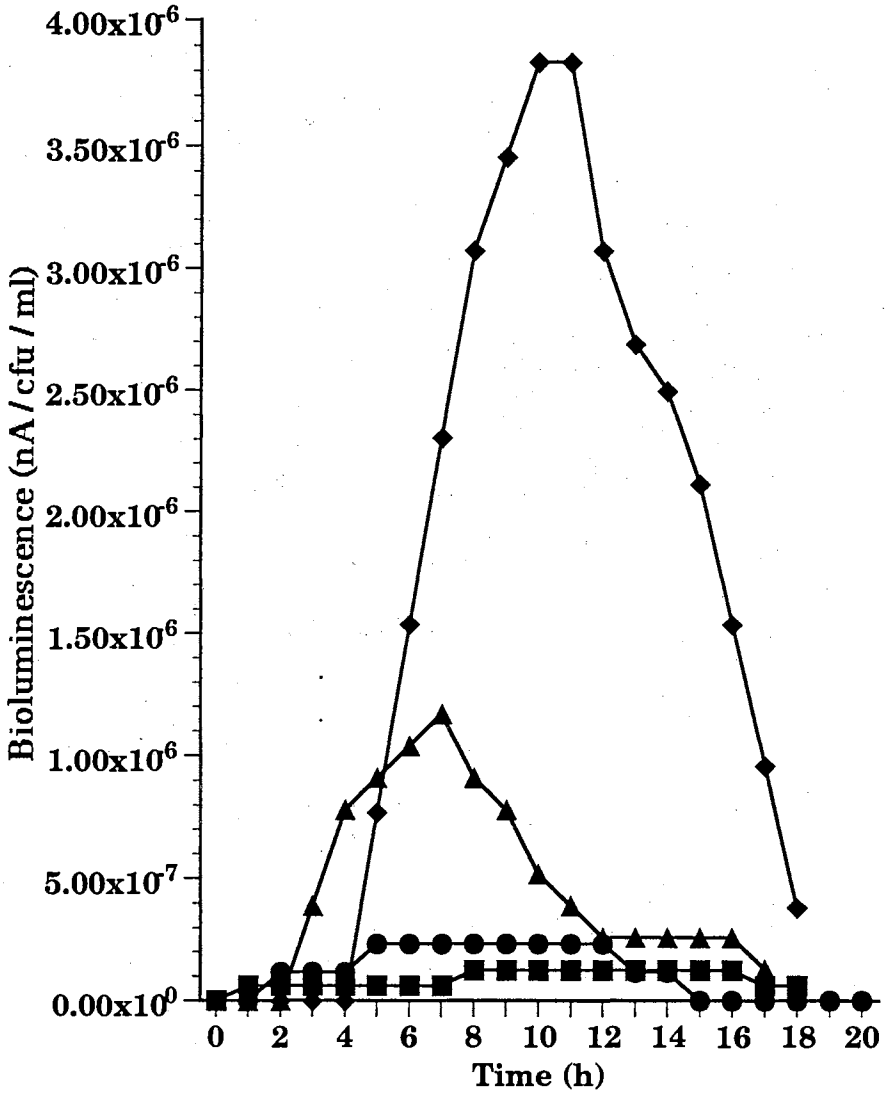


Figure 30. Normalized response of RM4440 to increasing concentration of methyl methane-sulfonate. ■, 0 %; ●, 0.02 %; ▲, 0.1 %; ◆, 0.2 %. The beads containing RM4440 were stirred in 100 PMM for 1 hour, before incubation. Bioluminescence was divided by the number of viable cells.

environmental organism *in situ* is far more accurate and useful than the methods available. Risk assessments in the release of chemicals into the environment can only be effective if the organisms concerned are studied in their natural niche. Biofilms have been shown to be an important state of existence for microorganisms in the environment and thus it is reasonable to develop an assay such as the alginate-RM4440 system described here to study them. Another potential use for RM4440 is the evaluation of different bioremediation processes by introducing this organism into polluted sites to be treated and following the bioluminescence response through the treatment process.

The SOS Chromotest has been shown to rival the Ames Test in specificity of detection of carcinogens and ease of assay (Quillardet and Hofnung, 1993). The SOS Chromotest relies on a quantitative colorimetric response through the fusion of a β-galactosidase gene to an SOS network member (*sfiA*). The response is obtained in a few hours which is very advantageous when compared to the Ames Test. The sensitivity ,i.e., the capacity to identify carcinogens, of both tests are 62% with the SOS chromotest and 77% with the Ames test. The Ames test shows and increased ability to identify carcinogens, however, the number of false positives is lower in the SOS chromotest. This improved specificity, i.e., capacity to dicriminate between carcinoges and non-carcinoges, of the SOS chromotest makes it a good test to complement the Ames test results (Quillardet and Hofnung, 1993). RM4440 has showed a strong correlation between with the SOS chromotest and the Ames test in its ability to identify carcinogens. RM4440 provides better sensitivity than the chromotest

because of the greater sensitivity of light measurement over colorimetric assays. RM4440 is an easier assay when compared to SOS chromotest and the Ames test since it does not require plating the tester strain and long incubation time. RM4440 also has the added advantage of real-time analysis. It is apparent that RM4440 has great potential for use in the screening for genotoxic agents, although, it is probably best to use more than one test to screen chemicals for genotoxic effects. Complementation of results of more than one test insures accuracy and increased specificity.

CHAPTER VII

CONCLUSIONS

This study was begun in an effort to develop a tool to detect and evaluate DNA damage in microbial communities in natural environments. Such a tool is long overdue given the increasing problems of pollution and the surge in bioremediation efforts. In order to be effective, cleanup efforts require a method to detect and quantify the health of natural bacterial communities in situ. During and after bioremediation, a method is also required to evaluate the efficacy of the bioremediative processes. In the first part of this study, a whole-cell biosensor for DNA damage was developed. This biosensor (RM4440) is an engineered *P. aeruginosa* strain. RM4440 carries a plasmid-based fusion of the *P. aeruginosa recA* promoter and the *luxCDABE* of *V. fisheri*. RM4440 responds to the presence of DNA damage by an increase in light production. The components of RM4440 were carefully chosen in order to end up with sensitive, quantifiable, and real-time stress biosensing. The choice of the host strain was based on the fact that *P. aeruginosa* is autochthonous to soil and fresh water habitats. These niches are host to a vast variety of microbial communities, they are also major targets for pollutants, and,

therefore, demand remediative efforts. The use of the *recA* gene as an indicator for DNA-damage stems from its regulatory role in the SOS system and its direct involvement in DNA repair. In response to DNA damage *recA* expression is increased from a low, constitutive level to a higher level that depends on the amount of injury to the genetic material requiring repair. If *recA* makes a fitting indicator of damage, bioluminescence is the most suitable reporter for the purposes designed for RM4440 as a biosensor. Bioluminescence permits easy and real-time assay since it can be measured remotely. This property is crucial in ecological studies since it averts invasion and destruction of microbial communities. Sensitivity in light quantification is only limited by the instruments used. Light measurement can be accomplished on-line which allows easy long-term monitoring. RM4440, as a whole-cell biosensor, can be incorporated into microbial communities to report on the health the ecosystem in an accurate and meaningful manner. After its construction, RM4440's competence was shown by testing its response to various doses of UVC in liquid cultures. In order to account for cell death the bioluminescence response was normalized by dividing it with number of colony forming units. This yields a specific response and allows quantification of light production from individual cells. RM4440 proved to be dose responsive and sensitive to DNA damage.

In the second part of this study, the ways in which microorganisms in biofilms cope with stress was investigated. Biofilms are very significant in environmental studies since they have been shown to be the preferred growth mode of bacteria in aquatic habitats when

nutrients are available. Biofilms are complex structures where cells are attached to a surface through exopolymers. To simulate a biofilm, RM4440 was immobilized in alginate and continuously fed the matrix with minimal medium. This biofilm was subjected to a series of different stresses using UV as the damaging agent. UVC was used because of its efficient DNA-damaging effects and because of its germicidal effects since destruction of biofilms is of great interest for some industries where microbial biofilms are responsible for fouling materials or contamination. In order to observe how microorganisms manage the daily stresses from sunlight, UVB and UVA doses that are environmentally relevant were used. Although there was not a strong correlation between the dose and the peak response, there seems to be a tendency for the bioluminescent response to UVC and UVB to increase with the dose. The rate of survival was higher than that observed in experiments done on planktonic cells. This elevated survival rate was also observed with UVB which produced a strong bioluminescent response. UVC and UVB responses showed a second smaller peak that appears few hours after the first peak response. This may be due to secondary damage that is caused by radicals formed during UV exposure that interact with DNA in a delayed fashion. A second possible explanation is the presence of two subgroups in the population after irradiation, a first subgroup that constitutes the majority of the cells that induce *recA* expression promptly and a second one that shows a delayed response. Another possibility is the beginning of a second round of replication that is halted by residual damage which would cause *recA* activation and induction of the SOS response.

Treatment with UVA failed to generate a bioluminescent response or any viability loss. These data clearly show that a shielding effect is produced by the alginate matrix. However, when psoralen was added to the cells and they were irradiated with UVA light, a bioluminescent response was observed. Psoralen is a photosensitizer that is produced by plants. It causes DNA damage when activated by UVA. These results illustrate the fact that UVA can be an important stressor for biofilm, as well as, planktonic bacteria. Alginate was shown to physically protect cells by only transmitting a small portion of the radiation (maximum transmittance of 13% of UVC, 31% of UVB and 33% of UVA). These results illustrate an added advantage for the formation of biofilms by microorganisms in the environment.

Finally, the last part of this study dealt with the use of potentially DNA-damaging chemical agents to test the response of RM4440 in biofilm. A set of chemicals was chosen to investigate the sensitivity and the specificity of RM4440 as a detector of DNA-damaging agents. This strain shows excellent specificity and sensitivity while demonstrating its potential use as a tester strain in screening for genotoxic chemicals as well as its use in *in situ* ecological studies.

This study has proven RM4440 a very useful and reliable system for studying bacterial communities in biofilms. This biosensor can be used in three different manners. First it can be immobilized in a matrix, as described in this study, and chemicals can be studied in a laboratory setting. RM4440 can also be used to study an environment in a contained fashion. By containing Rm4440 in a dialysis bag for example and

introducing it into an environment, the biosensor is exposed to the environment to be studied without the concern of releasing a genetically engineered microorganism in nature. Containment has the added advantages of ease of sampling to assess cell viability and light measurement. The third way, RM4440 can be released and allowed to colonize the site to be monitored. There are, however, limitations to the release of RM4440 *in situ*. For instance, in order to obtain quantitative data, there is a need to assess the number of viable cells that are contributing to the signal produced. Such assessments may require a certain level of invasiveness in order to count cells in a natural biofilm. This diminishes one of the advantages of this biosensor. Qualitative results on the other hand will still be valid and would benefit from the non destructive feature of RM4440. In the environment, the use of RM4440 to assess the damage of a chemical spill will require using a series of dilutions of the contaminated medium and testing them separately. This is necessary to standardize the response and acquire meaningful results. A negative control will also be essential in order to differentiate between the response to a specific new contaminant and the response to agents that are indigenous to the site. Such negative controls may be obtained by various ways depending on the nature of the site. In the case of a river one can simply use a sample upstream of the contamination site which should reflect on the state of the river prior to the spill. In the case of soil, the relatively slow diffusion of chemicals and the relatively constant nature of soil composition over large areas allows using a sample outside the spill site as a control. In the case of a lake, one can either relies on

data before the contamination or use a neighboring body of water as a negative control.

Another limitation to the use of RM4440 in situ is nutritional requirements for the bioluminescence reactions. Bioluminescence is relatively expensive from an energetic point of view and so in a nutrient-poor site, the biosensor may not be capable of supporting light production. In this case one can has to rely on the laboratory assay by treating a sample of the site as a agents and providing laboratory nutrients to support the biosensor. Presence of luminescent bacteria in the site to be studied may affect the results and thus there is a need to be acquainted with the microbial communities that inhabit the site to be studied. RM4440 is most useful within ranges of UV that cause less than 50% killing. As the data showed, high doses of UV (>5 J/m^2 of UVC and >50 J/m^2 of UVB) yield increasing variation in the response as evidence by the error bars. This gives the biosensor a dynamic range within the 60 % survival or above.

Many potential studies can be achieved using this biosensor. Future work should focus on the study of effects of combinations of agents on biofilm communities. As I showed with UVA and psoralen, the presence of one these agents alone has no effect on cells, while the presence of both yields a synergistic effect. In their natural environment, bacterial communities are exposed to more than one potentially-harmful agent at a time. In order to accurately study their coping mechanisms with such stresses, we should consider the effects of the coexistence of more than one agent. The combined effects may turn out to be more harmful or more

beneficial to cells than the effect of individual agents. The results of this study suggest that the effects of stressors in natural environments may be different than when they are studied in the laboratory. This leads future studies to a new direction, where RM4440 is incorporated into a consortium of different microbial species. This will more accurately mimic natural biofilms. In nature, biofilm microcolonies are tied together by many complex interactions, such as cooperation. We have just begun to appreciate the communal relationships of microorganisms. These studies require new approaches in the field of microbiology and new ways of thinking. In my opinion, microbiology is undergoing a parallel evolution as was the study of higher animals. Zoology had informal beginnings with observations of dead animals. It then evolved to capturing live animals and keeping them caged for better understanding of their behavior. Modern animal studies are conducted in the natural environment of animals with minimal intrusion. The contemporary thinking is that animals are part of a larger picture and that profound understanding of their behavior necessitate studies *in situ*. Microbiology, I believe, is in transition from studying "caged" microorganisms in the form of uni-species-batch cultures to analyzing multi-species communities in their natural environments. Such studies, are challenging and require new tools. Engineered reporter systems, such as RM4440, are crucial for the infiltration of microbial habitats and the study microorganisms their native form.

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