To the Graduate Council:

I am submitting herewith a dissertation written by Robert S. Burlage entitled "Bioluminescence as a Reporter of Gene Activity: Description of a Promoter from NAH7, a Naphthalene-Degradation Plasmid". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Gary S. Sayler, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

Bioluminescence as a Reporter of Gene Activity: Description of a Promoter from NAH7, a Naphthalene-Degradation Plasmid

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Robert S. Burlage

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Dedication

This thesis is dedicated to my parents,

John and Wilma Burlage,

who supported, nurtured, and encouraged me for so many years,

and to my wife,

Karen,

who has agreed to take over the job.

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this system. Mr. Louis Allen was the artist for several figures found in this manuscript.

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ABSTRACT

Reporters of gene expression have allowed scientists to describe genetic and physiological processes in great detail. Most bioreporters utilize a biochemical assay to quantitate the response. A method is described here for the use of a physical medium, bioluminescence, to describe expression. The advantages of this method are that it is non-destructive of the sample, non-disruptive of the containment vessel, and very rapid in response time.

A series of genetic fusions was constructed, culminating with the pUTK9 plasmid which fuses the promoter for the upper pathway of naphthalene degradation from plasmid NAH7 with a lux gene cassette from Vibrio fischeri. When this plasmid construction was moved into a Pseudomonas putida strain that also contained the intact NAH7 plasmid the strain RB1351 was created. RB1351 emitted light when exposed to naphthalene, a trait that was shown to be correlated with naphthalene degradation. Thus this strain is able to both degrade naphthalene and report photometrically on the continued presence of this substrate.

An anomolous result was found when RB1351 was grown in batch cultures and naphthalene added at an exponential rate of growth. Light production was substantially delayed until the end of the exponential phase. Although many theories could account for this result, it is probable that this is

due to either a growth rate-related effect or to catabolite repression. Evidence suggests that the growth rate is implicated, since a lag phase culture behaves substantially the same as the stationary phase culture. Variations in light output are eliminated when RB1351 is grown in continuous culture. A fragment of DNA from the nahA gene was implicated in both magnitude of expression and time of expression, since the removal of this sequence produces a different bioluminescent profile. However, more work remains to fully characterize this effect.

Expression of the nah and lux genes was followed by RNA extraction and quantitation during batch culture growth. Here again bioluminescence was correlated with transcription, as evidenced by increased levels of specific mRNA while light production was at a peak level. Levels of lux and nah specific RNA remained low in the absence of naphthalene, except during a brief period when light was produced for an unknown reason.

This work has demonstrated the utility of bioluminescence as a reporter of gene activity and should be more generally applicable to many systems besides the application to biodegradation described here. Light measurement is especially attractive for projects in which a closed system must be maintained, where constant monitoring is necessary, or when great sensitivity is desired.

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I. Introduction

The contamination of soil and groundwater with a variety of chemical compounds has recently been identified as a serious problem. In large measure this is a legacy of the rapid industrialization that has taken place in the past century, which produced and disposed of chemical contaminants as a by-product of commercial production. Examples of chemical waste can be found from a variety of industries in which the products are widely disseminated, such as energy production, herbicide, pesticide, and artificial fertilizer production, and the electronics industry. Among the identified contaminants are heavy metals, polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins, trichloroethylene (TCE), and various other chlorinated and non-chlorinated aromatics.

The U.S. government has recognized the importance of removing these hazardous wastes, creating legislation such as the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA, better known as the Superfund program) and the Hazardous Solid Waste Act of 1984 for implementation of existing technologies and the creation of new ones for remediation. Successful completion of this clean-up effort would have a great many important benefits for society, especially as regards public health.

Many of the substances mentioned above are suspected

carcinogens and teratogens, and occasional reports in the popular press highlight public anxiety over the fate of the environment. Notably, the cases of the Love Canal contamination in Niagrara Falls, NY and the dioxin contamination of Times Beach, MO have focused public attention on the growing health risk of hazardous waste pollution.

Many forms of remediation have been suggested, and no single method has proven effective for all types of contamination at all sites. In addition, cost effectiveness is an important consideration, given the magnitude of the problem. Incineration at high temperatures has been tried by several communities. This method has the advantage that organic substances can be completely oxidized to CO_2 and H_2O. However, incomplete combustion would pollute the air with potentially toxic by-products, and this scheme is opposed by many environmentalists for this reason. Incineration is also a very expensive process, especially when considering the many thousands of tons of topsoil that must be treated at a typical waste site. Concretion is an example of another method that is used to dispose of hazardous waste. Liquid waste is combined with a hardener such as concrete, with the benefit that the resulting solid is resistant to leaching. This method does not destroy the waste, it only removes it from contaminating a larger area. In addition, the volume of waste is appreciably greater after concretion and must still

be stored somewhere, a substantial problem in a country with dwindling landfill space.

One area of research that has been pursued aggressively in recent years is the use of microorganisms for the destruction of hazardous wastes. In the past twenty-five years there has been remarkable progress in the fields of genetics and physiology, and this progress has translated into important advances in microbiology, virology, cell biology, ecology, and related disciplines. As the general understanding of these biological processes begins to form a more complete concept of cellular metabolism it will become possible to design more effective clinical procedures for treatment of disease, to exploit new products and processes, and to explore more difficult research topics effectively, such as the developmental biology of eukaryotic organisms. It will also become possible to utilize microorganisms more effectively for bioremediation, utilizing both natural and recombinant strains.

The idea of bioremediation of hazardous waste has gained acceptance with the acknowledgement of the problems inherent in the other systems. Briefly stated, bioremediation is the use of biological processes for the degradation of recalcitrant man-made compounds. These processes are not usually specific for a particular chemical waste, but the relaxed specificity of certain enzymes of a biochemical pathway will often accept the man-made compound as a

substrate. This is especially true for cases of co-metabolism, in which the recalcitrant compound is degraded coincidentally by the organism and appears to have no benefit to the cell. The degradation of TCE by methylotrophs has been demonstrated to occur in this way (Wilson and Wilson, 1985). The methane monooxygenase enzyme has a greater affinity for methane than for TCE, but when methane is absent the enzyme will effectively oxidize TCE.

In many cases the products of catabolism can be utilized as a carbon and energy source for the degrading organism. In fact, using a compound as a sole carbon and energy is a good way to select for degradative organisms. There have been many reports of microorganisms that are able to degrade a variety of chemical compounds (Sayler et al., 1990), either partially or to completion (i.e. mineralized). Biodegradation is usually determined by the disappearance of a radiolabelled substrate, and the subsequent appearance of labelled CO_2. For a few of these reported degrading organisms the biochemical pathways have been determined, as well as the genetic analysis of expression of their genes. This has produced a better understanding of the natural processes that these organisms have developed to cope with environmental contaminants, as well as to suggest mechanisms by which the substrate range can be extended. Some success in this area has been achieved by Reineke et al. (1982) who mutagenized a 3-chlorobenzoate degrading organism so that it would also

degrade 4-chlorobenzoate.

Biodegradation is not, however, a simple matter of incubating a target chemical with the appropriate microorganism. Many hazardous compounds are relatively insoluble in aqueous media, and transport problems may also limit the ability of the microorganism to enzymatically digest the chemical compound. In addition, harmless substrates may be converted to toxic metabolites, killing off the very population that was depended on for remediation. This is especially true for halogenated compounds, and much research has centered on the ability of a species to remove the halogen group without producing toxic by-products.

Industrial waste samples are not amenable to biological remediation for other reasons as well. The samples are rarely pure chemicals, or even in a defined condition. Usually they are mixtures of a great variety of chemical species that may include heavy metals or toxic compounds. The contaminated sample may be soil or water, and may be difficult to handle due to its bulk. In considering the possible bioremediation of PCB contamination of the Hudson River, it is instructive to consider that vast quantities of river sediment would need to be treated.

Even when an organism or a consortium is identified in the laboratory that can degrade a particular chemical it is not certain that a suitable method can be found to decontaminate larger samples. It is, after all, one thing to

degrade a compound in a 100 ml batch culture, and quite another to perform the same task in a 100 l reactor. When large vessels of this kind are contemplated it becomes necessary to consider the chemical and bioengineering principles involved. All of the variables regarding chemical fate that were described above apply to large reactors also. Reduced oxygen tension in a large volume is always a problem, which necessitates a high rate of sparging with air or pure oxygen. However, this process also leads to air stripping of volatile organic compounds. Sorption of the chemical to suspended solids, turbulent mixing of the medium, and the insolubility of the chemical in an aqueous medium are also potential problems in reactor scale-up. As a generalization, biological systems are unpredictable in large reactors, and the principles that determine physiological response still need to be characterized.

The emerging concept of environmental biotechnology brings together several disciplines in an attempt to describe the complexity of natural ecosystems. Blackburn (1989) has identified four levels of complexity associated with environmental biotechnology: molecular, cellular, ecological, and reactor. The molecular level refers to the genetic interactions involved in induction and expression of genes, and of the kinetics of enzyme reactions. There are many intricate mechanisms of control that have been discovered in bacterial systems. In aggregate they present a sophisticated

interaction of sensors and effectors that allow the bacterial cell to react to environmental stimuli in a precise and advantageous manner.

These processes coalesce at the cellular level to describe factors which affect cell growth and maintenance. Growth rate, of course, is related to the supply of nutrients and other conditions of growth (e.g. temperature, pH, surface availability) and so the limitations on bioavailability of some substrates is very relevant here. But physical forces play a major role here as well, such as the mixing (turbulence) of the medium or the heat transfer from cellular respiration.

These problems are compounded at the ecological level, where many species of a consortium may be involved. The ecology of microbial systems has historically been overlooked, largely because it is too complex to describe easily. However, the importance of understanding ecological principles can not be ignored, and in the past several years techniques have been developed to analyze in situ bacterial systems and defined cultures of several species. The intractability of this problem is illustrated by the work of Shapiro (1988), who has shown that pure cultures of bacteria also show a complex ecology. In his studies of bacterial colony growth it is evident that zones of bacteria are present, giving the growing colony a ringed appearance. The reasons for this effect are completely unknown.

Finally, at the reactor level the physical processes that are involved in shaping of the environment are considered. Here engineering principles are most applicable, since the problems of material transport, behavior of gases, liquids and solids, and energy transfer are able to be described using mathematical equations. It has been observed that the efficiency of a process will change markedly when the volume of the reactor is changed, even though all other factors remain nominally the same. Bioengineering attempts to explain this effect based on the non-obvious physical and chemical factors.

As each level of complexity is encountered the ability to make predictions on the fate of the chemical compound of interest decreases steadily, with the result that the behavior of the cell culture in a large reactor is no more predictable that at the very start of the investigation. Attempting to optimize conditions at the bioreactor stage can be a lengthy and expensive prospect, though a necessary one if large scale projects are to be successful. In order to accomplish optimization of such processes, it is very helpful to make use of bioreporters that analyze quantitatively the activity of certain genes of interest, so that incubation conditions can be altered to find optimal parameters.

This report describes a novel use of bioluminescence as an indicator of genetic activity for a pathway of interest - that of naphthalene degradation. A specific genetic fusion

was created and introduced into a strain of Pseudomonas as a model degradative system. Experiments involving a set of defined conditions were performed, and unexpected results were investigated more thoroughly to determine whether bioluminescence was an accurate reporter of transcription in this bacterial strain. Results indicate that bioluminescence works well in this capacity and that more complex, pilot plant scale operations might be optimized with this approach.

II. Literature Review

Bioreporters.

The development of genetic markers, or bioreporters, has been central to the advance in the understanding of genetics and physiology. A genetic marker, loosely defined, is any genetic element that is used primarily for the study of an unrelated biological function, and for which an assay has been developed. In practice this usually means a genetic construction for which a reliable biochemical assay can be used. However, assays involving antibodies, either polyclonal or monoclonal, have also been used effectively. The antibodies are conjugated to a useful enzyme, and so an indirect assay for genetic expression is performed. This is the basis of the Enzyme-linked Immunosorbent Assay (ELISA) technique. Jain et al. (1988) identified four characteristics that make a gene suitable as a marker: (1) the gene should be expressed in the host, (2) the gene product should be stable in the host, (3) the gene product should be transported to its final location (e.g. cell wall, outer membrane), (4) the gene product should be readily detectable by a standard, reliable assay, or provide a selection technique, or both.

The more useful genetic markers are associated with a biochemical assay that is both inexpensive and easy to perform. The variety of genes used for this purpose has

increased with the advent of recombinant DNA techniques and applications, but the earliest experiments with genetic fusions made use of spontaneous deletion mutations. This is a not uncommon occurence whenever two operons are closely linked on the chromosome, and where the deletion is not deleterious. Jacob et al. (1965) took advantage of one of these spontaneous mutations that fused the purE and lacY genes. This construction put the expression of galactoside permease (the lacY gene product) under the control of the purine synthesis promoter. The same approach was used by Ketner and Campbell (1974) to study the fusion of the gal and bio operons. In this case the biotin promoter controlled expression of the fusion.

The power of this technique was evident in a study of transcriptional fusions resulting from spontaneous deletions between the trp and lac operons (Reznikoff and Beckwith, 1969, Reznikoff et al., 1969). In this fusion the trp promoter controlled expression of both the truncated trp operon and the intact structural genes of the lac operon. Using this fusion workers were able to describe not only the genes of the lac operon (which had already been studied extensively) but also the control of expression of the trp operon. Through this fusion and subsequent mutations the repressible nature of the operon was described, as well as providing evidence for the location of the trpR repressor gene. Related fusions (Figure 1) demonstrated the function of

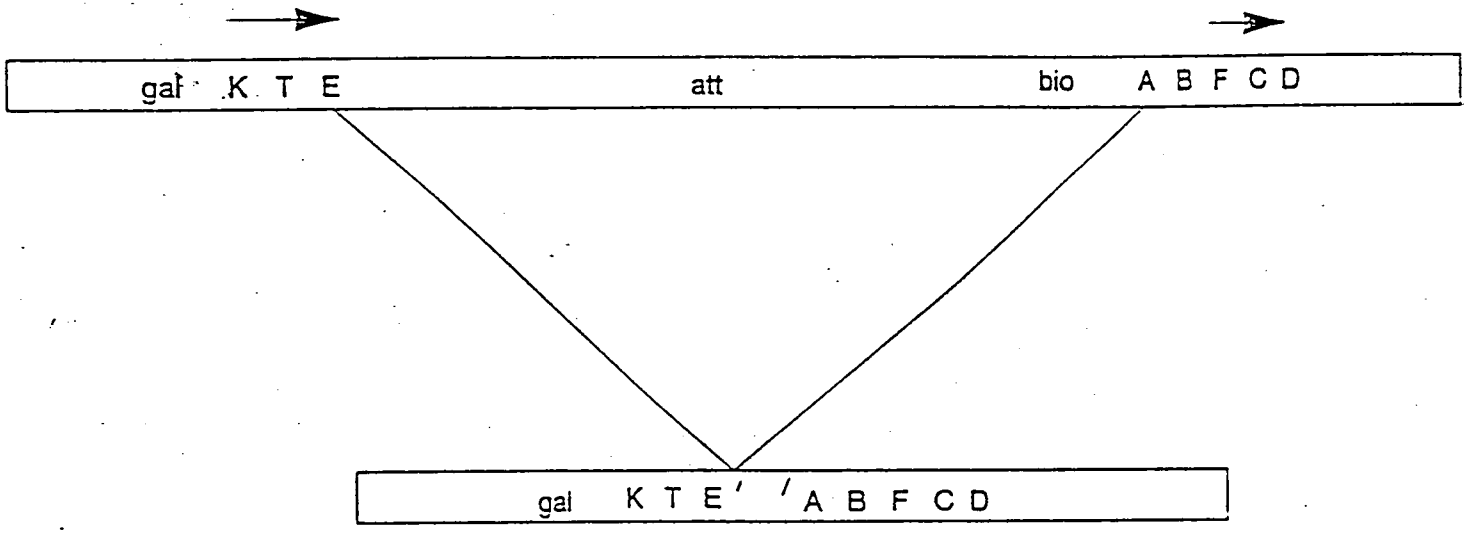


Figure 1. A spontaneous deletion mutation that results in a bioreporter. The deletion was between the gal and bio operons of E. coli results in a gal-bio fusion strain. The attachment site (att) for lambda phage is shown between the two operons. Based on Ketner and Campbell (1974).

the trp transcription terminator (Guarente and Beckwith, 1978).

One of the main reasons for the success of the fusions described above is that the lac operon was used. It would be difficult to exaggerate the importance of the lac operon to molecular genetic analysis, and particularly through the use of the lacZ gene, which encodes β-galactosidase. Both in the spontaneous deletions described above, and in recombinant genetic constructions that are literally too numerous to mention, lacZ fusions have proven their worth as a tool of genetic analysis. As with all good reporter genes, the lacZ gene product has an excellent assay. In fact, there are several techniques associated with the lacZ gene that are useful for diverse purposes. The β-galactosidase enzyme cleaves the disaccharide lactose into glucose and galactose. When the synthetic compound 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) is substituted the β-galactosidase is able to cleave the compound, creating an insoluble blue pigment that is very noticable on most agar media. This substrate can be used both as an indicator of β-galactosidase activity and for the absence of this activity, depending on the genetic construction employed. Interruptions of the lacZ gene give white colonies (as opposed to blue) on X-gal-containing media. Multiple cloning sites have been constructed at the 5′ end of this gene, allowing insertion mutations to be generated during cloning experiments. This

has been effectively exploited using the pUC family of plasmids and the related M13mp series of phage vectors (Vieira and Messing, 1982), which have been developed to facilitate nucleotide sequence analysis by the Sanger et al. (1977) dideoxy method. Recombinant phage clones appear as clear plaques on the indicator medium.

The presence of β-galactosidase activity can be utilized as a marker in a similar manner using transcriptional and, more commonly, translational fusions. Successful recombinant fusions appear blue against a background of white non-recombinant colonies. These fusions are particularly valuable as reporters of gene activity when the gene of interest can not be assayed directly. This is possible only because the fusion protein usually retains the β-galactosidase activity. Of course, the correct reading frame for β-galactosidase translation must be preserved, and various clever approaches to deal with this necessity have been developed, including the use of lacZ reporter transposons and cloning vectors which utilize all three reading frames. An important benefit of using the lacZ gene is that the fusion protein may stabilize an otherwise unstable protein (Goeddel et al., 1979). This extended protein stability has permitted analysis of certain proteins that have a short half-life, produced by a protein purification regime that allows selection for the β-galactosidase protein using either monoclonal antibodies or a β-galactosidase substrate in an affinity chromatography

preparation. Since X-gal is somewhat expensive, it is often preferable to screen these colonies on Lactose-MacConkey agar. Colonies that exhibit β-galactosidase activity appear red (as opposed to white) on this medium.

Successful β-galactosidase fusions can be assayed in liquid medium using o-nitrophenyl-β-D-galactoside (ONPG). This is a colorless substrate which can be hydrolyzed by β-galactosidase to o-nitrophenol, which is yellow. After cell disruption (β-galactosidase is a cytoplasmic enzyme) the enzyme activity can be determined spectrophotometrically at 420 nm. The assay procedure was described in great detail by Miller (1972) and for this reason activity is usually reported in Miller units. In the original assay there was no correction for total protein in the assay; subsequent modifications were made to yield a measurement of specific activity. One of the reasons the Miller assay is continually used is its great sensitivity. One unit of activity can be detected i.e. the amount of enzyme needed to produce 1 nmole of o-nitrophenol in one minute. It also means that, on average, one molecule per cell of β-galactosidase can be detected, although at least 10^4 - 10^5 cells must be assayed to arrive at this average value.

The versatility of the lacZ system makes it the most widely used reporter gene. It has even been used as a reporter of recombinant Pseudomonas in studies to determine whether genetically engineered microorganisms will survive

and disperse if released at an environmental site (Drahos et al., 1986). Pseudomonas strains that incorporate the lacZY genes are capable of cleaving the X-gal substrate to produce the characteristic blue color. This trait makes them very distinguishable from the non-recombinant Pseudomonas species that are indigenous to the site. Undoubtedly the lac genes will continue to be widely used for genetic analysis.

There are other reporter genes that are commonly used and which deserve mention. All have one or more of the attributes outlined above, and are useful for a specific task. The gene for chloramphenicol acetyl transferase (cat) mediates resistance to the antibiotic chloramphenicol. A promoterless cassette of this gene has been prepared that is effective for producing transcriptional fusions (Close and Rodriguez, 1982). This fusion is then used to accurately measure promoter activity under various conditions. Both a radioactive substrate, ^{14}C-labelled chloramphenicol, and a non-radioactive spectrophotometric have been used in CAT assays. In addition, there are several other fusion vectors available which utilize antibiotic resistance genes, such as for kanamycin and tetracycline.

In a similar manner the xylE gene of the TOL plasmid has been cloned for use as a transcriptional fusion reporter gene. This gene encodes catechol 2,3 oxygenase (C 2,3 O), and a simple aerosol spray technique has been used to find recombinant colonies. Colonies that turn yellow in the

presence of the catechol spray are expressing C 2,3 O, and produce 2-hydroxymuconic semialdehyde (Zukowski et al., 1983).

Bioluminescence: the *lux* Genes

The production of visible light by various organisms is an unusual trait that has been studied in great detail in recent years. The most obvious example is the firefly (*Photinus pyralis*) but there are other examples. The click beetle (*Pyrophorus plagiophthalamus*) (Wood et al., 1989b) can produce light of several wavelengths from different organs - a curious evolutionary development. Recently, cDNA clones of the various messages have been cloned, allowing investigators to compare the luciferases that generate green, yellow-green, yellow, and orange light (Wood et al., 1989b). The luciferase molecules are very similar, between 95-99 % homologous at the amino acid level, and thus the differences in wavelength produced are due to a relatively small number of changes in the protein. A review of the click beetle luciferase system and a comparison to the firefly system has recently been published (Wood et al., 1989a).

Several bacterial species can produce light, including members of *Photobacterium*, *Alteromonas*, *Xenorhabdus*, and *Vibrio*. The marine *Vibrio* can be found either free-living or living commensally with certain species of deep-sea fish. For

example, Vibrio fischeri lives commensally with Monocentris japonicus. These fish store the bacteria in specialized light-organs, and various theories have been advanced to explain how light generation is of use to the fish (Hastings, 1986). For instance, light production may be used in predation, to attract other fish close enough to be captured. Alternatively, it can be a hindrance in certain fish that have transparent skin. In these fish the gut microflora bioluminesce enough to make them a visible target for predators. Light production may be useful in species recognition prior to mating. It has even been suggested that these bacteria may bioluminesce once they are expelled in fecal pellets. These shining particles would then attract a copraphagous fish, and the ingested bacteria could then colonize the fish gut. In the absence of many facts all of these ideas retain some validity. However, this remains an area of speculation, as does the proposed biochemical signal from the fish host for induction of the bacterial light response.

Molecular analysis of the lux system.

The initial genetic analysis of the lux system of Vibrio fischeri was performed in a comprehensive study by Engebrecht et al. (1983). These workers were able to clone into E. coli the regions necessary for bioluminescence, and then

mutagenize the several genes. They mapped several complementation groups, including regions of regulation, luciferase and fatty acid reductase activity. This report was also notable for its examination of the exponential increase in light production after induction, and the description of autoinducer in a positive feedback regulatory loop. This report was followed by another from this group (Engebrecht and Silverman, 1984) that mapped the lux genes more precisely, and provided the first description of the gene products. These two reports provided the first genetic map of the lux genes and established the framework for subsequent analysis of their regulation.

According to this outline, the genes of the lux system in Vibrio fischeri are organized into two operons, usually termed the left and right operons for historical reasons (Figure 2). The left operon encodes a single gene, luxR, which has a regulatory function. The right operon contains all the other known lux genes, in the order luxICDABEG. The luxI gene product is involved in the production of the autoinducer molecule, N-(β-ketocaproyl) homoserine lactone, although its enzymatic activity is not known. The luxAB genes encode a heterodimeric luciferase, which catalyzes the light-producing reaction. Using immunogold labeling of a polyclonal antibody against the luciferase, Colepicolo et al. (1989) demonstrated that the luciferase is a cytoplasmic enzyme in V. harveyi, and does not appear to be associated with the

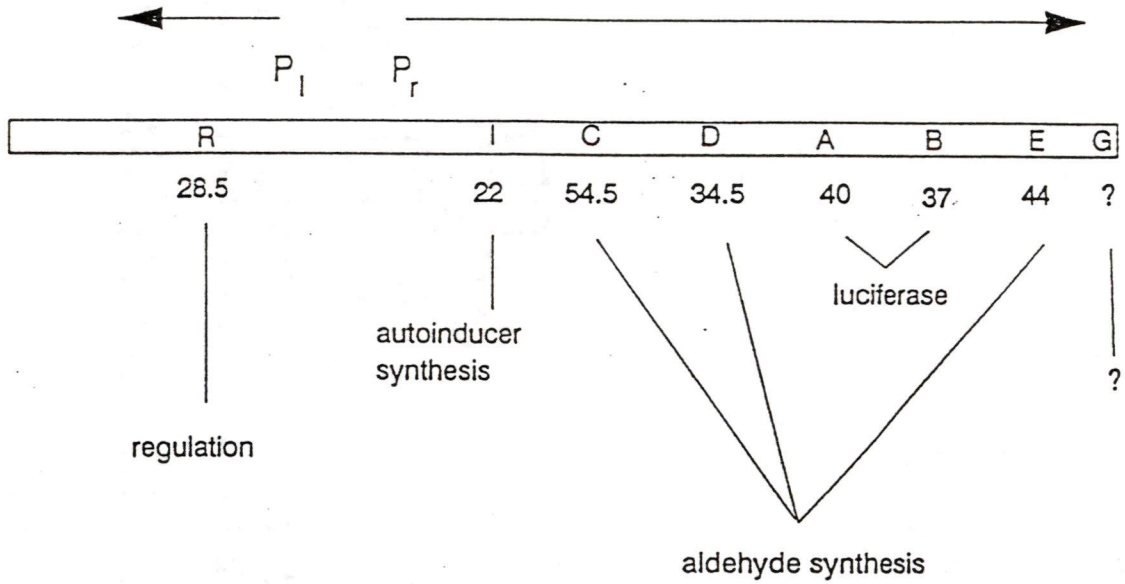


Figure 3. The lux genes of Vibrio fischeri. The molecular weights (in kdal) of the lux gene products are listed below their respective genes. Arrows show direction of transcription from the left (P_l) and right (P_r) promoters.

cytoplasmic membrane. At least two groups have attempted to fuse the luxA and luxB genes together to create a luciferase that is a single gene product. This luxAB fusion can then be used as a reporter gene in eukaryotic cells. Both Boylan et al. (1989) and Kirchner et al. (1989) used the lux genes from V. harveyi in their genetic fusions, and were successful in producing light in such disparate biological systems as E. coli, Saccharomyces cerevisiae, Nicotiana plumbaginifolia leaf protoplasts, and a lysate of rabbit reticulocytes. The advantage to using the bacterial system as opposed to the firefly luciferase system is that the two systems require different substrates. The components of the bacterial bioluminescence reaction are presented in equation form in Figure 3. The firefly luciferase requires the luciferin compound (Figure 4) along with O_2 and ATP, while the bacterial system requires an aldehyde substrate, $FMNH_2$ and O_2. While both luciferin and the aldehyde substrate are relatively toxic and must be used with great care, the aldehyde (usually decanal when added exogenously) crosses cell membranes much more easily than the luciferin, and thus can be used in smaller amounts. For use in plant cells, $FMNH_2$ must also be added exogenously, but this has not proved to be an impediment.

The luxCDE genes form a fatty acid reductase complex that regenerates the aldehyde substrate so that continuous light production is possible. These genes were described in

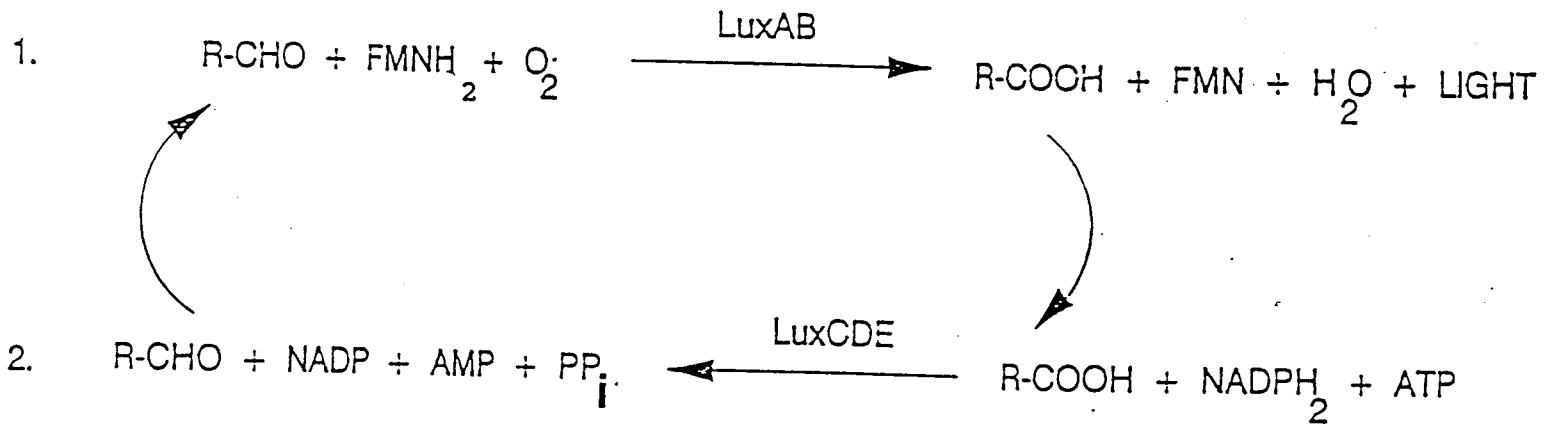


Figure 3. Components of bioluminescence in Vibrio fischeri. The cyclical nature of the bioluminescent reaction is shown here in equation form. R-CHO - a long chain aldehyde substrate.

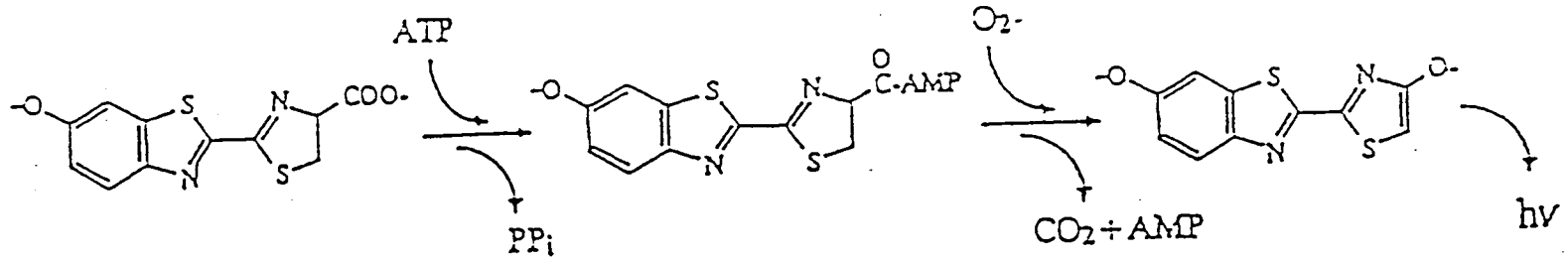


Figure 4. Firefly luciferin. the substrate and the light producing reaction are shown (Wood et al. 1989a).

great detail by Wall et al. (1984) in their study of protein acylation after induction of bioluminescence in V. harveyi. The luxG gene has only recently been described (Swartzman et al., 1990), and no function has been ascribed to it as yet. It is unnecessary for light production in Vibrio or in any of the several hosts into which these genes have been introduced. In fact, the constructions described in this thesis lack luxG. However, the commensal relationship of this microorganism and its host has not been explored in any great detail, and it is possible that this gene product plays some part in regulating light production in situ or in sensing a signal from the host. The recent description of a plasmid vector to re-introduce lux clones into Vibrio will undoubtedly aid in the elucidation of gene function in the natural host (Miyamoto et al. 1990). The lux genes and their products have recently been reviewed by Meighen (1988).

The molecular analysis of other bioluminescent organisms is worth discussing since there are interesting similarities and differences (Table 1). The lux genes of Vibrio harveyi have also been studied extensively, and most of these genes have been sequenced. The gene order of the structural genes is the same here as for V. fischeri, luxCDABE (Miyamoto et al. 1988a). It was in this organism that the extra genes luxG and luxH were discovered to be part of the polycistronic message of the right operon (Swartzman et al., 1990). These workers used the sequence data to make specific DNA probes

Table 1. Bacterial lux genes and their products

Gene	Function	Protein MW	Sequence ref.
Vibrio fischeri			
luxA	luciferase subunit	40,315	Foran and Brown, 1988 Baldwin et al., 1989
luxB	luciferase subunit	37,213	Foran and Brown, 1988 Baldwin et al., 1989
luxC	acyl-CoA reductase	54,469	Baldwin et al., 1989
luxD	acyl transferase	34,497	Baldwin et al., 1989
luxE	acyl-protein synthetase	44,088	Baldwin et al., 1989
luxG	?		Swartzman et al, 1990
luxI	autoinducer synthesis	21,937 21,937	Devine et al., 1988 Baldwin et al., 1989
luxR	regulatory	28,518 28,518	Devine et al., 1988 Baldwin et al., 1989
Vibrio harveyi			
luxA	luciferase subunit	40,108	Cohn et al., 1985
luxC	acyl-CoA reductase	55 kdal	Miyamoto et al. 1988b
luxD	acyl transferase	34,168	Miyamoto et al. 1988a
luxG	?	26,108	Swartzman et al. 1990
luxH	?	25,326	Swartzman et al. 1990
Photobacterium phosphoreum			
luxF	luciferase subunit?	26.6 kdal	Soly et al., 1988
luxG	?		Swartzman et al. 1990

Table 1 (continued)

Gene	Function	Protein MW	Sequence ref.
Photobacterium leiognathi			
luxA	luciferase subunit	40,290	Baldwin et al. 1989 Illarionov et al 1990
luxB	luciferase subunit	37,270	Baldwin et al. 1989 Illarionov et al 1990
luxG	?	26,180	Illarionov et al 1990
luxN	flavoprotein?	26,229	Baldwin et al. 1989

Legend: All molecular weights (MW) in daltons unless specified as kilodaltons (kdal).

for these genes, and subsequently discovered the presence of luxG in V. fischeri and Photobacterium phosphoreum. Recently P. leiognathi was added to the list of bioluminescent species bearing a homologous sequence (Illarionov et al., 1990).

The luxI gene that is found at the 5′ end of the right operon in V. fischeri is missing in V. harveyi (Miyamoto et al. 1988b). Instead there is a region of approximately 600 bp that does not have a significant open reading frame. Southern blot experiments with a luxI probe have shown that a luxI-homologous gene is present in this species, but its location has not been determined. The luxR gene of Vibrio harveyi has recently been located (Showalter et al., 1990), and it is clear that the locus is far from the lux structural genes. Also, there is no substantial homology between the luxR genes from V. harveyi and V. fischeri. When the regulatory system of V. harveyi is finally elucidated, it is likely that a significantly different system will appear.

Sequencing of the lux genes of Photobacterium revealed the presence of another gene that is not found in either Vibrio species. Soly et al. (1988) described the luxF gene of P. phosphoreum, placing it between luxB and luxE (Mancini et al., 1988). The other known structural genes are organized in the same order as for the Vibrio species (Mancini et al., 1988). The sequence of the 3′ end of this gene suggested to these workers that luxF was derived from a duplication of luxB, and had undergone a deletion near the 5′ end. This

finding would have interesting implications for bioluminescence, since it would be possible that, besides the LuxAB heterodimeric luciferase, a LuxAF luciferase could form. Perhaps this would result in a bioluminescence of a different wavelength, expressed under certain conditions. However, at about this same time a report from Baldwin et al. (1989) on the sequence of P. leiognathi described a gene they called luxN. This gene was in the same relative location as luxF, and a brief examination of both sequences convinces the viewer that the sequences are largely the same. However, this group likened luxN to a non-fluorescent flavoprotein, not to a possible luciferase subunit. This gene may actually be part of the reductase complex.

Regulation of the lux operons.

It is generally assumed that light production would be very tightly regulated, since this characteristic probably represents a very specialized function. Continuous production of light may lead to greater loss through predation, or otherwise disrupt the normal commensal relationship with its fish host. Light production may also be a metabolic burden for the cell, although careful experiments with Vibrio species growing in a calorimeter have shown that the fraction of energy devoted to light generation is a mere 0.007 % (Makemson and Gordon, 1989). Nevertheless, it would be

helpful to elucidate the control mechanism for lux gene expression. The nucleotide sequence of the left and right operons has been determined (Baldwin et al., 1989), and this information has given valuable clues to the control of lux gene expression. The 200 bp sequence between the operons may have several regulatory regions associated with it, and regulation is correspondingly complex (Figure 5). A putative SOS box has been identified, and it has been demonstrated that the LexA protein is able to bind to this site and repress bioluminescent activity in E. coli (Ulitzur, 1989). However, none of this work was performed in a Vibrio strain, and whether there is a similar system in this species is open to question. The report of Ulitzur (1989) also noted that the induction of the htpR gene resulted in an increase in bioluminescence. This gene encodes the sigma-32 factor that has been associated with the heat shock response, as well as to other stress responses (aerobic/anaerobic shift, starvation). Here again, this work was only performed in E. coli, and might be peculiar to this species. It was suggested that the light might be induced in response to certain physiological conditions, such as a starvation signal from the host, or a similar signal while floating freely in the planktonic state. It is not entirely clear what advantage would be gained if Vibrio bioluminesced after undergoing heat shock or DNA damage, or what the role of the autoinducer would be in this scheme. Obviously much more work remains to

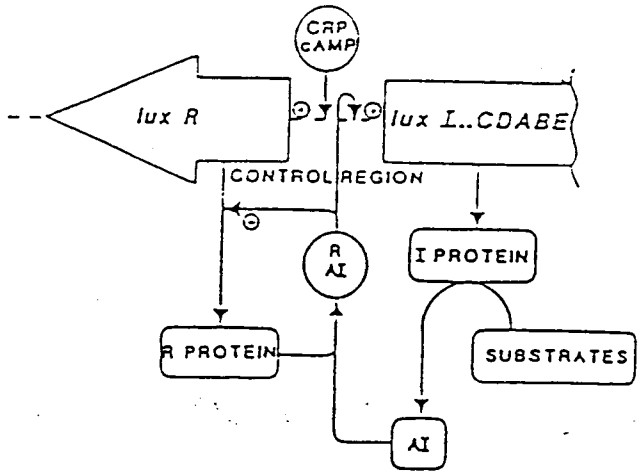


Figure 5. Proposed regulatory mechanism for lux gene expression. The pathway shown is from Vibrio fischeri. The luxI gene product converts a substrate to the autoinducer molecule, which conjugates with the LuxR protein. This stimulates transcription from P_r, and probably represses transcription from P_l. Catabolite repression is also thought to affect transcription activity (see text) (Baldwin et al., 1989).

be done.

A more satisfying explanation was proposed by Devine et al. (1989), who suggested that this palindromic region was actually the operator site for LuxR control of *lux* expression. By constructing a set of point mutations within this palindrome they were able to demonstrate the necessity for the LuxR-autoinducer complex at this site for full induction. It is possible that there is a similarity between the LuxR and LexA proteins, and that the binding of the latter to this site is just a coincidence. Here again, all the work was performed in *E. coli*, and it is also likely that both LexA and LuxR proteins are competing for binding at this site. This would, of course, complicate the study of the *lux* genes in *E. coli*, and presents a good reason for working with the original host strain.

The evidence for catabolite repression of the *lux* genes has also been developed through work in *E. coli*. It has been observed that glucose can repress bioluminescence under certain circumstances (Dunlap and Greenberg, 1985). The sequence of the region between the operons demonstrates an area that is homologous to the CAP (catabolite activating protein) binding site of *E. coli* (Devine et al., 1989). When a *lux* operon subclone is transformed into *E. coli*, it is apparent that bioluminescence is controlled by the level of cAMP and CAP. This control is mediated through the expression of *luxR*, since an overproduction of the LuxR protein can

overcome the catabolite repression (Dunlap and Greenberg, 1988). The relationship of a starvation signal to light production *in situ* is somewhat obscure, and has yet to be demonstrated conclusively in *Vibrio*.

Although it is not completely known how the regulatory system works, certain facts have been determined. It is known that an autoinducer substance is made on a more-or-less continuous basis, and is freely diffusable across the cell membrane. When a certain concentration of autoinducer is reached the right operon is induced to high levels of expression, and bioluminescence increases greatly (upwards of 1000-fold over uninduced (Eberhard, 1972)). The autoinducer varies between species; in *V. fischeri* it is N-(β-ketocaproyl)homoserine lactone (Eberhard, 1972), while in *V. harveyi* it is N-(β-hydroxybutyryl)homoserine lactone (Cao and Meighen, 1989).

This simple scheme explains why batch cultures of *Vibrio* express light in a density dependent manner. Since the autoinducer is freely diffusable, a critical concentration of autoinducer must accumulate before there is sufficient autoinducer to trigger the induction of the operon in all cells. The same pattern could be expected to occur in the fish host, since the light organ would concentrate the culture. In a planktonic state, or even in a gut-associated state, the explanation would seem to fall apart, since the surrounding medium would continually dilute the autoinducer

away from the cells. How the lux genes are expressed under these circumstances remains a mystery.

The metabolic pathway for the synthesis of the autoinducer, the starting material for its synthesis, and other genes for its synthesis (besides luxI) are all unknown. There may be other levels of regulation that are associated with this synthesis, and which would have a direct effect on the regulation of the right operon. It is known, for instance, that the autoinducer can be synthesized in the laboratory, and that by supplying it in excess to a culture the bioluminescence can be triggered at any cell concentration.

At this point a simple model of regulation for the lux genes can be described. Transcription of luxR may or may not be responsive to catabolite repression, but a pool of LuxR is created. The autoinducer molecule is produced continuously until a threshold quantity is present. At that time the autoinducer conjugates to the LuxR protein and binds to the palindromic region. Two recent reports have revealed the autoinducer-binding domain of LuxR (Slock et al., 1990, Shadel et al. 1990b) and the operator-binding domain (Slock et al., 1990). The binding of the conjugate to the operator stimulates transcription of the right operon and results in a positive feedback loop, generating light quickly. It has been suggested that luxR is autoregulatory, and that large amounts of the protein that are not conjugated to autoinducer

will repress the left promoter, which leads to repression of the right promoter.

Previous uses of the lux genes.

The use of bioluminescence as a reporter system has increased with genetic and physiological analysis of the lux genes, and with the availability of cloning vectors and other genetic constructions (Boivin et al., 1988) that facilitate their use. This has been especially true for work in eukaryotic systems, where the firefly luciferase is often employed (Nordeen, 1988). The cloned luciferase gene is usually introduced into a eukaryotic cell on a virus vector, and the production of light is used as an indication of gene expression during infection. This technique has been demonstrated using Vaccinia virus inoculated into mice (Rodriguez et al., 1988) and in Cauliflower Mosaic Virus when a cloned virus promoter was fused to the luciferase gene and introduced into cells of Daucus carota and Nioctiana tabacum (Ow et al., 1986).

Use of bioluminescence in prokaryotes is increasing (Stewart, 1990), and is more applicable to the work described below. Besides the lux vectors described here, other workers have developed lux constructions for their research uses. These include a transcription terminator probe (Peabody et al., 1989) that relies on decreased light production as a

screening method, the use of luxA as a translational fusion vector (Olsson et al., 1988), a Bacillus promoter probe vector, pOC1 (Carmi et al., 1987), that has been used to isolate promoters that are active during sporulation, and a promoter probe plasmid for use in cyanobacteria (Schmetterer et al., 1986). Using this latter vector, workers were able to form a transcriptional fusion with a promoter from Anabaena. These workers claimed that their construction would produce enough light so that the detection of a single cell's light output was possible. However, the lack of specific data to support this claim, as well as the absence of subsequent reports that would take advantage of the unique opportunities presented by this ability, cast doubt on the validity of this claim. In all likelihood a more powerful photometer than the one they describe would have to be used for such a purpose.

A particularly interesting set of reports utilizing lux gene constructions has investigated gene expression of plant pathogenic bacteria (Shaw and Kado, 1986, Rogowsky et al., 1987). In this work the induction of gene expression of bacterial virulence genes could be followed in planta by examining light production. These examples indicate the versatility of the lux system, suggesting that its use may be fairly compared to the lac operon. Bioluminescence research is not uniformly successful, however. For example, a lux fusion plasmid in a Bacillus subtilis strain showed a very unstable pattern of light emission (Karp, 1989). A strong

Bacillus promoter was used in this construction, with n-decanal added exogenously, and it is not readily apparent why the light output does not resemble that of other bacterial species.

The first practical application of bacterial bioluminescence was in 1985 when Engebrecht et al. constructed fusions between *E. coli* promoters from the *lac* and *ara* operons and the *lux* genes of *Vibrio fischeri*. A mini-Mu-*lux* vector was used to facilitate these constructions. Induction of the *lac*-*lux* fusion with IPTG or the *ara*-*lux* fusion with arabinose resulted in a significant increase in bioluminescence. This report promoted light measurement as a convenient *in vivo* assay for gene expression. This group extended their investigation to the *laf* genes of *Vibrio parahaemolyticus* in a subsequent report (Belas et al., 1986). The *laf* genes encode the production of lateral flagella in *Vibrio*, a response to growth on a solid substrate. Using *lux* as a reporter system, they were able to demonstrate control of *laf* gene expression at the level of transcription, and provided evidence that a physical signal was responsible for triggering expression, i.e. contact with a solid surface. It was noted in this report that cells at the center of a growing colony would no longer bioluminesce, suggesting that the *laf* genes become repressed when the attached surface is a bacterial colony. This report was very successful in demonstrating induction and repression of a specific gene in

response to controlled stimuli.

Since the lac operon has been studied in great detail, it is an obvious choice for a model transcriptional fusion. Besides the work of Engebrecht et al. (1985) another lac-lux fusion has been constructed to exploit this advantage (Korpela and Karp, 1988, Korpela et al., 1989). This E. coli strain produces light stably for several hours. These workers even proposed this strain as a biosensor for toxic substances. However, this is a crude assay in that it is non-specific - when the cells are in contact with a toxic substance they die and the light decreases. Usage would be limited to certain defined conditions for the test to be a reliable assay. This idea is similar to a proposal that emerged from a study of the effects of chlorinated phenols on a bioluminescent strain (Wagner et al., 1989). Certain isolates proved to be highly sensitive to the presence of these compounds, but this was also not a substrate-specific assay.

Another valuable usage of lux gene technology was exploited by Schauer et al. (1988), who studied differentiation in Streptomyces coelicolor. This group was able to fuse the lux genes to a variety of host promoters, some of which were only active during a specific phase of sporulation, or in a specific structure of the differentiating hyphal mass. Although this procedure involved a shotgun-cloning of Streptomyces DNA, and virtually all the

cloned promoters remain unidentified, this technique demonstrates the potential of the lux assay for isolation and study of gene expression in a unique manner.

An unusual application of lux technology was described by Nussbaum and Cohen (1988), who used bioluminescence as an indicator of recombination activity. They produced two different mutants of the luxA gene and placed both in an E. coli strain. Recombination of the two mutants would produce a functional luxA gene that would be observable, and quantifiable, by bioluminescence. Several strains of E. coli were used, each with one or more mutations in the host recombination genes. In this way the RedGam system of Lambda phage was examined in greater detail.

Biodegradation: the NAH7 plasmid.

State of knowledge

The NAH7 plasmid is the archetypical naphthalene-degradative plasmid, and one of the better studied degradation plasmids. It was first described by Dunn and Gunsalus (1973) when they examined a strain that had been selected for growth on naphthalene. The plasmid is approximately 83 kb in size, is transferred to other species conjugatively, and is a member of the Incompatibility group P-9 (Figure 6). Very little is known about the molecular

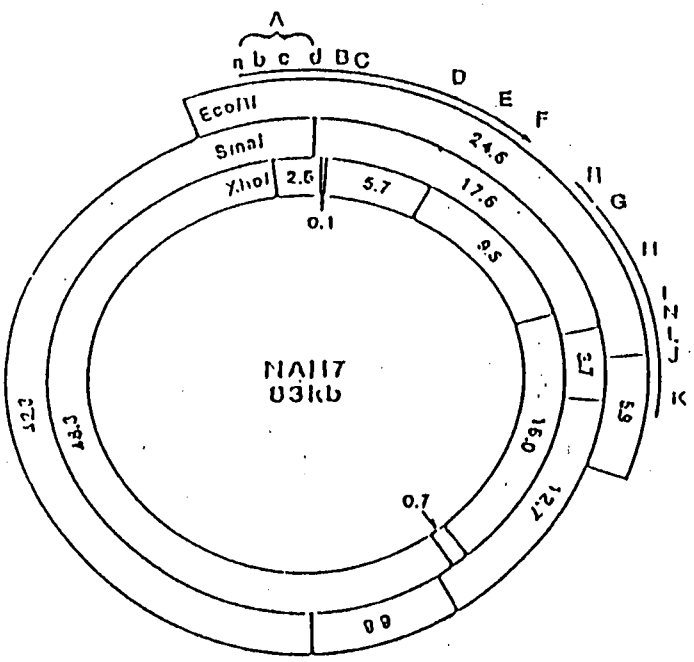


Figure 6. Map of the NAH7 plasmid. The nah genes are listed by letter designations. Arrows indicate direction of transcription. All SmaI and XhoI sites are indicated. Most of the 25 EcoRI sites are not mapped. Figure from Yen and Serdar (1988).

biology of this group, and so far the NAH7 plasmid is important only for the degradative genes it carries. NAH7 and other naphthalene degrading plasmids have recently been reviewed in great detail (Yen and Serdar, 1988).

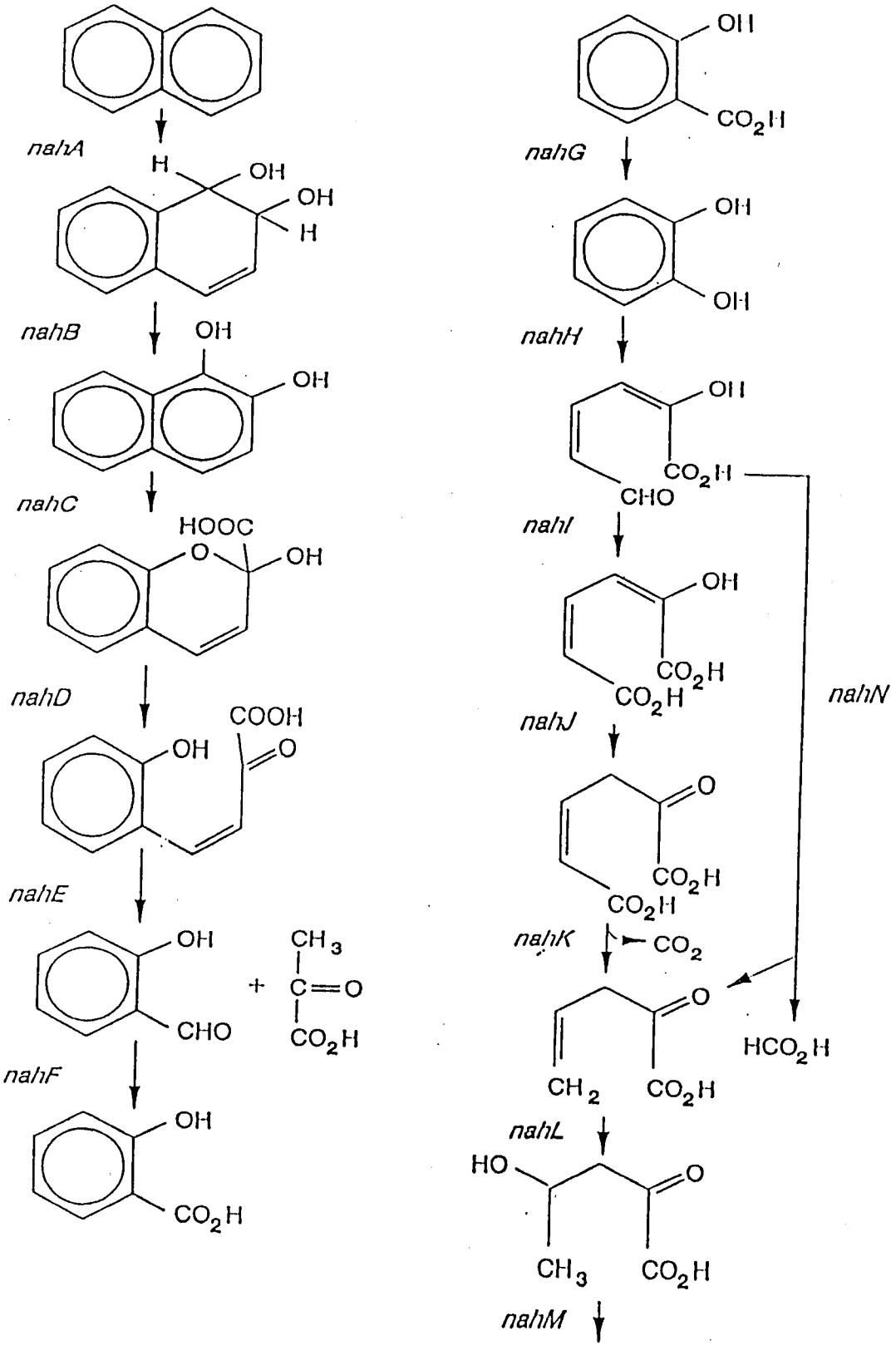
The degradative genes have been examined in some detail. By transposon mutagenesis and subcloning of these genes a map of the plasmid has been constructed (Yen and Gunsalus, 1982, Schell, 1983, Grund and Gunsalus, 1983). The degradative genes are arranged in two operons, comprising an upper and lower pathway that is reminiscent of the TOL plasmid (reviewed by Burlage et al., 1989). The upper pathway, nahABCDEF, encodes the enzymes for the degradation of naphthalene to salicylate (the inducing substrate), which results in the production of a molecule of pyruvate. The stable aromatic structure is disrupted through the action of a dioxygenase, much the same as is seen with other recalcitrant aromatic compounds. The gene order of nahE and nahF has not been established conclusively, raising the possibility that salicylaldehyde (the product of NahE activity) might also be an inducing substrate. The lower pathway, nahGHINLJK, encodes the degradation of salicylate to acetaldehyde and pyruvate. This pathway is also called the meta pathway since the catechol intermediate undergoes ring cleavage in the meta configuration; in this respect it is very similar to the meta pathway of TOL. The chromosomally encoded ortho pathway does not appear to be needed for

naphthalene degradation. An alternative pathway for degradation, the gentisate pathway, has been described for other naphthalene degraders but is not present in the host PpG7 strain. The lower pathway is bifurcated, again like the TOL plasmid pathway. Figure 7 demonstrates the intermediates of degradation and the genes that are responsible for the various steps.

Regulation

The degradative pathways of NAH7 have proven to be of less scientific interest, and inquiry, than the regulation of these operons. There is only one known regulatory gene, nahR, in contrast to the two genes that regulate TOL. This gene has been sequenced, and encodes a 36 kdal protein that has regions similar to other DNA binding proteins (You et al., 1988, Schell and Wender, 1986). NahR is a member of the LysR family of DNA binding proteins. Transposon mutants in the nahR gene render the strain unable to utilize either naphthalene or salicylate (Yen and Gunsalus, 1982). When this cloned region was supplied in trans the degradative capacity was restored (Yen and Gunsalus, 1982, Grund and Gunsalus, 1983). By determining the transcript quantity in both nahR$^+$ and nahR$^-$ strains it was shown that nahR controlled expression at the level of transcription, with the induced state approximately 30 fold greater than the uninduced state.

Figure 7. Degradation of naphthalene by NAH7. Naphthalene is in the upper left corner, salicylate is in the lower left and upper right corners. Gene products are as follows: A, naphthalene dioxygenase; B, cis-naphthalene dihydrodiol dehydrogenase; C, 1,2-dihydroxynaphtahlene oxygenase; D, 2-hydroxychromene-2-carboxylate isomerase; E, 2-hydroxybenzalpyruvate aldolase; F, salicylaldehyde dehydrogenase; G, salicylate hydroxylase; H, catechol oxygenase; I, 2-hydroxymuconic semialdehyde dehydrogenase; J, 2-hydroxymuconate tautomerase; K, 4-oxalocrotonate decarboxylase; L, 2-oxo-4-pentenoate hydratase; N, 2-hydroxymuconic semialdehyde hydrolase.


nahA
nahB
nahC
nahD
nahE
nahF
nahG
nahH
nahI
nahJ
nahK
nahL
nahM
nahN
Pyruvate + Acetaldehyde

This work also demonstrated that both nahR and the inducing substrate were required for full induction (Schell, 1985). This report described the constitutive expression of the nahR gene, both in Pseudomonas putida and E. coli. The nahR gene is located between the two pathways, and is transcribed divergently from them (see Figure 8). Some data have suggested that there are two regulatory genes at the nahR locus (You and Gunsalus, 1986), and not even DNA sequencing of this region has yielded unambiguous results.

The current theory of NAH7 regulation has evolved around the single known regulatory gene. Naphthalene is not an inducer of either the upper or lower pathway, but salicylate (and possibly salicylaldehyde) is. In addition, induction is possible with anthranilate (2-aminobenzoate), a gratuitous inducing compound that resembles salicylate. The constitutively expressed nahR gene ensures that an adequate supply of NahR protein is available. It is hypothesized that the NahR protein binds to a site upstream of the upper and lower pathway promoters (P_u and P_l, respectively). This site, the -70 site, is highly conserved, and the sequence is depicted in Figure 9 along with the sequences of the various promoter regions (Schell, 1986). From this position on the -70 site, NahR presumably binds to salicylate and undergoes a conformational change that facillitates RNA polymerase binding, or possibly attracts it to the site (Figure 10). Hydroxylamine mutagenesis of the nahR gene resulted in the

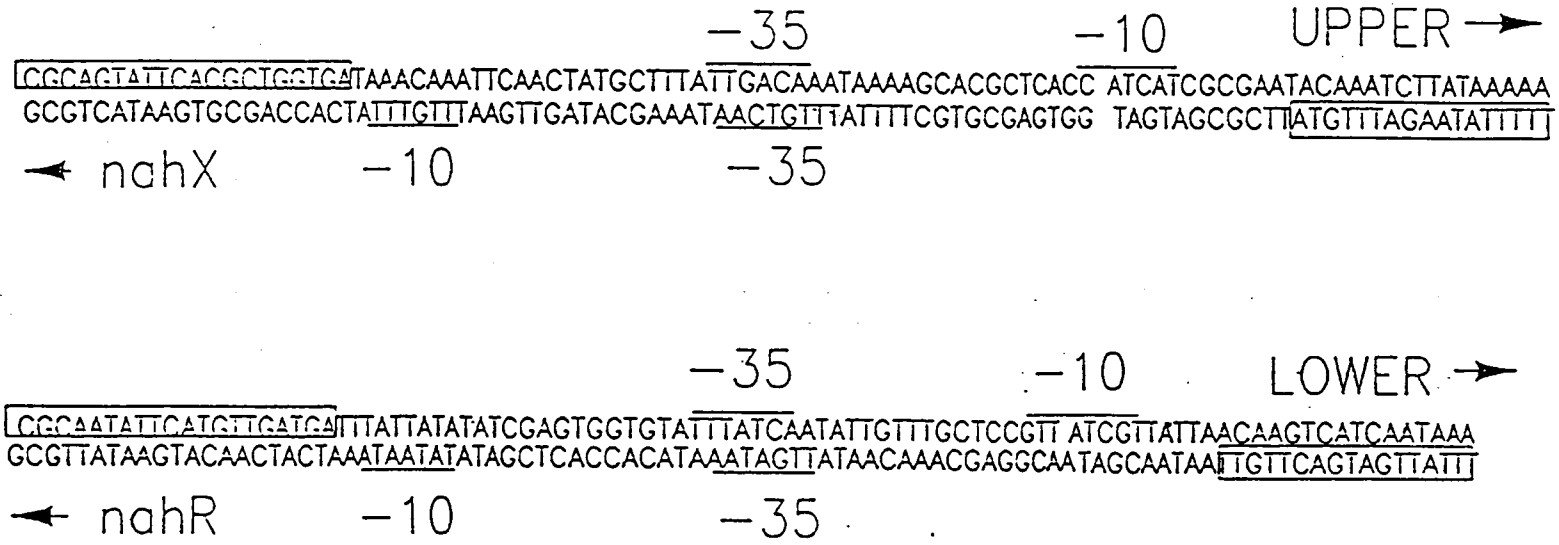


Figure 8. Relative orientation of promoters in NAH7. -10 and -35 sites of promoters are indicated. -70 sites are in boxes. Arrows show direction of transcription.

	−70	−35		−10
lower	CGCAATATTCATGTTGATGAT	TTATCA	15bp	TTATCGT
upper	CGCAGTATTCACGCTGGTGAT	TTGACA	17 bp	CATCAT
nahR	TTTATTGATGACTTGT	TTGATA	16 bp	TATAAT
nahX	TTTTTATAAGATTTGT	TTGTCA	16 bp	TTTGTT

Figure 9. Comparison of the promoters from NAH7. The promoters from Figure 8 are reoriented here for comparison. -70, -35, and -10 regions are denoted, as is the spacing between the -35 and -10 sites.

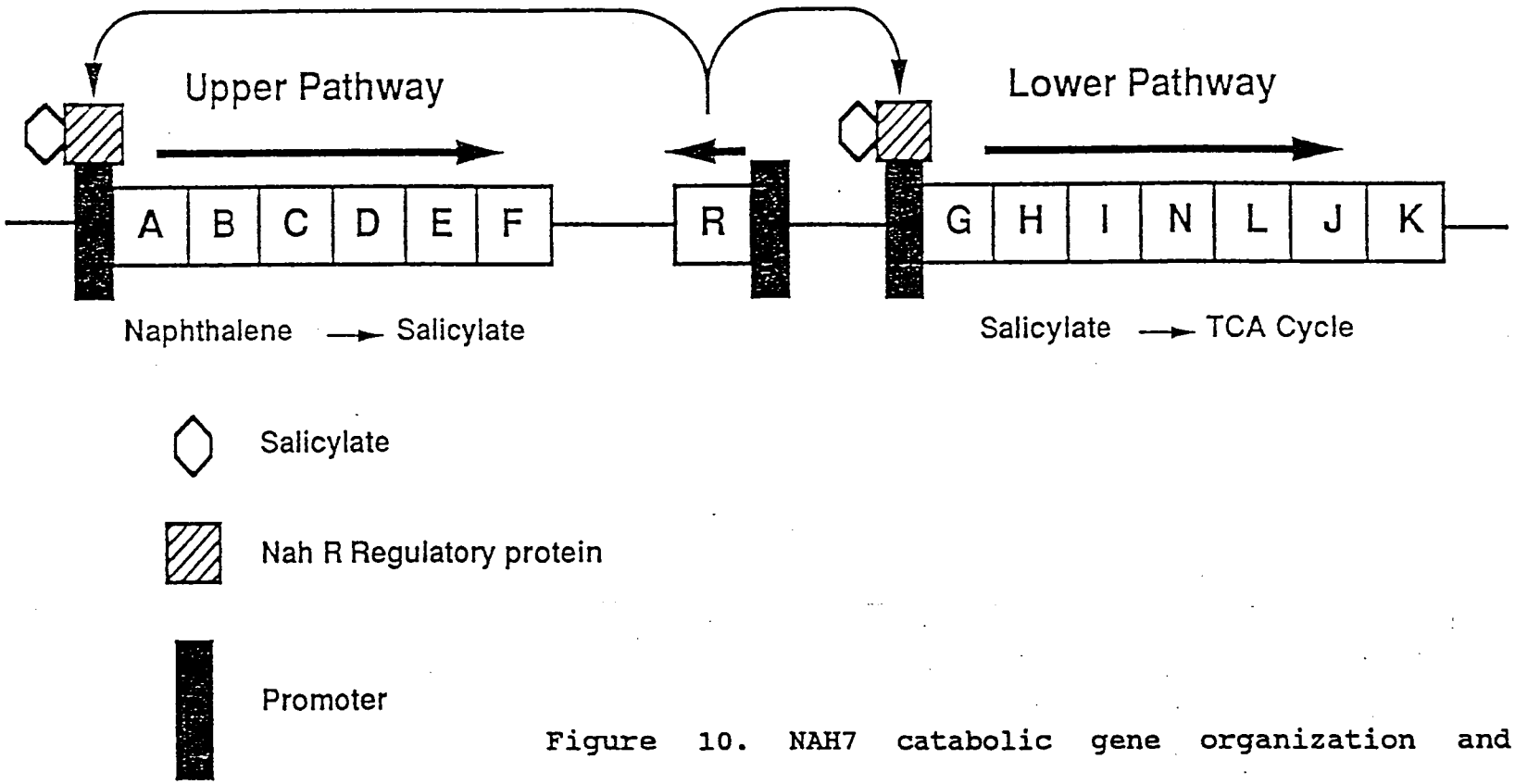


Figure 10. NAH7 catabolic gene organization and regulation. The NahR protein binds to the -70 site at both the upper and lower pathway promoters. When salicylate binds to NahR transcription is induced.

identification of three domains (Schell et al., 1990). Two domains were involved in the DNA-binding capacity of the protein, and this data supports the inclusion of nahR in the lysR family of regulators. The third domain is located towards the center of the gene, and appears to be the site for inducer binding or recognition.

The promoters for these various operons deserve special attention since their physical orientation plays a part in regulation. The sequence of these regions has been compared, and the transcription start sites have been accurately located (Schell, 1985). In all four cases represented in Figure 9 (P_u, P_l, P_r, and P_x) there is an easily identifiable promoter with good homology to described E. coli promoters. These promoters make up two divergent promoter pairs: the P_r and P_l promoters overlap at each others' respective -35 sites, as do the P_u and P_x promoters. This raises questions about the role of NahR, since binding to the -70 site for P_l would obviously block transcription of nahR. Of course, it is possible that a dynamic equilibrium exists which allows nahR to maintain its low level of expression and still permit NahR to be available often enough for induction of the lower pathway. Evidence is lacking for any definitive explanation. Although the -70 regions of P_r and P_x have not yet been shown to possess any regulatory function, they are included here to demonstrate the high degree of homology between them.

This description of the NAH7 pathway raises several

questions that have yet to be addressed, and which would seem to be fundamental to the utility of the naphthalene degradation system *in situ*. These questions will not be answered in this work since the task is very complex, but in raising these questions it may be possible to hypothesize about the origins of the naphthalene degradative pathway and its ecological function. Chief among these questions is why the inducing substrate, salicylate, induces both the upper and lower pathways when only the lower pathway is required for mineralization of the inducer. Prokaryotic genetic regulation is usually characterized by stringent control of expression, and it is unusual to find an operon whose expression is apparently wasted. The importance of this observation is accentuated by the knowledge that naphthalene, unlike salicylate, is a rare compound in nature. Salicylate is a fairly common plant metabolite, and was isolated by the Bayer Co. from Willow trees over 100 years ago for their synthesis of aspirin. It would not be unusual to find a bacterium that could utilize this potential source of energy. Naphthalene is not ordinarily found naturally, being confined to small amounts in coal and oil supplies, and thus not usually available to microorganism (Howard, 1989). The only appreciable concentrations are found near industrial centers, where the residue from oil and coal-powered machines is distributed over the land. Why would a microorganism develop a metabolic pathway to deal with such a rare energy source?

The next question deals with this last question in an oblique way. The diagram of the pathway shows that the lower pathway is bifurcated after the synthesis of 2-hydroxymuconic semialdehyde. Both the nahIJK and the nahN pathways yield the same compound, but use different methods. This is identical to the bifurcated lower pathway of the TOL plasmid (Burlage et al., 1989). When toluene or p-xylene are the starting substrates the xylGHI branch of the pathway degrades the intermediates, whereas the xylF branch is used when m-xylene is the starting substrate. A similar discrimination among metabolites has not been described for the NAH7 plasmid, and apparently the only substrate for the upper pathway is naphthalene. Attempts to degrade methylated derivatives of naphthalene have not been successful, and it is presumably the nahIJK pathway that is functioning during naphthalene catabolism. The role of nahN has not been effectively determined. These several questions will be addressed at greater length in the Discussion section.

III. Objectives

Although many reporters of gene expression have been described, they all suffer from the same limitations in application to certain experimental systems. For example, they require the destruction of part of the sample, they disturb the experimental apparatus in obtaining the sample, and they require a significant length of time to process the sample materials before an answer can be obtained. In addition, some assays are relatively expensive or require highly trained personnel to carry out the assay. Although these limitations are not significant for many practices in the study of genetics and physiology, there are applications for which the standard biochemical assays would not be optimal. For these applications an assay based on a physical characteristic, rather than a biochemical characteristic, would be more helpful. Specifically, the measurement of light is preferred because of the great sensitivity afforded by present technology, and because the response can be detected very quickly.

An example where a light (bioluminescent) response would be useful can be found in bioprocess engineering, where many parameters must be varied to obtain the optimum efficiency for the process involved. One of the unknowns in bioreactor design is whether the bioprocesses are functioning at the same efficiency throughout the reactor or are optimal at

certain locations. Another potential usage is in microbial ecology, in which the examination of consortia are necessarily complex. The contribution of a single species to the community is difficult to study under these conditions, especially when it is possible that several community members may share a particular biochemical marker. Finally, the rapidity with which these assays can be carried out and their relative economy make a bioluminescent reporter an attractive alternative to traditional means of assaying genetic expression.

The overall objective of this study is to demonstrate the utility of bioluminescence as a genetic reporter. In keeping with the research aims of this laboratory, bioluminescence will be used as a reporter for the presence and subsequent degradation of an aromatic compound that has been identified as a contaminant. Specifically, naphthalene will be the compound under study. To accomplish this objective in a logical progression, the following specific objectives will be accomplished.

1. A genetic fusion between a nah promoter and a lux gene cassette will be created.

2. This genetic fusion will be introduced into an appropriate bacterial strain, and the bioluminescent response of that strain to inducing substrates will be recorded.

3. A correlation will be made between light generation and substrate presence and catabolism.

4. A correlation between light production and transcriptional activity will be made.

5. Discrepancies between observations and the present theory of *nah* genetic expression will be investigated. A model will be suggested to account for any new data.

It is anticipated that this work will demonstrate the potential of bioluminescence as a reporter for genetic expression under a variety of conditions. It will be determined whether light measurement is an accurate tool for *in situ* biodegradation analysis, and for more generalized analysis of other bacterial systems.

IV. Materials and Methods

Bacterial strains and plasmids.

All strains used in this study are described in Table 2; plasmids that were either obtained from an outside source or constructed in this laboratory are described in Table 3.

Cultivation media and growth conditions.

Luria-Bertani (LB) medium contains (in g l^{-1}): tryptone, 10; yeast extract, 5; NaCl, 10; pH was adjusted to 7.5 with 1 N NaOH. E. coli strains were grown in LB broth at 37°C in a waterbath (Labline Bioengineering Co., Melrose Park, IL) with constant shaking at 150 rpm with antibiotic selection. Pseudomonas strains were grown in LB broth at 25°C in a growth chamber (Environmental Growth Chambers, Winnipeg, Manitoba) with constant stirring on a magnetic stir plate. Yeast extract-Peptone-Salicylate-Succinate (YEPSS) medium contains (in g l^{-1}): yeast extract, 0.2; polypeptone, 2.0; NH_4NO_3, 0.2; sodium salicylate, 0.5; and sodium succinate, 2.7. Plates were made by adding 1.5 % Bacto-agar. Basal Salts medium (BSM) (pH 7.2) contains (in g l^{-1}): NH_4Cl, 2; KH_2PO_4, 1.5; Na_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $NaNO_3$, 4.0; $FeCl_2$, 0.005; and $CaCl_2$, 0.01. Pseudomonas Isolation Agar (Difco Laboratories, Detroit, MI) with antibiotic selection was used

Table 2. Bacterial strains used in this study.

Strain	Relevant genotype or description	Source
Escherichia coli		
DH5	F-, *hsdR*17, *supE*44, *recA*1	BRL
DH5α	as DH5, with *argF-lacZYA* deletion	BRL
HB101	F-, *hsdS*20 (r_B-, m_B-) *recA*13	C. Kado
Pseudomonas putida		
PB2440	w.t. cured of TOL plasmid	M. Bagdasarian
PpG7	host for NAH7 plasmid	I.C. Gunsalus
Alcaligenes eutrophus		
A5	host for pSS50 plasmid	Shields et al., 1985

Table 3. Plasmids used in this study

Plasmid	Relevant charcteristics	Source or reference
pUC18	Ap^r, *lac*$^+$	BRL
pUCD615	promoterless *lux* cassette	C. Kado
pUCD623	TN 4431 host	C. Kado
pHF360	rRNA clone	Festl et al., 1986
pE317	NAH7 subclone	B. Ensley
pRK2013	TRA helper plasmid	Figurski and Helinski, 1979
pDTG113	NCIB 9816 subclone	D. Gibson
NA-15	pDTG113::Tn4431	This study
22c2	Nah7::Tn4431	This study
pUTK2	pSS50::TN4431	This study
pUTK4	*lac*-*lux*	This study
pUTK8	*nah* subclone	This study
pUTK9	P_u-*lux*	This study
pUTK10	P_x-*lux*	This study
pUTK11	pUTK9 deletion	This study

for certain procedures involving selection of Pseudomonas strains. Analytical reagent grade (>99% pure) glycerol (Mallinckrodt, St. Louis MO), 20 ml per liter, was added to this medium prior to autoclaving.

Other chemicals and antibiotics were purchased from Sigma Chemical Co., St. Louis, MO. except for naphthalene (scintillation grade, >99% pure) (Aldrich Chemical Co., Milwaukee, WI). Tetracycline (15 mg l^{-1}), kanamycin (50 mg l^{-1}) and ampicillin (50 mg l^{-1}) were added for selection where appropriate. Cell growth was followed using either plate counts after serial dilution, or by light scattering using a Klett photoelectric colorimeter (Klett Mfg. Co., New York).

For storage of bacterial strains, cultures were grown in appropriate liquid medium with antibiotic selection overnight. The culture was washed in medium without antibiotics and resuspended in one-tenth volume of medium. This was added to sterile glycerol to a final concentration of 15 %, and the tube was frozen at -70°C. Aliquots were sampled on medium without antibiotics to determine whether any contaminants were present. When needed, a few crystals of frozen culture were removed with a sterile toothpick and grown on selective medium.

Plasmid isolation.

The Anderson and McKay (1983) procedure was used for plasmid DNA preparations from Pseudomonas and Alcaligenes strains and whenever DNA for especially large plasmids was recovered. This procedure was originally intended for Streptococcus work, but has been slightly modified for Gram-negative species (Hooper et al., 1989) A cesium chloride-ethidium bromide density gradient centrifugation procedure was used to purify plasmid DNA.

An alkaline lysis procedure was used for most E. coli strains (Maniatis et al., 1982). These preps were also purified with cesium chloride centrifugation. The technique of Holmes and Quigley (1981) was used for screening plasmid mini-preps from E. coli. This is especially useful when screening high-copy-number plasmids and results in enough DNA for three restriction digests.

Molecular techniques.

Enzymes, reagents, and techniques.

The techniques of Maniatis et al. (1982) were followed for restriction enzyme digestion, mapping of fragments, dephosphorylation of DNA, and related techniques. Restriction enzymes were used with the 10X buffers provided by the

manufacturers. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Labs, Gaithersburg, MD, except for Xho II (Boehringer Mannheim, Indianapolis, IN).

Dephosphorylation of vector DNA was performed in a 50 μl volume with two units of calf intestinal alkaline phosphatase (Sigma) using a commercial 10X reaction buffer. The reaction was stopped after one hour by adding 1/10 volume of 10X STE buffer and 1/20 volume 10% SDS and heating at 68°C. The DNA was subsequently extracted with phenol-chloroform and ethanol precipitated. 10X STE buffer is composed of 100 mM Tris HCl, pH 8.0; 1 M NaCl; and 10 mM EDTA. Ligation was performed in a 20 μl volume with one unit of T4 DNA ligase and approximately 0.5 μg total DNA. One unit is defined here as the amount needed to catalyze the transfer of 1 nmol of labeled pyrophosphate into ATP in 20 minutes at 37°C. The ligation incubation was at 15°C for approximately 16 hours (overnight). Ligation conditions recommended by Dardel (1988) were used throughout.

For some cloning procedures, DNA fragments were separated by agarose gel electrophoresis and bands were excised from the gel. The DNA was extracted using the Gene-Clean (Bio 101, La Jolla, CA) procedure or the Spin-X procedure (Costar, Cambridge, MA). For this work a Tris-acetate-EDTA buffer (Maniatis et al., 1982) and a low percentage agarose gel was used. For other electrophoresis work a Tris-borate-EDTA buffer was used (Maniatis et al.,

1982). Molecular size markers were used to estimate DNA fragment lengths. Lambda phage digested with HinDIII and a DNA ladder made up of increments of 1 kb fragments were obtained from BRL. Horizontal gels were typically run for 1.5 hours at a constant 100 volts. All gels were stained with ethidium bromide at a concentration of 0.5 $\mu g\ ml^{-1}$. A permanent photographic record of these gels was obtained using a UV transilluminator and Polaroid type 57 black and white film.

Transformation, conjugation and electroporation.

The procedure of Hanahan (1983) was used for transformation of E. coli with plasmid DNA. E. coli cells (DH5 or DH5α) were grown to an exponential growth phase in SOB medium. SOB contains (in $g\ l^{-1}$): tryptone, 20; yeast extract, 5; NaCl, 0.58; KCl, 0.18; $MgCl_2$, 2.0; $MgSO_4 \cdot 7H_2O$, 2.5. TFB buffer was used to make the cells competent; it is composed of 10 mM Potassium 2-(N-Morpholino) Ethane Sulfonic acid (MES), 100 mM RbCl, 45 mM $MnCl_2 \cdot 4H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 3 mM $CoCl_3$. Conditions of the original assay were followed for each of the incubation steps. A heat pulse at 42°C for 90 seconds was followed by resuspension of the cells in SOB medium supplemented with glucose. A one hour recovery time was allowed at 37°C with vigorous shaking and aliquots were spread on selective agar.

Conjugation was frequently employed to move plasmids from E. coli strains to Pseudomonas. Both donor and recipient strains were grown to a mid-exponential growth phase in rich medium. If the plasmid was mobilizable but not self-transmissable, the helper plasmid pRK2013 was used. The E. coli HB101 strain was grown to a mid-exponential phase before use. Cultures were kept on ice until all strains were ready.

Filter mating was found to be the most efficient conjugation procedure. Sterile Whatman filters with a pore size of 1 μm were arranged in a filter tower apparatus and attached to a vacuum source. The ratio of recipients/donors/(helpers) was approximately 10/1/(1). After concentration on the filter, it was removed to a non-selective agar medium and incubated overnight at a permissive temperature for all strains involved. The filter was then washed in minimal medium and aliquots were inoculated onto selective agar plates. These plates were kept at the appropriate temperature for the recipient strain until colonies formed (about 36 hours for Pseudomonas).

Electroporation was used to introduce plasmids into Pseudomonas species, and is especially valuable in cases where conjugation fails. A BTX Transfector 100 with a 400 microFarad capacitor was coupled with an Optimizer (BTX, San Diego, CA) and set for 14 kvolt cm^{-1} with a pulse length of 5 msec. The preparation procedure for Pseudomonas strains is outlined in Table 4. Although it is commonplace to freeze

Table 4. Electroporation protocol for Pseudomonas.

1. 1 Liter of LB medium is inoculated with a 1:100 dilution of an overnight culture of Pseudomonas.

2. The culture is incubated with constant shaking until it reaches an exponential rate of growth.

3. The cells are harvested by centifugation at 7000 rpm for 15 minutes.

4. The cells are washed twice in sterile distilled water, first resuspending in 100 ml, centrifuged, then 20 ml.

5. The cells are centrifuged and washed once in sterile 10 % glycerol, then centrifuged a final time and resuspended in 3 ml of 10 % glycerol. This suspension should be very thick, about 10^{10} cells ml^{-1}, and resemble a paste.

6. Use 50 μl in each aliquot. Add approximately 1-2 μg of DNA for each tube.

electroporation-competent E. coli cells for future work, with Pseudomonas strains it is important to use a fresh preparation of cells. When frozen cells are used the efficiency is extremely low.

In a typical procedure the competent cells are briefly mixed with 1-2 μg of plasmid DNA. Approximately 10^8 mid-log phase cells were resuspended in 10% sterile glycerol and layered between the electrodes. A typical pulse demonstrated a medium-specific resistivity of 25 kohm cm with an exponential decay curve. After pulsing, the cells were diluted in SOB broth supplemented with 20 mM glucose and incubated for 1 hour at 25°C. Selection for successful transformants was accomplished on LB media with antibiotic selection.

Colony hybridization.

Colony hybridization was used to demonstrate the presence of plasmid DNA, in conjunction with the Genius (Boehringer-Mannheim, Indianapolis, IN) system of non-radioactive detection. A 2.0 kb fragment of the lux operon obtained from restriction digestion of pUCD615 with HinDIII was used as the probe for the presence of pUTK9 in Pseudomonas strain PB2440. The fragment was excised from an agarose gel and labeled with a digoxigenin-conjugated dUTP by the random priming method.

Transformed Pseudomonas colonies were removed to Biotrans nylon membranes, the cells were quickly lysed and the DNA was bound to the membrane by baking at 80°C for one hour. Prehybridization proceeded according to Genius manufacturers specifications in a 68°C water bath for at least one hour, followed by hybridization with the labeled lux probe DNA at 68°C. Incubation proceeded overnight.

Washing of the filters was performed at 68°C with a solution of SSC and SDS. The final step is the color reaction; manufacturers reagents and methods were followed throughout. An anti-digoxigenin antibody that is conjugated to alkaline phosphatase is reacted with the filter. The color reagents are nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate. After addition they allow the production of a blue color where the antibody has attached to the digoxigenin labelled probe DNA.

Light Measurement.

Photomultiplier equipment.

An Oriel (Stratford, CT) digital display model 7070 with a photomultiplier tube model 77340 connected to a flexible liquid-light pipe and collimating beam probe was used for all light measurements. This unit has its highest efficiency of signal acquisition in the color range from blue to yellow.

Light detection is measured as a current from a photoelectric effect, and is sensitive to 10^{-12} amps of induced current. The amperage is convertible to power units (watts) by multiplying the amps by 3.08×10^{3} amps·watt^{-1}, the conversion factor at the peak wavelength for the expressed light (about 500 nm). For convenience, the light measurements in this work are reported as amperes.

The photomultiplier probe was set inside the growth chamber mentioned above through an equipment portal. The interior of the chamber was maintained in the dark and remained light-tight. A constant temperature of 25°C was used throughout these experiments; humidity inside the chamber remained at approximately 25%. A magnetic stir plate was used when mixing was required.

The photomultiplier was connected to an IBM PS/2 computer through a RS232 port, allowing continuous sampling of photomultiplier output over long periods of time. The software was written by Mr. Rod Bunn, University of Tennessee. This software program typically records light output at 900 second intervals and averages 3 readings for greater accuracy. The equipment described in this section and their interactions are presented schematically in Figure 11.

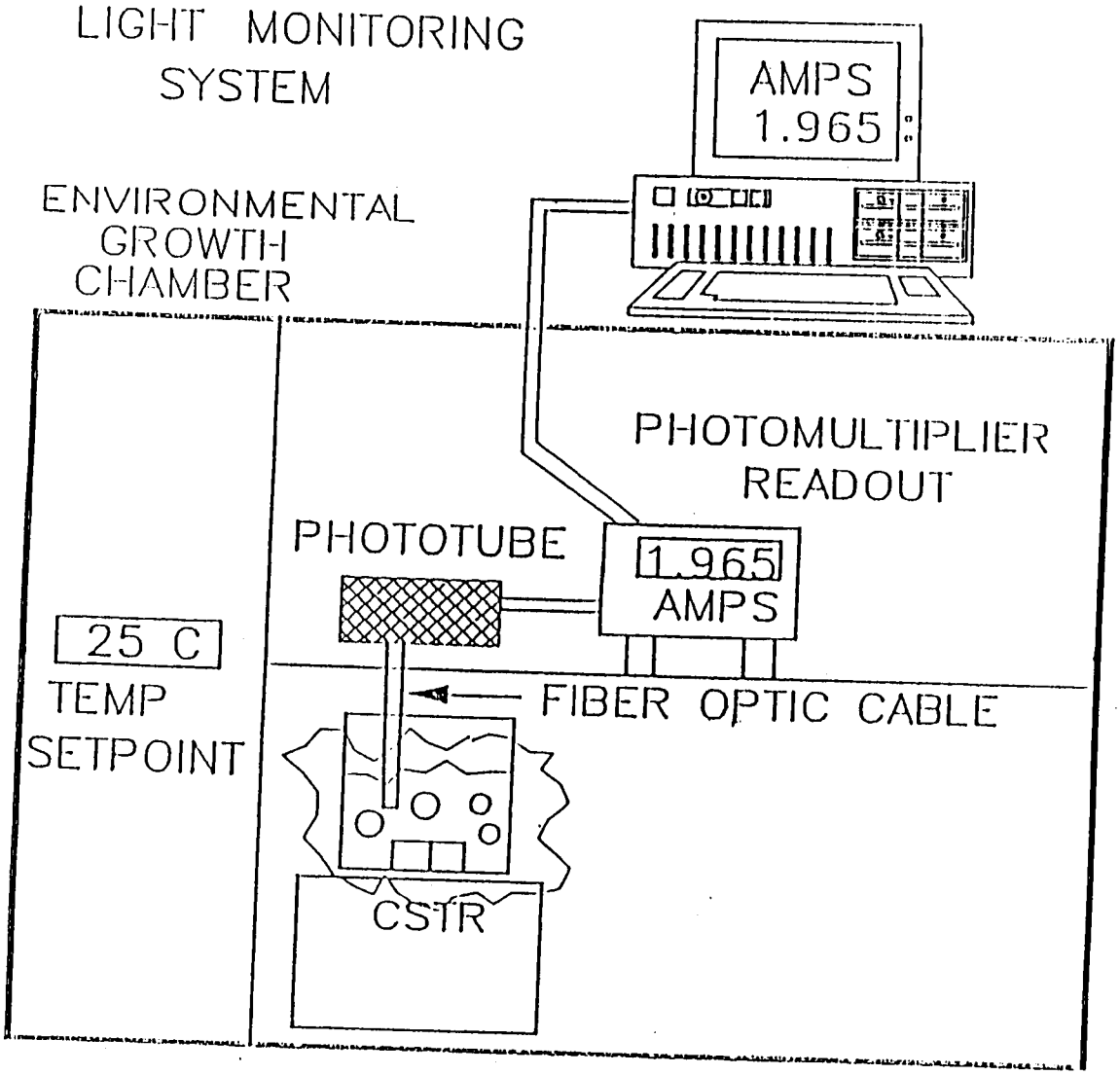


Figure 11: Description of the growth chamber and photomultiplier used in this study. The sample here is contained within a Continuously Stirred Tank Reactor (CSTR) or chemostat.

Continuous Culture.

The reactor system consisted of a New Brunswick Scientific BiOFLO Model C30 chemostat modified with a teflon top and tubing. The flow rate was adjusted with a peristaltic pump model RP-SY (Fluid Metering, Inc., Oyster Bay, NY). All parts of this apparatus were sterilized by autoclaving. The reactor liquid volume was 350 ml, with a 10 l feed tank. The medium was added at a typical flow rate of 72 ml Hr^{-1}. Agitation was maintained at 100 rpm, and sterile aeration at 0.1 l min^{-1}.

Mineralization.

Naphthalene metabolism was followed using a mineralization procedure to measure conversion of ^{14}C-naphthalene to $^{14}CO_2$. [1-^{14}C] naphthalene with a specific activity of 8.0 mCi $mmol^{-1}$ and a purity in excess of 98% was purchased from Sigma. Overnight cultures RB1351 were washed once in Basal Salts medium and then resuspended at a concentration of 10^7 ml^{-1} in either LB or Basal Salts in 25 ml EPA vials with teflon-lined silicone septa and caps (Pierce Chemical C., Rockford, IL). Unlabelled naphthalene was added to the vials at a concentration of 100 mg l^{-1} and 100,000 dpm of ^{14}C-naphthalene was dissolved in 2 μl of acetone and added before sealing the vials. $^{14}CO_2$ was collected in a 7.5 ml

glass vial containing 0.5 ml 0.4 M NaOH maintained inside the larger cultivation vial. The vials were shaken at 100 rpm in the dark at 25°C. Duplicate vials were taken at each timepoint, after determining the light output as described above. The assay was terminated by addition of 0.5 ml of 0.2 N H_2SO_4 injected into the large vial. After 30 minutes the NaOH solution was removed to 10 ml of scintillation fluid (Beckman Instruments, Fullerton, CA) and counted in a Beckman LS3801 scintillation counter. The H# method as described by Beckman Instruments was used for automatic quench compensation and dpm conversion of cpm data based on standard quench curves. The incubation medium was then extracted with 5 ml of hexane. A 0.5 ml sample from the hexane phase was used to determine the remaining naphthalene by counting the sample in 10 ml of Econofluor (NEN, Dupont). The ^{14}C-polar metabolites were determined by taking a 0.5 ml sample of the aqueous phase and counting in Beckman Ready-Protein scintillation cocktail.

RNA Quantitation.

The specific mRNA in each sample was determined according to the set of procedures outlined below. Total RNA was first isolated from samples at discrete timepoints. The extraction procedure is outlined in Table 5 (Maniatis et al., 1982). The total amount was determined in a Beckman

Table 5. RNA extraction procedure for Pseudomonas.

1. 1 ml aliquots of culture are collected in eppendorf tubes at discrete timepoints.
2. Centrifuge at 10,000 rpm for 10 minutes.
3. Resuspend in 1 ml of protoplasting buffer, with lysozyme at a final concentration of 0.4 mg ml^{-1}. Incubate on ice for 15 minutes.
4. Centrifuge at 7,000 rpm for 5 minutes.
5. Resuspend in 50 μl of lysing buffer. Incubate 5 minutes at 37°C.
6. Add 25 μl of saturated NaCl solution and mix well. Incubate on ice for 10 minutes.
7. Centrifuge at high speed for 15 minutes and transfer supernatent to a fresh tube.
8. Ethanol precipitate overnight at -20°C.
9. After drying pellet, resuspend in water and quantitate.

Protoplasting Buffer: Tris HCl, pH 8.0, 15 mM; sucrose, 0.45 M; EDTA, 8 mM.

Lysing Buffer: Tris HCl, pH 7.4, 30 mM; NaCl, 100 mM; EDTA, 5 mM; SDS, 1 %; proteinase K, 100 μg ml^{-1}.

Saturated NaCl: 40 g in 100 ml of water.

All solutions use distilled water with diethyl pyrocarbonate (0.1%) and are sterilized by autoclaving.

spectrophotometer model DU-70 by examining absorbance at 260 and 280 nm. This procedure is designed to minimize contamination with DNA; treatment of samples with RNase-free DNase reveals no change in nucleic acid concentration. The A_{260}/A_{280} ratio was between 1.7 and 2.0; this ensured that the sample was not heavily contaminated with other cell constituents. Samples that were not in this range were re-extracted with phenol, washed with chloroform/isoamyl alcohol, and ethanol precipitated. The A_{260} value was multiplied by 40 $\mu g\ ml^{-1}$ and then by the sample volume to find the total RNA.

Samples of equal volume were applied to Biotrans nylon membranes by slot or dot blot (see below). Each membrane was examined by hybridization analysis with either the nah or lux gene probes. These probes were collected and labelled as described below. Pre-hybridization and hybridization conditions are outlined below. Autoradiography was used to produce a permanent record of the strength of the signal from each sample. To accurately measure the relative intensity of the spots a Bio-Image model Visage 60 densitometer with standard software was used. Data were normalized using the concentration of the RNA samples.

Slot and dot blots.

For slot blots a Bio-Rad slot blotter apparatus was used. Whatman 3M filter paper was saturated with distilled water containing diethyl pyrocarbonate (DEPC) and placed on the apparatus, followed by the pre-wetted nylon filter. The unit was then sealed and connected to a vacuum pump. Samples of 50 μl were layered into the appropriate slots with a pipetter and the vacuum applied for a short time until most of the volume was pulled through. Each slot was further washed with DEPC water and the vacuum was applied again until all the liquid was gone. The filter was then removed, dried at room temperature for one hour, and then dried completely in a drying oven for 30 minutes.

Dot blots were much simpler to prepare. One μl dots of sample were applied directly to nylon membranes and dried as outlined above. For both slot and dot blots it was found that no denaturation of the RNA sample was necessary (results from other workers in this laboratory indicate that sensitivity is greater if the denaturation step is omitted).

Probe DNA for hybridization.

Two probes were used to measure transcript quantity from Pseudomonas strains. In each case the probe was available as a subclone on a convenient plasmid vector. The pE317 plasmid

contains part of the upper pathway of the NAH7 plasmid (Ensley et al., 1983), and was used to clone the upper pathway promoter. A 2.7 kb PstI fragment that contained part of the nahA gene was used as the nah-specific probe. A 2.0 kb fragment of the pUCD615 plasmid was used as the lux probe. This fragment was obtained by HindIII digest and represents part of the luciferase (luxAB) genes.

The plasmid pHF360 contains a 360 bp fragment from the 5′ end of the Pseudomonas aeruginosa 23S rRNA gene, which is cloned into plasmid vector pHF1.1 at the HinDIII site (Festl et al., 1986). This fragment was used as a probe for the total 23S rRNA in each of the samples.

After restriction digestion the fragments were separated on an agarose gel as outlined above. The appropriate bands were excised from the gel and the DNA removed by the Spin-X column procedure. After ethanol precipitation the DNA was resuspended in water. The DNA was labelled with ^{32}P-dCTP by nick-translation as outlined above. 1μl samples were suspended in 10 ml of Econofluor and quantitated in a Beckman liquid scintillation counter. Excess DNA probe was used with each hybridization.

Hybridization conditions.

The dried filter described above was placed into a sealable plastic bag and pre-hybridized for at least one hour

at 42°C with 4 ml of hybridization solution for every 100 cm^2 of membrane. The hybridization solution contained 5X Denhardt's solution, 5X SSC, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 250 $\mu g\ ml^{-1}$ salmon sperm DNA and 50 % formamide. The formamide had previously been deionized by incubating with Bio-Rad AG501-x8 resin (50 g in 1 l formamide) at room temperature for one hour with constant stirring. The resin was removed by filtration through Whatman #1 filter paper.

After pre-hybridization the solution was drained off and replaced with fresh solution. The DNA probe was denatured by boiling for 10 minutes and then quickly cooling in an ice bath. It was then added to the filters. Incubation was again at 42°C, and allowed to proceed overnight.

The filters were washed in two phases. The first wash solution was composed of 2X SSC and 0.1 % SDS. The filters were washed four times at room temperature for five minutes each time. The second wash consisted of 0.1X SSC and 0.1 % SDS. Two washes at 50°C, each for 15 minutes, were performed. After this final wash the filters were exposed to Kodak X-OMAT AR film to produce an autoradiograph. Exposure time at -70°C varied from 2 to 7 days.

A Kodak (Rochester, NY) Bio-Image Visage 60 densitometer was used to quantitate all autoradiograph data. The Scan mode was used to compare the relative darkness of each spot. Figures were normalized against the least-dark spot.

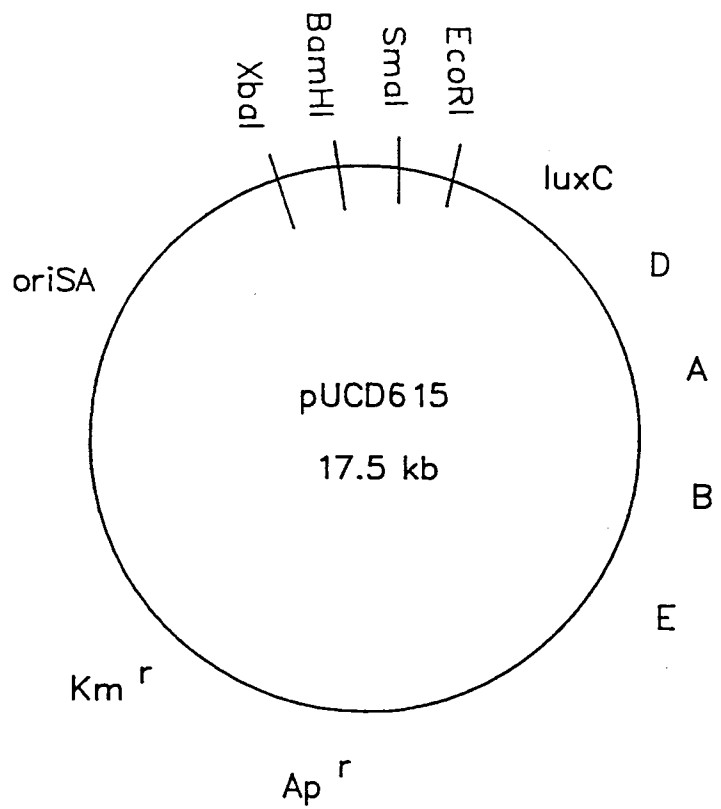
V. Results

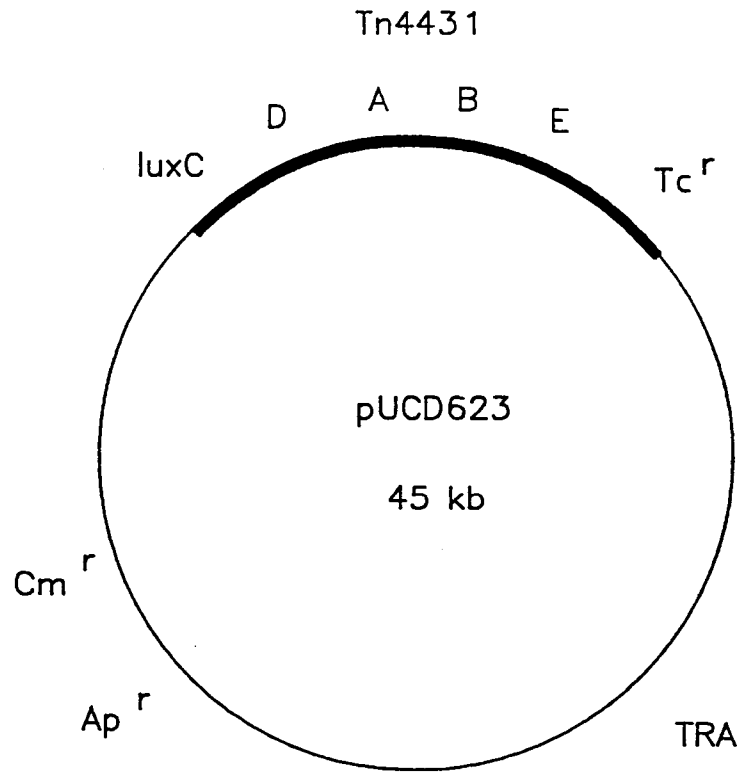
Construction of useful bioluminescent strains.

Two plasmids containing the lux genes of Vibrio fischeri were obtained from Dr. C. Kado, University of California, Davis (Figure 12). The pUCD623 plasmid contains a transposon, Tn4431, which incorporates a promoterless luxCDABE gene cassette into a derivative of Tn1721. Tn4431 also encodes a tetracycline resistance gene as a selectable marker. Transposition of Tn4431 into an appropriate position within an operon allows a transcriptional fusion to be formed. The pUCD623 plasmid has a narrow host range replicon (ColEI) that can not be maintained in most hosts. The pUCD615 plasmid contains the promoterless lux gene cassette downstream from a multiple restriction site, which allows cloning of promoter fragments from other sources. This plasmid also has ampicillin and kanamycin resistance genes as selectable markers, and a broad host range replicon (IncW). It is mobilizable using the conjugation helper plasmid pRK2013. Both of these plasmids were used to create genetic constructions.

Alcaligenes strain A5 was isolated in this laboratory from a lake sediment sample. The original strain was shown to mineralize 4-chlorobiphenyl, and contained a very large plasmid (approximately 75 kb) that encoded the degradation

Figure 12. Maps of plasmid vectors used in this study. pUCD615 is designed to allow cloning of promoters at the several restriction sites. pUCD623 is a "suicide" vector that delivers Tn4431 (thick line). Tc - tetracycline, Ap - ampicillin, Cm - chloramphenicol, Km - kanamycin, r - resistance, TRA - region of conjugative transfer genes, oriSa - origin of replication from an IncW plasmid.


XbaI
BamHI
SmaI
EcoRI
luxC
D
A
B
E
oriSA
pUCD615
17.5 kb
Km r
Ap r


Tn4431
D
A
B
E
luxC
Tc r
pUCD623
45 kb
Cm r
Ap r
TRA

genes. Recent experiments have revealed that the genes for the upper pathway of degradation are now located chromosomally, probably as a result of homologous recombination, and the lower pathway appeared to be deleted. Consequently, the strain no longer mineralized 4-chlorobiphenyl, but converted it to 4-chlorobenzoate. The size of this truncated plasmid, pSS50, was estimated at 53 kb, and may still contain a regulatory gene for induction of 4-chlorobiphenyl degradation. This plasmid was mutagenized with Tn4431. A biparental mating between A5 and E. coli HB101 (pUCD623) was performed, with a subsequent mating to E. coli DH5 to enrich for mutations in the highly transmissible pSS50 plasmid. Using this method it was very easy to generate many mutants, and several were selected for plasmid analysis by restriction digest. One such plasmid, pUTK2, is depicted in Figure 13. pUTK2 produced light constitutively in E. coli DH5, and was not inducible under any experimental conditions. Other examined mutants from various mating experiments demonstrated that Tn4431 often inserted in the same general region, as exemplified by pUTK2, and this suggested that there was an insertion "hotspot" for Tn4431. A mutant that responded to an inducer substrate was not found and so the use of pSS50 was abandoned. However, pUTK2 proved to be useful for other reasons. Investigations into the nature of this plasmid revealed that it is a member of the Incompatibility group P (Pseudomonas group P-1). Using

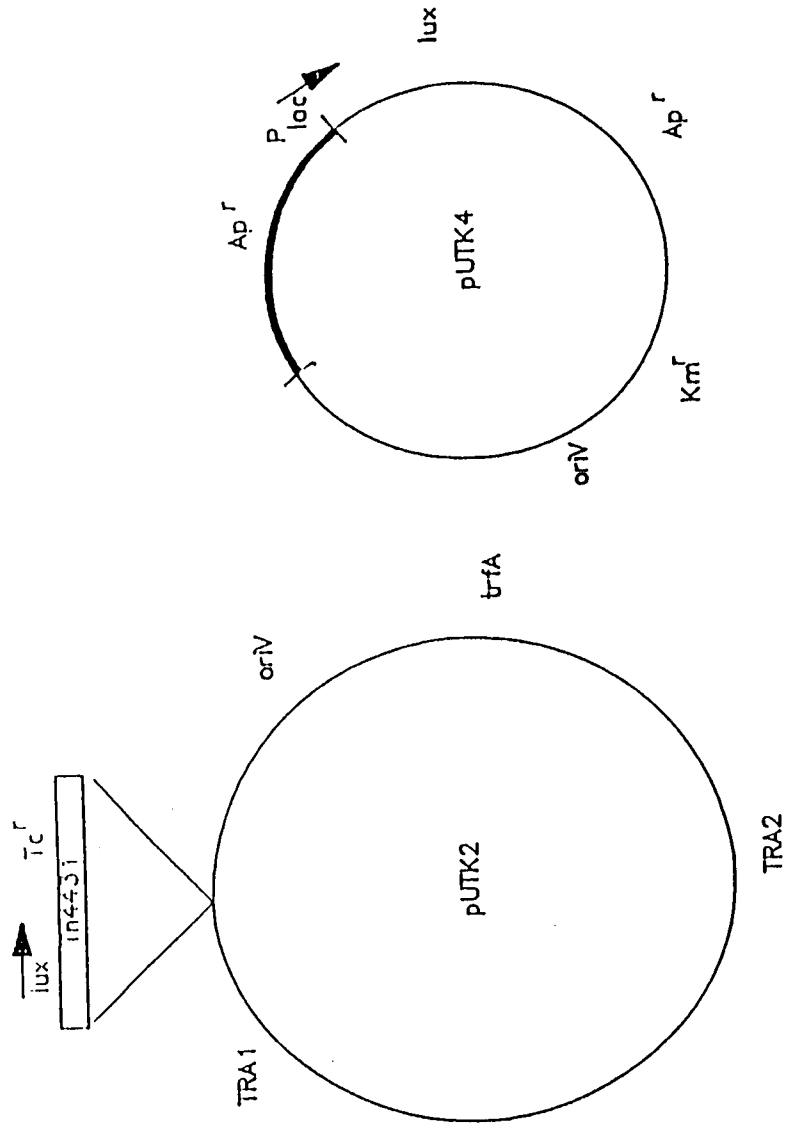


Figure 13. Maps of pUTK2 and pUTK4. two early lux+ plasmid constructions. See text for full details. Arrows show direction of transcription. oriV - origin of replication, trfA - trans-acting replication function, TRA1 and TRA2 - regions encoding conjugative transfer genes, Tc - tetracycline, Km - kanamycin, Ap - ampicillin, r - resistance, P_{lac} - lac operon promoter.

nucleotide probes derived from other known IncP plasmids, it was shown that pUTK2 had the Tn4431 transposon inserted in the TRA1 region of the plasmid (Burlage et al., 1990). At the present time, however, it is not known why light production is constitutive at high levels.

The pUCD615 plasmid was used to produce a genetic lux construction with a known promoter, in the expectation that this promoter would be more predictable. The lac promoter of E. coli is contained on the pUC18 plasmid, and both this plasmid and pUCD615 were digested with EcoRI. The linearized pUCD615 was dephosphorylated with calf intestinal alkaline phosphatase, and ligated to the linearized pUC18, producing a cointegrate plasmid, pUTK4 (Figure 13). Restriction digestion showed that the appropriate orientation of the pUC18 fragment was obtained to create a transcriptional fusion, a lac-lux fusion. In DH5α (the transformation host) light production was constitutive at a high level since the lacI repressor gene has been deleted from this host. When the pUTK4 plasmid was transformed into E. coli DH5 (which has an intact lacI gene) the light production was slightly lower in the absence of an inducing substrate. When isopropyl-β-D-thiogalactoside (IPTG), the commonly used gratuitous inducer of the lac operon, was added to a culture of DH5 (pUTK4) the light production increased to the level of the DH5α (pUTK4) strain. Uninduced light production was higher than expected since the pUTK4 plasmid was present in many copies, and the

lacI gene is present as a single chromosomal copy, which probably resulted in the titration of the Lac I repressor protein.

In order to restore control of light production to the Lac repressor system an F′ plasmid containing the *lacI*^{q} gene was introduced into the pUTK4-containing strains. This mutation of the *lacI* gene overproduces the LacI repressor protein. In each case the light production was lower than the corresponding strain without the *lacI*^{q} gene, but was still emitting bioluminescence at an appreciable level. Here also, addition of an inducer restored light production approximately to the level seen for the DH5 (pUTK4) strain. Since the copy number of the various plasmids was much higher than in a wild-type strain, and since a *lac*-*lux* fusion had already been reported by two other groups, further investigation with these strains was not performed. However, this work demonstrated that bioluminescence was practical as a medium for genetic analysis, was easily observable with the naked eye, and that differences in gene expression were identifiable by a specific induction event.

One important outgrowth of this work was the determination of the resolution of the photomultiplier used in this study i.e. the least quantity of bioluminescence that could be detected with the apparatus. A confluent culture of DH5 (pUTK4) was grown in LB broth with antibiotic selection. The photomultiplier probe was easily able to hold 10 μl on

the glass tip, where it would spread out to a thin film. A mirror was then placed over the top of the probe to reflect as much light as possible back towards the probe. Serial dilutions were performed, and successive dilutions were tested on the photomultiplier for significant light output over background current fluctuations. Similar aliquots were spread on agar medium to determine viable counts. For this experiment the power of the photomultiplier was increased to its maximum, 750 volts instead of the usual 700 volts, to increase sensitivity. The current fluctuations were greater at this level, though, and so a value of twice the average value of the background was used as the lower limit of detection. At this value a minimum of 9000 colony forming units were detected. It is unknown how many of these bacteria were actively bioluminescent at the time of the assay, but it was assumed that all of the cells were producing approximately the same level of light. This is an important distinction, since it would be desirable to use the lux assay to examine a single cell's response in vivo. There are more powerful (and more expensive) systems available for the detection of light, and it is theoretically possible to examine a single cell with one of these machines. The charge-coupled device (ccd) is a good example of this technology. A ccd camera uses a liquid nitrogen-cooled photoreceptor to detect very low intensities of light, and integrates the signal over relatively long periods of time (minutes rather

than seconds) to obtain a significant result. In effect, the ccd sacrifices speed for sensitivity.

Construction of a nah-lux strain.

Although the strains described above had the capability to produce light, and the lac-lux strain was clearly inducible to a higher level of bioluminescence, they were unsuitable for further work. The criteria for an optimum bioreporter strain includes the following: (1) the construction should be novel, (2) the construction should be inducible under defined conditions, (3) the sequences involved should be well-characterized, and (4) in keeping with the research aims of this laboratory, the construction should employ genes involved in the biodegradation of a hazardous waste material. Naphthalene was chosen since it is easy to handle and since the literature already provides a wealth of information on microorganisms that degrade it.

Initial experiments to create a nah-lux fusion utilized the plasmid pDTG113, a recombinant plasmid that contains a subclone of the naphthalene degradation pathway from Pseudomonas NCIB 9816 in the broad-host-range cloning vector pKT230. This plasmid was mutagenized with Tn4431 in a manner similar to the mating procedure described above. Unfortunately, it was discovered after the construction of this fusion that pDTG113 no longer has the nah promoter, and

expression was actually dependent on the promoter from the streptomycin resistance gene on the vector. Obviously this rendered these constructs useless for further study.

The Tn4431 transposon was used to create nah-lux fusions in Pseudomonas putida PpG7, the strain which contains plasmid NAH7. PpG7 was mated with HB101 (pUCD623) and colonies were selected on Pseudomonas Isolation Agar supplemented with tetracycline. When naphthalene crystals were added to these plates several colonies produced a measureable increase in bioluminescence. The light production increased substantially when n-decyl aldehyde was added at a final concentration of 0.001%, signifying that PpG7 was deficient in a suitable aldehyde substrate for the lux reaction.

One of these clones, 22c2, was examined in greater detail. The Tn4431 insertion was mapped to the lower pathway of NAH7, with the correct orientation for a successful nah-lux fusion. A series of experiments was performed with this strain, but it was quickly noted that certain experiments were not repeatable; the light production gradually diminished with successive experiments. Plasmid analysis of this strain revealed that the culture was mixed, and a large fraction of the cells contained a plasmid that was smaller than the 22c2 plasmid. It was apparent that this construction was unstable, and spontaneous deletions and/or rearrangements are frequent. This instability made the strain unsuitable for further experimentation.

A possible explanation for this instability might include the recent discovery that the naphthalene degradation genes of NAH7 are contained within a degenerate transposable element (M. Tsuda, pers. comm.). This element, Tn4655, is a 37 kb sequence that only shows active transposition and instability of the nah phenotype in the presence of Tn1721. Tn4431 is a derivative of Tn1721, and it is possible that the presence of this lux transposon provides a factor involved in the instability seen with the 22c2 experiments.

pUTK9, a nah-lux fusion plasmid.

Since nah::Tn4431 constructions seemed to be of limited usefulness, it was decided to take advantage of the pUCD615 cloning vector. There were several advantages in doing this: (1) the construction will be more defined than a transposon mutation in terms of sequences involved, (2) the response of the strain in the absence of other NAH7-related genes can be examined, and (3) when supplied with the intact NAH7 plasmid, the strain will degrade naphthalene as well as report on the presence of the substrate.

The nucleotide sequence of the upper pathway promoter and the nahA gene have been determined (B. Ensley, pers. comm.) The nahA gene encodes a naphthalene dioxygenase, and is composed of four cistrons in this order: a flavoprotein, a ferredoxin, and the α and β subunits of an Iron-Sulfur

protein (nahAa-Ad, respectively). This information was used to obtain the appropriate promoter fragment. The subcloning of the upper pathway promoter of NAH7 proved to be more difficult than expected. The pUCD615 plasmid has a limited number of restriction sites for direct cloning, and this influenced the selection of the fragment to be cloned. A 5.3 kb EcoRI - XmaI fragment was selected for cloning. Despite many attempts using both pUCD615 and pUC18 plasmids this fragment could not be isolated. The same result occurred when a shorter (3.4 kb) EcoRI - XhoI fragment was selected. Both of these fragments included a substantial region upstream of the nah promoter, and it is possible that the presence of a deleterious gene from this region could be lethal to the E. coli host. The presence of an E. coli-like promoter divergent from the nah promoter raised the possibility that the suspected deleterious gene might be intimately associated with the degradation system. Although no activity has yet been described for this putative promoter, this may be due to a lack of specific inquiry. It is perhaps significant that other workers have been able to clone very large fragments of nah that include this upstream region, but that smaller subclones (less than 5 kb) that contain this region have not been reported.

Figure 14 shows the subcloning procedure for construction of plasmids used in this study. A 2.7 kilobasepair (kb) PstI fragment of pE317 that contains the

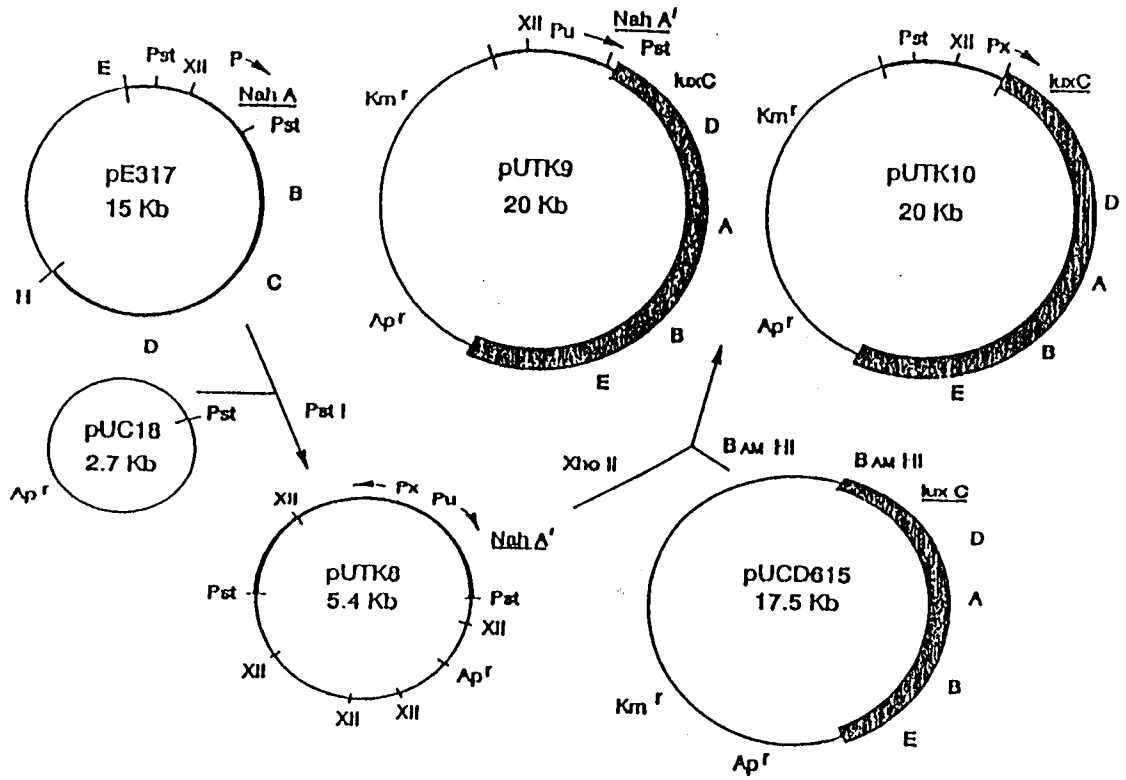


Figure 14. Subcloning procedure for pUTK9. A PstI fragment of pE317 was cloned into pUC18, forming pUTK8. A XhoII fragment of pUTK8 was then cloned into pUCD615 at the BamHI site in both orientations to form pUTK9 and pUTK10. P - upper pathway promoter, P_x - putative divergent promoter, XII - XhoII, E - EcoRI, Ap - ampicillin, Km - kanamycin, r - resistance. Arrows show direction of transcription.

upper pathway promoter was cloned into pUC18, with selection for colonies growing on LB plates with ampicillin and X-gal. White colonies were screened for the correct fragment by restriction digest. This construction, pUTK8, was used to subclone a 2.3 kb XhoII fragment into the dephosphorylated lux plasmid vector, pUCD615. This step also required the cloning of the pUC18 fragment between the PstI and BamHI (XhoII) sites, but this added only a few basepairs of vector DNA to the fragment and should not affect the fusion. Both orientations were obtained in this manner: the nah promoter and the first three cistrons of nahA were transcriptionally fused to the lux genes, forming pUTK9; pUTK10 has the opposite orientation. The identity of these plasmid constructions was confirmed by restriction digest and subsequent Southern blotting of the fragments (Figure 15). DNA hybridization with NAH7 showed the expected pattern in each construction.

The natural host for the NAH7 plasmid is Pseudomonas putida PpG7. This strain is a poor host for light generation as evidenced by the NAH7::Tn4431 results. The n-decyl aldehyde substrate could be added exogenously, although the difficulties inherent in the use of this aldehyde are great (including toxic effects on the cells). Therefore, it was decided to utilize Pseudomonas putida PB2440, which had previously demonstrated light-producing potential in the absence of added aldehyde. The NAH7 plasmid was mated into

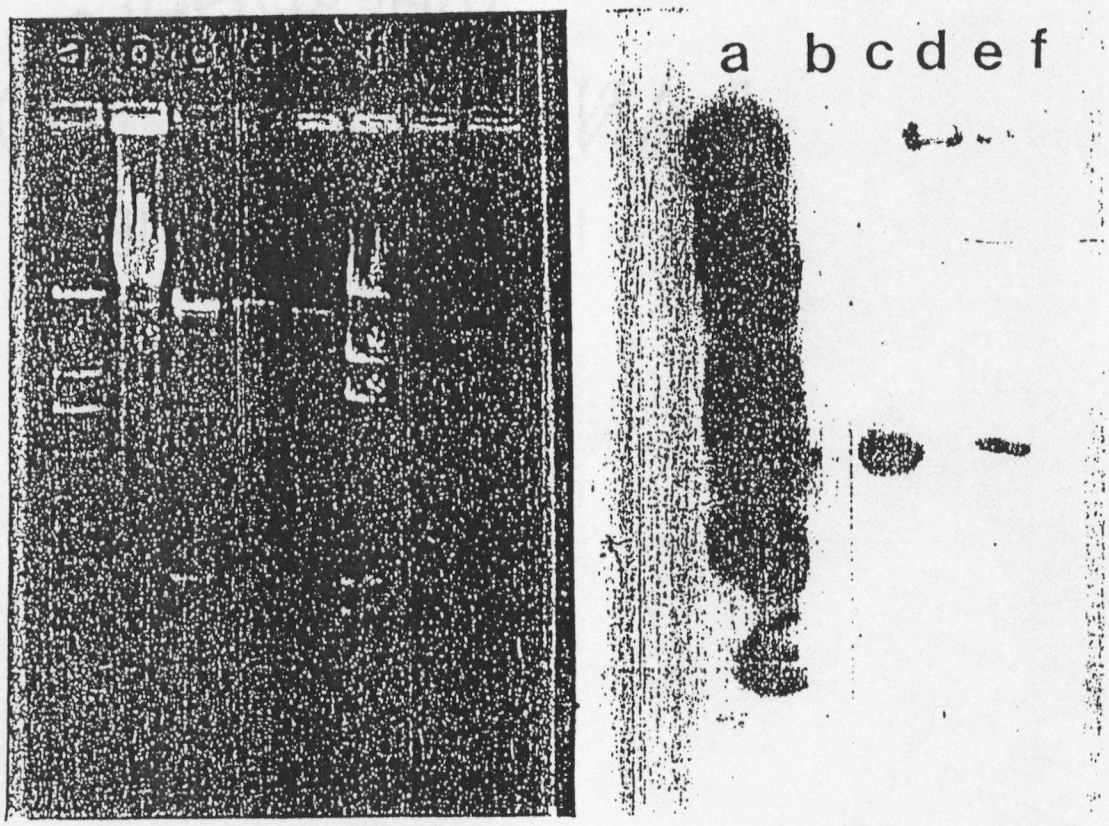


Figure 15. Southern blot of pUTK9 and pUTK10. The left panel shows restriction digests of pUTK9 and pUTK10. The right panel shows the autoradiograph of this gel when NAH7 is used as a probe. Lanes: a. NAH7 digested with EcoRI, b. pUCD615 digested with EcoRI and BamHI, c. pUTK10 digested with EcoRI and BamHI, d. pUTK9 digested with EcoRI and BamHI, e. pUTK9 digested with EcoRI and XbaI, f. lambda phage digested with HindIII.

PB2440, forming strain HK53. This strain grew on naphthalene or salicylate as a sole carbon and energy source, and so the catabolic pathways appeared to function normally in this host. This strain and the parent strain PB2440 were used as hosts for the pUCD615 plasmid and for the constructed plasmids pUTK9 and pUTK10, which were introduced by electroporation. Strains were maintained on LB agar with kanamycin selection or YEPSS agar medium.

Each of these Pseudomonas strains was grown overnight as a single colony on LB agar medium. Table 6 reports the light output from isolated colonies on plate assays both in the presence and absence of naphthalene, which was supplied as vapor from naphthalene crystals. As expected, most strains do not produce any measureable amount of light, either because they do not contain the lux genes or because they lack the nahR regulatory gene from the NAH7 plasmid. It has been demonstrated that the nahR gene is necessary for induction of the upper pathway promoter. Strain RB1351, which harbors both pUTK9 and NAH7, contrasted sharply with these other strains. It was evident from these data that expression of the upper pathway was constitutive at low levels, and that expression increased substantially (about 24 fold) when naphthalene vapor was present. Figure 16 demonstrates the rapid bioluminescent response of the strain after naphthalene induction. An increase in light production was apparent within three minutes, and reached its peak within ten

Table 6. Light production by Pseudomonas strains

Strain	Naphthalene	
	absence	presence
PB2440	0	0
HK53 (=PB2440(NAH7))	0	0
RB1356 (=PB2440(pUCD615))	0	0
RB1359 (=PB2440(NAH7, pUCD615))	0	0
RB1358 (=PB2440(pUTK10))	0	0
RB1364 (=PB2440(NAH7, pUTK10))	0.060	0.080
RB1357 (=PB2440(pUTK9))	0	0
RB1351 (=PB2440(NAH7, pUTK9))	0.860	21.0
RB1369 (=PB2440(pUTK11))	0	0.060
RB1368 (=PB2440(NAH7, pUTK11))	0.060	2.7

All numbers are in amperes x 10^{-9} of induced current in an Oriel photomultiplier (see Materials and Methods). Naphthalene was supplied as vapor from crystals, and measurements were taken after approximately 30 minutes. Each strain was present as a single colony grown overnight at 25° C on LB agar. A value of 0 indicates that less than 10^{-12} amps were detectable on the photomultiplier.

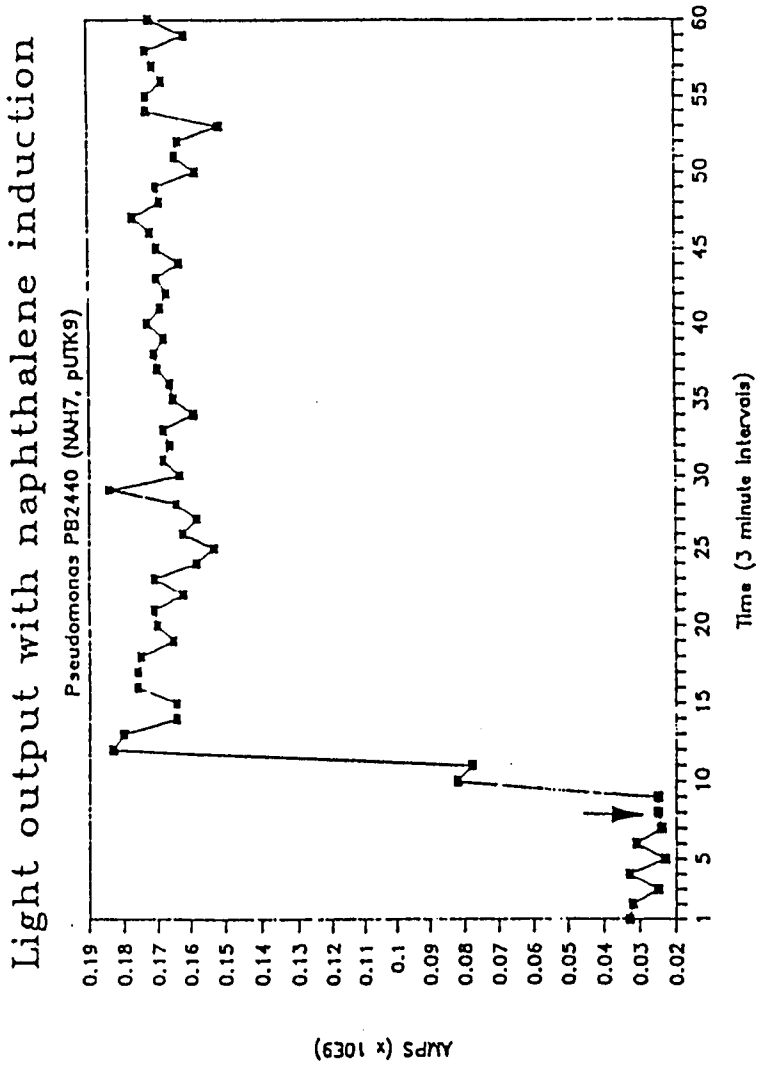


Figure 16. Light output with naphthalene induction. Strain RB1351 was grown to mature colonies on LB agar plates. Naphthalene was supplied as vapor from crystals at timepoint 8 (arrow). Light is reported in amps. E - exponent.

minutes. Light production was stable for at least 24 hours on these plate assays, varying by approximately 10 % during this time. The pUTK10 plasmid was created to determine whether a divergent promoter overlaps P_u, much as the promoters for the lower pathway and nahR overlap. Strain RB1364, which contains both NAH7 and pUTK10, produced a very low level of light (less than 10% of the uninduced P_u) that was apparently unaffected by the presence of naphthalene (Table 6). There does not appear to be sufficient evidence for a promoter in this orientation.

Light production of RB1351 in liquid media.

It was anticipated that liquid batch cultures of RB1351 could be induced with naphthalene in the same way that colonies were induced. RB1351 was grown in LB broth with kanamycin selection to a mid-log growth phase, at which time naphthalene was added. Figure 17 demonstrates the growth curve and light output of this strain. Two observations are significant from this experiment. The first is that light was not produced until several hours after naphthalene addition, which corresponded to a late logarithmic phase of the growth curve. This was true whether naphthalene was added during early or mid-log phase. Addition of naphthalene at a stationary phase resulted in a gradual increase in light production, much the same as was seen for the colonies. The

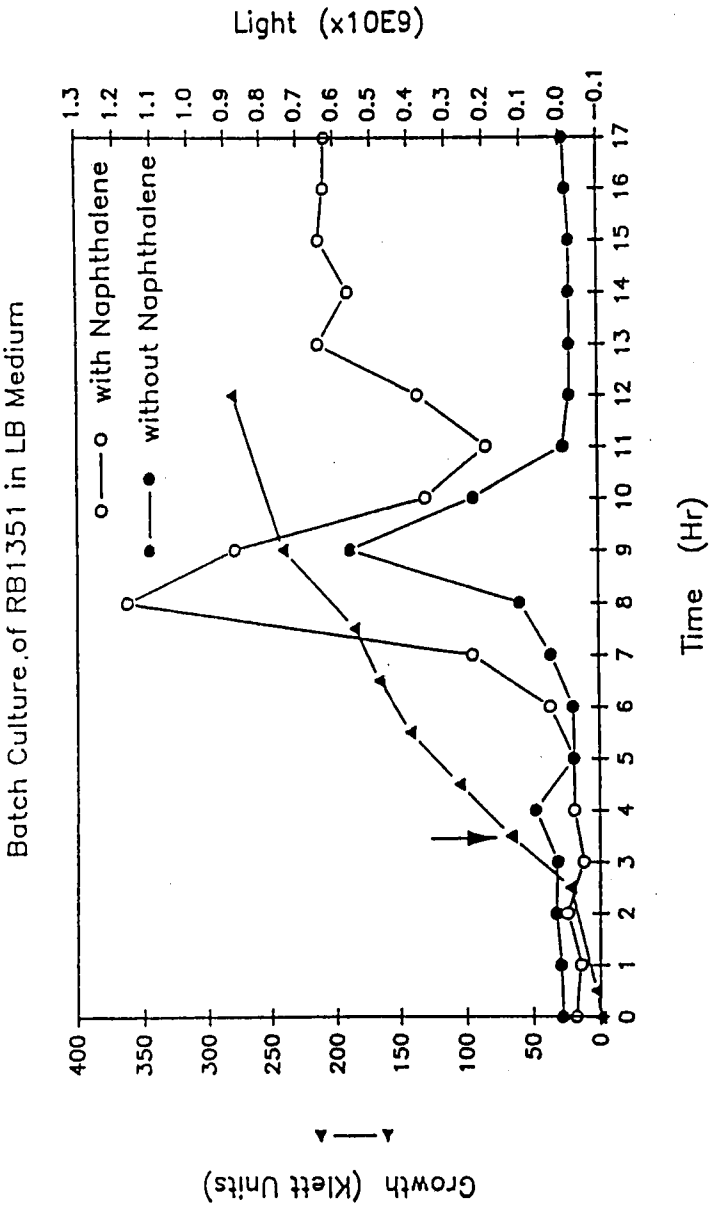


Figure 17. Batch culture of RB1351 in LB medium. RB1351 was grown in LB broth with kanamycin selection at 25°C with constant stirring. Arrow indicates when naphthalene crystals (1 % w/v) were added. E - exponent.

second observation was that the light response curve was a complex pattern. A control culture to which no naphthalene was added exhibited a light pulse during the late-log phase; it was apparently due to the induction of the upper pathway promoter, even though an inducing substrate had not been added. When naphthalene was added a large, relatively short-lived peak of light production occurred first, followed by a smaller, more stable plateau. The response of this short-lived peak was approximately the additive value of the control peak and the naphthalene-specific plateau. Light production during the plateau was very stable, remaining almost unchanged as long as naphthalene was present.

It was expected that the bioluminescent response would occur soon after addition of the substrate. A trivial explanation of these results would be that the aldehyde substrate required for the light generating reaction was not available at the time of naphthalene addition. However, addition of n-decyl aldehyde to growing cultures as described previously at a final concentration of 0.001 % produced no significant changes in light output (data not shown). This raised the possibility that bioluminescence was not correlated with naphthalene metabolism in this system, which would be surprizing but possible. The other major possibility was that bioluminescence is correlated with naphthalene metabolism, but that there is a catabolite repression of the upper pathway in rich medium that is exhausted by late-log

phase, or that perhaps naphthalene catabolism is growth rate-regulated. To distinguish between these possibilities the experiments described below were performed.

Naphthalene mineralization.

To determine when naphthalene was being utilized during culture growth on rich medium, and by extension whether bioluminescence was correlated with naphthalene metabolism, a series of mineralization experiments was performed using radiolabelled naphthalene. This arrangement allowed the determination of the fraction of naphthalene converted to CO_2 (mineralized), the fraction of unused naphthalene, and the fraction that was still cell-associated (biomass, or naphthalene uptake). Excess headspace in the vials provided ample oxygen during the experiment. Figure 18 describes the fate of naphthalene when Basal Salts medium is used. Naphthalene utilization was readily apparent, both from the removal of initial naphthalene and the increase in radiolabelled CO_2. Light output increased gradually throughout the experiment, as expected. However, the viable cell count was unchanged throughout the course of the experiment. Catabolism of the naphthalene substrate appeared to be used for cell maintenance. A separate experiment in which the growth medium was supplemented with 6 mM glucose showed similar results. When this experiment was repeated

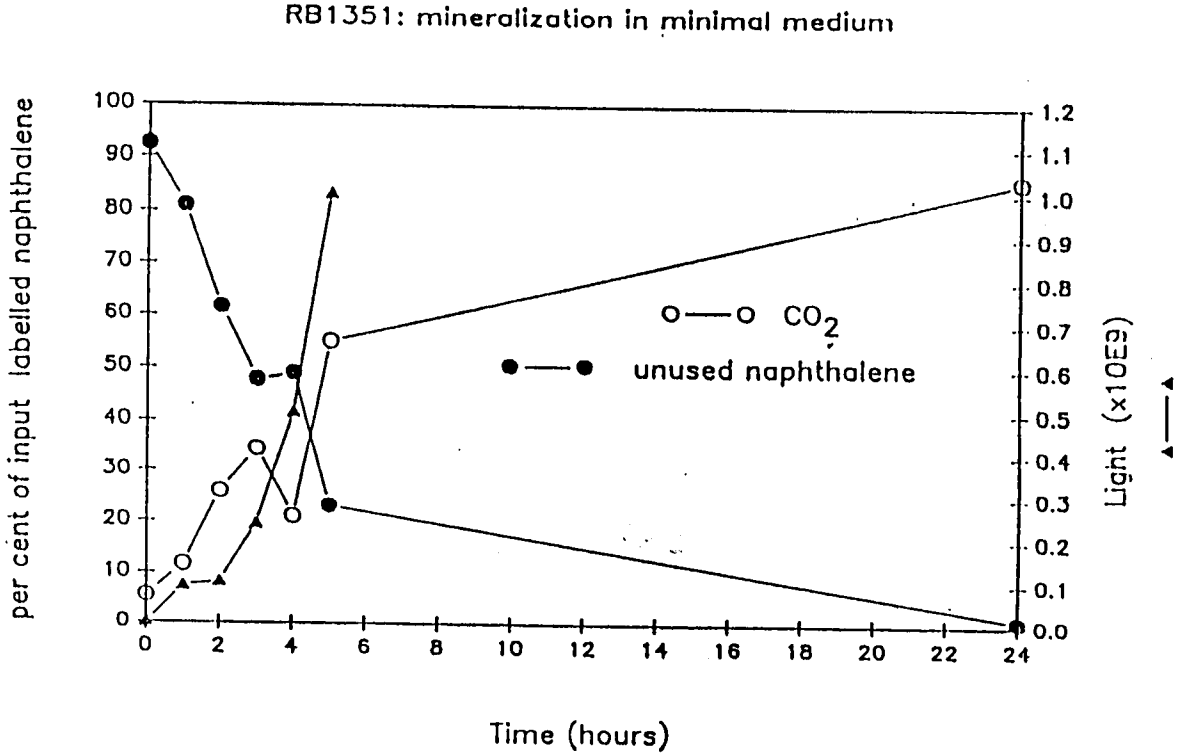


Figure 18. RB1351: mineralization in minimal medium. Light production was determined immediately before fraction analysis. Viable count remained at 3 x 10^7 cells ml^{-1} throughout the experiment. E - exponent.

using LB broth a very different pattern appeared (Figure 19). Cell growth was evident throughout this experiment, as was observed with the batch experiments described above. The production of light was delayed until the late log phase, also as expected from previous results. It is evident from the data on the utilization of naphthalene, however, that there was very little catabolism occurring during the log phase. This is supported by the data from the unused naphthalene and from the generated CO_2 fractions. These data demonstrate that naphthalene metabolism is closely correlated with bioluminescence under these conditions, whether in rich or minimal medium.

Bioluminescence at a slow growth rate.

The correlation of bioluminescence with naphthalene utilization leaves open two major possibilities: that there is a catabolite repression of the upper pathway, or that there is a preferred growth rate for the utilization of naphthalene. It has been suggested that there is no catabolite repression of the NAH7 pathway (Schell and Wender, 1986), although data are lacking to demonstrate this effect in LB medium. In order to determine whether catabolite repression was present a culture of RB1351 was grown to a mid-log phase in LB broth, washed twice with Basal Salts medium, and resuspended in Basal Salts with naphthalene. No

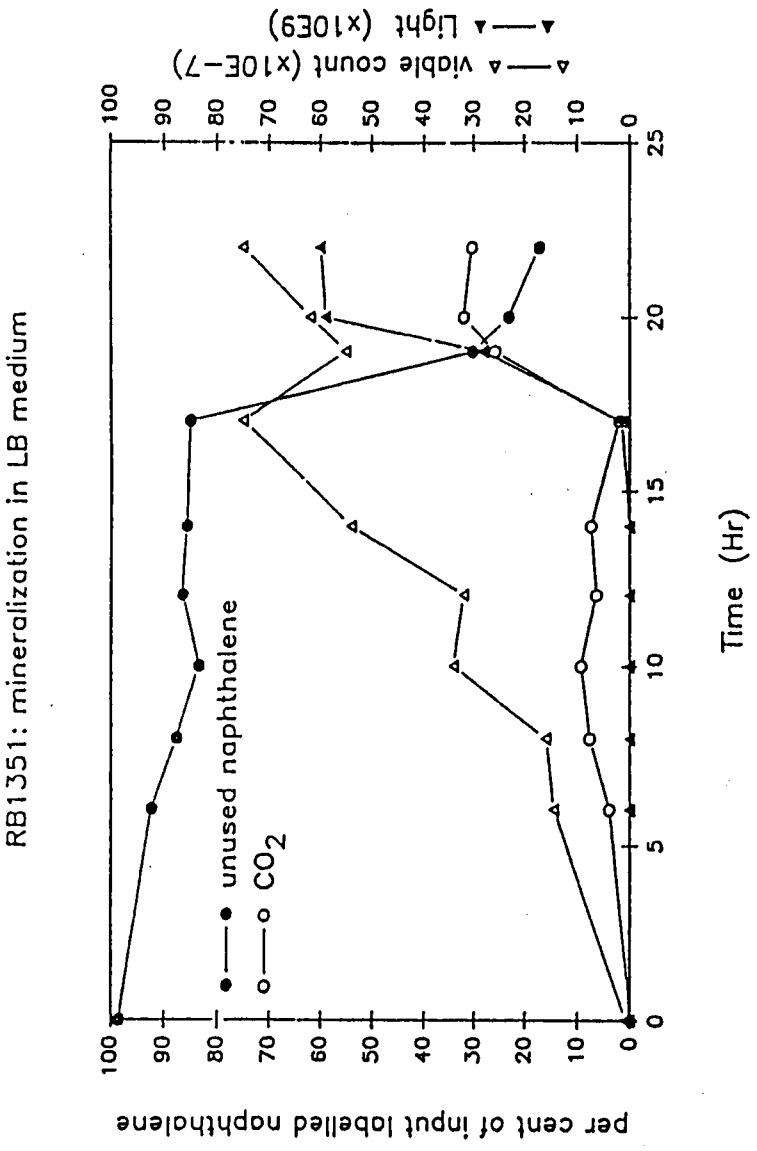


Figure 19. RB1351: mineralization in LB medium. Light output was measured immediately before fraction analysis. E - exponent.

light was observed from this culture even after several hours (data not shown). It was suggested that this procedure could induce a stringent response that might mask true catabolite repression, so a second method was employed. A batch culture of RB1351 was grown overnight in LB medium saturated with naphthalene in order to reach a stationary phase, and to deplete the medium. Identical 0.5 ml aliquots were collected and centrifuged to form a pellet. One aliquot was resuspended in 9 ml of spent medium while the other was resuspended in 9 ml of fresh LB-naphthalene medium. Both flasks were shaken at 25°C and periodically examined for light output and cell growth (using a Klett meter). Figure 20 shows the outcome of this experiment. It is evident that the spent medium was unable to support much additional growth, but that there was sufficient naphthalene present to permit high levels of light output. The fresh medium, in contrast, allowed the culture to reenter exponential growth, but the light output decreased dramatically. Interestingly, while both cultures were in the lag phase the light output per cell was almost identical, but the light output from the fresh medium culture fell off sharply with the first timepoint clearly in log phase. If catabolite repression was functioning in this system, it would be expected to have a noticeable effect during the first hour after addition of fresh medium. These results strongly suggest that there is no catabolite repression, but that a slow rate of growth is preferred for activation of the

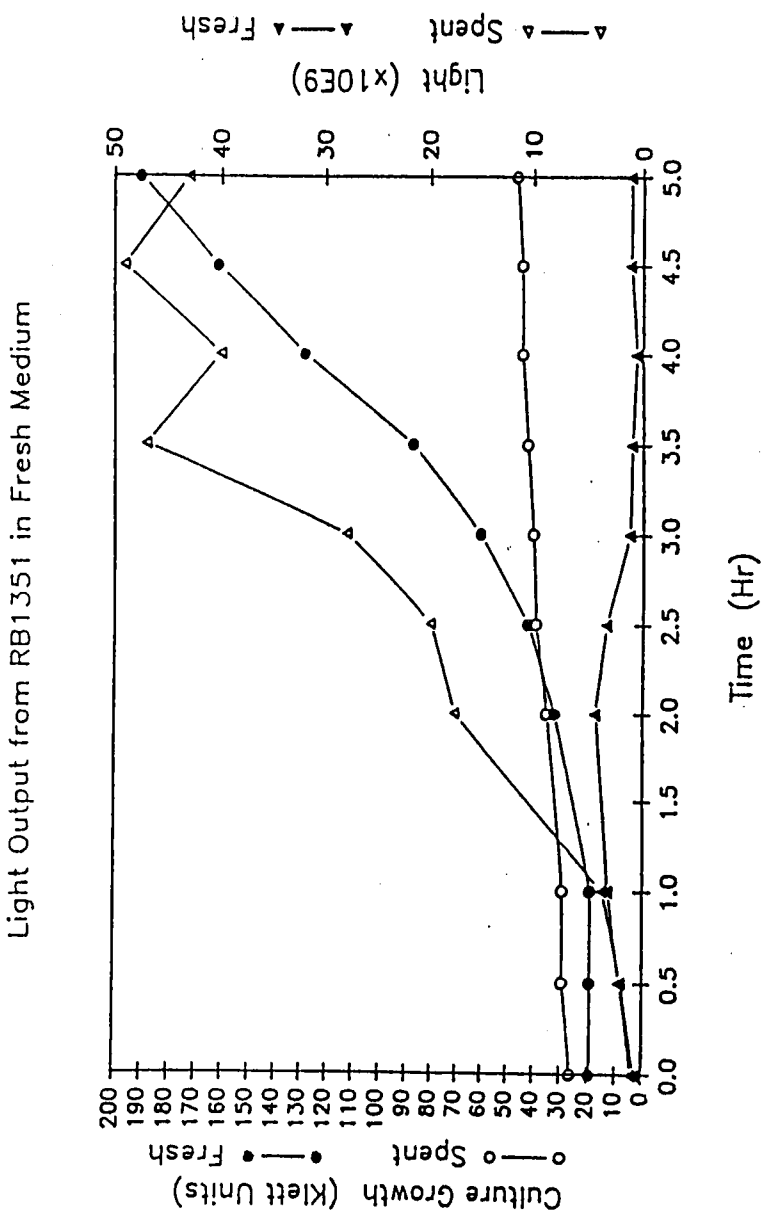


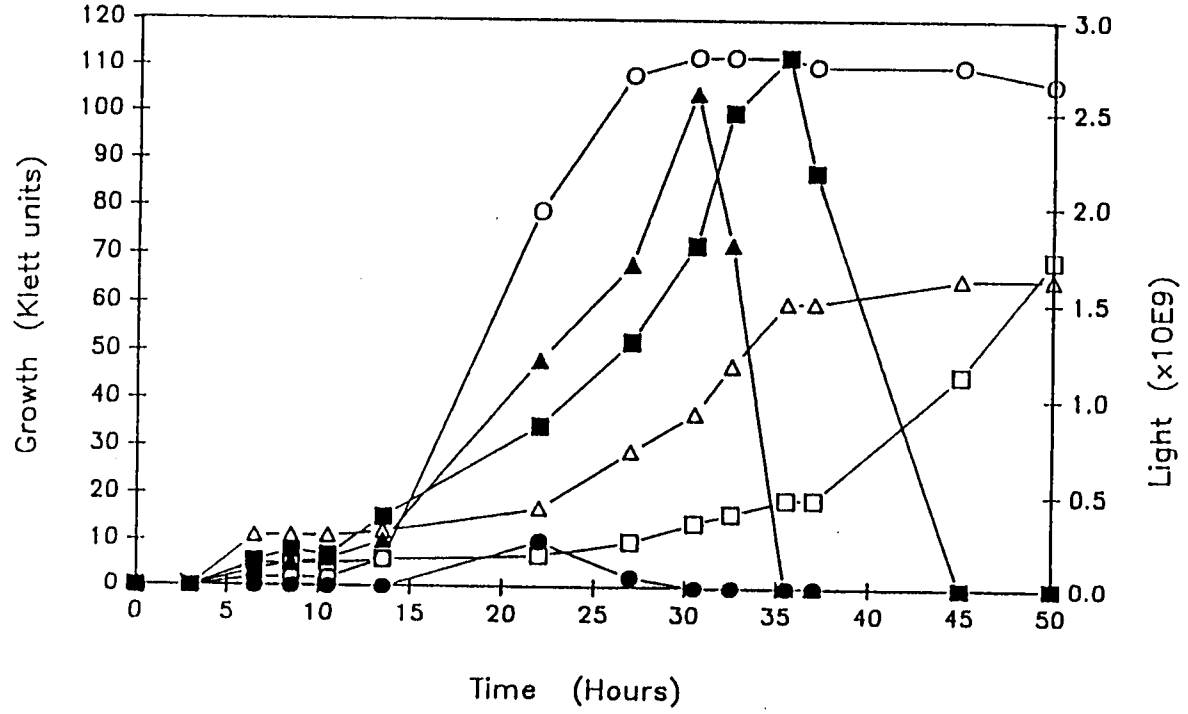
Figure 20. Light output from RB1351 in fresh medium. Aliquots from a stationary phase culture of RB1351 in LB medium supplemented with naphthalene were resuspended in fresh medium and a filtered spent medium. Light output and culture growth were examined at each timepoint. E - exponent.

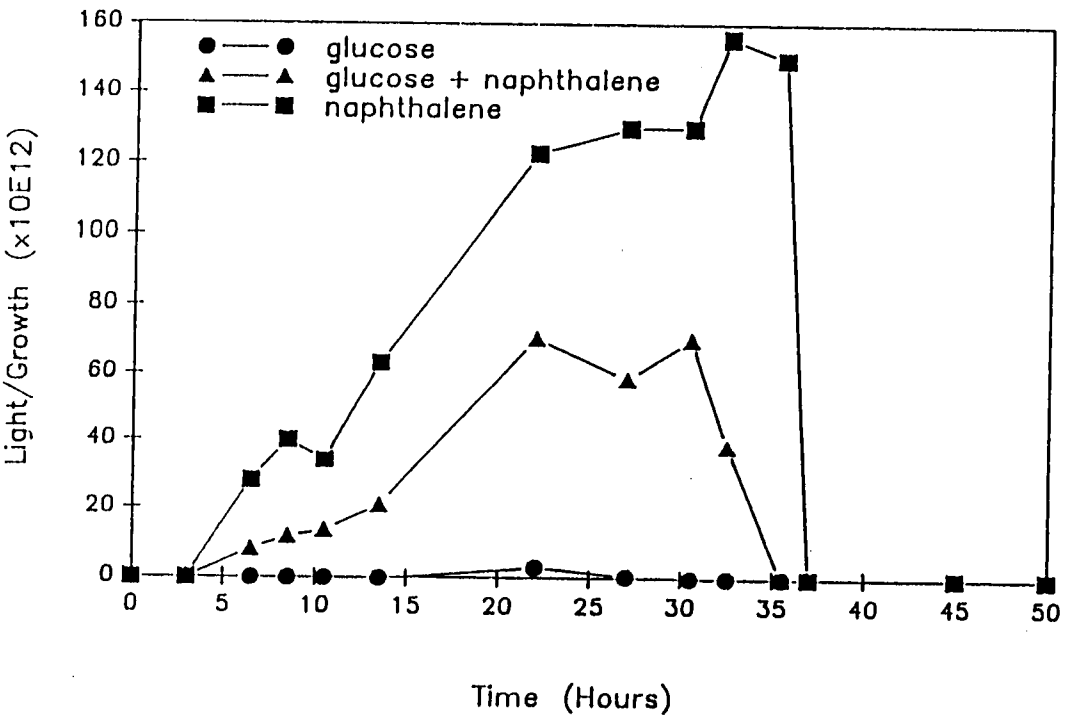
nah genes.

In order to firmly identify the nature of this effect, the RB1351 strain was grown in a defined Basal Salts medium (BSM, see Materials and Methods). RB1351 was grown overnight in BSM with glucose (10 mM final concentration) as sole carbon and energy source. This culture was used to inoculate three flasks: one with the same medium, one with naphthalene (1% w/v final concentration) as sole carbon and energy source, and one with both naphthalene and glucose. Batch culture growth and light output were determined throughout this experiment. Results are shown in Figure 21 (top).

Growth was initially very slow in this medium, a fact that was noticed also during the mineralization experiments described above. After this extended lag phase the culture grown in glucose alone grew exponentially. The culture grown in naphthalene alone grew very slowly at first, went through a period of linear growth, and then finally entered exponential growth. This growth continued for some time: a timepoint at approximately 90 hours was omitted from this figure, but showed that growth peaked at 360 Klett units (light production had ceased at this time). It is not known why the lag phase is so long, although it is known from the mineralization experiments and from the light production during this time, that naphthalene degradation is in progress. The growth of RB1351 in both glucose and naphthalene was apparently intermediate between the other

Figure 21. Light output from RB1351 in Basal Salts Medium. Top. Empty symbols show growth, filled symbols show light output. Circles, glucose added; triangles, glucose and naphthalene added; squares, naphthalene added.
Bottom. Light production normalized for cell number. Symbols are as listed. Both top and bottom graphs have abscissas that are synchronous for easy comparison. E - exponent.


Growth (Klett units)
Light (x10E9)
Time (Hours)
120
110
100
90
80
70
60
50
40
30
20
10
0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
40
45
50


glucose
glucose + naphthalene
naphthalene
Light/Growth (x10E12)
Time (Hours)
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50

two, and this was somewhat surprizing. The culture had both 10 mM glucose and 1% naphthalene available for use, more total substrate than was available to the other two cultures. If there had been a simple preference for glucose over naphthalene the growth curve would have assumed a biphasic shape; this was not seen. Instead it appeared that the presence of the naphthalene inhibited growth on glucose.

The light production from these cultures was also examined. It was apparent that the glucose-grown culture had a slight increase in light production in the late log phase, although this was small compared to the other strains. Light production from the other two cultures appeared to be substantially the same in the top part of this figure. However, the number of cells emitting light was very different in the two cultures. To obtain a more realistic value the light was divided by the growth to approximate a light per cell value (Figure 21, bottom). The data from the early timepoints are obscured by the presence of the naphthalene crystals in the medium but the later points are more likely to be accurate. At these times it was evident that the naphthalene-only culture was producing substantially more light on a per cell basis than the naphthalene and glucose culture. This light output was fairly stable throughout several hours, but then decreased abruptly at a time that corresponds to the start of exponential growth. Light ouput from the culture with both glucose and

naphthalene increased substantially, then diminished rapidly until no light can be detected. Light production did not resume after the onset of stationary phase, suggesting that an accumulation of toxic metabolites may be contributing to the weak culture growth.

A nah deletion mutant

An attempt was made to determine the region of DNA that was responsible for this putative growth-rate related effect. It was observed that a nearly perfect 18 bp palindromic region was located within the flavoprotein (nahAa) gene (Figure 22). It is possible that the mRNA folds into a hairpin structure at this point, which could interfere with translation. Secondary structures that are responsible for growth-rate related control have been described (Carter-Muenchau and Wolf, 1989). It was therefore decided to remove the palindromic structure and determine whether any qualitative or quantitative effects could be observed.

The deletion of the palindromic region was accomplished by subcloning a 0.75 kb DNA fragment from pUTK9 that did not contain the palindrome, and re-inserting it into pUCD615. The details of this procedure are shown in Figure 22. This new plasmid, pUTK11, was then introduced into Pseudomonas strains both with and without the co-resident NAH7. A comparison of the bioluminescent activity of colonies of this strain with

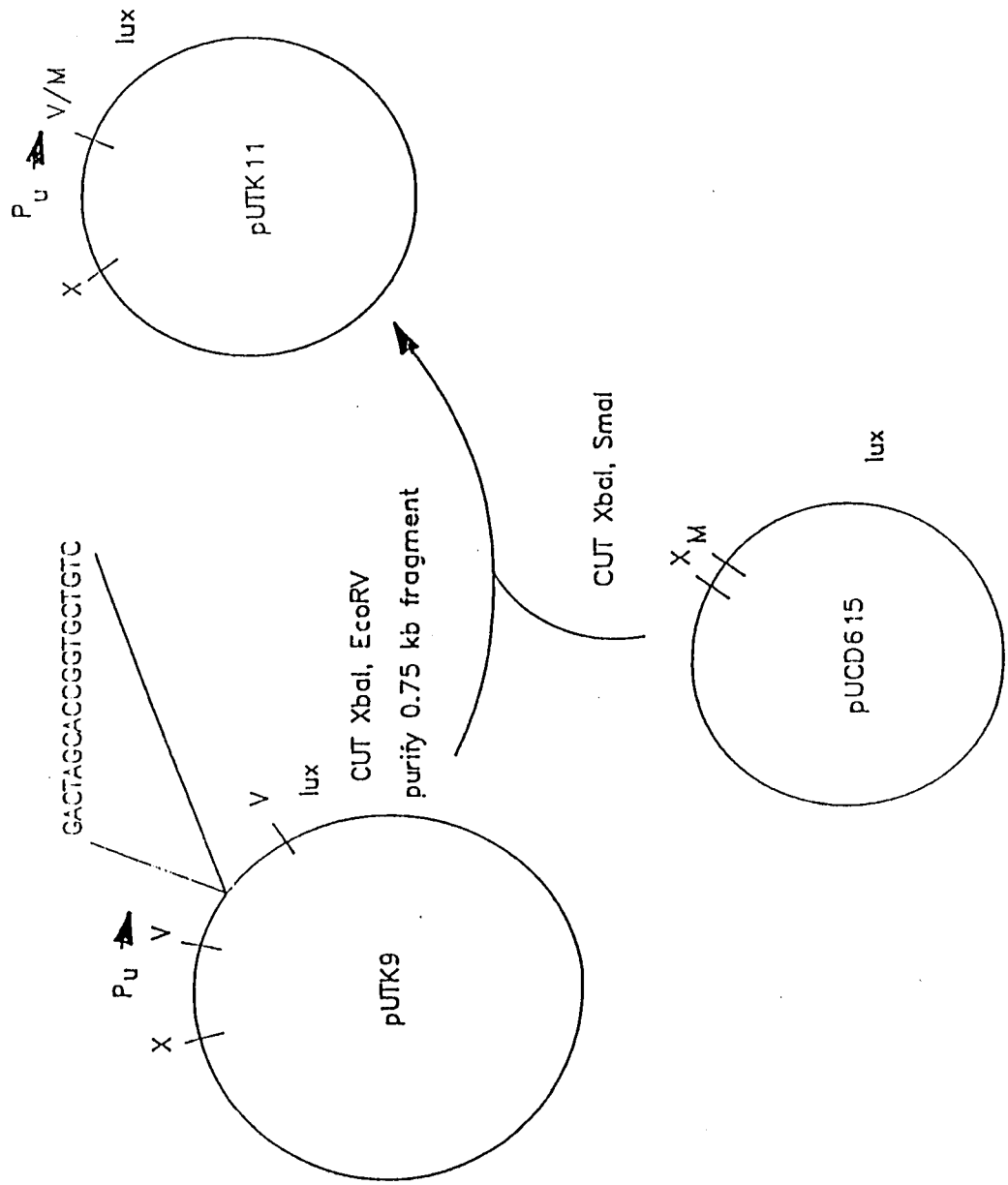


Figure 22. Construction of the pUTK11 plasmid. Location and sequence of the putative palindrome are shown. X - XbaI, V - EcoRV, M - SmaI.

other lux strains in both the presence and absence of naphthalene vapor is presented in Table 6 (p. 90). It is clear from these data that strain RB1368 (with NAH7 and pUTK11) increased light output substantially after exposure to naphthalene, and that in the absence of inducer the light output was negligible. Thus it appears that both RB1368 and RB1351 respond to the naphthalene inducer in the same manner on solid medium. Batch culture growth of RB1368 is depicted in Figure 23. After the addition of naphthalene during exponential growth there was a rapid rise in light output, in marked contrast to the results using RB1351 (Figure 17, p.93). A broad peak was reached at the end of the log phase, and light output decreased to a roughly steady-state condition that persisted for many hours. In summary, the response peaks of RB1368 and RB1351 look substantially the same, although the delay in the bioluminescent response with RB1351 is not seen with RB1368. Light output in the absense of added naphthalene remained low, and did not demonstrate the transient burst of light that the RB1351 strain did. However, these two strains are not strictly comparable, and care must be taken in interpreting these data. These data only suggest that the deleted region may contain important regulatory regions.

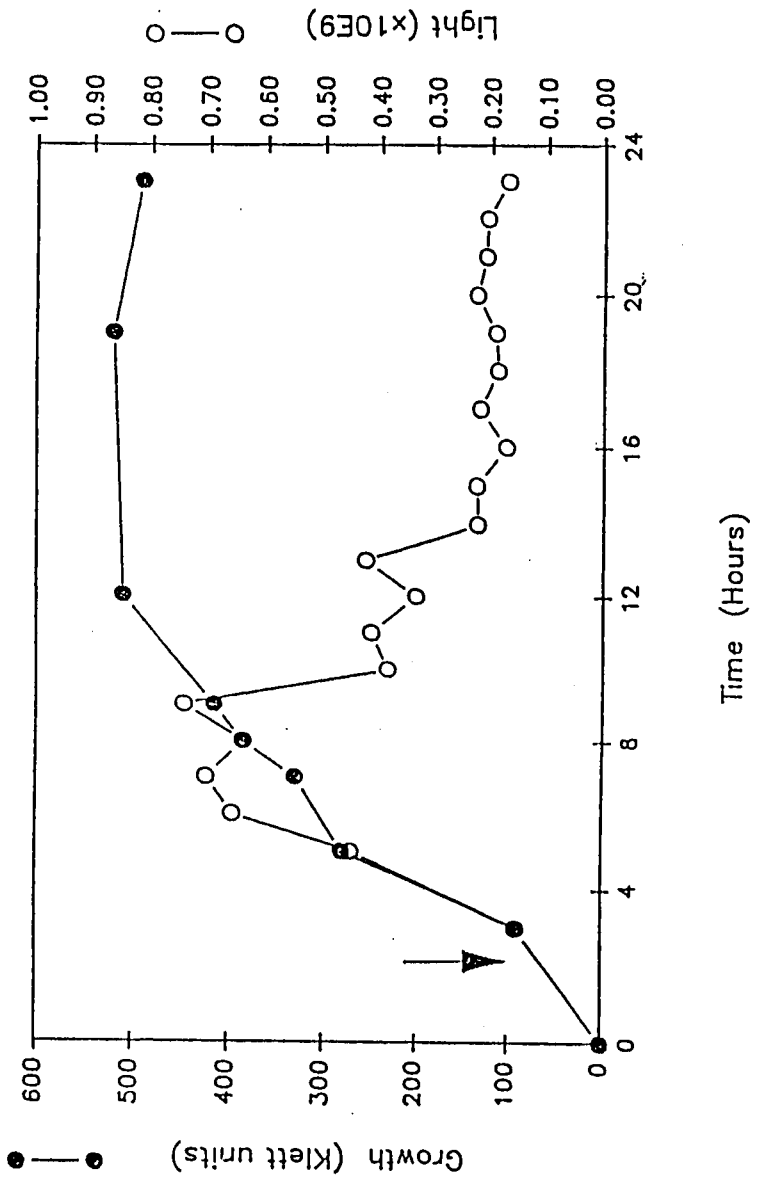


Figure 23. Growth curve of RB1368 in LB medium. Naphthalene crystals (1 % w/v) were added at time indicated by arrow. E - exponent.

Chemostat cultures can generate stable light production.

If nah pathway induction is optimal during stationary phase, a chemostat culture growing at a sufficiently slow rate should allow the maximum level of light production from RB1351. A chemostat culture of RB1351 using a basal salts-succinate (20 mM) medium (Figure 24), and an LB medium (Figure 25) was investigated. The dilution rate was very low, approximately 0.21 Hr^{-1}, with a doubling time of approximately 3.3 Hr instead of 0.7 Hr during exponential growth. Addition of either naphthalene or salicylate to the reaction vessel gave the same bioluminescent response, both in magnitude and duration. Substrate can be lost by cell metabolism, by washout from the vessel, or through air-stripping, and so this response was transient. However, when the reservoir was amended with naphthalene the response was continuous and stable. This response was very similar to that seen in the naphthalene-induced colonies, and the physiological state of cells in colonies and in this continuous culture may be similar as well. The difference in magnitude between the LB and Basal Salts medium is attributable to the difference in cell concentration; after a correction for this factor was made the response was identical.

The chemostat was also useful to determine the sensitivity of the nah-lux system for the detection of naphthalene. Dilutions of naphthalene in acetone were

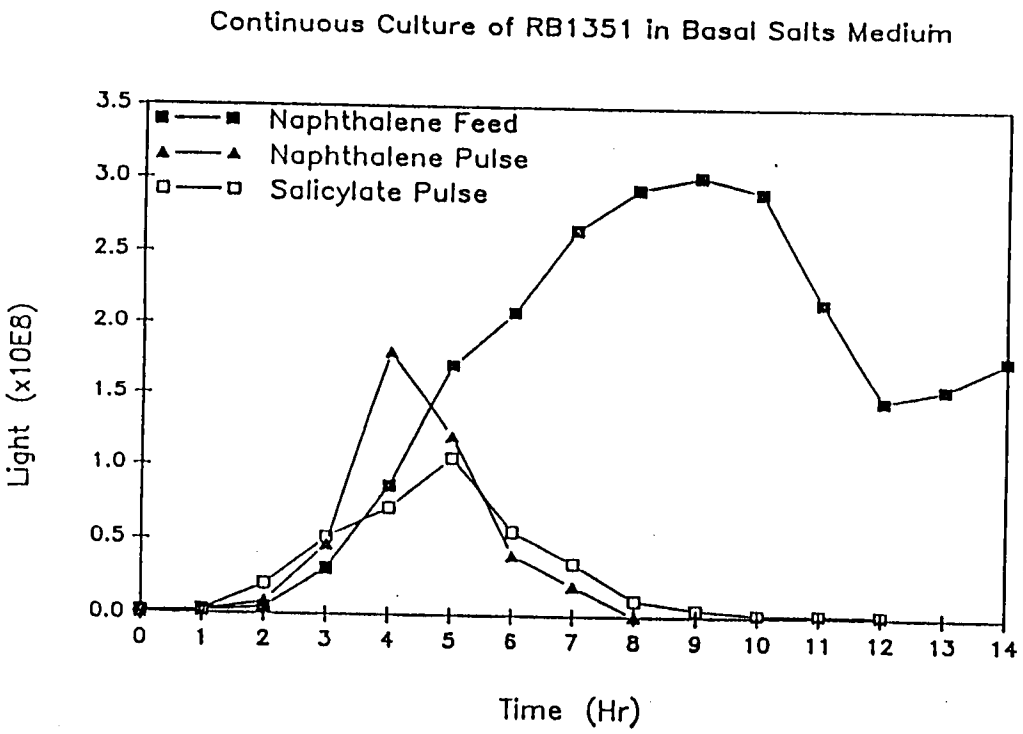


Figure 24. Continuous culture of RB1351 in Basal Salts Medium. Naphthalene or salicylate were added to the chemostat (pulse) or to the 10 l feed tank (feed). Light was monitored continuously. E - exponent.

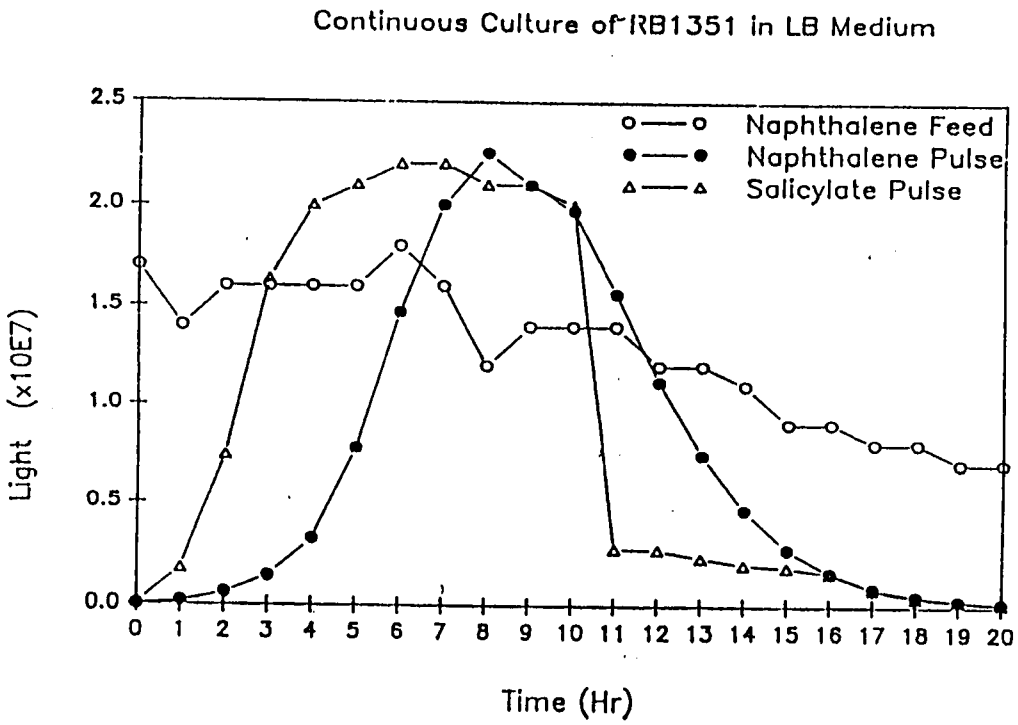


Figure 25. Continuous culture of RB1351 in LB medium. See Figure 24 legend for details.

injected into the chemostat and light production was followed (Figure 26). Bioluminescence in this apparatus will vary somewhat, creating background that can be mistaken for a true response. Therefore it was decided that an increase of 100% over the average background would be considered the minimum requirement for a positive increase in light. Using this criterion an addition of 100 parts per trillion of naphthalene was sufficient to induce a measureable bioluminescent response. The two points late in this assay that are far from the other points are aberrations that occur when the computer must sample and average many independent readings at a single timepoint.

Transcript level from RB1351.

The complexity of the bioluminescent response curve from a batch culture of RB1351 was investigated by isolating total cellular RNA and quantifying the specific transcripts at various timepoints. This was done to determine whether the addition of naphthalene was sufficient to induce transcription, or whether a delay in transcription was responsible for the delay in bioluminescence. This would provide valuable clues to the control of expression of the nah pathway.

At each selected timepoint the growth and light production were noted, and an aliquot was taken for RNA

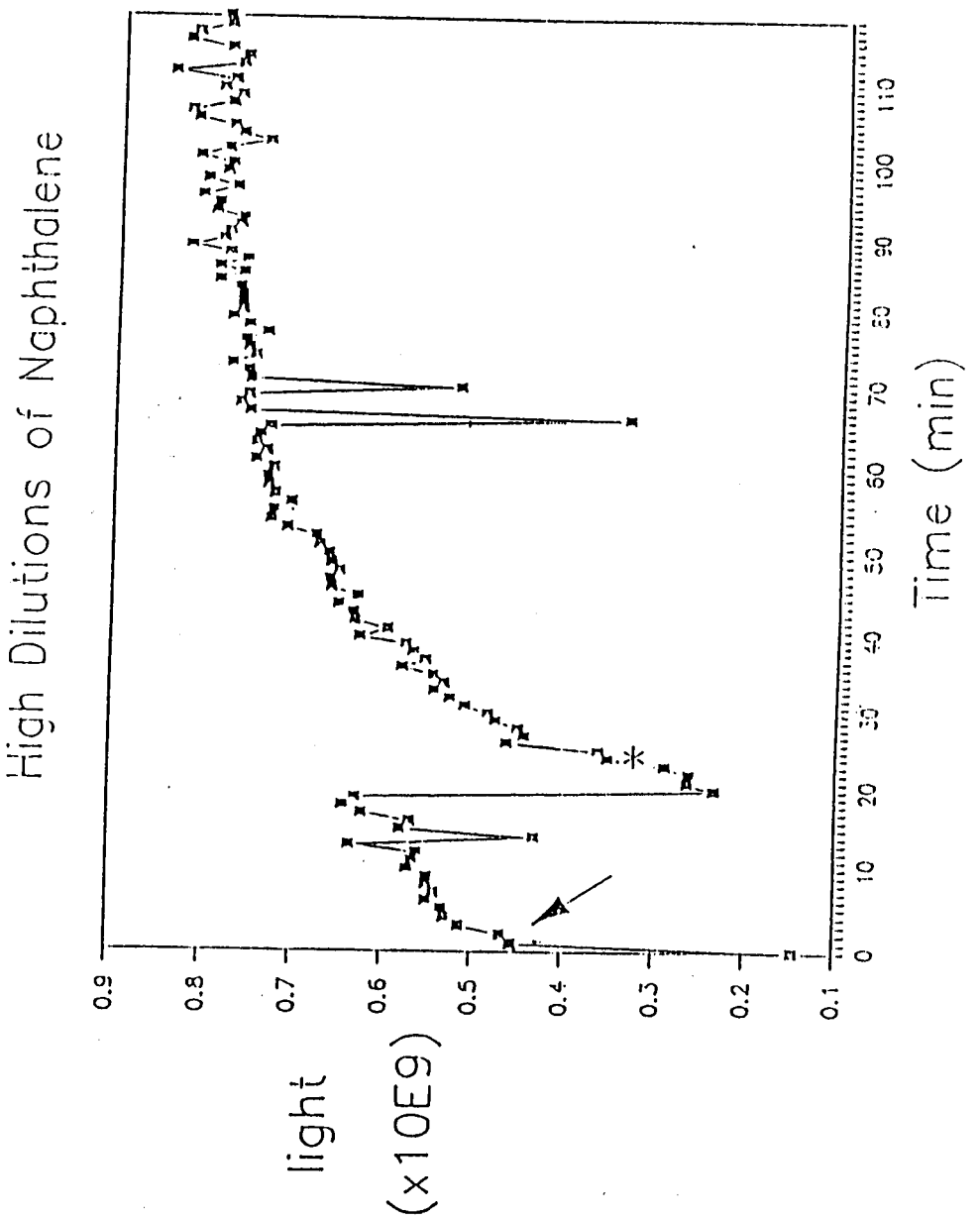


Figure 26. High dilutions of naphthalene. Naphthalene was dissolved in acetone and added to a chemostat culture of RB1351 at the indicated points. Arrow - added 1 part per trillion (ppt). Asterisk - 100 ppt.

extraction. The RNA was probed for the presence of specific transcripts with nah and lux probes. Typical results for the induced RB1351 culture are shown in Figure 27, while the uninduced culture is shown in Figure 28.

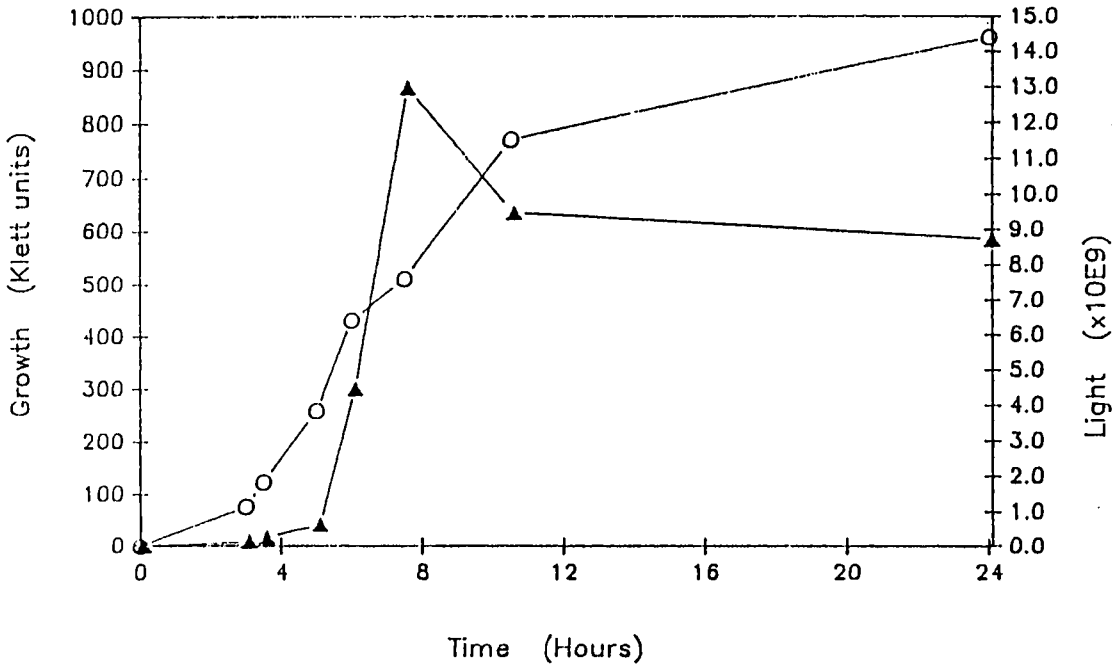
The first sample was taken immediately before the addition of naphthalene (3 Hr.), a time that corresponds to early log phase. After 30 minutes with the inducer present the second sample was taken (3.5 Hr). The data clearly show that there was no appreciable difference in light output during this time, as would be expected from previous results. There was also no increase in either nah or lux-specific mRNA, as shown in Figure 27 (bottom). In fact, the data at these points look very similar to the corresponding points in the control culture (to which no naphthalene was added).

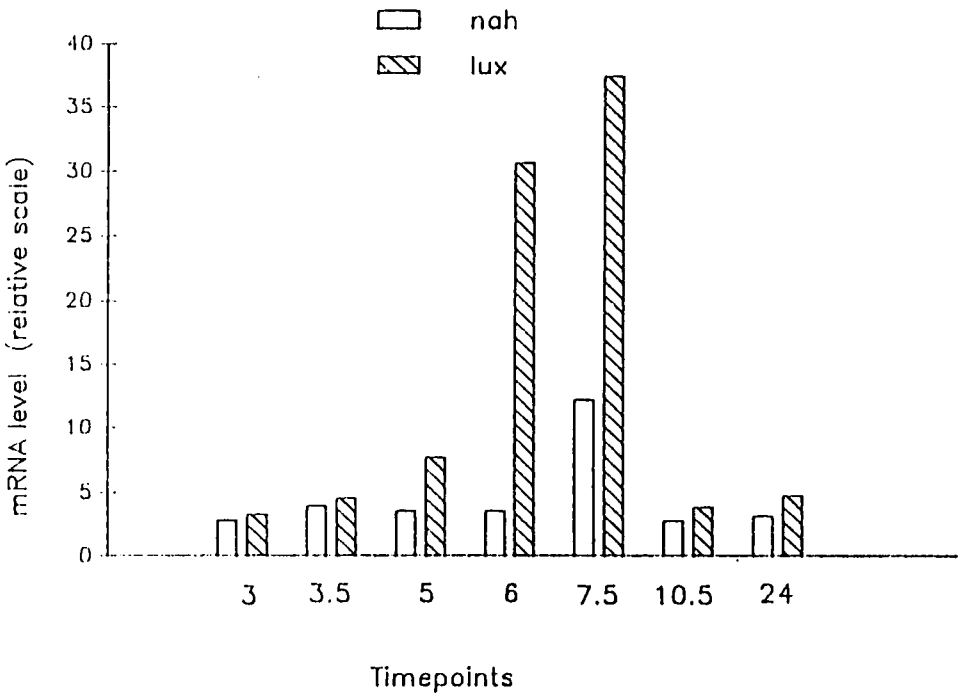
Throughout the control culture experiment the mRNA level remained low - approximately at the uninduced level. The only exception came at the same time as the slight increase in bioluminescence that is characteristic of batch culture growth. At this time (the 6 Hr. timepoint in the bottom panel of Figure 28) there was a slight rise in measureable lux mRNA, approximately a doubling of the 3 Hr. timepoint level. There was not a comparable rise in nah mRNA evident at this time.

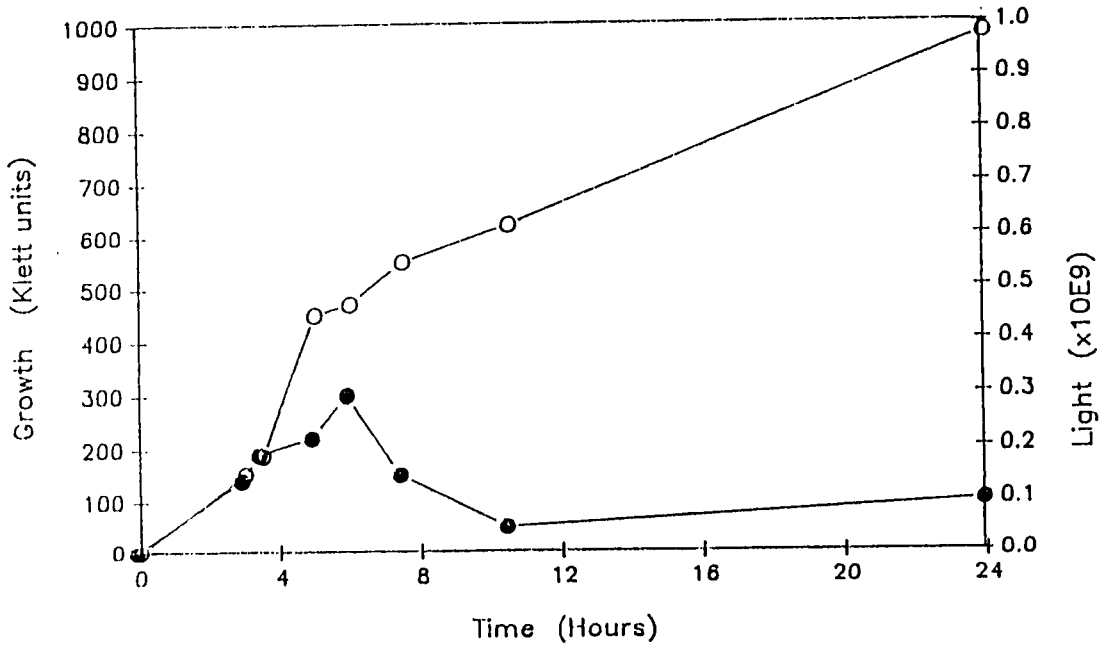
When the data from the induced culture is examined it is apparent that there were only slight increases during the mid-log phase, which coincided with the low light output at

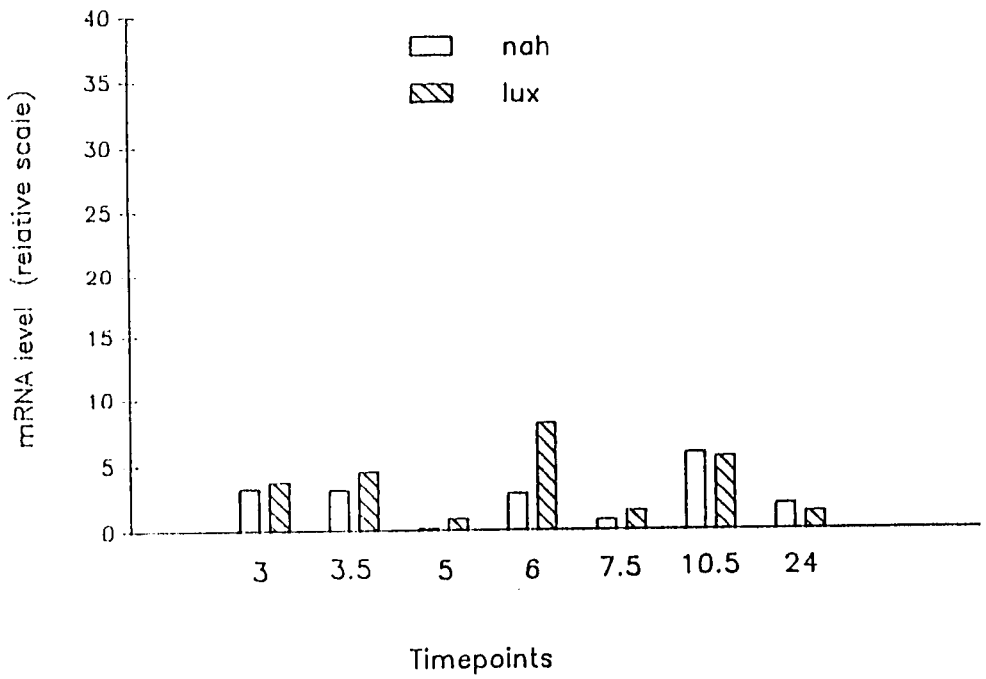
Figure 27. mRNA levels with naphthalene induction. Top. Growth curve timepoints (circles) and light measurement (triangles) are presented. Arrow indicates time of naphthalene addition. Only the timepoints at which RNA samples were obtained are shown. E - exponent. Bottom. Relative amounts of specific mRNA found at each timepoint, determined with nah and lux probes. Timepoints are noted (in hours) on the abscissa.

Figure 28. mRNA levels without naphthalene induction. See Figure 27 for details.


Growth (Klett units)
1000
900
800
700
600
500
400
300
200
100
0
Light (x10E9)
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
4
8
12
16
20
24
Time (Hours)


nah
lux
mRNA level (relative scale)
40
35
30
25
20
15
10
5
0
3
3.5
5
6
7.5
10.5
24
Timepoints


Growth (Klett units)
1000
900
800
700
600
500
400
300
200
100
0
0
4
8
12
16
20
24
Time (Hours)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Light (x10E9)


mRNA level (relative scale)
40
35
30
25
20
15
10
5
0
nah
lux
3
3.5
5
6
7.5
10.5
24
Timepoints

this time. When light output increased at the end of exponential growth (timepoints 6 and 7.5 Hr.) the specific mRNA levels increased significantly as well. This effect was especially apparent with the lux results. Peak lux mRNA production equaled a value that is ten times the uninduced (3 Hr.) level. At this time the increase in nah mRNA production was also evident, although the fold-increase was not as dramatic here. After the peak of light (and mRNA) production the mRNA level fell abruptly, despite the high level of light that was still being produced. This is an unusual observation, but it is apparent that a relatively high light output was produced despite mRNA levels that were similar to uninduced levels. It is possible that the nah mRNA produced constitutively by an uninduced culture is subject to a post-transcriptional regulatory mechanism which effectively prevents light production. This must remain speculative until further work is performed.

In summary, these data suggest that the peak of bioluminescence seen at the beginning of the stationary phase of an induced batch culture of RB1351 is correlated with an increase in transcription. A similar correlation is evident during the transient light burst seen in the stationary phase of an uninduced batch culture of RB1351. As expected, both the mRNA levels and the light output are not as high as with the induced culture.

VI. Discussion

The introduction of a gene as a new bioreporter of gene activity carries with it both the promise of unique usefulness and the responsibility for establishing the biological principles under which this gene is to function. This can all be gathered under the heading of "groundwork" and it is precisely to perform this groundwork that this study was initiated.

The results of this study are very favorable. In the specific example of the study, bioluminescence has been shown to be a useful method of describing the nah upper pathway promoter. These results should be encouraging to other workers who are considering the measurement of light as a reporter in their experimental systems. This laboratory is presently considering or actively creating other lux fusion strains designed to exploit this technology.

In accomplishing this research project certain departures from anticipated results were observed, and in reconciling these observations with gene activity two important results were obtained. First, the genetic and physiological evidence supporting bioluminescence as a bioreporter was strengthened by the additional weight of supporting data. Second, the model operon used here, the nah system, has been explored in greater detail, and important observations regarding the functioning of this system have

been made. These two results are presented, in order, in greater detail in the sections that follow. Comparisons with other systems are suggested where applicable, and certain speculations have been made that hopefully will stimulate future lines of inquiry.

The lux genes as a bioreporter

During the course of this research several light-producing strains of bacteria have been created, some of which are more valuable than others. The most useful strain is RB1351, the naphthalene bioreporter. Through many experiments this strain has demonstrated that bioluminescence is correlated with promoter expression. This correlation was first suggested by the induction of colonies of RB1351 by naphthalene vapor. It was evident in a few minutes that the light production increased substantially. This was a heartening result, since any other result would have been difficult to explain in terms of the currently understood theory of nah promoter function. As such, this experiment, though crude in execution, was an important prelude to other, more demonstrative experiments.

The pattern of light production that occurred when the RB1351 strain was grown in batch culture was completely unexpected. The significant delay between exposure to inducer and onset of bioluminescence was reproducible, and gave cause

for concern. If the delay were due to some event peculiar to the lux system (e.g. the absence of a suitable aldehyde substrate) then the assay would probably be of limited usefulness. Several trivial explanations were briefly investigated and dismissed. Subsequent mineralization experiments demonstrated that bioluminescence was correlated with naphthalene degradation, an important result that gave great support for the use of light production as an indicator of promoter activity. Determination of the transcription activity during batch culture growth further demonstrated that bioluminescence was correlated with transcription activity, at least during the initial peak of activity. The low levels of nah and lux mRNA that was seen while light production was stable was surprising. There may be other control mechanisms for nah expression that are as yet undescribed. The possible explanations for the long delay observed in induced batch cultures are explored below.

There was one instance in which bioluminescence was observed without naphthalene degradation. This event was manifest in the peak of light production that was seen during batch growth of RB1351 without added naphthalene. This light production is also correlated with a brief increase in transcriptional activity from the nah promoter, and it is possible that unique conditions arise during this time which can induce transcription. It can be hypothesized that a pool of anthranilate (a gratuitous inducer of nah) might be formed

during the transition from exponential to stationary phase growth. However, in the absence of any evidence this must remain a speculation.

From a utilitarian aspect, it is clear from this work that bioluminescence is very easy to assay, and that it is useful as a non-destructive, real-time assay of experimental conditions. These traits make it an attractive alternative for future work in complex systems, such as bioreactors, besides the simple assays that have already been reported in the literature. The biggest drawback to the continued use of bioluminescence is that the ambient light must be completely excluded. Even small light leaks can lead to very significant aberrations. During several procedures in this laboratory light output was seen to be very high - always an exciting result - and repeated experiments showed that the photomultiplier probe was not properly sequestered. However, with a little practice these mistakes become rare.

Expression of the nah promoter

Evidence has accumulated during this investigation that the expression of nah is a growth-rate regulated response, and a strong case can now be made for this effect. This was first suggested by the batch culture results and the mineralization experiments, in which bioluminescence and naphthalene degradation were present only during a slow rate

of culture growth. When two cultures of RB1351 were compared for light production it was evident that the stationary phase culture produced more light than the rapidly growing culture. Furthermore, the two cultures had produced the same light output when one was in stationary phase and the other was in lag phase, during which growth is also very slow. This experiment gave strong evidence for a growth rate-regulated control rather than a catabolite repression control.

If it can be established that expression of the upper pathway of NAH7 is optimal during a slow rate of growth, what would be the purpose of this limitation? In the absence of any great body of data regarding this specific case the reason must remain the object of speculation. But not all speculation is unwelcome, and sometimes provides a useful framework for future projects. Bearing this in mind, a line of reasoning can be advanced.

There are many other examples of genes that are expressed only during a specific growth phase, or that is preferentially expressed at certain growth rates. For instance, it was recently shown that production of K99 fimbrial subunits (the fanA-H genes) in E. coli is substantially increased during exponential growth (Van der Woude et al., 1990). A dual promoter system accounts for this phenomenon. One promoter appears to be active constitutively at low levels, while the other promoter is growth-rate-regulated. Although some theories have been advanced to

account for this control system, the purpose for the system and the other components of control remain a subject of further inquiry.

Another gene that is under a dual promoter control is the osmB gene. This gene encodes an outer membrane lipoprotein that appears to be important in maintaining the correct osmolarity of the cell. By separating the two promoters and making appropriate reporter gene fusions, Jung et al. (1990) were able to demonstrate that transcription increases from the main P2 promoter when the cell encounters hyperosmolar conditions (i.e. excess salt in the medium) or when a batch culture of the E. coli strain enters stationary phase. The P1 promoter apparently is functional only when both of these conditions are met. A batch culture growing in medium containing 0.3 M NaCl undergoes a biphasic response in osmB expression. The first peak is due to the presence of the added salt; the second peak appears as the culture reaches stationary phase. Although these workers were able to associate a short (7 bp) region in the promoter with the response to hyperosmolarity, they were unable to discover the signal that triggers stationary phase expression. Several attempts were made to study this response under nutrient-starved conditions, with a low pH medium, and with a reduced oxygen tension. In none of these cases where growth was slowed or stopped did they observe increased expression of osmB. They concluded "... the stationary phase stimulus may

be more complex than a simple chemical signal".

The examples cited above describe a set of gene products that have been loosely categorized as secondary metabolites. These are products that are produced by microorganisms, but are non-essential for growth. This is a very contrived definition, of course, since the definition of non-essentiality is in relation to man-made culture conditions, and ignores the natural ecology of these organisms. Nevertheless it is a useful definition in that it lumps together a set of unrelated but functionally similar genes. They are interesting for this discussion because they also are distinguished by their activity during slow growth of the organism. Secondary metabolism is exemplified by the production of antibiotic compounds, which has been studied in great detail due to their commercial and clinical importance. Other compounds which are created during secondary metabolism include bacterial spores, pigments (that may actually be used as antibiotic substances) and some lipids that appear to be made preferentially during stationary phase. Many of these compounds are produced by Pseudomonas species (Leisinger and Margraff, 1979). Unfortunately, sequence analysis of the promoter regions of growth-rate controlled genes do not provide any indication of a common sequence that might be implicated in control (Martin and Liras, 1989). There may be several control circuits involved in secondary metabolism, or the control region might be far from the promoter.

How does secondary metabolism relate to naphthalene degradation? It is possible that the naphthalene pathway evolved to degrade a secondary metabolite, such as an antibiotic compound. The bacterial strain might produce this compound itself and use the catabolic pathway as a means of avoiding toxic effects in the event any of the antibiotic was released inside the cell. Alternatively, the antibiotic might be produced by another microorganism, and the destruction of this compound would give the catabolic organism a competitive advantage. From the aspect of microbial ecology this would not seem surprising. If some species, like Streptomyces, can produce antibiotics to increase their competitiveness in a soil microcosm, then other species in the soil should be able to counteract these antibiotics by degrading them.

The substance that NAH7 was designed to degrade is open to question. It is unlikely that the pathway evolved to degrade naphthalene, since naphthalene is not found in uncontaminated environments. The origin of this strain is somewhat murky, having gone through some name changes; apparently the isolation of the strain was never formally reported. However, it is known that the organism was obtained from a soil sample through enrichment with naphthalene. Thus it is possible that this strain is the product of a mutation that was able to utilize naphthalene. The original pathway may have been designed to degrade a naphthalene-like substance with which the organism came into contact and found

it beneficial to destroy. There may have been several compounds that were degraded, since NAH7 has a bifurcated lower pathway. By using the nah genes as a probe of microorganisms in pristine environments, it may be possible to isolate the original pathway from which NAH7 evolved. It may then be possible to determine the compound it was designed to degrade and the changes that allow it to degrade other compounds.

In summary, the lux reporter gene system that has been described has allowed important characteristics of a biodegradation pathway to become evident. In the future it is anticipated that other lux constructions will yield equally important and interesting results, and allow the worker to examine gene expression from a culture in a continuous, reliable assay system.

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Vita

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