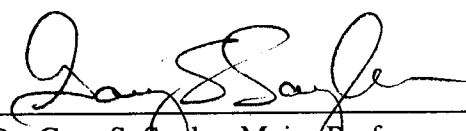
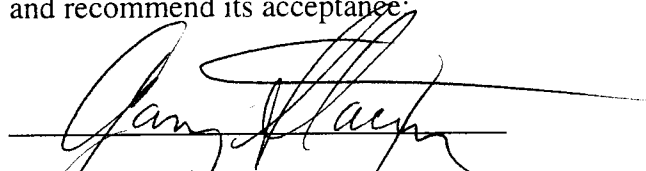
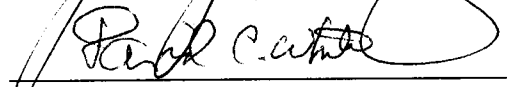
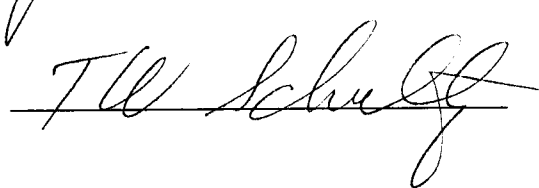


To the Graduate Council:

I am submitting herewith a dissertation written by Wade Hampton Johnston entitled "Fate of *Pseudomonas fluorescens* 5RL and its Reporter Plasmid for Naphthalene Biodegradation in Soil Environments." 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.


Dr. Gary S. Saylor, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Associate Vice Chancellor
and Dean of the Graduate School

FATE OF *PSEUDOMONAS FLUORESCENS* 5RL AND ITS REPORTER PLASMID
FOR NAPHTHALENE BIODEGRADATION IN SOIL ENVIRONMENTS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Wade Hampton Johnston
May 1996

DEDICATION

This dissertation is dedicated to

my wife

Pamela Johnston

my son

Andrew Johnston

and my parents

Mr. Arthur Johnston and Mrs. Pearl Johnston

whose love and support have made my graduate education possible.

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ABSTRACT

The capability of bioluminescence reporter strains for naphthalene biodegradation, to maintain reporter plasmids and compete against wild type degrading populations was examined. Plasmid maintenance was examined in non-selective continuous culture and competitiveness was examined in naphthalene limited continuous culture using two or three member mixed cultures. Microcosms consisting of a polyaromatic hydrocarbon contaminated soils were used to examine strain survivability in a relevant soil. A unique chromosomal marker was developed to monitor possible gene transfer events in soil microcosms. Individual populations were monitored with specific DNA probes for plasmids and organisms and by dilution spread-plating on selective media.

The wild type naphthalene degradative plasmid (pKA1) and the *lux* transposon (Tn4431) mutagenized version of this plasmid (pUTK21) were maintained in host strains, *P. fluorescens* 5R and *P. fluorescens* 5RL, at 100% of the population under the dilution rates tested. When the reporter plasmid was transferred to another host, *P. fluorescens* 18H, pUTK21 was segregationally unstable. The reporter plasmid from *P. putida* RB1351 (promoter probe vector with NAH7 upper pathway promoter) was segregationally unstable. In continuous culture competition experiments, strain 5RL was outcompeted at both dilution rates, strain HK44 was maintained at the lower dilution rate, and strain RB1351 was maintained at all dilution rates tested when competing against the wild type naphthalene degrader, strain 5R.

In soil microcosm experiments, a chromosomally marked version of strain 5RL (strain 5RLA) was used to determine persistence in soil as well as monitor possible gene transfer events. Strain 5RLA could survive, as determined by dilution spread plating, for up to 25 days in sterile and non-sterile soil at a useful level for a biosensor. When naphthalene was added to the soil, strain 5RLA was outcompeted after one week and fell below the level of a useful biosensor. Transfer of pUTK21 could only be detected when a plasmid-free recipient was added. Transconjugants were confirmed by DNA probing and occurred at low frequencies (10^{-5} - 10^{-6} per donor). The transconjugant level fell below detection after 17 days. These studies demonstrate that one reporter strain, 5RLA, could compete in soil for 25 days at a level useful as a biosensor. The strain maintains the reporter plasmid which is transferred to indigenous soil bacteria at a level too low to detect under the conditions tested except when an ideal recipient was added.

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PART 1

GENERAL INTRODUCTION

General Background and Research Objectives

The potential of using biotechnology for aiding in the cleanup of environmental pollutants has been a cornerstone of the hazardous waste remediation industry. In many instances, contaminated sites already contain microorganisms capable of degrading the target pollutant compounds. Little is known concerning the activity of these microorganisms in relation to the biodegradation of a specific or class of pollutants. A cellular reporter system was recently constructed that is capable of reporting on the biodegradative capacity for naphthalene and salicylate (Burlage et al., 1990; King et al., 1990). Naphthalene biodegradation has been well characterized, both biochemically and genetically, and is used as a model system for the biodegradation of polycyclic aromatic hydrocarbons (PAH's). The system utilizes bioluminescent reporter technology, consisting of a *lux* transcriptional fusion. This type of reporter technology offers advantages in that it is a non-invasive and non-destructive process offering high sensitivity and real time analysis. The long term goal of such technology is the *in situ* monitoring of contaminant biodegradation in waste streams and soil systems.

A major obstacle in the realization of the potential of such recombinant organisms is the lack of knowledge concerning the fate and effects of genetically modified organisms (GMO's) in the open environment, including the persistence of introduced

organisms and the transfer of recombinant DNA to indigenous populations. From a utility standpoint, the issues of strain survival/persistence and the molecular integrity of the recombinant DNA are critical. A substantial amount of research has been undertaken to examine the types and the extent of gene transfer (Miller and Levy, 1989) and to develop methods to monitor specific genotypes (Jain et al., 1988). Still, the question has been raised as to whether our current state of knowledge is sufficient to assess the effects of widespread dissemination of recombinant genotypes in the environment (Dixon, 1991). In this regard, the objectives of this research are:

- Determine the segregational stability of bioluminescence reporter plasmids under non-selective conditions.
- Determine the persistence of bioluminescence reporter strains in mixed continuous culture in direct competition for naphthalene.
- Determine the ability of reporter strains to maintain bioluminescence when competing against wild-type degrading strains.
- Construct a marker system for monitoring the persistence in soil of an introduced host strain (*P. fluorescens* 5RL).
- Determine the persistence of *P. fluorescens* 5RL and the *nah-lux* reporter plasmid for naphthalene degradation and monitor the molecular stability of the

reporter plasmid in naphthalene contaminated and uncontaminated soil microcosms.

- Determine the extent of transfer of the *nah-lux* reporter plasmid pUTK21 in naphthalene contaminated and uncontaminated soil microcosms.

Literature Review

Naphthalene Biodegradation

Introduction.

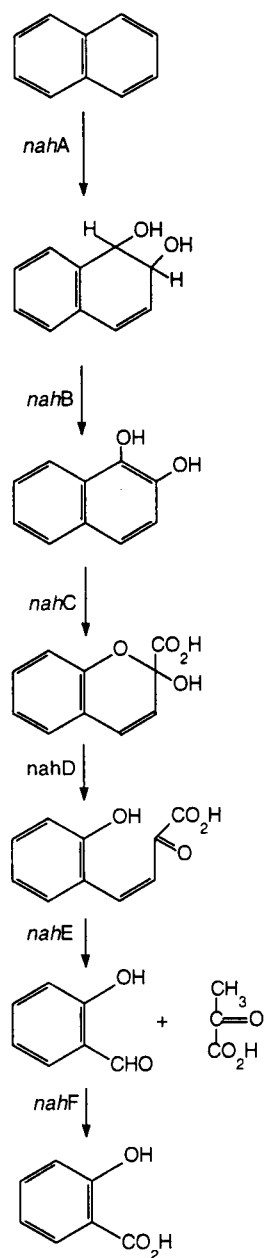
Naphthalene is a dicyclic aromatic compound that is a common component found in soils contaminated by hazardous wastes. The biodegradation of naphthalene is the best established of the PAHs and serves as a model compound for the PAH class (Cerniglia, 1984). The compounds of the PAH class are persistent in the environment and are formed as the result of incomplete combustion of organic matter (Cerniglia, 1984). PAHs are produced by natural and anthropogenic processes. Natural sources of PAH's include forest and prairie grass fires and volcanic eruptions. Anthropogenic sources of PAHs include petroleum leakage, vehicle emissions, and coal liquefaction and gasification processes. Manufactured gas plants (MPG) generated gas from coal and oil from the mid-19th century through the mid-20th century. This gas was used primarily for public lighting and heating as well as fuel for industrial furnaces. The major waste products from the gasification process were tars and sludges, tar liquors, and off-grade coal and coke. The soil at MGP sites were contaminated by the PAHs which are the major components of the tars and sludges. Many PAH's are carcinogenic and are relevant environmental pollutants (Cerniglia, 1984). It is for these reasons that naphthalene is on the US-EPA priority pollutant list (National Research Council, 1991)

Biochemistry.

In bacteria, naphthalene degradative genes have been found exclusively on plasmids (Boronin et al., 1985; Cane & Williams, 1982; Connors & Barnsley, 1982; Yen & Gunsalus, 1982). There was also one unsubstantiated report of a possible chromosomal encoded naphthalene pathway (Zuniga et al., 1981). The bacterial degradative pathway for naphthalene has been best characterized molecularly for the NAH7 plasmid in *Pseudomonas putida* G7 (Dunn and Gunsalus, 1973). The NAH7-encoded pathway for the metabolism of naphthalene through salicylate involves thirteen enzymatic steps (Davis and Evans, 1964). These enzymatic steps and gene indications are illustrated in Figure 1-1. The pathway encodes the genes to oxidize naphthalene to salicylate by enzymes encoded in the upper *nah* operon pathway. Salicylate is oxidized by enzymes encoded in a second operon in the lower *sal* pathway.

In the upper *nah* pathway, naphthalene is first oxygenated by naphthalene dioxygenase (*nahA*). Both atoms of molecular oxygen are incorporated into naphthalene to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (Catteral et al., 1971; Jerina et al., 1971). Naphthalene dioxygenase in *Pseudomonas* sp. NCIB9816 consists of a three-component protein complex (Ensley et al., 1982). These components consist of a flavoprotein reductase; a two-iron, two-sulfur ferredoxin; and an iron-sulfur protein. Naphthalene dioxygenase has a molecular weight of 158,000 and is composed of two subunits of 55,000 and 22,000 M_w . Due to the subunit molecular weights, a $\alpha_2\beta_2$ quaternary structure was proposed (Ensley and Gibson, 1983). In the presence of oxygen, NADH, and the other two components of the dioxygenase, the iron-sulfur protein forms

Naphthalene Oxidation



Salicylate Oxidation

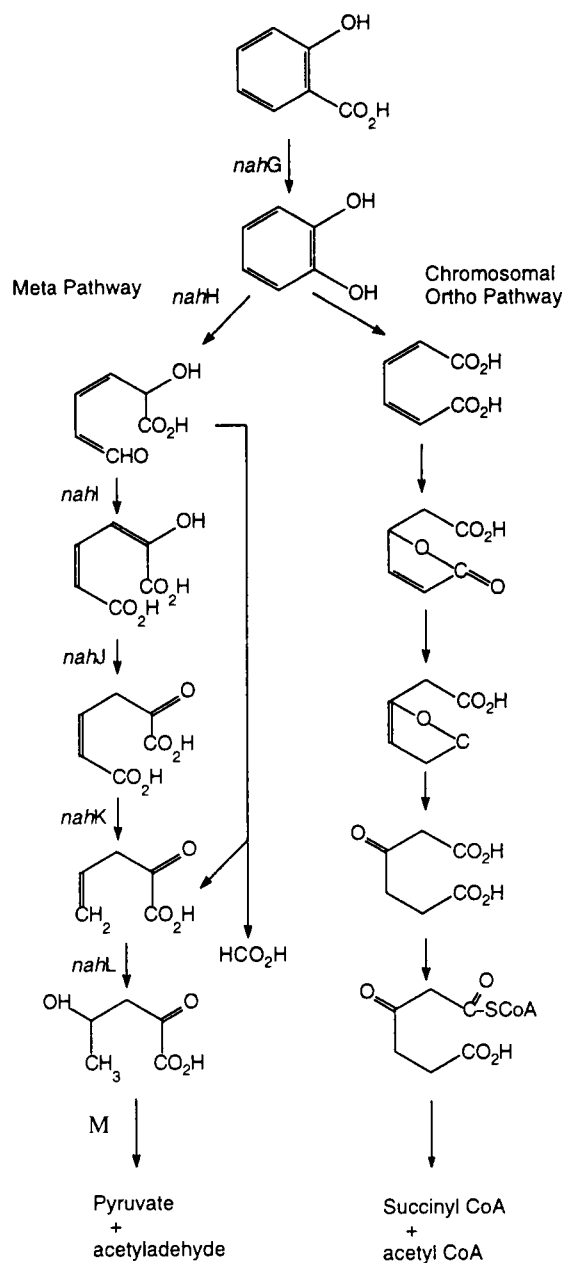


Figure 1-1. Enzymatic steps in the catabolism of naphthalene by genes encoded on NAH7. The individual *nah* genes are discussed in the text. The M gene is an aldolase not encoded by *nah* or *sal*. Catechol may be metabolized by a chromosomally encoded *ortho* pathway that is indicated.

naphthalene-*cis*-dihydrodiol as shown in Figure 1-2. The ratio of oxygen and NADH to product formation is a 1:1:1 stoichiometry (Ensley et al., 1982). Additionally, the NADH-ferredoxin reductase has been purified and characterized (Haigler and Gibson, 1990).

The second step in naphthalene oxidation involves the conversion of the naphthalene-*cis*-dihydrodiol to 1,2-dihydroxynaphthalene. The enzyme responsible for this conversion is naphthalene-*cis*-dihydrodiol dehydrogenase (*nahB*). This enzyme requires NAD as an electron acceptor (Patel & Gibson, 1974).

The remaining steps of naphthalene oxidation are based primarily on the work of Barnsley (1976) and more recent work by Eaton and Chapman (1992) which differs slightly from the original work of Davies and Evans (1964). In the third step, 1,2-dihydroxynaphthalene is cleaved by 1,2-dihydroxynaphthalene dioxygenase (*nahC*). An unstable ring cleavage product is formed and is spontaneously cyclized to give 2-hydroxychromene-2-carboxylate. This ring cleavage compound is converted in the fourth step to *cis-o*-hydroxybenzylidenepyruvate by 2-hydroxychromen-2-carboxylate isomerase (*nahD*). At the fifth step, *cis-o*-hydroxybenzylidenepyruvate is cleaved by *cis-o*-hydroxybenzylidenepyruvate aldolase (*nahE*) to give salicylaldehyde and pyruvate. Salicylaldehyde dehydrogenase (*nahF*) forms salicylate from salicylaldehyde in step six. This step requires NAD⁺.

Salicylate is degraded via oxidation by the catechol meta-cleavage pathway to pyruvate and acetaldehyde by a second operon in the lower pathway (Yen and Gunsalus, 1982). Salicylate is hydroxylated by salicylate hydroxylase (*nahG*) to form

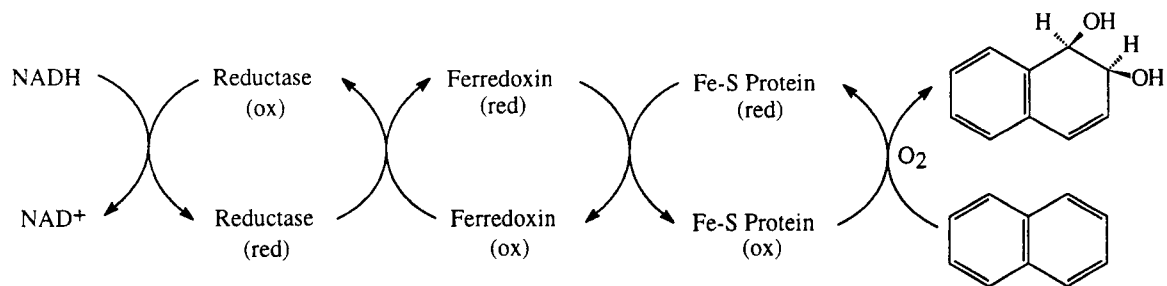


Figure 1-2. Naphthalene dioxygenase multicomponent enzyme system for *Pseudomonas* sp. NCIB9816 (Haigler et al., 1985).

catechol.

Catechol is oxidized to 2-oxo-hydroxypentanoate through a *meta*-cleavage pathway (Figure 1-1). The final step, which produces pyruvate and acetaldehyde, is catalyzed by an aldolase. The aldolase is not encoded by *nah* or *sal* (Yen & Gunsalus, 1982).

Catechol may also be oxidized by a chromosomally encoded *ortho* pathway (Schell, 1990). Salicylate may be alternatively oxidized via gentisate. The formation of gentisate during growth on naphthalene has been demonstrated for *Pseudomonas fluorescens* (Starovoitov et al., 1975) and *P. alcaligenes* (Monticello et al., 1985). These two additional routes of oxidation from either salicylate or catechol represent additional metabolic routes.

Regulation.

The genetic organization of the *nah/sal* catabolic system was first described by Yen & Gunsalus (1982) using Tn5 insertions and is shown in Figure 1-3. Polar mutations formed by Tn5 mutagenesis showed the transcriptional directions of the two catabolic

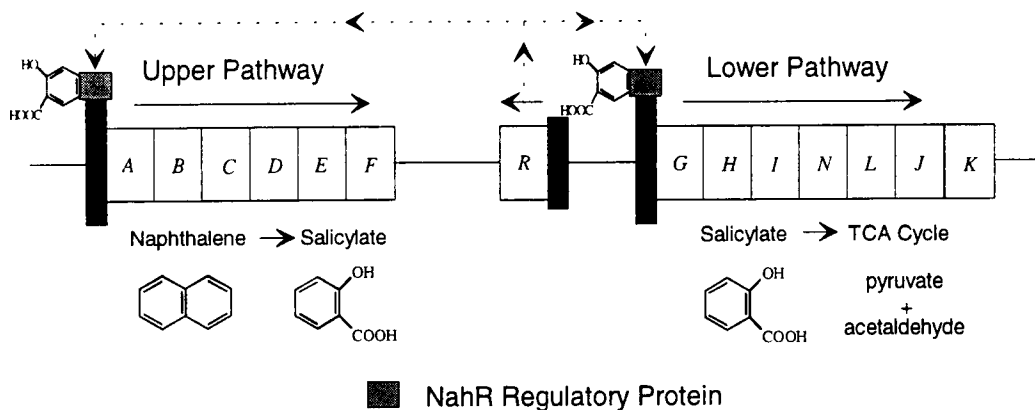


Figure 1-3. A schematic representation of the genetic organization and regulation of the NAH7 naphthalene catabolic pathway where (→) represents direction of transcription and (- - -) transcriptional regulation.

operons separated by a regulatory region. Barnsley (1975 & 1976) first recognized that salicylate might be the inducer of the NAH pathway. The work of Barnsley also showed that 2-aminobenzoate is a gratuitous inducer of the upper and lower operons. It was later shown that transcription of *nah* and *sal* is increased 20- to 50-fold by salicylate (Schell, 1985). This increase in transcription is consistent with the enzyme levels determined by Barnsley (1976). Schell (1985) also showed that salicylate is probably the natural inducer of both operons because only salicylate induced expression of *sal* in cells unable to metabolize naphthalene or salicylate. Also 2-methyl-naphthalene and 4-methylsalicylate have been shown to induce the Nah system (Leblond, 1995).

The two operons are coordinately controlled by the *nahR* regulatory protein in a positive fashion (Schell, 1985; Huang & Schell, 1991). The *nahR* gene is divergently

transcribed relative to *nal* and *sal*. Transposon mutagenesis of the *nahR* region using Tn5 showed that such mutations blocked the induction of both the *nah* and *sal* operons. The defect was complemented by supplying the *nahR* gene in *trans*. Subsequently, the *nahR* protein was shown to confer salicylate inducible transcription activation of both *nah* and *sal* (Schell, 1985). The *nahR* gene product has been shown to bind the promoters of both the *nah* and *sal* operons by DNA footprinting (Schell & Poser, 1989; You et al., 1988). The *nahR* protein is constitutively produced (Schell, 1986). The complete naphthalene degradative pathway encompasses 30 kb of the 83 kb plasmid (Yen & Serdar, 1988).

There is a need for methods to monitor the biodegradation of PAH's in environmental matrices. Methods that are non-intrusive and provide real time analysis are advantageous. A *nah-lux* reporter plasmid, capable of reporting on the degradation of naphthalene and salicylate, was made by transposon mutagenesis of the plasmid pKA1 in strain *Pseudomonas fluorescens* 5R with the promoterless *lux* transposon Tn4431 (Figure 1-4). *P. fluorescens* 5R is an environmental isolate from a Manufactured Gas Plant (MGP) soil. The plasmid pKA1 (101Kb) contains both the upper and lower pathway as determined by southern blotting and probing with various fragments of the upper and lower pathways of NAH7 and *nahR* (unpublished results). *P. fluorescens* 5R has been shown to mineralize naphthalene, phenanthrene, and anthracene (Sanseverino et al., 1993).

The plasmid pUCD623, carrying Tn4431 which contains the promoterless *luxCDABE* cassette as well as tetracycline resistance, (Shaw et al., 1987) was introduced into strain 5R by conjugation. The transposon has been shown to have inserted into the

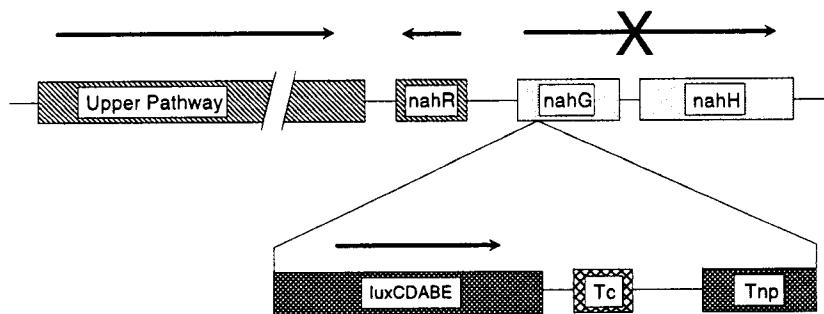


Figure 1-4. Tn4431 mutagenesis of the naphthalene catabolic plasmid pKA1 from *P. fluorescens* 5R. The resulting *nah-lux* plasmid is pUTK21.

nahG gene of the lower pathway as shown in Figure 4 (King et al., 1990). The *nahG* gene codes for salicylate hydroxylase and the inactivation of this gene prevents degradation of salicylate. The resulting plasmid is referred to as pUTK21 and the strain *P. fluorescens* 5RL. The plasmid pUTK21 has been shown to be self-transmissible to other *Pseudomonas* sp. (Sanseverino et al., 1993) which is consistent with the findings on other catabolic plasmids (Yen & Serdar, 1988).

Bioluminescence

Introduction

Bacterial bioluminescence is a popular reporter of gene expression as well as a phenotype and genotype for tracking microorganisms (Prosser, 1994). Being 2-3 times more sensitive than assays for other enzymes, bioluminescence offers the advantage of high sensitivity (Meighen, 1991). Light emission is measurable in seconds to minutes in

a non-invasive manner. It also offers a linear dependence of light intensity on the amount of luciferase over a wide range of enzyme concentration.

As a reporter of gene expression, bioluminescence (or light emission) can be used to test the strength and regulation of promoters (Shaw et al., 1987). Bioluminescence was used in this manner to examine environmentally responsive promoters during sporulation and germination of *Bacillus* sp. (Carmi, 1987), *Streptomyces* (Schauer, 1989), and Cyanobacteria (Wolk et al., 1991). *Lux* insertions were used to identify promoters involved in lateral flagella (*laf*) in *Vibrio parahaemolyticus* (Engbrecht et al., 1985). Bioluminescence was also used to screen for promoter activities in the *vir* region of pTi58 (Shaw et al., 1986; Rogowsky et al., 1987). *Lux* constructions were recently used in *nah-lux* reporter systems for monitoring naphthalene and salicylate biodegradation (Burlage et al., 1990; King et al., 1990) and bioavailability (Heitzer et al., 1992; 1994).

Ecology and Taxonomy of Bioluminescent Organisms

Bioluminescent organisms are ubiquitous in the environment and include bacteria, dinoflagellates, fungi, fish, insects, and squid (Hastings et al., 1985). These organisms are found in marine, freshwater, and soil environments. Bioluminescent bacteria are the most abundant of the light-emitting organisms (Meighen, 1991). The most common species are free-living in marine habitats. The marine species are commonly saprophytes that grow on dead fish, gut symbionts in marine fish, and light organ symbionts in teleost fishes and squid. These bioluminescent marine species are Gram-negative motile rods and are facultative anaerobes.

Most bioluminescent bacteria have been classified into three genera: *Vibrio* (formerly *Beneckeia*), *Photobacterium*, and *Xenorhabdus* (formerly *Heterorhabdus*). These genera are composed of mostly marine species. The bioluminescent symbionts in shallow waters are *P. leiognathi* or *V. fischeri*. *Photobacterium* species have been isolated as light organ symbionts. The deep-water fish contain mostly *P. phosphoreum*. *Xenorhabdus* is the only genera containing species that infect terrestrial organisms. *X. luminescens* has been found acting in symbiosis with nematodes and caterpillars.

Biochemistry of the *lux* System.

The most studied bioluminescent bacteria are *Vibrio harveyi*, *V. fischeri*, *Photobacterium phosphoreum*, *P. leiognathi*, and *Xenorhabdus luminescens*. These studies show that there are significant differences between the *lux* systems of different genera in terms of the number of genes involved, their order, and the regulation of the system. The enzymes that catalyze the bioluminescence reaction are referred to as luciferases, while the substrates are referred to as luciferins. The light-emitting reaction also requires O₂, cellular reducing power in the form of FMNH₂, and ATP. The overall reaction sequence involves the oxidation of reduced riboflavin phosphate (FMNH₂) and a long-chain fatty aldehyde with emission of blue-green light as shown in Figure 1-5.

Bacterial luciferase is a monooxygenase consisting of an α (40 kDa) and a β (37 kDa) subunit. The two subunits are encoded by the *luxA* and *luxB* genes, respectively. Luciferase is highly specific for FMNH₂. The natural aldehyde substrate is tetradecanal

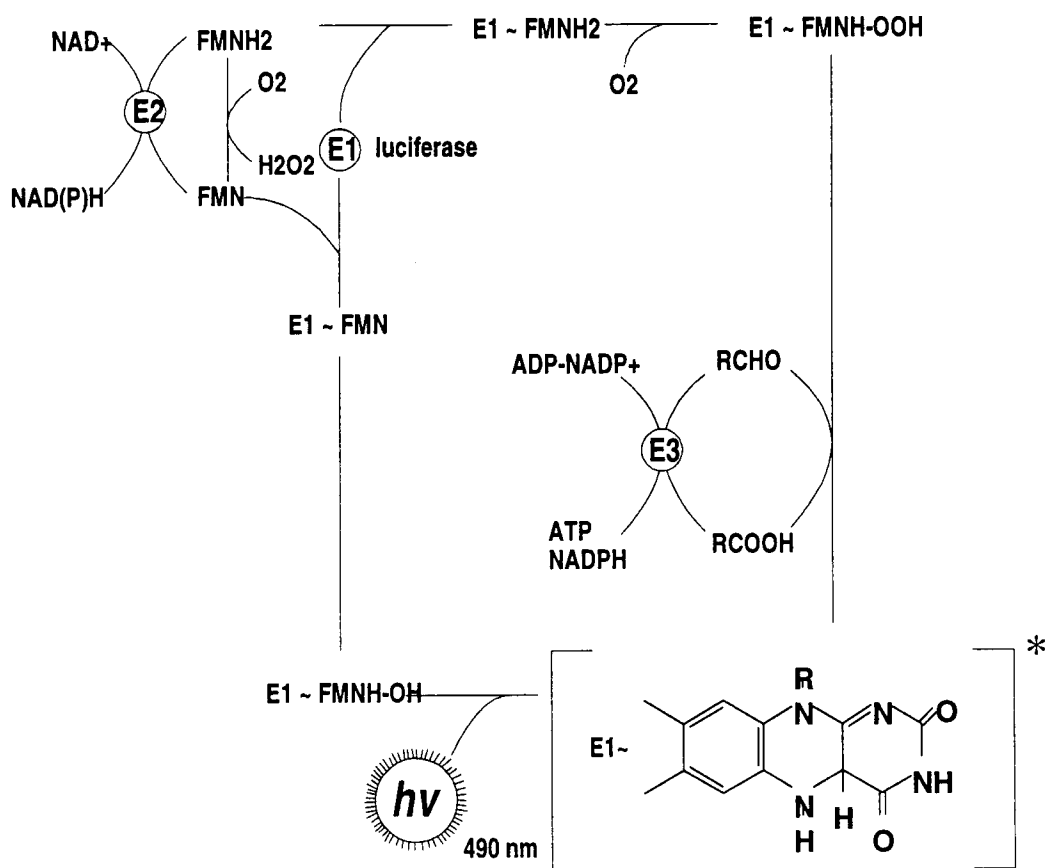


Figure 1-5. The steps of the bacterial bioluminescence reaction. Formation of FMNH₂ is catalyzed by FMN reductase (E2). Luciferase (E1) binds with FMNH₂ and reacts with oxygen to form the 4a-peroxy-flavin, that reacts with the long-chain aldehyde to form the excited species (*). The fatty acid is formed and released and reconverted to aldehyde by reductase (E3). Adapted from Hastings et al. (1985).

as determined by lipid extracts (Ulitzur & Hastings, 1979). The mechanism of luciferase has been well studied. When bound to luciferase, the reduced flavin, FMNH₂ reacts with oxygen to form a 4a-peroxy-flavin. This enzyme complex interacts with the long-chain aldehyde to form a highly stable excited intermediate. The intermediate decays slowly, resulting in emission of light concomitant with oxidation of the substrate.

The synthesis of aldehyde is catalyzed by a multienzyme fatty acid reductase complex. This complex is composed of a reductase, a transferase, and a synthetase encoded by *luxC*, *luxD*, and *luxE*, respectively. These genes are found in the *lux* operons of all bioluminescent bacteria. The primary reaction catalyzed by this system is the reduction of fatty acids by the reductase and synthetase. The synthetase activates the fatty acid, forming a fatty acyl-AMP intermediate that is bound to the enzyme. With the reductase, the acyl group is transferred to the synthetase and then to the reductase, where it is reduced by NADPH to the corresponding aldehyde. The transferase subunit catalyzes the transfer of the activated fatty acyl groups. The fatty acid is presumed to be recycled by the multienzyme reductase complex.

Relationship Between Selection Pressure and Plasmid Frequency

Microbial systems respond to environmental flux by a process called adaptation which occurs when microbial populations acclimate to a particular set of environmental conditions. There are three basic ways in which microbial populations can adapt to a new environmental stress (Spain et al., 1980): (1) specific enzymes may be induced or

expressed, (2) the number of organisms able to catalyze a needed function may increase, and/or (3) new metabolic capabilities may be selected or produced by genetic changes. Horizontal gene transfer via conjugation is one way adaptation can occur through the latter process (Trevors et al., 1987). There is little evidence showing that the presence of a pollutant influences the appearance of strains containing degradative genes as a result of gene transfer. Direct evidence is needed to show that populations of bacteria adapt to environmental stress by acquiring genetic information through conjugation.

The effect of selection pressure as a result of pollution is recognized in the spread of antibiotic resistance. Levine et al. (1983) showed that the proliferation of transconjugants in the intestinal tract could be linked to antibiotic selection pressure. This work showed that pBR325 was transferred to indigenous microbiota in the presence of tetracycline. These data provided direct evidence of the effect of selection pressure by antibiotics. Examples of how impressive the effect may be are discussed by Farrar (1981).

In contrast, this type of selection advantage has not been demonstrated for catabolic plasmids. It has been shown that strains carrying plasmids are more prevalent in polluted versus unpolluted environments. Glassman and McNicol (1981) examined 218 aerobic heterotrophs from three locations of Chesapeake Bay. They demonstrated that organisms isolated from cleaner sites contained a single, small plasmid (~3 Mdal), whereas more polluted areas contained bacteria harboring multiple, larger plasmids (≥ 30 Mdal). The proportion of plasmid-containing bacteria did not differ between sites.

Hada and Sizemore (1981) determined the incidence of plasmids in marine *Vibrio* spp. from an oil field in the Gulf of Mexico. They examined 440 isolates of *Vibrio* spp. for the presence of plasmids. In 31% of plasmid-bearing strains, the majority contained plasmids of ≤ 10 Mdal, a substantial number harbored plasmids ranging from 10-80 Mdal, and none contained plasmids larger than 100 Mdal. Half of the plasmid-containing strains possessed multiple plasmids. *Vibrio* sp. isolated from the oil field area had a higher plasmid incidence (35%) than the control area (23%). Also the bacteria isolated from the polluted areas contained more plasmids per cell than the unpolluted site.

Burton et al. (1982) isolated 400 aerobic heterotrophic bacteria from polluted and unpolluted river sediments. They found no significant differences in the number of plasmid containing isolates between polluted and unpolluted sites. When cultured on media, the nutritionally versatile population did have higher proportions of plasmid-bearing isolates at the polluted site. This observation was attributed to the effect of pollution.

These studies correlating plasmid frequency with pollution do not directly indicate that selection resulted in the presence of plasmid-containing bacteria at polluted sites because it is not known what functions these plasmids encode. From the limited data presented, it was determined that most plasmids were small and probably did not encode transfer functions, although such plasmids may be mobilized by helper plasmids from other strains. These plasmids may too small to encode catabolic or resistance functions. These data provide compelling circumstantial evidence that the presence of a pollutant

selects for plasmid-containing bacteria, but the studies were limited to cultivable and easy to manipulate bacteria, thus biasing the results.

There have been numerous studies on the effect of pollutants on bacterial community adaptation and structure. One study demonstrated the effects of adaptation on biodegradation rates of methyl parathion and *p*-nitrophenol in sediments (Spain et al., 1980). Biodegradation rates were faster when bacterial communities were pre-exposed to the pollutant. Another study on the effect of long-term application of sewage sludge contaminated with heavy metals on soil bacterial communities resulted in a more diverse and resistant community (Barkay et al., 1985). The effect could not be attributed to dissemination of resistance plasmids. Aquatic microbial communities were shown to adapt to mercuric ion stress (Barkay, 1987). Acid mine drainage was shown to lower community diversity in river water and sediment communities, although tolerance to heavy metals was higher than the control site (Wassel & Mills, 1983). These studies demonstrated adaptation in phenotypic terms. The observations were based on the activities of the communities represented by cultured isolates. The activities may have resulted from any of the three previously discussed mechanisms for adaptation.

If the mechanisms promoting the inheritance of the phenotypes could be described, an understanding of adaptation in terms of the molecular mechanisms discussed earlier could be developed. Mechanisms such as DNA rearrangements, transposition, and gene transfer may promote adaptation. Barkay & Olson (1986) demonstrated the effects of mercury contamination of lake sediments in terms of phenotypic and genotypic resistance to mercury stress. They found a strong correlation

between mercury concentration and the frequency of mercury resistance genes. The determinants for mercury resistance are known to be carried on plasmids. This phenomenon is observed for antibiotic resistance, resistance to other heavy metals, and pollutant catabolic genes. The occurrence of plasmids as carriers for such resistance genes and catabolic genes has led to the hypothesis that gene transfer by conjugation is important in assisting adaptation (Davey & Reanney, 1981). Top (1993) has demonstrated the effect of zinc stress on selection of transconjugants receiving the *czc* genes (cadmium-zinc-cobalt resistance) on pDN705. In sterile zinc-polluted soil, the transconjugant ratio was 10^{-2} versus the 10^{-6} ratio in non-polluted soil. Survival experiments of transconjugants and plasmid-free recipient bacteria showed an increase in the population of transconjugants in sterile-polluted soil. This increase was attributed to growth of early transconjugants and may have biased the study. This observation however does not rule out the influence of heavy metals on conjugal transfer.

There have been few reports of the transfer of catabolic plasmids in soil. Ramos-Gonzalez et al. (1991) showed the influence of *p*-ethylbenzoate on the transfer of the TOL plasmid, pWWO-EB62, which encodes *p*-ethylbenzoate biodegradative genes. Transfer of pWWO-EB62 was examined in soil where recipient populations were co-inoculated. The chemical negatively affected plasmid transfer, and this was attributed to the toxicity of the chemical to host bacteria. It was shown that TOL transferred to intergeneric recipients at a frequency of 10^{-2} transconjugants per recipient but the intergeneric transfer rate was 10^{-3} to 10^{-7} transconjugants per recipient. When

transconjugants were detected, outgrowth seemed to establish high numbers of transconjugants in soil.

The transfer of the 2,4-dichlorophenoxyacetic acid degradative plasmid pJP4 was studied in soil microcosms where a recipient population was added (Neilson et al., 1994). The frequency of transfer was 10^{-5} in sterile soil and 10^{-6} in 2,4-D amended soil. There have been few reports of transfer of plasmids to indigenous soil bacteria. The transfer of a marked RP4 (60 kb) plasmid harbored in *P. fluorescens* was studied in the rhizosphere (Smit et al., 1991). They observed a transfer frequency of 10^{-4} when using 10^7 donor cells. Transfer of a 3-chlorobenzoate degradative plasmid was observed in sediment mesocosms (Wyndham et al., 1988; Fulthorpe & Wyndham, 1991). Transconjugants were observed at the conclusion of their experiment that were not the original host but were able to express the 3-chlorobenzoate degradative genes.

PART 2

THE MAINTENANCE AND STABILITY OF *NAH-LUX* BIOLUMINESCENCE REPORTER PLASMIDS AND THEIR HOST STRAINS IN CONTINUOUS CULTURE

Introduction

Bioluminescence has been used in a number of systems as a cellular reporter of physiological processes (Selifonova et al., 1993; Meighen, 1991). The bioluminescence (*lux*) genes were used in the construction of reporter gene fusions (*nah-lux*) for naphthalene biodegradation (Burlage et al., 1990; King et al., 1990). These reporter strains are capable of reporting on naphthalene biodegradation through detectable bioluminescence production. The goal of this technology is the *in situ* measurement of biodegradation and bioavailability processes. Bioluminescence can be determined remotely and is a non-invasive and non-destructive measurement. Such technology makes sensitive and real-time environmental biodegradation analysis possible.

Bacterial bioluminescence is encoded by the *luxCDABE* genes (Meighen, 1988). A heterodimeric luciferase, which catalyzes the bioluminescence reaction, is encoded by the *luxAB* genes. Luciferase is a monooxygenase that requires oxygen, FMNH₂, and a long chain fatty aldehyde. The fatty aldehyde is oxidized to the corresponding fatty acid by luciferase, producing light as a by-product. The recycling of the aldehyde from the fatty acid requires ATP and NADPH. The multienzyme fatty acid reductase responsible for this recycling is encoded by *luxCDE*.

Naphthalene biodegradation has been well characterized genetically (Yen & Serdar, 1988). The genes responsible for naphthalene biodegradation are plasmid encoded and organized in two distinct operons (Yen & Gunsalus, 1982). Naphthalene is degraded to salicylate by enzymes of the upper (*nah*) *meta*-cleavage pathway, while salicylate is degraded to TCA cycle intermediates by the lower (*sal*) *meta*-cleavage pathway (Yen & Serdar, 1988). The expression of both operons is positively regulated by the constitutively produced and divergently transcribed *nahR* gene product (Schell, 1985; Schell, 1990). Salicylate and its structural analog, 2-aminobenzoate, were until recently the only known inducers of either operon (Yen & Gunsalus, 1982). Also 2-methylnaphthalene and 4-methylsalicylate have been shown to induce the Nah system (Leblond, 1995). Induction of both operons by salicylate has been shown to result in increased synthesis of specific mRNA (Shell, 1985).

In the construction of these *nah-lux* bioluminescence reporter strains, two strategies were used to create *nah-lux* transcriptional gene fusions. The first strategy involved the cloning of the promoter for the upper pathway for naphthalene biodegradation. The promoter was derived from NAH7 and inserted upstream of the *lux* cassette (*luxCDABE*) obtained from *Vibrio fischeri* and contained in a promoter probe vector (Burlage et al., 1990). The *nahR* gene product is supplied by a co-residing NAH7 plasmid for this system which also allows complete naphthalene biodegradation. The second strategy involves the insertion of Tn4431 into the *nahG* (salicylate hydroxylase) gene of the NAH7-like plasmid pKA1 resulting in a transcriptional *nahG-luxCDABE* fusion (King et al., 1990). The degradation of salicylate is provided by a separate and

uncharacterized *ortho*-cleavage salicylate degradative pathway (unpublished data).

Transposon Tn4431 is a derivative of Tn1721 containing a *luxCDABE* insertion along with inducible tetracycline resistance (Shaw et al., 1988).

Initial studies with these reporter systems established that the bioluminescence induction was concomitant with naphthalene biodegradation (Burlage et al., 1990). Simultaneous light production and naphthalene biodegradation was demonstrated directly with the use of ^{14}C -naphthalene mineralization assays. Continuous culture studies showed that the bioluminescence was responsive to the dynamic range of naphthalene exposure (King et al., 1990). Additionally, this study demonstrated the bioluminescence response to naphthalene in contaminated soils. The reporter system produced sufficient bioluminescence to act as a biosensor for naphthalene and as a reporter for naphthalene biodegradation.

A standardized bioassay was developed for the assessment of both naphthalene and salicylate bioavailability (Heitzer et al., 1992). With respect to bioluminescence, a linear relationship for aqueous solutions of naphthalene and salicylate was found over a two order of magnitude range of pollutant concentration. A detection limit of 45 ppb was determined for aqueous solutions of naphthalene. Additionally, a reproducible bioluminescent response was determined in experimentally contaminated soil slurries.

In order to determine the utility of these strains to report on long-term naphthalene biodegradation in mixed culture, the stability of the *nah-lux* reporter plasmids and the ability of the host strains to compete for available naphthalene was studied. Continuous flow chemostat cultivation has been shown to be a useful model system for studying

plasmid stability and maintenance (Proctor, 1994) as well as studying microbial interactions such as competition (Bungay & Bungay, 1968). In this study, continuous culture was used to examine maintenance and stability of *nah-lux* reporter plasmids and the ability of the *nah-lux* reporter strains to compete with the wild type naphthalene degradative strains for naphthalene.

The specific objectives of this work were to determine the maintenance of the two *nah-lux* reporter plasmids under non-selective conditions, to evaluate the persistence of reporter strains in mixed culture, and to evaluate the ability of *lux* reporter strains to maintain bioluminescence when competing against wild type degradative strains.

Materials and Methods

Strains

The characteristics of individual strains are shown in Table 1. *Pseudomonas fluorescens* 5R is an environmental isolate from a manufactured gas plant (MGP) soil, harboring pKA1, a NAH7-like naphthalene degradative plasmid (King et al., 1990). This strain can grow on naphthalene as a sole source of carbon. *Pseudomonas fluorescens* 5Rrif is a rifampicin resistant strain of *P. fluorescens* 5R. *Pseudomonas fluorescens* 5RL is *Pseudomonas fluorescens* 5R containing a Tn4431 mutagenized version of pKA1 (pUTK21) and is capable of degrading naphthalene to salicylate (King et al., 1990). *Pseudomonas fluorescens* 18H is an MGP soil isolate capable of degrading salicylate through an uncharacterized *ortho*-cleavage pathway (King et al., 1990; unpublished data). *Pseudomonas fluorescens* HK44 is *Pseudomonas fluorescens* 18H containing pUTK21.

This strain has a separate *ortho*-cleavage pathway for salicylate biodegradation that is separate from pUTK21 and is capable of complete naphthalene mineralization (King et al., 1990; unpublished results). Also *P. fluorescens* 18H does not possess a salicylate hydroxylase that has a high similarity with *nahG* of NAH7 (unpublished results). *P. putida* HK53 is *P. putida* PB2440 containing the NAH7 plasmid from *P. putida* G7 (King et al., 1990). *Pseudomonas putida* RB1351 is *P. putida* HK53 containing a *lux* reporter construct (pUTK9) as well as the NAH7 plasmid, and is capable of using naphthalene as a sole carbon source (Burlage et al., 1990). The 'undefined assemblage' of PAH degraders was isolated and previously described by DiGrazia (1991).

Individual Strain Generation Time Determinations

The generation times of individual strains were determined as previously described (Zund & Lebek, 1980). One-tenth strength LB medium was inoculated from freezer stocks and grown overnight at 26°C with agitation. Each culture was grown with and without salicylate (200 µg/ml) to induce the *nah* or *sal* pathways as appropriate (Table 1). Antibiotics were included as to assure maintenance of reporter plasmids. The overnight cultures were centrifuged at 3,840 x g at 25°C for 10 min. and the pellet washed twice with one-tenth strength LB medium. Each strain was inoculated into one-tenth strength LB medium with and without salicylate (200 µg/ml) with each treatment done in triplicate. The starting A_{595} was around 0.02-0.05. The cultures were incubated at 26°C in a orbital shaker at 200 rpm (New Brunswick Scientific). The A_{595} was monitored over time on a DU-70 spectrophotometer (Beckman Instruments, Inc.).

Table 1. Listing of strains and relevant characteristics.

Strain	Phenotype	Genotype	Plasmid	Source
<i>P. fluorescens</i> 5R	¹ Nah ² Sal	<i>nah, sal</i>	pKA1	Environmental Isolate
<i>P. fluorescens</i> 5RL	Nah Sal 3Lux ⁴ Tc ^R	<i>nah, sal, lux</i>	pUTK21	King et al. (1990)
<i>P. fluorescens</i> 18H	⁵ PSI Nah Sal		multiple	Environmental Isolate
<i>P. fluorescens</i> HK44	Tc ^R PSI Nah Sal Lux	<i>nah, sal, lux</i>	pUTK21	18H + pUTK21
<i>P. putida</i> HK53	PSI Nah Sal		NAH7	Burlage et al. (1990)
<i>P. putida</i> RB1351	PSI Nah Sal Lux ⁶ Km ^R	<i>nah, sal, lux</i>	pUTK9 NAH7	Burlage et al. (1990)
<i>P. putida</i> PB2440	PSI		none	Burlage et al. (1990)

¹Oxidation of naphthalene to salicylate

²Oxidation of salicylate to 2-oxo-hydroxypentanoate

³Produces bioluminescence when induced by salicylate

⁴Resistance to tetracycline (14 µg/ml)

⁵Ability to grow on *Pseudomonas* isolation agar

⁶Resistance to kanamycin (50 µg/ml)

Continuous Culture

A Bioflow C30 chemostat (New Brunswick Scientific) with a Teflon headplate and Teflon tubing was used for continuous cultures. Medium was supplied using a peristaltic pump equipped with a Teflon head (Fluid Metering, Inc.). Cultures were aerated continuously at 0.2 L/min and agitated at 250 rpm at 25°C. A buffered mineral salts medium (Meynell & Meynell, 1965) was used with saturating concentrations of 99+% naphthalene (Aldrich Chemical Co.), or salicylate (ACS grade, Mallinckrodt) and D-glucose (USP, Mallinckrodt) as indicated. In experiments where bioluminescence was measured, a light-tight chamber (Environmental Growth Chambers) housed the chemostat. A Model 7070 digital display with a Model 77340 photomultiplier tube (Oriel Corp.) and a liquid light pipe with a collimating beam probe was used to detect bioluminescence online (Figure 2-1). Data was collected online by an IBM PS/2 computer using data acquisition software (Rod Bunn, Biological Services, Univ. Tennessee). All inocula for chemostat cultivation were started from freezer cultures and grown under antibiotic selection to ensure retention of plasmids. Cultures were centrifuged at $3,840 \times g$ at 25°C for 10 min. and the pellet washed twice with minimal salts. In competition experiments, the inocula were adjusted with minimal salts to the same O.D. or by direct counting with a Petroff-Hausser counting chamber. Chemostat experiments were inoculated aseptically by syringe.

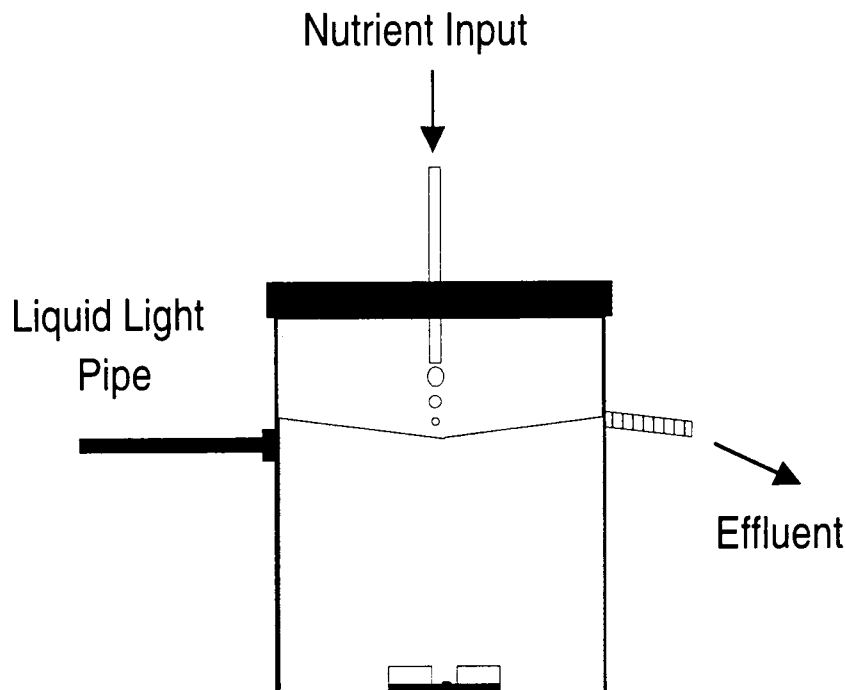


Figure 2-1. Continuous culture apparatus showing the placement of the liquid light-pipe.

Strain Monitoring

Strains were routinely monitored in the chemostat by spread plating on YEPG agar containing antibiotics (Table 1). YEPG is composed of (g/l): yeast extract, 0.2; peptone, 2.0; glucose, 1.0; and NH_4NO_3 , 0.2. *Pseudomonas putida* RB1351 was monitored on YEPG containing ampicillin and kanamycin (both 50 $\mu\text{g/ml}$). *P. fluorescens* 5Rif numbers were determined by plating on YEPG containing rifampicin (50 $\mu\text{g/ml}$). *P. fluorescens* HK44 numbers were monitored on YEPG containing tetracycline (14 $\mu\text{g/ml}$). The loss of plasmid pUTK9 from *P. putida* RB1351 was monitored as the difference between the colony forming units on pseudomonas isolation

agar (PSI) and PSI containing ampicillin and kanamycin. The oxidation of indole to indigo was used as a presumptive indicator of naphthalene dioxygenase (Ensley, 1983) and was incorporated into YEPG agar plates at 100 µg/ml. Culture density was monitored spectrophotometrically by absorbance at 595 nm on a DU-70 spectrophotometer (Beckman Instruments, Inc.).

DNA Probing

Colonies from spread plates were blotted onto 0.45 µm Biotrans nylon filters (ICN Biomedicals, Inc.). The colonies were lysed and the DNA denatured with 0.2 N NaOH, and the filters were baked at 80°C for 1 hr. All filters were prehybridized using the method of Church and Gilbert (1984). Colony blots were probed with single stranded ³²P-*luxAB* specific DNA. Probe was generated using the polymerase chain reaction (PCR) using a model 9810 thermocycler (Perkin Elmer Applied Biosystems) and with a GeneAmp PCR kit (Perkin Elmer). Previously published sequences (Baldwin et al., 1989) were used to design the oligonucleotides for the forward primer (*lux1*) 5'-GGGGGTTGTTATTCCAACAGC-3' and the reverse primer (*lux2*) 5'-GTCATCATGAGACCCTACTGC-3' (Genosys Biotechnologies, Inc.). The plasmid pUCD623 (Shaw et al., 1988) was used to obtain vector free double stranded template DNA. This double stranded template was used along with one of the oligonucleotide primers and ³²P-αdCTP to produce single stranded probe. The *luxAB* DNA was amplified using an initial denaturing of 92°C for 5 minutes coupled with a 1 min. denaturing at 92°C, 2 min. annealing at 55°C and a 2 min. extension at 72°C for 2 min. for 38 cycles. Previously

published sequences were used to design the oligonucleotides for the forward primer (*nahA* 3.2) 5'-CTCCTGAGCATGCGCGTCC-3' and the reverse primer (*nahA* 4) 5'-GGTCCAGACCTCGGTGGTG-3' for the production of *nahA* specific DNA probe (Genosys Biotechnologies, Inc.). The plasmid NAH7 was used to obtain vector free double stranded template DNA (Simon et al., 1993). This double stranded template was used along with one of the oligonucleotide primers and ^{32}P - αdCTP to produce single stranded probe. The *nahA* DNA was amplified using an initial denaturing of 92°C for 5 minutes coupled with a 1 min. denaturing at 92°C, 2 min. annealing at 50°C and a 1 min. extension at 72°C for 2 min. for 38 cycles. Probes were purified through a NucTrap column using a Pushcolumn apparatus following the manufacturers protocol (Stratagene Cloning Systems). All hybridizations and washings were done at high stringency at 65°C (Sayler et al., 1985). Positive signals were detected using autoradiography.

Plasmid Isolation and Analysis

The molecular stability of reporter plasmids was determined by endonuclease cleavage profiles. Colonies were randomly picked from chemostat culture platings and subject to mini-prep plasmid isolation. The mini-prep procedure was a modification of Technical Bulletin #009 (Promega Corp.). The resulting plasmid DNA was of sufficient purity for restriction endonuclease digestion. The plasmid preparations were treated with the endonuclease *Bam*H1 which will excise the *luxABCDE* genes from Tn4431 (Shaw et al., 1987). The DNA fragments were resolved on a 0.7% vertical agarose gel and visualized by staining with ethidium bromide. The gel was vacuum blotted following the

manufacturers protocol (Pharmacia Biotech, Inc) and probed with *luxAB* specific DNA probe.

Mineralization Experiments

Endpoint batch ^{14}C -mineralization assays were performed in 25 ml glass EPA vials (Pierce Chemical Co.) as the culture vessel in which a 8 ml vial containing 0.5 ml of 0.5N NaOH was used as the $^{14}\text{CO}_2$ trap. The outer vial was sealed with a Teflon-lined cap. Exponentially growing cultures were used for inocula. The cultures were centrifuged at $3,840 \times g$ at 25°C for 10 min. and washed twice with minimal salts (Meynell & Meynell, 1965). The cells were resuspended in minimal medium and the volume adjusted to give an A_{595} of 0.13 in a 1:20 dilution of which 0.5 ml was added to the outer vial containing 2 ml of minimal salts. Each outer vial also received 60,000 dpm of [UL- ^{14}C -ring] salicylate (Sigma Chemical Co.) dissolved in distilled water (specific activity of 10.4 mCi/mmol) as well as 50 $\mu\text{g/ml}$ (final conc.) unlabeled salicylate. The cultures were incubated at 26°C and were shaken at 200 rpm on a rotary shaker (Lab-Line Instruments, Inc.). Vials sacrificed at each time point and killed controls were stopped by adding 200 μl of 2N H_2SO_4 with shaking continued for at least one hour. The NaOH was removed from the trap and 1 ml of water along with ReadySafe scintillation cocktail (Beckman Instruments, Inc.). The trapped $^{14}\text{CO}_2$ was quantified by scintillation counting on a LS3801 counter (Beckman Instruments, Inc.). The H number method was used for automatic quench correction and calculation of disintegrations per minute was based on standard quench curves.

The protein concentration at each time point was determined using the enhanced BCA protein assay following the manufacturers recommendations (Pierce Chemical Co.). Bovine serum albumin was used as a standard.

Substrate Monitoring

Chemostat samples were analyzed for naphthalene and salicylate by HPLC. A 3ml aliquot of each sample was extracted against an equal volume of hexane-isopropanol (4:1). The extract was analyzed on a series 410 HPLC (Perkin-Elmer) with a C18 column using UV detection at 254 nm. The mobile phase was methanol:water (85:15) and delivered isocratically at 1.5 ml/min.

Glucose was measured colorimetrically using a glucose analysis kit based on glucose oxidase and O-toluidine (Sigma Chemical Co.). The analysis was done following the manufactures protocol.

Statistics

All error bars on graphs indicating bacterial populations represent one standard deviation of the mean of triplicate platings from duplicate dilutions series. Significant differences were determined using a paired t-test at a level $\alpha=0.05$.

Results

Plasmid Maintenance Under Non-selective Conditions in Continuous Culture

Plasmid maintenance studies were conducted in continuous cultures growing on non-selective media. These studies were designed to monitor the segregational stability of the *nah-lux* bioluminescence reporter plasmids harbored in *P. fluorescens* 5RL (pUTK21), *P. fluorescens* HK44 (pUTK21) and *P. putida* RB1351 (pUTK9) as compared to *P. fluorescens* 5R (pKA1).

Wild type pKA1

The wild type naphthalene degradative plasmid pKA1, harbored in *P. fluorescens* 5R, was maintained in 100% of the *P. fluorescens* 5R culture growing on minimal glucose media in continuous culture. Chemostat samples were monitored for presumptive plasmid maintenance by spread plating on YEPG agar with indole (100 µg/ml). The plasmid was maintained for 94 generations at a dilution rate of 0.171 hr^{-1} and an additional 27 generations at a dilution rate of 0.257 hr^{-1} at a cell density of $1.2\text{-}2.4 \times 10^8$ (Figure 2-2). All colonies on the YEPG plates containing indole were indigo positive, indicating presumptive maintenance of pKA1. All colonies from platings done at the beginning of the experiment and at the conclusion were *nahA* positive, confirming 100% maintenance.

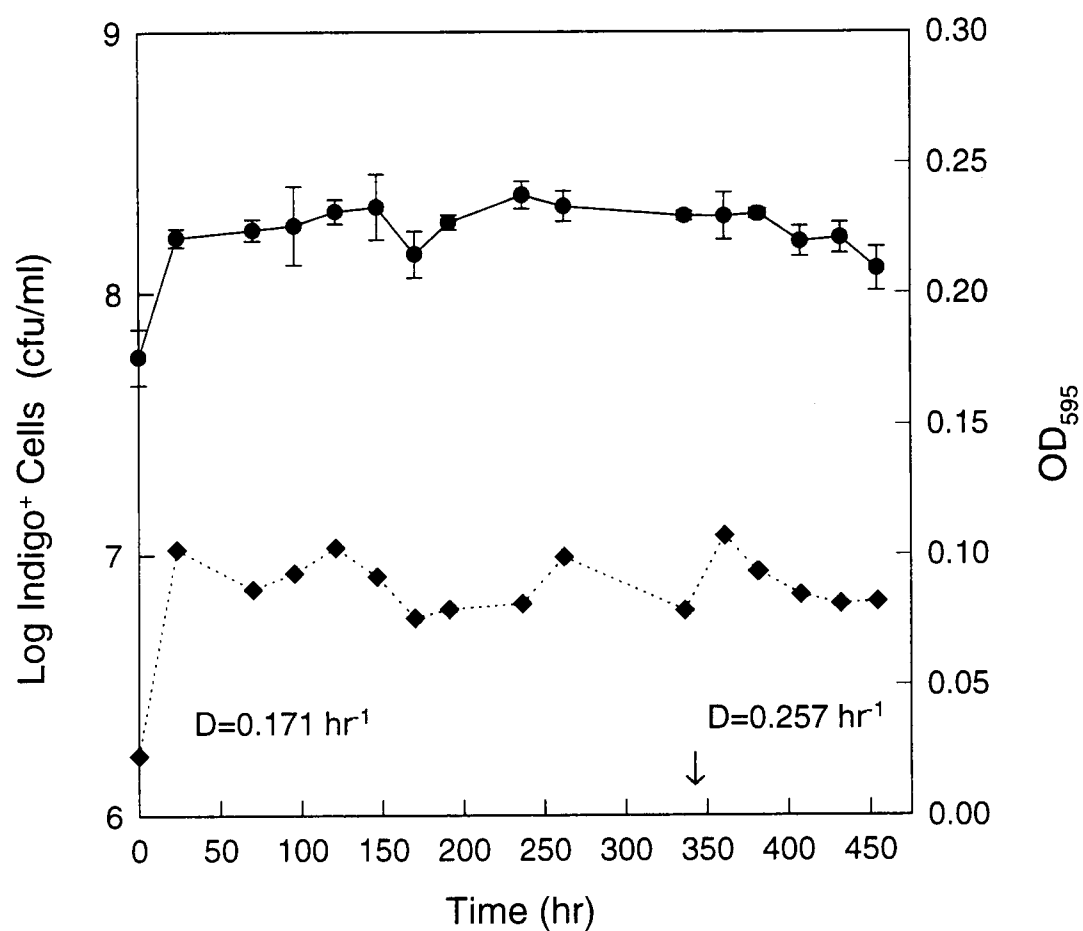


Figure 2-2. Maintenance of pKA1 (●) in *P. fluorescens* 5R growing in continuous culture on 100 ppm glucose in minimal media. Colony forming units were determined by spread plating on YEPG agar containing indole (100 µg/ml). The OD₅₉₅ is shown (◆).

pUTK21

The naphthalene bioluminescence reporter plasmid pUTK21, harbored in *P. fluorescens* 5RL, was maintained in 100% of the culture growing on glucose minimal media in continuous culture at a dilution rate of 0.171 hr^{-1} (Figure 2-3). The total culture population was monitored on YEPG plates and the population retaining pUTK21 was monitored on YEPG plates plus tetracycline ($14 \text{ } \mu\text{g/ml}$). The use of YEPG plates containing tetracycline to monitor the population of 5RL retaining pUTK21 was not reliable since this method underestimated the pUTK21 retaining population. The plates counts from tetracycline selective media, indicating *P. fluorescens* 5RL cells retaining pUTK21, are significantly different over time from the total colony forming units determined on YEPG plates. This would suggest that a portion of the total population did not maintain pUTK21. The *luxAB* probed colony blots taken from the plates without tetracycline (total CFUs) more closely follows the total CFU curve. All colonies at the last time point produced indigo when indole was supplied in the plate lid. The difference between the total CFUs and the *luxAB* positive colonies can be accounted for by the inability to blot all the colonies from the edge of the plate. This small difference in the number of colonies could account for the difference since all colonies produced indigo, indicating dioxygenase activity.

The naphthalene bioluminescence reporter plasmid pUTK21, harbored in *P. fluorescens* HK44 was not completely maintained over time when the HK44 culture was growing on salicylate media at a dilution rate of 0.086 hr^{-1} (Figure 2-4A). This strain was cultured on salicylate as a non-selective medium since the salicylate pathway is blocked

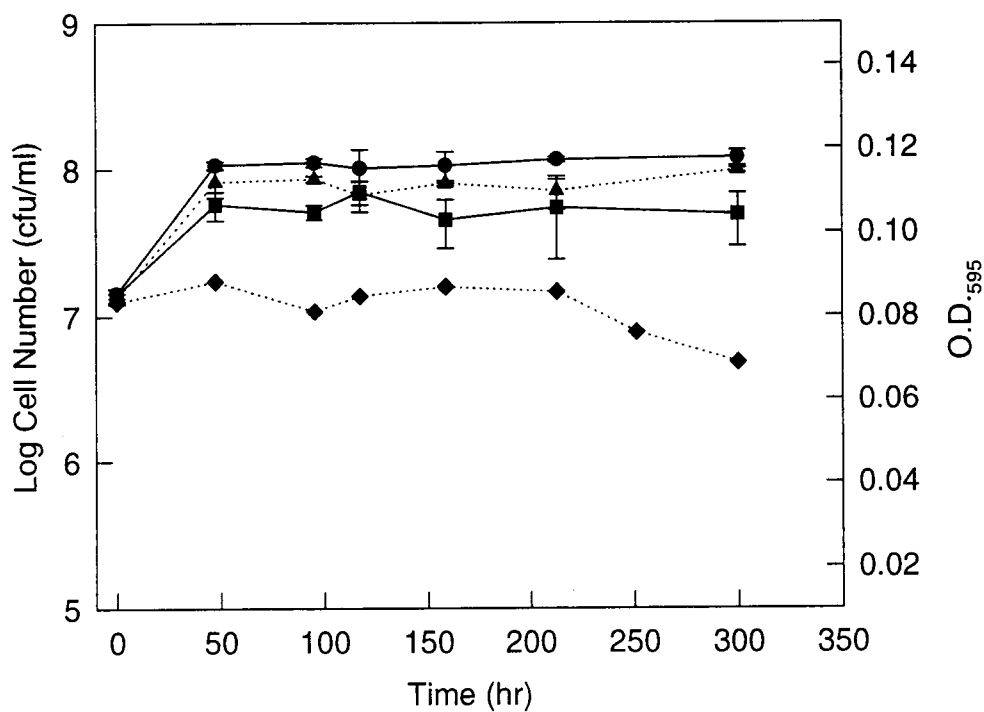


Figure 2-3. Maintenance of pUTK21 in *P. fluorescens* 5RL growing in continuous culture on 100 ppm glucose in minimal media at a dilution rate of 0.171 hr^{-1} . The total culture population was monitored on YEPG plates and the population retaining pUTK21 was monitored on YEPG plates plus tetracycline ($14 \mu\text{g/ml}$). Total cells (●), cells retaining pUTK21 (■), *luxAB*⁺ cells (▲), and OD₅₉₅ (◆) are shown.

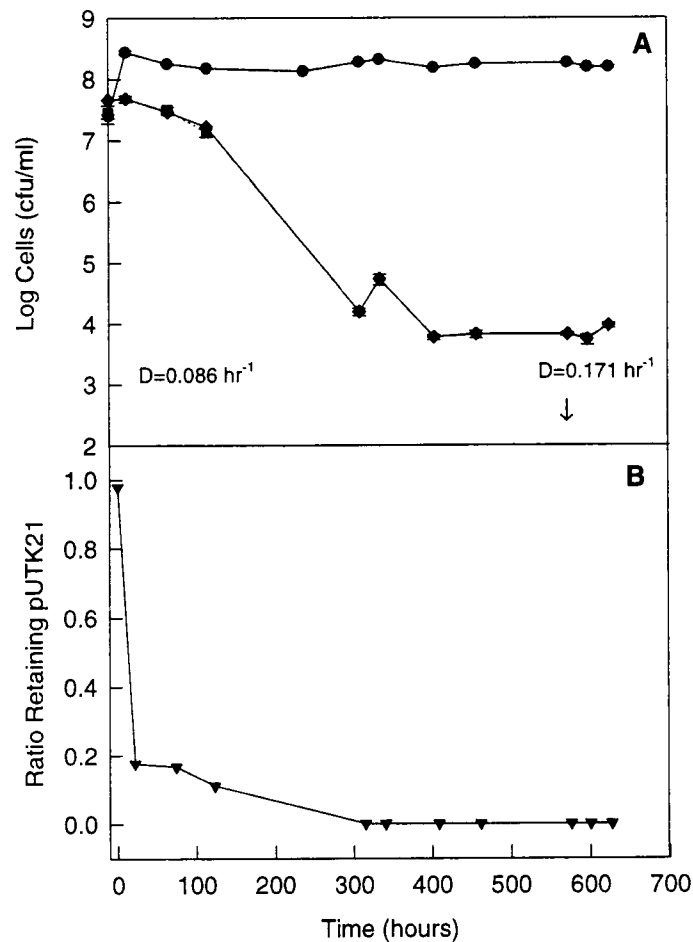


Figure 2-4. Maintenance of pUTK21 in *P. fluorescens* HK44 growing in continuous culture on 100 ppm salicylate in minimal media at a dilution rate of 0.086 hr^{-1} . The dilution rate was increased to 0.171 hr^{-1} as indicated by the arrow. (A) Maintenance of total population as determined by spread plating on YEPG plates and the population retaining pUTK21 was monitored on YEPG plates plus tetracycline ($14 \mu\text{g/ml}$). Maintenance of the individual population are indicated as: total cells (●), cells retaining pUTK21 (◆), and *luxAB*⁺ (■). (B) The ratio of cells retaining pUTK21 is shown (▼).

by Tn4431 in pUTK21 and *P. fluorescens* HK44 has a separate *ortho*-degradative pathway for salicylate degradation. This was done to minimize the chance of contamination of the continuous culture. The data show that the portion of the population retaining the reporter plasmid declined to 1.6×10^4 cfu/ml after 39 generations at a dilution rate of 0.086 hr^{-1} as determined by spread plating culture samples on YEPG plus tetracycline agar. This decrease coupled with a 1.6×10^8 cfu/ml increase in total cells resulted in 99% loss of pUTK21 in the HK44 culture. The plasmid bearing portion of the culture was maintained at the same level for another 23 generations. An increase in the dilution rate to 0.171 hr^{-1} had no significant effect in plasmid maintenance for an additional 13 generations. The *luxAB*⁺ portion of the population, determined from colony blots from YEPG plates, closely followed the YEPG plus tetracycline plate counts for the 3 data points available. The portion of the *P. fluorescens* HK44 culture retaining pUTK21 was also determined as the ratio of colonies on YEPG plates plus tetracycline to those on YEPG plates (Figure 2-4B).

Plasmid preparations derived from random continuous culture isolates from YEPG plates at the conclusion of the experiment showed that the Tn4431 transposon in pUTK21 remained stable. The plasmid preparations from individual strains were *Bam*HI digested, vacuum blotted onto a nylon membrane and probed with a *luxAB* specific DNA probe (Figure 2-5). Whole pUTK21 was used as the positive control and a plasmid preparation from *P. fluorescens* 18H was used as the negative control. Cleavage with *Bam*HI excises the *luxCDABE* cassette yielding a 7.5 kb fragment. All isolates retaining pUTK21 (lanes 4-5, 8-9, 11) also retained the *lux* insertion consistent with the expected

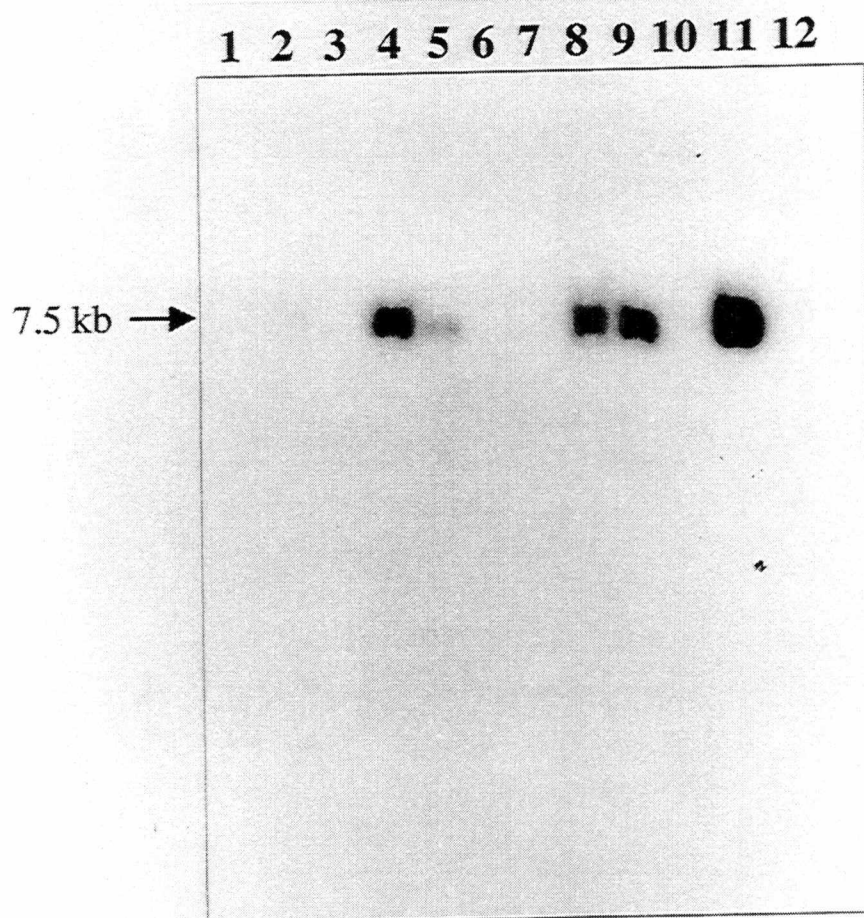


Figure 2-5. Southern blot of *Bam*H1 digested plasmid isolates from a continuous culture of *P. fluorescens* HK44 growing in continuous culture on salicylate as a sole source of carbon. Random isolates were picked from YEPG plates at the conclusion of the experiment. Lanes: (1) 1Kb ladder, (2) whole pUTK21 (pos. control), (3) plasmid preparation from *P. fluorescens* 18H, (4-12) plasmids from reactor isolates.

7.5 kb fragment compared to standard pUTK21 DNA. All isolates retaining pUTK21 were tetracycline resistant whereas those isolates that lost pUTK21 were tetracycline sensitive. This indicates that the transposon did not insert in a chromosomal site. Insertion in a chromosomal location would have resulted in tetracycline resistance. Since no excision events were determined in the small sampling noted above, this type of event was not deemed responsible for the loss of the *luxAB* genotype but rather the plasmid pUTK21 was segregationally lost from the culture. Excision events may occur at a low frequency but were not observed in this experiment.

Although this blot was not probed for naphthalene degradative genes, there did not appear to be any large deletions or rearrangements in any of the plasmid isolations since each maintained the same DNA fragment pattern (data not shown). Additionally, all isolates that lost pUTK21 had the plasmid bands that are present in the plasmid preparation from the parent *P. fluorescens* 18H strain. This precludes the possibility that the isolates lacking pUTK21 were merely contaminants.

pUTK9

The maintenance of the reporter plasmid pUTK9 in *P. putida* RB1351 fluctuated over time when RB1351 was growing on saturating levels of naphthalene in continuous culture (Figure 2-6A). These data were derived from a naphthalene competition experiment and do not represent data from a RB1351 culture grown on glucose minimal media. Since naphthalene minimal media does not select for pUTK9 (contrast to pUTK21), there was no selection for pUTK9 and this experiment served the purposes of demonstrating

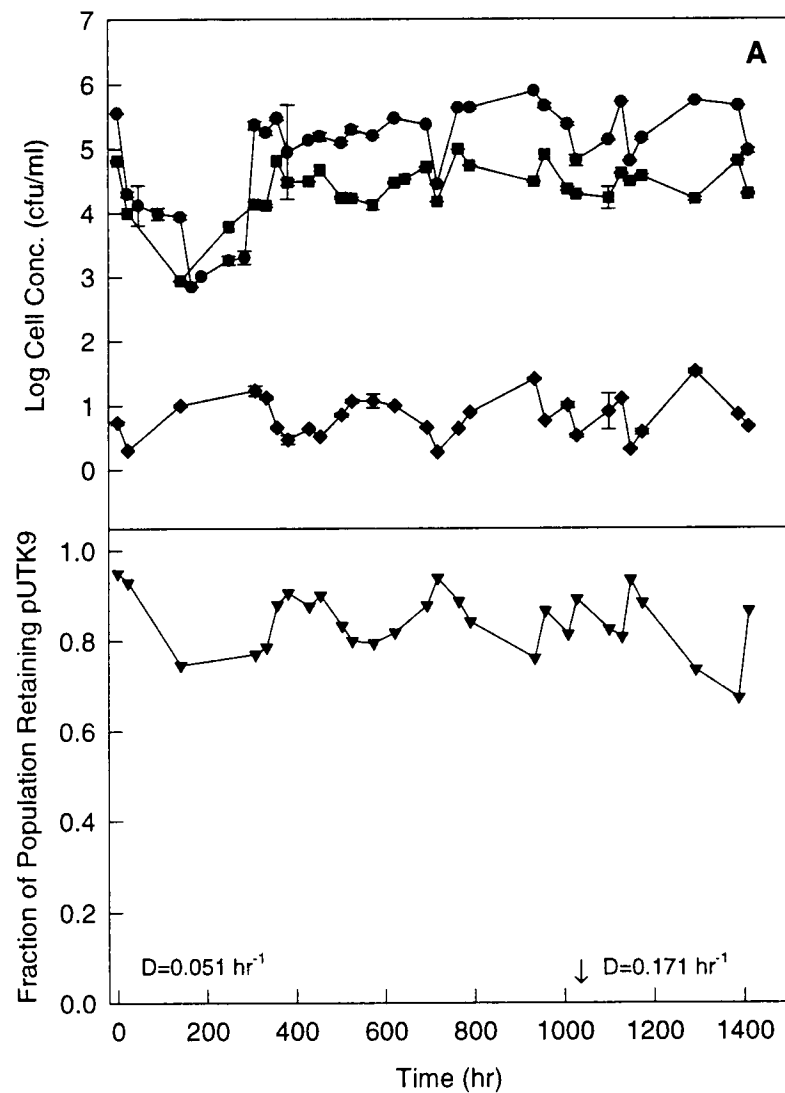


Figure 2-6. (A) Maintenance of pUTK9 in a chemostat culture containing *P. putida* RB1351 growing on saturating levels of naphthalene in minimal media. (A) The total RB1351 population (●), the RB1341 population retaining pUTK9 (■) and the RB1351 population not retaining pUTK9 (◆) are shown. (B) The plasmid retention ratio of pUTK9 in *P. putida* RB1351 (total CFUs/CFUs retaining pUTK9) is shown (▼).

maintenance in a non-selective medium. These data show the total RB1351 population and the population retaining pUTK9. There was a measurable difference between the two populations. The population that did not retain pUTK9 was low and determined as the difference between the plate counts of the total RB1351 population and the counts of the portion retaining pUTK9. The reporter plasmid pUTK9 was maintained in 75-95% of the *P. putida* RB1351 population for 74 generations at a dilution rate of 0.051 hr^{-1} and 99 generations at a dilution rate of 0.171 hr^{-1} (Figure 2-6B). The population of *P. putida* RB1351 lacking pUTK9 fluctuates, but there is no identifiable trend in plasmid loss. This lack of a trend may in part be due to the difficulty in monitoring these populations at such low numbers. The portion of the *P. putida* RB1351 culture retaining pUTK9 was also determined as the ratio of colonies on PSI agar with kanamycin (50 $\mu\text{g/ml}$) to those on PSI agar minus the PSI agar with kanamycin (Figure 2-6B).

Competitive maintenance of bioluminescence reporter strains vs. *P. fluorescens* 5R

Tri-culture of *P. fluorescens* 5R, *P. fluorescens* 5RL and *P. putida* RB1351

Population maintenance of the wild type degrading strain *P. fluorescens* 5R and two engineered reporter strains (*P. putida* RB1351 and *P. fluorescens* 5RL) growing on saturating levels of naphthalene minimal medium demonstrated that *P. fluorescens* 5RL was not maintained at the highest dilution rate tested but *P. putida* RB1351 was maintained at both dilution rates tested (Figure 2-7). These data show that the wild type strain, *P. fluorescens* 5R, was maintained at a level 3-5 orders of magnitude higher than either engineered strain. The wild type strain *P. fluorescens* 5R, *P. fluorescens* 5RL, and

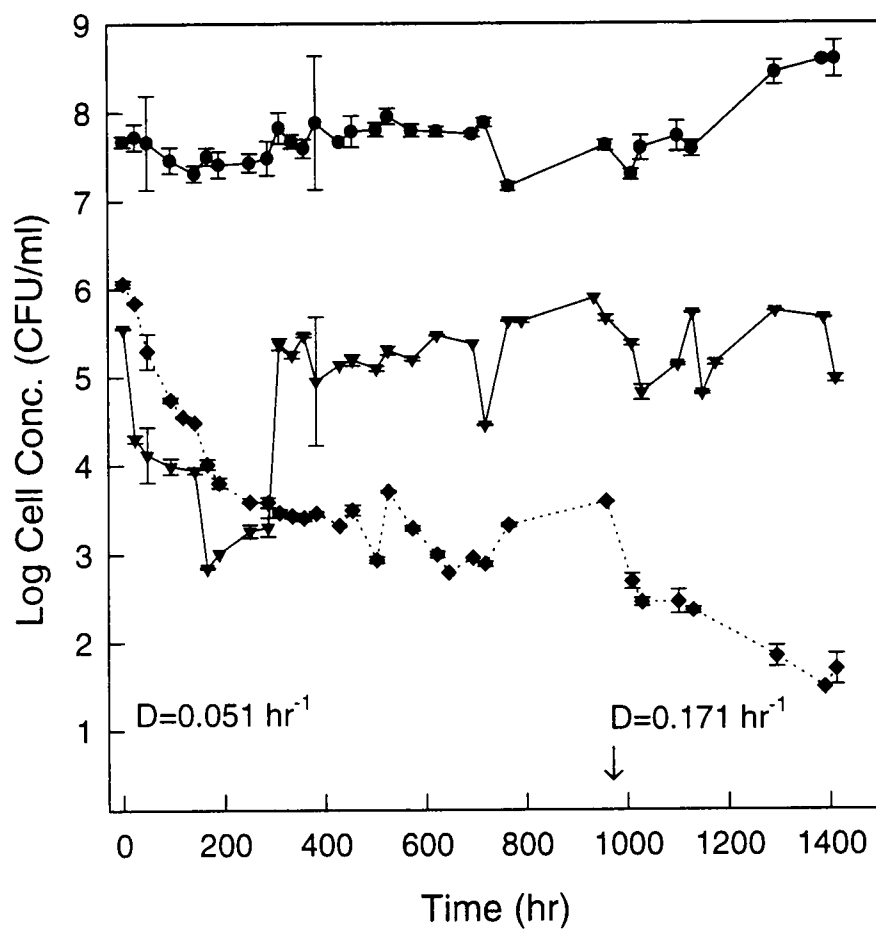


Figure 2-7. Population maintenance of a wild type PAH degrading strain *P. fluorescens* 5R (●), and two engineered reporter strains; *P. putida* RB1351 (▼), and *P. fluorescens* 5RL (◆) growing on saturating levels of naphthalene in minimal media.

P. putida RB1351 were maintained at around 1×10^8 , 1.3×10^3 and 5×10^4 cfu/ml respectively at a dilution rate of 0.051 hr^{-1} for 75 generations. At a dilution rate of 0.171 hr^{-1} , the *P. fluorescens* 5R and *P. putida* RB1351 were maintained as before for an additional 99 generations, but *P. fluorescens* 5RL was nearly washed out. The results do show that *P. putida* RB1351 was maintained at 10^4 - 10^5 cfu/ml even at higher dilution rates. *Pseudomonas fluorescens* 5RL was nearly washed out at higher dilution rates, possibly due to the inability of the strain to mineralize naphthalene completely (accumulates salicylate). This inability to completely mineralize naphthalene places strain 5RL at a competitive disadvantage. The sharp increase in total cell number and in the population level of *P. fluorescens* 5R is unexplainable. No naphthalene or salicylate were detected in the culture effluent as determined by HPLC. The fact that no salicylate was detected in the culture effluent indicates that *P. fluorescens* 5RL provided an additional substrate for growth to *P. fluorescens* 5R and *P. putida* RB1351 if it could exit the cell, thus providing a competitive advantage to those strains.

Tri-culture of *P. fluorescens* 5R, *P. fluorescens* HK44 and *P. putida* RB1351

Because *P. fluorescens* HK44 has a complete salicylate degradation pathway, it was hypothesized that this strain could compete with the other strains for naphthalene and be maintained in continuous culture. Population maintenance of the wild type naphthalene degrading strain *P. fluorescens* 5R and two engineered reporter strains (*P. putida* RB1351 and *P. fluorescens* HK44) growing on saturating levels of naphthalene minimal medium demonstrated that *P. fluorescens* HK44 was not maintained over time

but *P. putida* RB1351 was maintained at both dilution rates tested along with *P. fluorescens* 5R (Figure 2-8A). The wild type strain *P. fluorescens* 5R declined from 5.6×10^6 to 1.4×10^5 cfu/ml and *P. putida* RB1351 declined from 3.6×10^6 to 4.2×10^5 cfu/ml for 5 generations at a dilution rate of 0.017 hr^{-1} . The population of *P. fluorescens* HK44 dropped from 4.5×10^5 to 2×10^4 cfu/ml. At a dilution rate of 0.034 hr^{-1} , *P. fluorescens* 5R increased to 3.4×10^6 cfu/ml over 8.2 generations. *Pseudomonas putida* RB1351 increased to 5×10^6 cfu/ml over 8.2 generations. *P. fluorescens* HK44 gradually decreased until no CFUs could be detected. This eventual washout of *P. fluorescens* HK44 may have been due to the 10-fold lower inoculum of this strain even though there was an attempt to inoculate all strains at the same concentration. This situation would provide a competitive advantage to the strains present in higher numbers.

The bioluminescent response of the mixed culture was dynamic in character (Figure 2-8B). Upon inoculation of the respective strains, bioluminescence increased to 0.2×10^{-9} amps and remained constant until the continuous addition of medium after 24 hr. At this time, the bioluminescence increased to 0.63×10^{-9} amps. The response fluctuated over the next 120 hours. Upon increasing the dilution rate from 0.017 hr^{-1} to 0.034 hr^{-1} , the bioluminescence increased 2.5 fold, fluctuated, then decreased to 0.6×10^{-9} amps. This data demonstrates that the bioluminescent response is sensitive to perturbations such as a change in dilution rate. A steady state was regained, indicating that the bioluminescent response is probably not related to growth rate. The bioluminescence data ends at 260 hours due to power failure and subsequent loss of data.

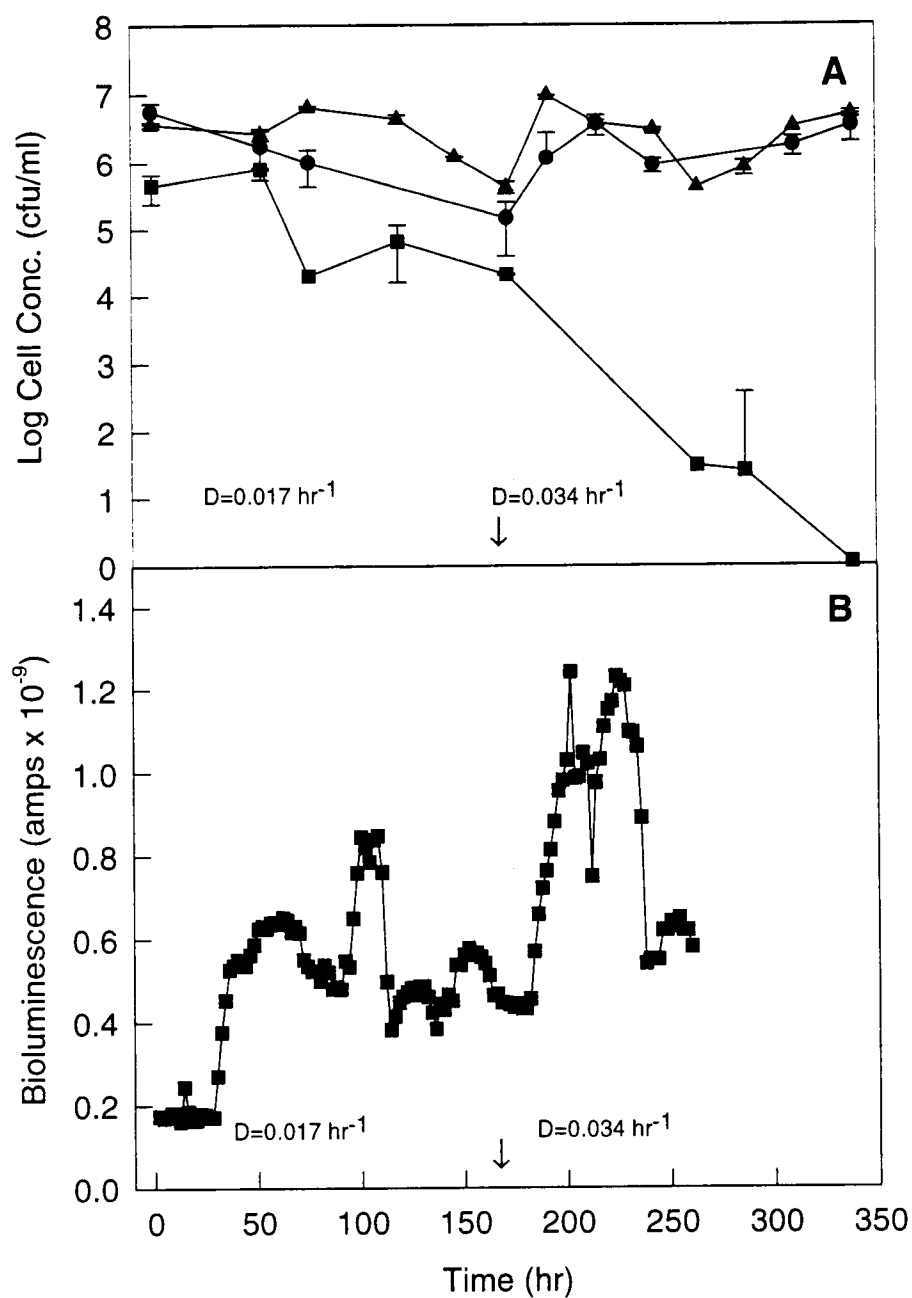


Figure 2-8. (A) Population maintenance of a wild type PAH degrading strain *P. fluorescens* 5Rif (●), and two engineered reporter strains *P. putida* RB1351 (◆), and *P. fluorescens* HK44 (▼), growing on saturating levels of naphthalene in minimal media. (B) Bioluminescence is indicated (■).

Co-culture of *P. fluorescens* 5R and *P. fluorescens* HK44

Because *P. fluorescens* HK44 was not maintained in the previous experiment, this strain was continuously cultured alone with naphthalene as a sole source of carbon. The experiment was set up to test the hypothesis that *P. fluorescens* HK44 was not maintained in mixed continuous culture due to an inability to maintain pUTK21. Although naphthalene serves as selective pressure for pUTK21 in that an individual bacterium cannot grow and divide if it cannot use naphthalene as a sole source of carbon, it is conceivable that the plasmid is not maintained and the host strain washes out of the culture. The culture was monitored using YEPG plus tetracycline plates (selective) and YEPG plates (non-selective) media. If a large portion of the population were losing pUTK21, then large differences in plate counts on the respective media would be expected if the plasmid-free cells were not washed out as fast as produced. There were no significant differences in numbers between the two plate counts and pUTK21 maintained when *P. fluorescens* HK44 was grown in continuous culture on saturating levels of naphthalene (Figure 2-9A). The population maintenance data of *P. fluorescens* HK44 shows that the culture started at 1.2×10^6 cfu/ml. This level increased to $1 \times 10^8 - 7 \times 10^7$ cfu/ml and was maintained for 9 generations at a dilution rate of 0.026 hr^{-1} . The culture was maintained for another 17 generations at a dilution rate of 0.06 hr^{-1} . Upon addition of *P. fluorescens* 5R (rif⁺), the population of *P. fluorescens* HK44 decreased but was co-maintained along with *fluorescens* 5R (rif⁺) for another 21 generations at a dilution rate of 0.06 hr^{-1} .

Colony blots taken from spread plating culture samples on YEPG agar were

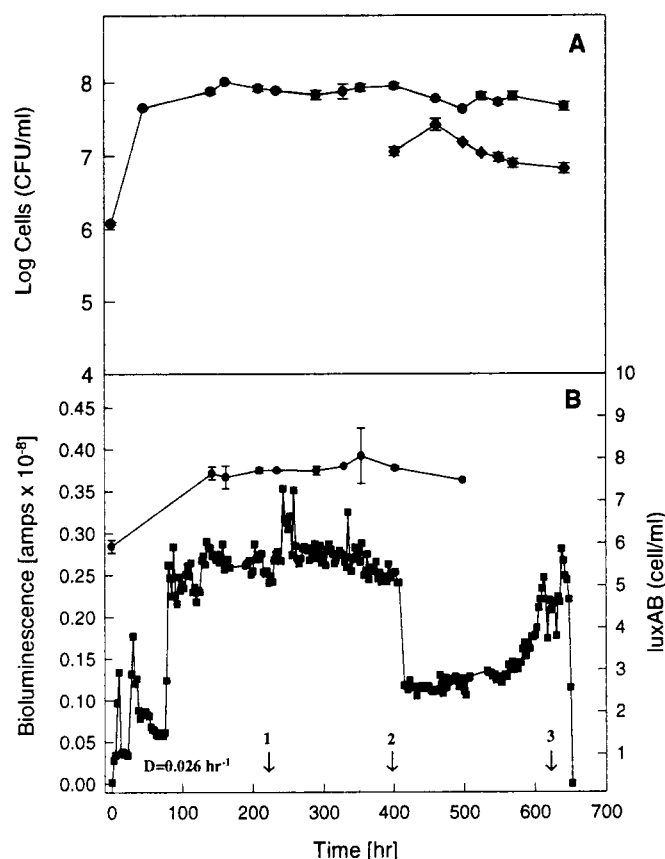


Figure 2-9. (A) Population maintenance of *P. fluorescens* HK44 (●) growing on saturating levels of naphthalene in minimal media in continuous culture. The wild type PAH degrading strain, *P. fluorescens* 5Rif (◆), was inoculated into the culture after 17 days of cultivation. (B) Maintenance of pUTK21 (●) in *P. fluorescens* HK44 growing in continuous culture on saturating levels of naphthalene in minimal media. The bioluminescent response is indicated (■). The system was perturbed in the following ways: (1) The dilution rate was increased to 0.06 hr⁻¹; (2) The strain *P. fluorescens* 5Rif was inoculated into the system; (3) The culture was poisoned with the detergent Quatsyl[®] 256.

probed with *luxAB* specific DNA and the maintenance of pUTK21 determined (Figure 2-9B). The results agree with the viable count data shown in Figure 2-9A. These data indicate that the reporter plasmid pUTK21 is not lost during continuous culture on naphthalene. The *lux* genotype was stably maintained for 9 generations at a dilution rate of 0.026 hr^{-1} . Upon increasing the dilution rate to 0.06 hr^{-1} , the *lux* genotype continued to be maintained for another 17 generations. When the wild type PAH degrading strain *P. fluorescens* 5R (rif^+) was added to the culture, the *lux* genotype continued to be maintained for another 21 generations.

The bioluminescent response of the culture is also shown in Figure 2-9B. Over the first 3 generations, the bioluminescence was highly variable but reached a steady-state at 0.27×10^{-8} amps. These data agree with the viable counts shown in Figure 2-9A with respect to cell number and bioluminescent output. The bioluminescence becomes steady about the same time the viable count of *P. fluorescens* HK44 becomes steady. The bioluminescence remains steady for 9 generations at a dilution rate of 0.026 hr^{-1} .

Upon increasing the dilution rate to 0.06 hr^{-1} , the bioluminescent response shows a brief fluctuation and then attains its former level for another 6.5 generations. This fluctuation was much smaller in magnitude than the one in Figure 2-8. The dilution rates were different and it is impossible to judge whether such a perturbation is more pronounced in a mixed culture than in a pure culture. Upon addition of *P. fluorescens* 5R (rif^+) to the culture, the bioluminescence dropped two fold. The bioluminescence regained its former level after an additional 21 generations. It seems that the sudden burden of competition has a pronounced effect on the bioluminescent response in this

situation. This drop in bioluminescence output may have been due to a drop in oxygen concentration in the when strain 5R was added to the culture. If this were the case, it would not have taken 200 hr to reach the former oxygen level since the culture was continuously aerated. It seems more likely that a shift in metabolism occurred relating to FMNH₂, NADPH, ATP or fatty acid levels in the cell. These cellular constituents are all important in the bioluminescence reaction and a perturbation in the available pools may have caused the drop in bioluminescence. At the conclusion of the experiment, the culture was poisoned with the detergent Quatsyl® 256. The bioluminescence dropped to 0×10^{-8} amps. This demonstrates that the response was indeed due to light from the culture and not due to light leakage or dark current.

Persistence of an engineered reporter strain in mixed culture

The naphthalene bioluminescence reporter strain *P. putida* RB1351 could successfully compete with a wild type undefined assemblage derived from a MGP soil (Figure 2-10A). Population maintenance of *P. putida* RB1351 in a mixed culture containing a community derived from a PAH contaminated soil showed that after 24 generations at a dilution rate of 0.171 hr^{-1} , the RB1351 reporter strain was maintained at a level of $1.1\text{-}2.8 \times 10^6$ cfu/ml. The wild type assemblage was maintained at $3.5\text{-}8.5 \times 10^6$ cfu/ml for 35 generations. *Pseudomonas putida* RB1351 was inoculated at a concentration sixteen times higher than the undefined assemblage but still dropped below the level of the wild type assemblage after 24 generations. No naphthalene was detected after day one, and phenanthrene could not be detected after day six. The abrupt rise in the

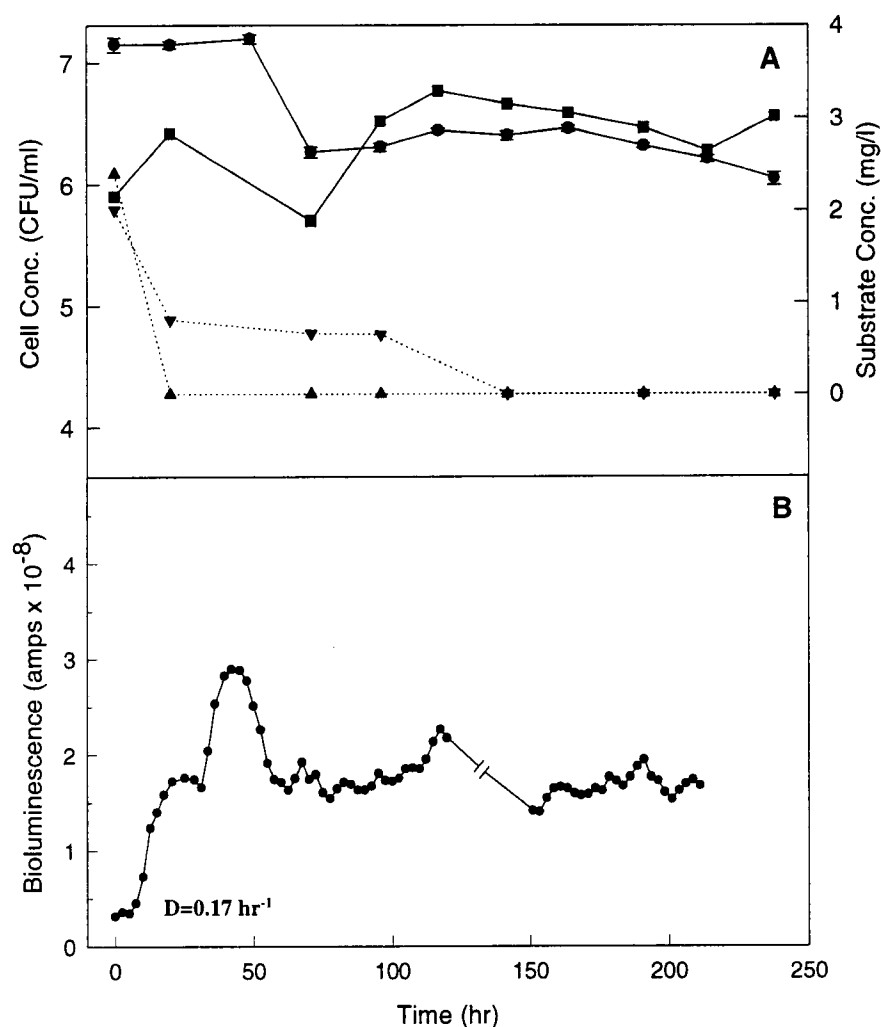


Figure 2-10. (A) Maintenance of an engineered *lux* reporter strain *P. putida* RB1351 (■) in a mixed culture containing a community derived from a PAH contaminated soil. The total CFUs (●) are indicated. Naphthalene (▲) and phenanthrene (▼) were supplied at saturating concentrations in minimal media supplemented with a soil extract (DOC = 10 mg/ml). (B) Bioluminescence from a *nah-lux* reporter strain *P. putida* RB1351 in a mixed culture containing a community derived from a PAH contaminated soil.

level of the wild type assemblage tails after phenanthrene has disappeared from the culture effluent. Although bacteria harboring NAH7 and NAH7-like plasmids have been shown to mediate metabolism of phenanthrene (Sanseverino et al., 1993; Menn et al., 1993) it may have been used as a carbon source by the wild type assemblage of bacteria, thus giving that population a competitive advantage.

Pseudomonas putida 1351 was capable of maintaining a detectable output of bioluminescent light for long periods of time (Figure 2-10B). Bioluminescence from *P. putida* RB1351 in the mixed culture was not steady but was maintained at $1.4\text{--}2.3 \times 10^{-8}$ amps after 17 generations. The gap in the data was due to a power outage and subsequent loss of data.

Growth Experiments

^{14}C -Salicylate mineralization

A possible explanation for the inability of HK44 to compete with 5R is the different salicylate degradation pathway in HK44. It was hypothesized that HK44 mineralized salicylate slower than 5R, either due to copy number or pathway regulation. The specific mineralization rates of 5R and HK44 were determined. Mineralization of ^{14}C -salicylate occurred at a measurably faster rate for *P. fluorescens* 5R than for *P. fluorescens* HK44 (Figure 2-11). The rate of mineralization was $148 \text{ dpm mg}^{-1} \text{ protein hr}^{-1}$ (± 12) for *P. fluorescens* HK44 and $198 \text{ dpm mg}^{-1} \text{ protein hr}^{-1}$ (± 34) for *P. fluorescens* 5R. The rates were determined not to be significantly different by t-test (significant at an α of 0.05).

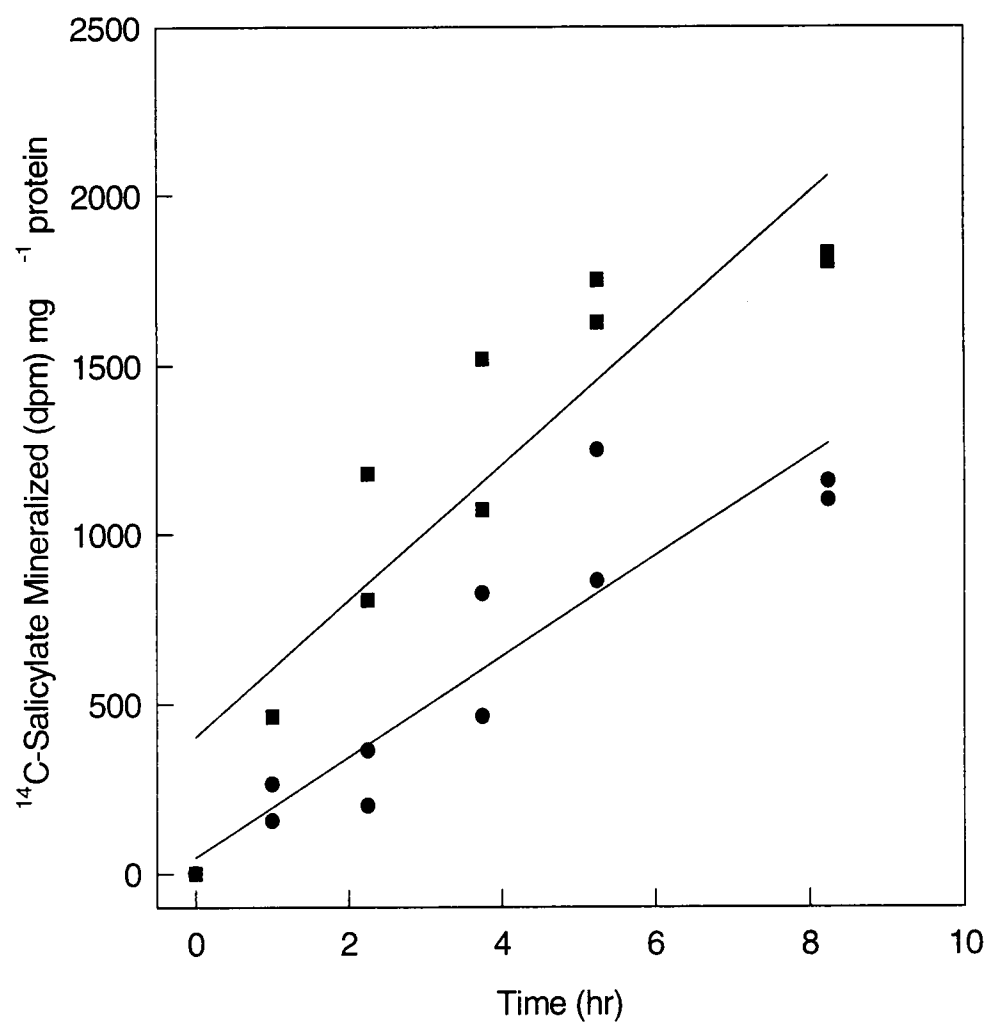


Figure 2-11. Specific mineralization rate of salicylate by *P. fluorescens* 5R (■) and *P. fluorescens* HK44 (●).

Strain Doubling Times

The doubling times of individual strains listed in Table 1 were determined for cultures growing on one-tenth strength LB with and with out *nah* induction with salicylate (Figure 2-12). The induced culture of *P. fluorescens* 5RL had a longer doubling time than the uninduced culture. The parental strain, *P. fluorescens* 5R, showed no statistical difference between induced and uninduced cultures. *P. putida* RB1351 and its parental strain, *P. putida* HK53 did not show measurable differences in doubling times between induced and uninduced cultures. Both *P. fluorescens* HK44 and the parental strain, *P. fluorescens* 18H, had longer doubling times in induced cultures. Significant differences were determined by t-test (significant at an α of 0.05).

DISCUSSION

Plasmid maintenance as determined in non-selective continuous culture differed according to strain. The wild type strain *P. fluorescens* 5R harboring pKA1, maintained the plasmid in 100% of the culture as shown by conversion of indole to indigo and DNA probing with a *nahA* specific probe. This was not a surprising finding since previous attempts to cure this strain were unsuccessful (unpublished results). The bioluminescence reporter plasmid pUTK21 (pKA1::Tn4431) in strain *P. fluorescens* 5RL, was also maintained in 100% of the culture as shown by conversion of indole to indigo and probing with a *nahA* specific probe. It has been shown that the insertion of large fragments of DNA reduces the segregational stability of plasmids (Warnes & Stephenson,

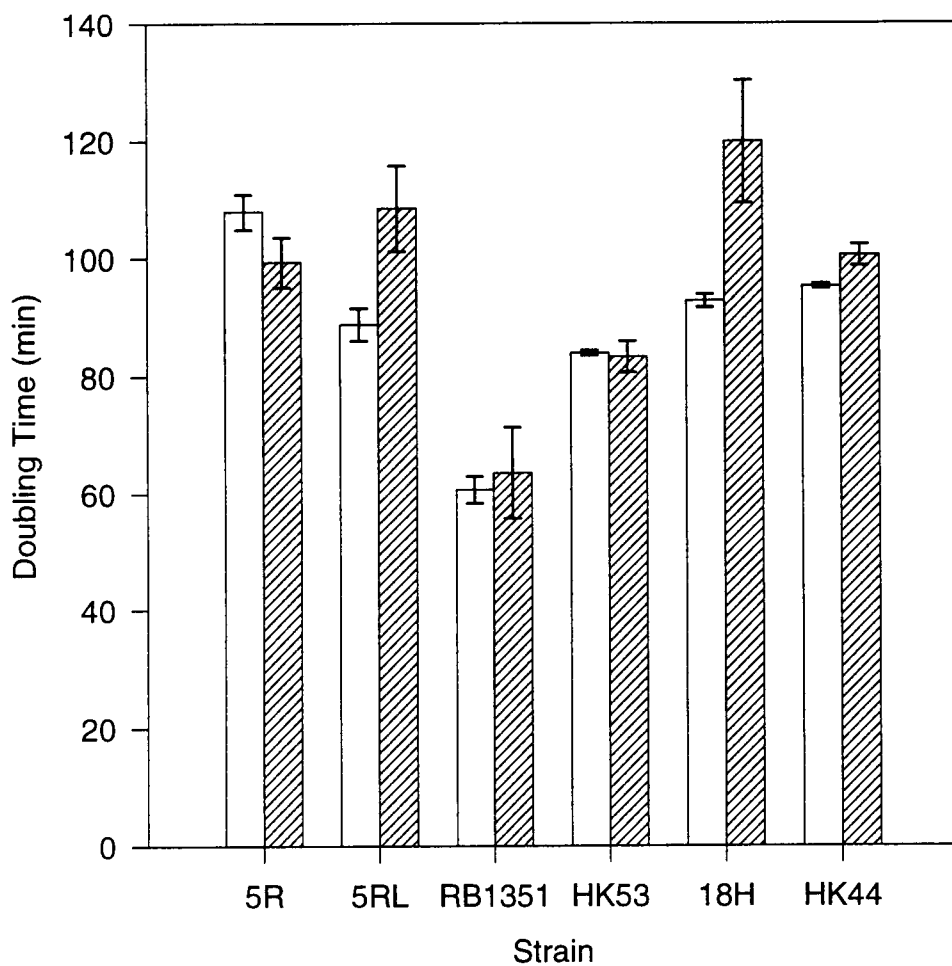


Figure 2-12. Doubling times of the bioluminescence reporter strains and their parental strains. Cultures were grown in one-tenth strength LB and induced with 100 $\mu\text{g/ml}$ salicylate. Open bars represent uninduced cultures and hatched bars represent induced cultures.

1985). This effect was not observed for pUTK21 under the conditions tested. The lone previous study on the stability of naphthalene biodegradative plasmids demonstrated stable maintenance of the *nah* plasmid under glucose limitation in continuous culture but an instability when cultured under salicylate limitation (Boronin et al., 1985). Under salicylate limitation, plasmid-free cells were evident as well as plasmid-bearing cells containing deletion of the *nah* genes in the plasmid. It was unclear how such strains would persist in a continuous culture under salicylate limitation with the ability to utilize the limiting substrate. An *ortho*-pathway for salicylate utilization was not discussed. A similar *nah* plasmid with constitutive expression of the naphthalene biodegradative genes was not maintained under glucose limited continuous culture (Boronin et al., 1985). These results indicate that the plasmids were segregationally and structurally unstable under non-selective conditions and were selected against due to a slower growth rate of plasmid bearing cells.

Plasmid pUTK21 in *P. fluorescens* HK44 was not maintained in a non-selective culture under salicylate limitation. A lack of maintenance was apparent only after a few generations. After 50 generations of culture, 99% of the HK44 population was pUTK21 deficient as determined by spread plating on antibiotic selective agar and DNA probing for *luxAB*⁺ colonies on non-selective agar. There were two possibilities for this observation (Summers, 1991). Either the plasmid was segregationally unstable or the Tn4431 transposon was unstable. Since the population retaining pUTK21 was determined on antibiotic selective media, the measurement was only presumptive for plasmid maintenance. The *luxAB*⁺ cfu/ml did follow the YEPG plus tetracycline cfu/ml

for the three available determinations. This would indicate segregational instability. Colonies chosen at random from the final YEPG plates, were subject to plasmid analysis by endonuclease cleavage and Southern blotting. All colonies harboring pUTK21 were *luxAB*⁺ and those colonies that were pUTK21 deficient were *luxAB*⁻. Also the colonies retaining pUTK21 were tetracycline resistant whereas the colonies that were pUTK21 deficient were tetracycline sensitive. This indicates that the transposon was relatively stable and had not inserted in the chromosome at a measurable frequency, or the isolates would have been tetracycline resistant. The plasmid NAH7 has been shown to harbor a defective transposon, Tn4655, which could be mobilized in the presence of the transposase of Tn1721, the parent transposon of Tn4431 (Tsuda & Iino, 1990). The Tn4655 contained a *cis*-acting (*res*) site and the gene for a *trans*-acting resolvase. The NAH pathway on pKA1 is not contained on a similar transposon since PCR amplification of pKA1 with primers for the inverted terminal repeats of Tn1721 showed no amplified product (Menn et al., 1993). No large scale deletions or rearrangements were noted in the above plasmid isolates cleaved by *Bam*HI also indicating that the plasmid and Tn4431 were stable. Certain catabolic plasmids harboring transposons have been shown to be unstable under non-selective culture due to excision of transposons (Brenner et al., 1993; Keshavarz et al., 1985; Williams et al., 1988; Duetz & van Andel, 1991).

In a situation where the plasmid is not faithfully partitioned to the daughter cell during cell division, the occurrence of a plasmid-free bacterium can lead to take-over of the culture by the plasmid-free population. In general, plasmid bearing bacteria have longer doubling times as compared to plasmid-free bacteria. It has been shown that R-

plasmids larger than 80 kb in size increase the generation time by 15 percent (Zund & Lebek, 1980; Modi et al., 1991) and that naphthalene biodegradative plasmids increase the generation times of *Pseudomonas putida* (Boronin et al., 1985). A mixed culture of plasmid-bearing and plasmid-free bacteria can coexist only if the growth rate of the plasmid-bearing cells is larger than that of the plasmid-free cells (Stephanopoulos & Lapidus, 1988). When considering the growth rate differences between plasmid-bearing and plasmid-free cells, plasmids would not be maintained unless they encoded functions that enhance the fitness of the strain (Dykhuizen & Hartl, 1983; Sayler et al., 1990). This implies that under non-selective conditions plasmid-free cells would have the growth advantage. It seems that pUTK21 in HK44 is not segregationally stable and the occurrence of a plasmid-free daughter cell resulted in takeover of the culture. This contrasts with the maintenance of pUTK21 in 5RL and pKA1 in 5R. No measurable number of plasmid-free cells could be determined under non-selective conditions. This would indicate that faithful partitioning of pKA1 and pUTK21 occurs even under conditions when maintenance does not seem to offer enhanced fitness. No other functions have been described on NAH plasmids other than catabolic pathways. Unknown functions may exist on this plasmid that confer an advantage in the natural host strain, *P. fluorescens* 5RL.

The *nah-lux* bioluminescence reporter plasmid pUTK9 (pUCD615 Ω P_{nah}) harbored in *P. putida* RB1351 demonstrated unstable maintenance in continuous culture. The portion of the RB1351 population retaining pUTK9 fluctuated between 75-95% of the total population. The parent plasmid of pUTK9, pUCD615, contains both a pBR322

origin of replication and a pSA origin of replication. In pseudomonads, the pSA origin allows replication and a copy number of around 4 copies per chromosome equivalent in *Agrobacterium tumefaciens* (Rogowsky et al., 1987). The pSA plasmid belong to the IncW group of plasmids and little is known about their maintenance functions (Valentine & Kado, 1989). The pUCD615 is missing the putative plasmid stability functions present on pSA and as a consequence segregation occurs readily in non-selective medium (Close et al., 1984). It was shown for pUCD2, the precursor of pUCD615, a 2.38 segregants per generation as opposed to stable maintenance of pSA in non-selective medium. The pSA derived plasmids also occur in only 90-95% of cells even under selective pressure. With such a low copy number, the probability of a plasmid-free cell arising is 1 in 8 per cell division (Williams & Thomas, 1992). Low copy number plasmids have been shown to possess active partitioning systems to insure stable inheritance (Williams & Thomas, 1992; Austin, 1988). Such a system has not been described in IncW plasmids but surely must exist since the copy number is so low. The observation that pUTK9 is maintained in a large portion of the population contrasts with the results of pUTK21 in *P. fluorescens* HK44 and the results of Close et al. (1984). The results would suggest that the growth rates of plasmid-bearing and plasmid-free strains may be similar under the continuous culture conditions tested (Fredrickson & Stephanopoulos, 1981). It has been demonstrated for pBR322 grown under non-selective medium, the constitutive expression of tetracycline resistance has an adverse effect on plasmid maintenance in glucose-limited continuous culture but constitutive expression of ampicillin resistance determinants did not exhibit such an effect (Lee & Edlin, 1985). It is possible that the constitutive

expression of kanamycin resistance on pUCD615 does not effect fitness of RB1351. The range in maintenance (75-95%) should be regarded as the deviation of the mean maintenance since measurements of plasmid-bearing and plasmid-free populations were so similar.

There has been much discussion of bacterial competition in substrate-limited continuous cultures (Frederickson & Stephanopoulos, 1981). The outcome of competition experiments for a single limiting substrate is thought to depend on several parameters. These parameters include the maximum specific growth rate, affinity of the competitors for the limiting substrate, and the dilution rate. It has been demonstrated theoretically and empirically that two species cannot coexist in a continuous culture when limited for growth by a single substrate (Gottchall & Thingstad, 1982). Coexistence is possible if noncompetitive interactions exist. The interplay of these factors above can allow coexistence under conditions of periodic transients such as in dilution rate (Pavlou et al., 1990).

Competition experiments were done in order to compare the competitiveness of *nah-lux* bioluminescence reporter strains relative to a wild type naphthalene degradative strain. The hypothesis tested was that the bioluminescence reporter strain could compete with the wild type strain while producing detectable bioluminescence. A tri-culture of 5R, 5RL and RB1351 exhibited maintenance of all three strains. The reporter strains were initially maintained although their numbers declined from the starting inoculum. The wild type strain 5R was maintained throughout the experiment. An increase in dilution rate caused 5RL to nearly washout. This reporter strain was at a competitive

disadvantage since it could only mineralized naphthalene to salicylate. The strains 5R and RB1351 were co-maintained then in a dual substrate limitation, naphthalene and salicylate.

A similar tri-culture was composed of 5R, RB1351 and HK44. The strains 5R and RB1351 were inoculated at similar concentration and were maintained throughout the experiment. *P. fluorescens* HK44 did persist until the dilution rate was increased, upon which time HK44 was washed out. The bioluminescence response to the mixed culture indicated that bioluminescence was sensitive to perturbations in the culture including changes in dilution rate.

Since HK44 was not maintained in the previously described experiment, it was decided to culture HK44 alone to determine maintenance, then add 5R as a competitor. *P. fluorescens* HK44 attained a steady state and was maintained even when the dilution rate was increased. The bioluminescence response was less noisy but did show fluctuation as a response to the change in dilution rate. Upon addition of 5R to the culture, HK44 was co-maintained with 5R. The bioluminescence response decreased and was due to a shunting of reducing power, ATP to other cellular functions, decrease in oxygen concentration in the culture or the long chain fatty aldehyde was required for other cellular purposes, or both.

These results indicated that all three reporter strains could be maintained under certain conditions. Strain RB1351 was tested in a more realistic conditions under which such a strain might be used to monitor pollutant waste streams. A dual substrate culture growing on naphthalene and phenanthrene consisting of RB1351 and an undefined wild

type assemblage. Strain RB1351 was able to compete successfully when the culture was begun with RB1351 having the numerical advantage. Both the undefined assemblage and RB1351 were conceivably competing for both substrates in NAH7 has been shown to mediate biodegradation of phenanthrene (Menn et al., 1993). The bioluminescence response was variable over time at the same dilution rate.

Strain HK44 seemed to be less competitive than RB1351. One distinct difference between the two strains was the manner in which they catabolize salicylate. *P. putida* RB1351 uses the *meta*-pathway of the NAH7 pathway (as does 5R use pKA1), and HK44 uses an undescribed *ortho*-pathway. A mineralization experiment was designed to test the specific rates at which HK44 and 5R mineralize salicylate. Most likely HK44 uses a single copy chromosomal pathway and 5R uses a plasmid pathway with a copy number of 1-6 copies per chromosomal equivalent. Although 5R had a consistently faster rate than HK44, they were statistically equal. The difference between the two rates however small could be considered a competitive disadvantage.

One of the principle reasons for plasmid-bearing strains having slower growth rates over plasmid-free strains has been proposed to be the metabolic burden of plasmid encoded protein production (Bentley et al., 1990). There are several reasons why NAH7, pKA1, and pUTK21 may be a metabolic burden including transfer functions which have been shown to be derepressed in TOL (Bradley & Williams, 1982) and for the reporter strains the production of *lux* associated protein. The energy for bioluminescence production was shown to be 0.007% of the total energy expended in a culture of *Vibrio harveyi* (Makemson & Gordon, 1989). Instead the metabolic burden would seem to be

the production of the *luxCDABE* proteins. Growth experiments were conducted to determine the effect of induction of the bioluminescence genes had on growth rate. Although there was no statistical difference between induced and uninduced cultures, 5RL had a longer generation time over 5R (not different) as did 18H and HK44. There were no differences for HK53 and RB1351. These paired comparisons demonstrate differences that could make bioluminescence reporter strains less competitive.

The results presented demonstrate that bioluminescence reporter strains can successfully compete under certain defined conditions with concomitant production of bioluminescence. The bioluminescence response was sensitive to perturbations such as dilution rate and competition by other bacteria. The naphthalene biodegradative plasmid isolated from the environment and mutagenized with Tn4431 to produce a reporter plasmid was the most segregationally stable in the natural host as compared to a foreign host or the use of promoter probe vectors. This work should lay a foundation in stability and maintenance for future work using bioluminescence as a reporter of biodegradation in mixed substrate and mixed culture environments.

PART 3

CONSTRUCTION OF A CHROMOSOMAL MARKER SYSTEM FOR STUDYING THE FATE OF A NAPHTHALENE DEGRADING STRAIN AND CATABOLIC PLASMID IN SOIL ENVIRONMENTS

Introduction

Studying the fate of individual bacterial strains and plasmids in different environments has become necessary with respect to risk assessment issues in the utilization of genetically modified microorganisms (GMOs). Risk assessment of GMOs in the environment requires information about persistence, survival, growth, and transfer of recombinant DNA to indigenous microbiota. Such information deals with fundamental issues in microbial ecology and technological advances in gathering these data will greatly enhance our understanding of microbial ecology (Prosser, 1994). An area largely neglected in microbial ecology has been the study of the fate and transfer of catabolic plasmids in the environment. This lack of information is largely due to the difficulty in distinguishing the plasmid of interest from others in the same environment as well as the host strain from indigenous bacteria.

Many methods for monitoring and following microbial populations and plasmids have been reported. Methods conferring a distinctive phenotype include GUS (Jefferson, 1989), antibiotic resistance, *lacZ* (Drahoš et al., 1986; Hofte et al., 1990), *xyIE* (Winstanley, 1989; Macnaughton et al., 1992), TFD monooxygenase (King et al., 1991) *moc* (Hwang & Farrand, 1994) and bioluminescence (Amin-Hanjani et al., 1993; Shaw & Kado, 1986; Shaw et al., 1992). Immunodetection has been proposed (Zaat et al., 1994;

Ramos-Gonzalez et al., 1992) although the utility of this approach in high organic carbon content soil remains to be proven. Other methods depend on DNA hybridization for detection and include strain-specific rRNA probes (Boye et al., 1995), colicin-A (Zaat et al., 1994) and a synthetic DNA sequence (Amici et al., 1991). Regardless of the marker system chosen, it must be unique to the environment studied. Much has been made of direct selection based on a unique phenotype. Phenotypic expression may underestimate population numbers (Genthner et al., 1990) is generally dependent on culture methods. Phenotype may also confer a fitness disadvantage to the marked strain and make hypothesis testing difficult. Also the marker system must remain molecularly stable in the host strain.

A dual marker system consisting of a chromosomal marker composed of a eucaryotic segment of DNA consisting of an actin intron from a yeast and a plasmid marker composed of a *lux* cassette is proposed that will enable sensitive detection of host and plasmid DNA in a naphthalene degrading bacterium. The naphthalene degrader, *Pseudomonas fluorescens* 5R, is an environmental isolate from a manufactured gas plant (MGP) soil. Strain 5R harbors pKA1, a NAH7-like naphthalene degradative plasmid (King et al., 1990). This strain can grow on naphthalene as a sole source of carbon.. A bioluminescence reporter strain, *P. fluorescens* 5RL, is strain 5R containing a Tn4431 mutagenized version of pKA1 (pUTK21) and is capable of degrading naphthalene to salicylate (King et al., 1990). The bioluminescence cassette is inserted into *nahG* (Tn4431::*nahG*) and creates a reporter system for naphthalene and salicylate biodegradation (Menn et al., 1994). The bioluminescence genes were derived from

Vibrio fischeri and are not found in soil. The Tn4431 (*lux*) insertion makes a sensitive marker for pUTK21. The Tn4431 insertion in pUTK21 has been shown to be stable in this host (Part 2).

The marking of the chromosome of *P. fluorescens* 5RL with a eucaryotic DNA sequence that is not found in MGP type soils is demonstrated in this paper. The marker sequence is a actin intron fragment (referred to as AI) from *Candida albicans* that will be delivered on a mini-Tn5 transposon. This DNA sequence was chosen because it is from a non-coding region and is not conserved between *Candida albicans* and *Candida tropicalis* which are not found in soil environments. This marker will provide the ability to examine strain persistence and transfer of pUTK21 *in situ* where no information is needed about possible recipient populations. Additionally there is no phenotype associated with the AI and there should be no reduction in strain fitness due to the marker. Although broad spectrum mercury resistance is associated with the mini-Tn5 transposon and tetracycline resistance is associated with Tn4431, both are inducible systems. Both AI and *lux* are detectable by hybridization and PCR allowing differing levels of sensitivity as required.

Materials and Methods

Strains

All strains and plasmids used in this study as well as their sources are described in Table 1.

Table 1. Relevant Characteristics of Strains and Plasmids.

Strain	Plasmid	Description	Source
<i>E. coli</i> S17 λ <i>pir</i>	none	contains λ <i>pir</i> for R6K replication	Herrero et al. (1990)
<i>E. coli</i> DH5 α	none	ϕ 80d <i>lacZ</i> AM15 <i>endA1</i> <i>hsdR</i> 17 <i>recA1</i> <i>deoR</i> <i>relA1</i>	Gibco/BRL
	M13mp9-AI	Ap ^r ; contains 2 kb Actin intron/exon	Bagash (1989)
	pCR-Actin	Ap ^r ; Km ^r ; ¹ contains 0.385kb AI	This study
	pUC18-AI	Ap ^r ; AI flanked by <i>NotI</i> sites	This study
	pUTHg	Ap ^r ; Hg ^r , Tn5-based delivery plasmid;	Herrero et al. (1990)
	pUTHg-AI	pUTHg containing AI	This study
<i>P. fluorescens</i> 5RL	pUTK21	Tc ^r ; ² Nah ⁺ ; ³ Sal ⁻ ; ⁴ Lux ⁺	King et al. (1990)
<i>P. fluorescens</i> 5RLA	pUTK21	derivative of 5RL::Tn5(Hg-AI); ⁵ Nal ^r	This study

¹Actin intron fragment

²Able to oxidize naphthalene to salicylate

³Salicylate oxidation pathway blocked by Tn4431 in *nahG*

⁴Bioluminescence genes (*luxCDABE*) contained in Tn4431 300 bp downstream of *nahG*

⁵Resistance to nalidixic acid (50 μ g/ml)

Media and Reagents

Yeast extract-peptone-glucose medium (YEPG) contains (g/l): yeast extract, 0.2; peptone, 2.0; glucose, 1.0; and NH_4NO_3 , 0.2. Luria-Bertani (LB) medium has been described previously (Maniatis et al., 1989). Plates were made by adding 1.6% Bacto-Agar (Difco Laboratories). Mineral salts medium has been described previously (Meynell & Meynell, 1965). D-glucose (USP grade, Mallinckrodt) was used in minimal medium at 100 $\mu\text{g/ml}$. Ampicillin and tetracycline (Sigma Chemical Co.) were used in plates at a concentration of 100 $\mu\text{g/ml}$ and 14 $\mu\text{g/ml}$ respectively. Mercuric chloride (EM Science), 99.5+ % pure, was used in liquid medium (15 $\mu\text{g/ml}$) and agar plates (5 $\mu\text{g/ml}$) in concentrations based on calculation of Hg^{2+} ion. The oxidation of indole (Aldrich Chemical Co.) to indigo was used as a presumptive indicator of naphthalene dioxygenase (Ensley, 1983) and was incorporated into YEPG agar plates at 100 $\mu\text{g/ml}$.

Recombinant DNA Techniques

Endonucleases, T4 DNA ligase and DNA molecular size marker (Gibco/BRL) and calf intestinal alkaline phosphatase (Promega Corp.) were used according to the manufacturers protocols. Polymerase chain reaction (PCR) was done using a GeneAmp PCR kit (Perkin Elmer Applied Biosystems). The PCR product was directly cloned into the pCR vector containing a T-overhang with the TA Cloning Kit (Invitrogen Corp.) using the manufacturers protocol. Transformants from ligations were plated onto LB agar containing 100 $\mu\text{g/ml}$ Ampicillin with 2 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) spread over the surface (BioWorld) for α -complementation with

blue/white screening for inserts. White colonies were screened for inserts by plasmid mini-preparation (Holmes & Quigley, 1981). DNA fragments for cloning were gel purified and recovered by electroelution (Maniatis et al., 1989). Large scale plasmid DNA extractions were done using alkaline lysis (Promega Corp.) and preparations were purified by cesium chloride-ethidium bromide density gradient centrifugation (Maniatis et al., 1990). Transformation of *E. coli* DH5 α was done following the manufacturers protocol (Gibco/BRL). Electroporation was used to introduce the final construct (pUTHg-AI) into *E. coli* S17 λ pir using a Model ECM 600 electroporator (BTX Inc.) following the manufacturers suggestions. The plasmid pUTHg-AI was mated into *P. fluorescens* 5RL in liquid culture. Log phase cells of the recipient and donor were adjusted to an A₅₉₅ of 0.6 and mixed in a ratio of 1:10 respectively. The cells were pelleted in a microfuge tube and resuspended in 50 μ l of YEPG and incubated at 28°C overnight. The culture was spread plated onto YEPG containing mercuric chloride (5 μ g/ml) and tetracycline (14 μ g/ml) and incubated overnight at 28°C.

Construction of Delivery Plasmid and Chromosomal Insertion

A 0.385 kb portion of the 2 kb actin intron/exon of M13-AI (Bagash, 1989) was obtained by PCR. Amplification was done using a model 9810 thermocycler (Perkin Elmer Applied Biosystems) and with a GeneAmp PCR kit (Perkin Elmer Applied Biosystems). The previously published DNA sequence of the actin intron/exon (Bagash, 1989) was used to design the forward primer (#621) 5'-GTCGACATAAATAGATCTC GTTTGGG-3' and the reverse primer (#907) 5'-TGGAGCCGGGAGATCTGTATAGT

CTAGA-3'. Oligonucleotides were made on an Oligo-1000 DNA synthesizer (Beckman Instruments, Inc.). The PCR amplification conditions consisted of an initial melting at 95°C for 5 min. followed by strand melting at 95°C for 1.0 min., primer annealing at 55°C for 30 sec. and extension at 72°C for 30 sec. for 38 cycles. A 0.38 kb fragment was confirmed by electrophoresis on a 2% agarose gel. The PCR product was directly cloned into pCR to give pCR-Actin. Blue-white screening was used to detect transformants containing inserts which were confirmed by endonuclease digestion with *EcoRI*. The actin intron fragment contained in pCR-Actin was subcloned as an *EcoRI* fragment into dephosphorylated pUC18-Not to flank it with *NotI* sites. Blue-white screening was used to detect transformants containing inserts which were confirmed by endonuclease mapping with *XbaI*. The actin intron fragment was then cloned into the unique *NotI* site in pUTHg. The pUTHg was linearized with *NotI* and dephosphorylated. Transformants were screened by endonuclease digestion with *XbaI* (pUTHg and AI have a unique *XbaI* site). A clone containing the actin intron fragment was picked and the plasmid, pUTHg-AI, purified for further endonuclease mapping for confirmation. The delivery plasmid was mated into *P. fluorescens* 5RL and transconjugants were screened on YEPG containing mercuric chloride (5 µg/ml) and tetracycline (14 µg/ml). Confirmation of a chromosomal insertion achieved by slot blotting plasmid and chromosomal DNA with a Bio-dot SF manifold (Bio-Rad Laboratories) and probing with AI specific DNA probe. Chromosomal DNA was obtained by the method of Wilson (1990) and purified by ethidium bromide-caesium chloride density gradient centrifugation (Maniatis et al., 1989).

Stability of Chromosomal Insert

A Bioflow C30 chemostat (New Brunswick Scientific) with a Teflon headplate and Teflon tubing was used for continuous culture of *P. fluorescens* 5RLA to determine the stability of the actin intron chromosomal marker. Medium was supplied using a peristaltic pump equipped with a Teflon head (Fluid Metering, Inc.). Cultures were aerated continuously at 0.2 L/min and agitated at 250 rpm at 25°C. A buffered mineral salts medium (Meynell & Meynell, 1965) containing 100 µg/ml D-glucose (USP, Mallinckrodt) was used as a feed stock. The culture vessel, pump head and associated tubings were sterilized by autoclaving. Inoculation was done aseptically by syringe. The culture was monitored by spread plating culture aliquots on ¼-strength YEPG agar alone and containing indole or tetracycline. The ¼-strength YEPG plates containing indole were colony blotted onto 0.45 µm Biotrans nylon filters (ICN Biomedicals, Inc.). The colonies were lysed and the DNA denatured with 0.2 N NaOH, and the filters were baked at 80°C for 1 hr. All filters were prehybridized using the method of Church and Gilbert (1984). Colony blots were probed with single stranded ³²P-AI specific DNA. The plasmid pCR-Actin was used to obtain vector-free double stranded template DNA. This double stranded template was used along with one of the oligonucleotide primers and amplification conditions described above and ³²P-αdCTP to produce single stranded probe. The probe was purified through a NucTrap column using a Pushcolumn apparatus following the manufacturers protocol (Stratagene Cloning Systems). All hybridizations and washings were done at high stringency at 65°C (Sayler et al., 1985). Positive signals were detected using autoradiography.

Results

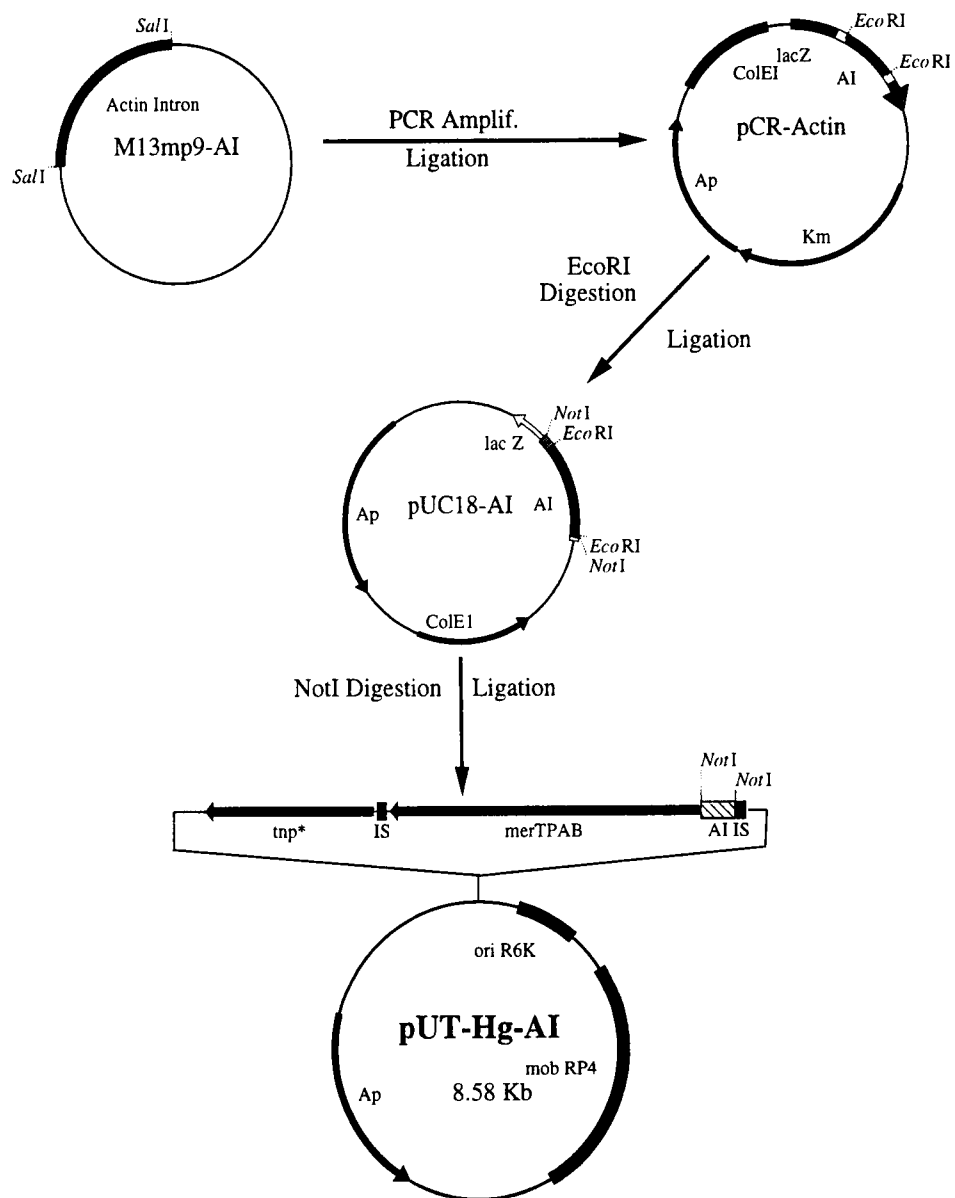
Characterization of *P. fluorescens* 5RLA

The actin intron marker delivery plasmid, pUTHg-AI was constructed as shown in Figure 3-1. The plasmid pUTHg-AI was mated into *P. fluorescens* 5RL and putative chromosomally marked isolates screened. The isolates were exposed to naphthalene to ensure that bioluminescence was produced (King et al., 1990). Three colonies produced visible light when exposed to naphthalene vapor and were chosen for further analysis. All three isolates were also capable of growing on minimal agar containing 10 mM succinate. This observation indicated that the transposon carrying AI did not insert into biosynthetic or catabolic pathways required for growth on minimal media.

The three putative insert strains were colony blotted and probed with AI specific DNA probe. The results are shown in Figure 3-2. All three insert strains probed positive for actin intron. The positive control (*E. coli* pUTHg-AI) and negative control (*P. fluorescens* 5RL) demonstrated that the probe was specific for the AI marker and did not hybridize with bacterial DNA sequences.

Chromosomal and plasmid DNA preparations were prepared from putative insert strain number one from Figure 3-2 as well as *P. fluorescens* 5RL. Six micrograms of each DNA was slot-blotted and probed with AI specific DNA probe (Figure 3-3). The 5RLA chromosomal DNA was positive for AI whereas the 5RL chromosomal DNA was negative. The reporter plasmid, pUTK21, was also negative in *P. fluorescens* 5RL and

Figure 3-1. PCR amplification and subcloning of a portion of the actin intron to produce a suicide delivery plasmid pUTHg-AI. A 0.385 portion of the actin intron/exon was obtained by PCR and direct cloned into pCR creating pCR-Actin. A *EcoRI* fragment was subcloned into pUC18-Not to flank the fragment with *NotI* sites creating pUC18-AI. The AI fragment was cloned into the unique *NotI* site in pUTHg to create pUTHg-AI. Abbreviations: AI, actin intron; Ap, ampicilin resistance; Km, kanamycin resistance; Hg, broad spectrum mercury resistance; tnp, transposase; IS, insertion sequence. Arrows indicate direction of transcription.



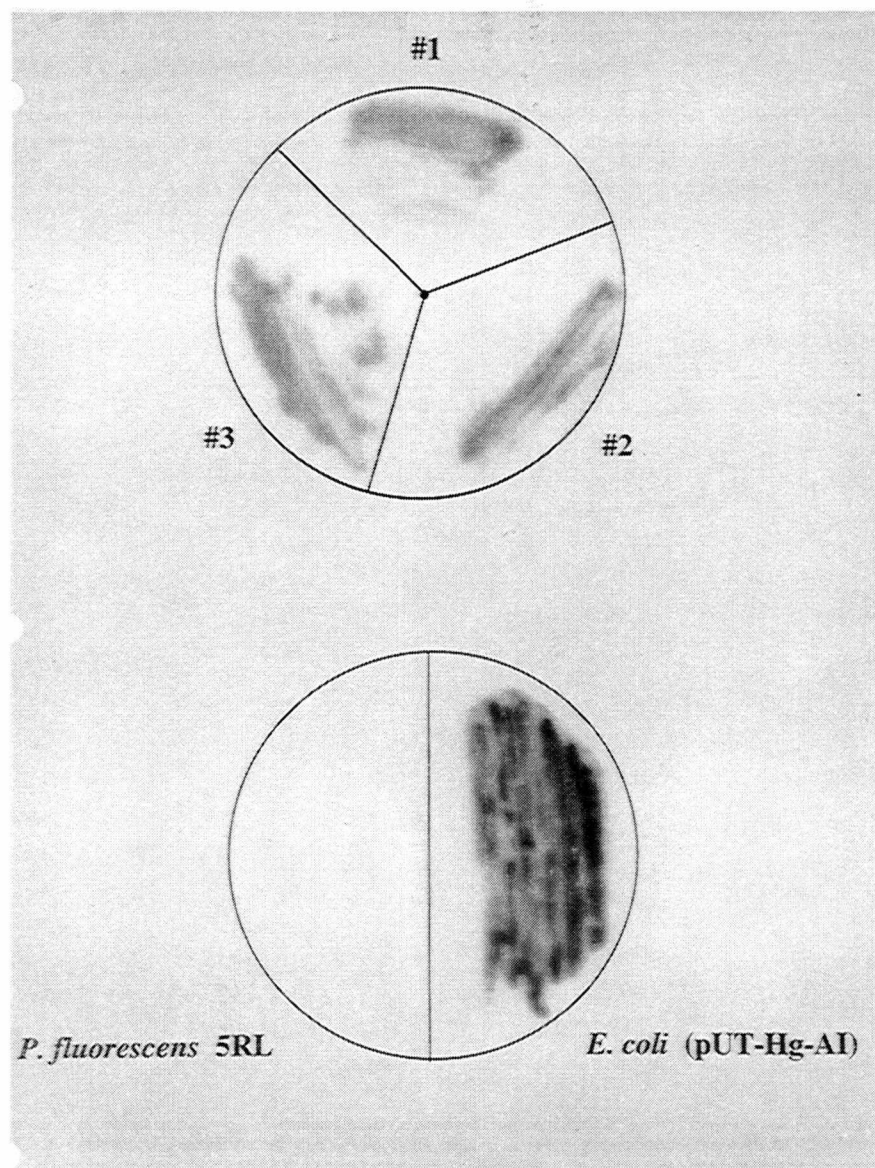


Figure 3-2. Colony blots of putative AI chromosomal insert strains of *P. fluorescens* 5RL probed with AI specific DNA probe. The positive control was *E. coli* (pUTHg-AI) and the negative control was *P. fluorescens* 5RL.

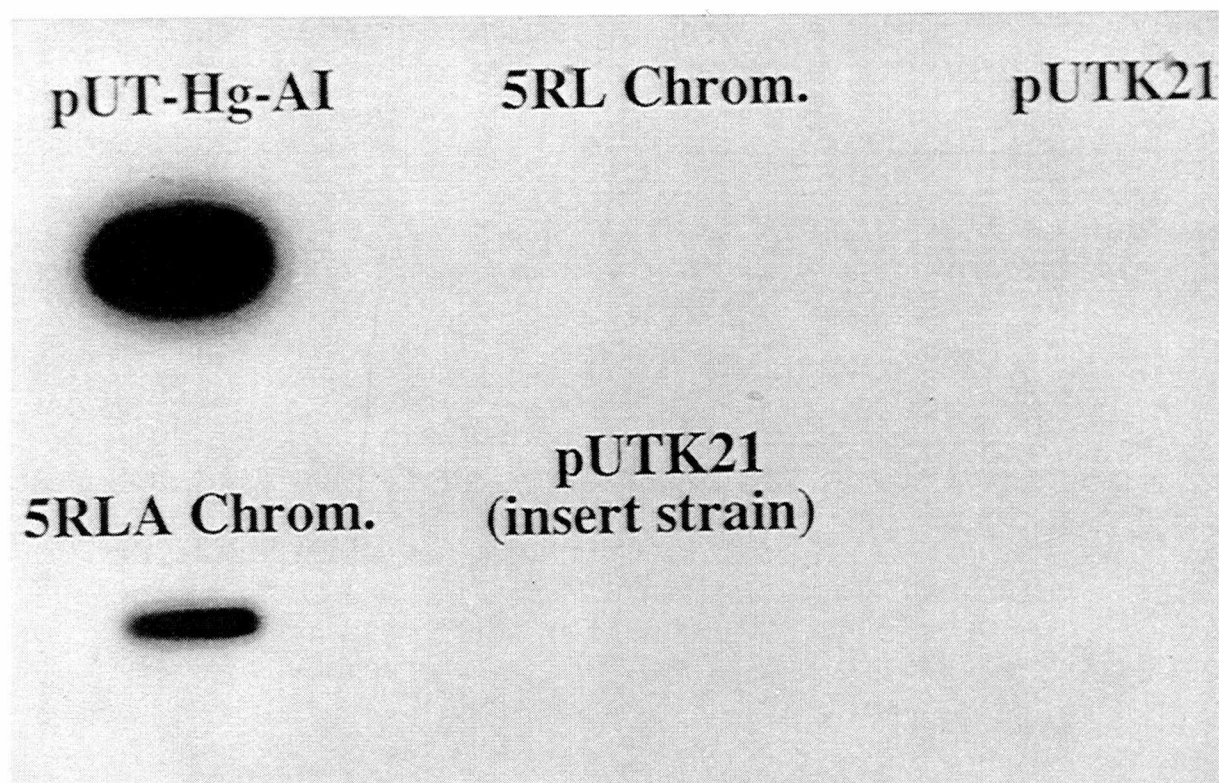


Figure 3-3. Slot blot of total chromosomal and plasmid DNA from insert strain P.

fluorescens 5RLA and the parent strain P. fluorescens 5RL. The blot was hybridized with actin intron specific DNA probe. The Tn5Hg-AI delivery plasmid was used as a positive control.

5RLA. These results indicate that the insert was in a chromosomal location in 5RLA and not in the plasmid (pTUK21) or in both locations.

Stability of the AI Chromosomal Insert

The AI chromosomal marker was maintained in *P. fluorescens* 5RLA in glucose limited continuous culture for 160 generations at a dilution rate of 0.171 hr^{-1} . The population of 5RLA was maintained at a concentration of 7.4×10^6 to 5.3×10^7 cfu/ml (Figure 3-4A). All colonies on YEPG plates containing indole were indigo positive indicating complete maintenance of pUTK21. The YEPG plates containing indole were colony blotted and probed with AI specific DNA probe (Figure 3-4B). The AI⁺ population correlated with the total population in Figure 3-4A and all colonies probed AI positive at the conclusion of the experiment. The population determination based on tetracycline resistance conferred by pUTK21 is shown to underestimate the *P. fluorescens* 5RLA population (Figure 3-4A).

Discussion

The successful introduction of a unique eucaryotic DNA segment (AI) into the chromosome of *P. fluorescens* 5RL was demonstrated. The AI marker was introduced into the chromosome on a mini-Tn5 transposon conferring broad spectrum mercury resistance (Herrero et al., 1990). The transposase is external to the transposon on the delivery plasmid. The mini-Tn5 transposon is contained on a suicide delivery plasmid

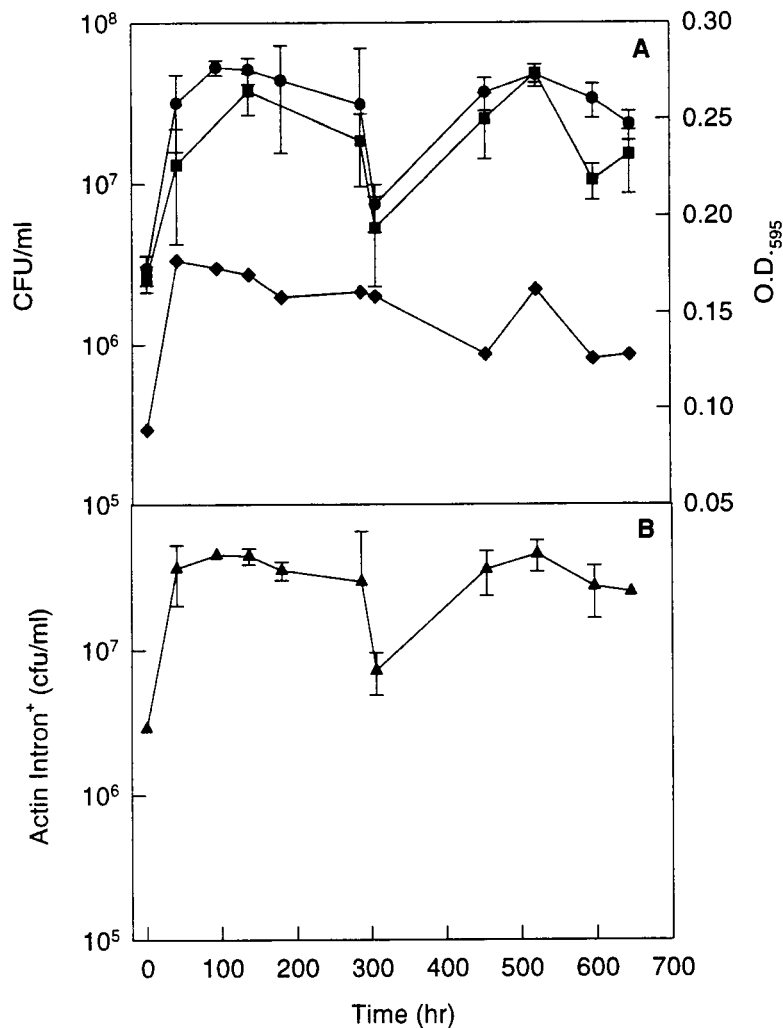


Figure 3-4. Maintenance of the AI chromosomal marker and the reporter plasmid pUTK21 in *P. fluorescens* 5RLA growing in continuous culture on 100 ppm glucose in minimal media at a dilution rate of 0.171 hr^{-1} . A. The total culture population was monitored on YEPG plates containing 100 $\mu\text{g/ml}$ of indole and the population retaining pUTK21 was monitored on YEPG plates plus tetracycline (14 $\mu\text{g/ml}$). Total cells (●), cells retaining pUTK21 (■), and OD₅₉₅ (◆) are shown. B. The AI marker was monitored by colony blotting YEPG plates.

based on the R6K system of replication. The π -protein required for R6K replication is maintained as a lysogenic insert on the chromosome of *E. coli* S17 λ pir. Insert strains retained the ability to produce bioluminescence when induced by naphthalene, a first indicator that the transposon had not inserted into pUTK21. The probing of colony blots with AI specific DNA demonstrated that the strains contained the AI marker and that there were no DNA sequences in 5RL that cross hybridize under high stringency conditions. Slot blots of total chromosomal and plasmid DNA from strains 5RL and 5RLA showed that the AI insert was contained in the chromosome of 5RLA and not pUTK21 or both locations. These data do not however allow determination of copy number, which will be determined in the future. Thus the system is composed of two highly specific DNA markers detectable by hybridization or PCR.

Direct selection by specific markers as a means of detecting GMOs *in situ* has a number of drawbacks such as expression of a phenotype which if not tightly regulated, may confer a reduced fitness to the host bacterium. Also the use of phenotype to detect populations may result in an underestimate of the population concentration which was observed in this work and reported by others for antibiotic resistance (Genthner et al., 1990). This underestimation was not observed during plating experiments for a unique catabolic marker (Hwang and Farrand, 1994) but also was not demonstrated for recovery from soil. Also phenotypic markers tend to be present in many environments and thus limit specificity.

The strategy put forth in this work utilizes a bioluminescence (*lux*) marker on a naphthalene biodegradative plasmid (pUTK21) and a eucaryotic DNA sequence from *C.*

albicans as a chromosomal marker. Neither the AI marker nor *lux* has been found in MGP soil (data not shown). The only bioluminescence encoding DNA found in soil is from *Xenorhabdus luminescens*, an insect pathogen (Schmidt et al., 1989). The percent similarity between the subunits of the luciferases from *Vibrio fischeri* and *Xenorhabdus luminescens* is 66% for *luxA* and 52% for *luxB* (Meighen, 1991). Thus *lux* genes from *Vibrio fischeri* can provide a unique DNA marker in soil. Both markers are associated with a selectable marker. The AI marker is associated with broad spectrum mercury resistance on a mini-Tn5 transposon and *lux* is associated with tetracycline resistance on Tn4431. Additionally, exposure to naphthalene or salicylate results in production of bioluminescence. Since it is often desirable to have estimates of cultivable bacteria, these antibiotic and mercury resistance markers coupled with colony blotting and DNA probing with AI and/or *lux* specific probes make such estimates possible and with good specificity. Such direct selection may underestimate the population of 5RLA as shown in figure 4A for tetracycline resistance and as shown by other workers (Genthner et al., 1990). Bioluminescence would be a good phenotypic marker for pUTK21 only in the host 5RLA strain. Plasmid transfer to indigenous bacteria may result in the plasmid not being detected by inducible light since not all bacterial strains are good hosts for the bioluminescence reaction (Burlage et al., 1990).

The AI insertion is stable as demonstrated in figure 3-4A-B where the insertion was maintained for 160 generations in glucose limited continuous culture. The AI marker is carried on a mini-Tn5 transposon and does not contain a transposase within the composite transposon. The use of an inactivated transposon does not preclude excision

since a related transposon could provide transposase in *trans*. Also the pUTK21 lux marker contained on Tn4431 has been shown to be stable both molecularly and segregationally under non-selective conditions when *P. fluorescens* 5RL was grown in continuous culture (Part 2). Since the AI insert is in the chromosome, transmission to other bacteria would be unlikely.

The marker system described in this work meets the criteria for a sensitive, stable and specific tracking system. The AI chromosomal marker is derived from a phylogenetically distant species, *Candida albicans*, which is a medically important yeast and not common to soil environments. The bioluminescence marker is derived from a marine species of bacteria and is also not found in soil environments. Bioluminescence is found in terrestrial habitats in *Xenorhabdus luminescens*, an insect pathogen. Therefore the markers are unique to the soil environment and provide a specific tracking system.

The AI marker is a non-coding segment of DNA and should not affect the fitness of the host. The *lux* marker is transcribed when induced by naphthalene or salicylate. The mercury and tetracycline resistance markers are inducible as well. Additionally both the Tn5-AI and Tn4431 (*lux*) markers have been shown to be stable. Both markers are detectable by DNA hybridization on colony blots or slot blots of total DNA extracts from the environment as well as by PCR. This marker system will allow studies on the fate of an environmentally derived naphthalene degrading bacterium and its biodegradative plasmid in soil environments.

PART 4

FATE OF *PSEUDOMONAS FLUORESCENS* 5RLA AND ITS REPORTER PLASMID FOR NAPHTHALENE BIODEGRADATION IN A MANUFACTURED GAS PLANT SOIL

Introduction

Genetically modified organisms (GMOs) have been proposed for the past decade for use in remediation of contaminated soil and subsurface sites. Most, if not all, of these GMOs were rationalized to be enhanced degraders or contain hybrid catabolic pathways. A different approach was proposed that involved monitoring of naturally occurring biodegradation processes *in situ*. Bioluminescence offers an attractive means of achieving this goal. A cellular reporter system utilizing bioluminescence was developed (King et al., 1990; Burlage et al., 1990) to monitor the biodegradation of naphthalene and salicylate. The plasmid based system utilizes *lux* transcriptional gene fusions where the bioluminescence gene cassette from *Vibrio fischeri* is under the control of the positively regulated *sal* promoter on plasmid pUTK21. This technology offers the advantage of being a non-invasive and non-destructive process offering high sensitivity and real time analysis. The long term goal of this technology is the *in situ* monitoring of PAH biodegradation in waste streams and soil systems. Naphthalene biodegradation has been well characterized, both biochemically and genetically, and is used as a model system for the biodegradation of polycyclic aromatic hydrocarbons (Yen & Serdar, 1988; Schell, 1990; Eaton & Chapman, 1992). Recently NAH7 has been shown to additionally mediate the catabolism of phenanthrene and anthracene (Menn et al., 1993). Also 2-methyl-

naphthalene and 4-methylsalicylate have been shown to induce the Nah system (Leblond, 1995).

A major obstacle in the realization of the potential of such GMOs is the lack of knowledge concerning their fate and effects in the open environment, including the persistence of introduced organisms and the transfer of recombinant DNA to indigenous populations. From a utility standpoint, the issues of strain survival and the persistence and molecular stability of the recombinant DNA are critical for successful utilization of GMOs. A substantial amount of research has been undertaken to examine the types and extent of gene transfer (Miller & Levy, 1989) and develop methods to monitor specific genotypes (Jain et al., 1988; Prosser, 1994). After years of studying gene transfer and strain survival/persistence, field research using GMOs is evaluated on a case-by-case basis. Official risk assessments ask questions concerning survival, growth, dispersal, effects on indigenous species, and about the genetic modification (Williamson, 1992). The use of defined molecular markers for strains and recombinant DNA should provide clear information about these concerns. The use of molecular ecology to examine these concerns would provide information to satisfy risk assessment questions but would also be far reaching in advancing microbial ecology (Williamson, 1992).

A marker system was developed for the naphthalene bioluminescence reporter strain *P. fluorescens* 5RL (Part 3). The reporter plasmid already contained a unique marker for soil studies by use of the *lux* genes. A unique marker has long been a technical barrier to studying transfer of catabolic plasmids in the environment. Chromosomal markers for the host strains have included mutations for rifampicin and

nalidixic acid resistance and auxotrophic markers. While useful for many studies, these markers are not well suited for environmental studies. Markers with a distinct DNA sequence are useful for environmental studies and an actin intron fragment from *Candida albicans* was used to mark the chromosome of 5RL to produce strain 5RLA (Part 3). With markers for the host strain and the reporter plasmid, questions concerning strain persistence and plasmid transfer could be addressed in an environmentally relevant system.

Factors affecting the persistence and survival of bacteria in soil have been examined for some time (Stotzky & Babich, 1986). Survival and plasmid transfer have been studied in many different soils under many different conditions. The survival of a GMO should be studied in a soil in which it would be used. The use of non-sterile, pollutant impacted, soil should give a reasonable estimate of how a strain would compete and survive against indigenous microbiota. The bioluminescence reporter strain *P. fluorescens* 5RL would best be studied in a manufactured gas plant (MGP) soil, which would be the type of soil in which the strain would be applied.

The work described here concerns the survival of strain 5RLA and plasmid transfer of pUTK21 in an MGP soil. Simple homogenous microcosms (Trevors, 1988) were used to examine the effect of naphthalene on these processes. The specific objectives of this work were to determine the persistence of *P. fluorescens* 5RLA and its reporter plasmid for naphthalene biodegradation in a environmentally relevant soil system and determine the possibility and extent of transfer of pUTK21 to indigenous soil bacteria.

Materials and Methods

Strains

The strains used in this study are shown in Table 1. *Pseudomonas fluorescens* 5RLA is an environmental isolate from a manufactured gas plant (MGP) soil. This strain harbors a NAH7-like naphthalene degradative plasmid, pUTK21 (King et al., 1990). The plasmid contains a Tn4431 insertion in *nahG* within the lower *nah* pathway (Menn et al., 1994). This strain can grow on naphthalene as a sole source of carbon through the metabolism of naphthalene to salicylate. *Pseudomonas fluorescens* 5RLA also contains a chromosomal marker composed of a eucaryotic segment of DNA (mini-Tn5 actin intron insertion) unique to the MGP soil used in this study (Part 3). *P. putida* PB2440(Nal) is a spontaneous nalidixic acid resistant mutant of *P. putida* 2440 (Franklin et al., 1981). This strain is restriction minus and has been shown to be a good recipient for large plasmids (Franklin et al., 1981; King et al., 1990; Sanseverino et al., 1993).

Soil

Soil from a MGP (soil N) site in the northeastern United States was used in the microcosm experiments (Sanseverino et al., 1993). The soil was air dried (sufficient to sieve but not completely dry) and sieved (3.35 mm). Sodium bromide (25 mg/kg) was used as a tracer for homogenization. The soil was homogenized for 6 hr in a Hobart commercial kitchen mixer. Four random 5 g soil samples were removed and combined with 45 ml distilled water in a 50 ml centrifuge tube and vortexed for 5 min. The mixture was centrifuged at 1,520 x g at 25°C for 10 min. in a Model TJ-6 centrifuge (Beckman

Table 1. Relevant Characteristics of Strains and Plasmids.

Strain	Plasmid	Description	Source
<i>P. fluorescens</i> 5RLA		¹ Al ⁺ ² Hg ^R	Part 3
	pUTK21	³ lux ⁺ ⁴ Tc ^R	King et al. (1990)
<i>P. putida</i> PB2440	None	⁵ NaI ^R <i>hsdR1 hsdM</i> ⁺	Franklin et al. (1981)

¹ Actin intron fragment

² Resistance to broad spectrum mercurial compounds

³ Produces bioluminescence when induced by salicylate

⁴ Resistance to tetracycline (14 µg/ml)

⁵ Resistance to nalidixic acid (50 µg/ml)

Instruments Inc.). Ten milliliters of supernatant were removed and the bromide concentration determined with a bromide specific electrode (Orion Corp.) on a Model EA920 ISE/pH meter (Orion Corp.). Analytical reagent grade sodium bromide (Mallinckrodt) was used as a standard for calibration.

Particle analysis was done by standard method (Plant and Soil Science Manual). One hundred grams (dry wt.) of sieved soil was covered with ddH₂O and oxidized with 20 ml of 30% H₂O₂ combined with heating to destroy the organic matter. The soil particles were dispersed with 20 ml of 2.5 N sodium hexametaphosphate. The soil was transferred to a sedimentation cylinder and shaken to disperse the soil particles. Specific gravity was measured over time with a hydrometer.

Artificial naphthalene contaminated soil

A portion of the MPG soil was impacted with naphthalene using supercritical CO₂ deposition of naphthalene (Justin Shingleton, Chemical Engineering, Univ. Tennessee). The final naphthalene concentration was ca. 5000 µg/g of soil. The soil was stored at 4°C in a screw-cap bottle.

Microcosms

To minimize sample error, microcosms were designed in an end-point batch fashion as shown in Figure 4-1. The moisture of the sieved soil was adjusted with ddH₂O

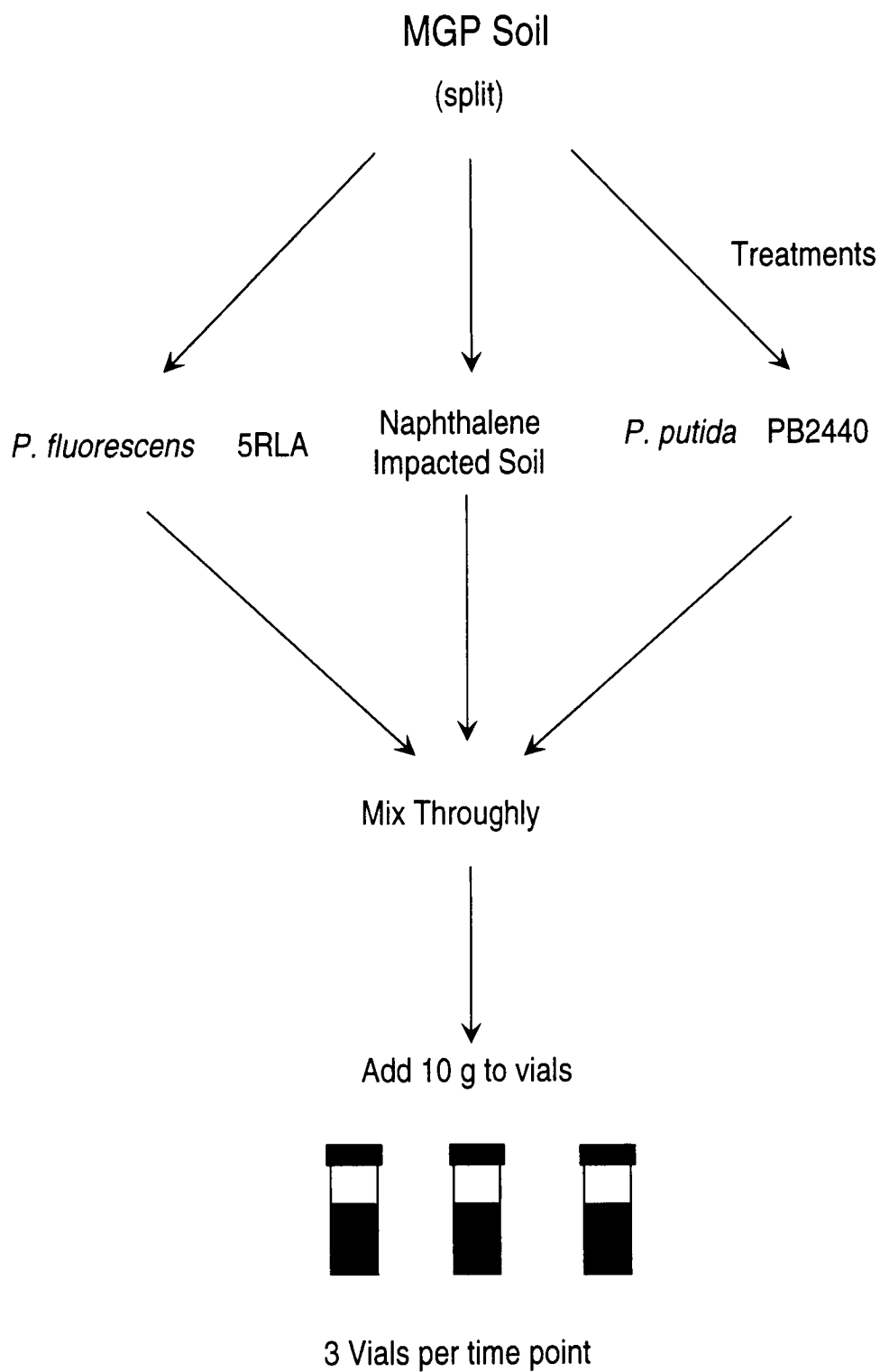


Figure 4-1. Generalized diagram of strain inoculations and amendments to MGP soil.

to give a final moisture content of 18% (final moisture including amendments) and divided into portions to receive treatments. Soil was then placed in plastic trays lined with polypropylene bags. *P. fluorescens* 5RLA was grown in YEPG with tetracycline (14 µg/ml) to an OD₅₉₅ of 0.3 - 0.35 and *P. putida* PB2440 in YEPG with nalidixic acid (50 µg/ml) to an OD₅₉₅ of 0.3. The cells were harvested by centrifugation at 3,840 x g at 25°C for 10 min. and the pellet washed twice with minimal salts (Meynell & Meynell, 1965) and resuspended in minimal salts. Strains were inoculated into the soil at 10⁷-10⁸ cfu/g by pipetting the cell suspension over the soil and mixing thoroughly. Each strain was mixed into a separate portion of soil and the two portions mixed together so as to prevent mating associated with inoculation. The naphthalene impacted soil was incorporated the same way by mixing in a portion of pre-moistened impacted soil to give a final naphthalene concentration of 1000 µg/g of soil. MGP soil for sterile control microcosms was gamma irradiated at greater than 5 Mrad total dosage (Isomedix, Groveport OH). Ten grams of soil (wet wt.) were placed in 50 ml glass EPA vials (Pierce Chemical Co.) with Teflon lined caps and incubated at 25°C in the dark.

Population Monitoring

Soil samples (10 g) were combined with 90 ml of 0.1% sodium pyrophosphate (Balkwill, et al., 1977) in milk dilution bottles (Kimax) and agitated on a wrist-action shaker for 10 min. (Ron Burrell Corp., Pittsburgh PA). Individual bacterial populations were monitored in soil microcosms by spread plating on ¼-strength YEPG agar containing antibiotics (Table 1). A dilution series was performed in 0.1% sodium

pyrophosphate on each of three microcosms for each treatment at each time point. For experiments where a recipient strain (PB2440) was added, a separate dilution series was prepared for each individual microcosm and nalidixic acid (50 µg/ml) was added to prevent dilution tube and plate mating artifacts when plating onto nalidixic acid containing agar medium (Walter et al., 1991). In microcosm experiments where no recipient was added, PB2440 was added to a 10^{-1} dilution blank (10^8 cfu/ml) of one microcosm. An aliquot was plated onto YEPG agar containing nalidixic acid (50 µg/ml) and tetracycline (14 µg/ml) to detect the possibility of dilution tube mating. DNA probing of colony blots was used to enumerate *P. fluorescens* 5RLA and possible transconjugants. YEPG is composed of (g/l): yeast extract, 0.2; peptone, 2.0; glucose, 1.0; and NH_4NO_3 , 0.2. Agar plates were made with 1.6% Bacto Agar (Difco Laboratories). Antibiotic plates contained tetracycline (14 µg/ml), nalidixic acid (50 µg/ml), cycloheximide (50 µg/ml) and mercuric chloride (5 µg/ml) as Hg^{+2} ion as indicated.

DNA Probing

Colonies from spread plates were blotted onto 0.45 µm Biotrans nylon filters (ICN Biomedicals, Inc.). The colonies were lysed and the DNA denatured with 0.2 N NaOH, and the filters were baked at 80°C for 1 hr to fix the DNA to the membrane. All filters were prehybridized using the method of Church and Gilbert (1984). Colony blots were probed with single stranded ^{32}P -*luxAB* specific DNA. Probe was generated using the polymerase chain reaction (PCR) using a model 9810 thermocycler (Perkin Elmer) and

with a GeneAmp PCR kit (Perkin Elmer Applied Biosystems). Previously published sequences (Baldwin et al., 1989) were used to design the oligonucleotides for the forward primer 5'-GGGGGTTGTTATTCCAACAGC-3' and the reverse primer 5'-GTCATCATGAGACCCTACTGC-3' (Genosys Biotechnologies, Inc.). The plasmid pUCD623 (Shaw et al., 1987) was used to obtain vector free double stranded template DNA. This double stranded template was used with one of the oligonucleotide primers and ^{32}P - α dCTP to produce single stranded probe. The *luxAB* DNA was amplified using an initial denaturing of 92°C for 5 minutes coupled with a 1 min. denaturing at 92°C, 2 min. annealing at 55°C and a 2 min. extension at 72°C for 38 cycles. Blots were stripped using the manufacturers protocol and re-probed with ^{32}P -AI specific probe. The previously published DNA sequence of the actin intron/exon (Bagash, 1989) was used to design the forward primer (#621) 5'-GTCGACATAAATAGATCTCGTTTGGG-3' and the reverse primer (#907) 5'-TGGAGCCGGGAGATCTGTATAGTCTAGA-3'. Oligonucleotides were made on an Oligo-1000 DNA synthesizer (Beckman Instruments, Inc.). The PCR amplification conditions consisted of an initial melting at 95°C for 5 min. followed by strand melting at 95°C for 1.0 min., primer annealing at 55°C for 30 sec. and extension at 72°C for 30 sec. for 38 cycles. Probes were purified through a NucTrap column using a Pushcolumn apparatus following the manufacturers protocol (Stratagene Cloning Systems). All hybridizations and washings were done at high stringency at 65°C (Sayler et al., 1985). Positive signals were detected using autoradiography.

Results

Soil

The soil was homogenized using bromide as a tracer. The average bromide concentration after mixing was 83.8 µg/ml with a CV of 5.9 indicating well mixed soil. The pH was determined to be 7.1 and the total organic content was 585 g/kg of soil (Sanseverino et al., 1993). The particle analysis showed 56.6% sand, 26.4% clay and 17% silt.

Soil background populations

A 10^{-1} dilution of MGP soil that was sampled in the same manner as microcosms was spread plated onto YEPG plates containing tetracycline, nalidixic acid or mercuric chloride. There was no growth observed on these plates after one week of incubation. The total colony forming units (CFUs) as determined on YEPG agar was 1.8×10^7 cfu/g with a standard deviation of 3.5×10^6 cfu/g. DNA from direct extraction of the MGP soil coupled with slot blotting and probing with *luxAB* and AI specific probes showed no background of the two markers in the soil (data not shown).

Survival of *P. fluorescens* 5RLA

P. fluorescens 5RLA as the sole introduced species

P. fluorescens 5RLA was able to persist under several different conditions in an MPG soil. Figure 4-2A shows strain persistence in unamended MGP soil. The initial concentration was 5.5×10^7 cfu/g as determined by *lux* and AI probing of colony blots taken from non-selective media. The concentration remained the same for 7 days and

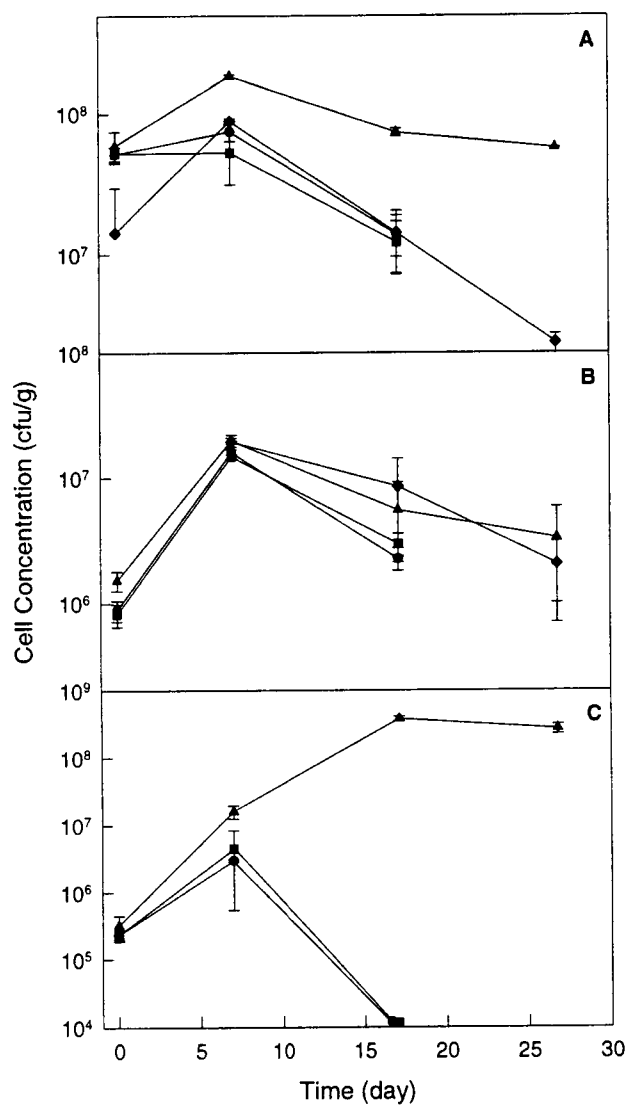


Figure 4-2. Persistence of *P. fluorescens* 5RLA and pUTK21 in an MGP soil.

Conditions: A. unamended soil; B. sterile soil; C. MGP soil amended with naphthalene impacted MGP soil. Symbols: (●) *lux* positive CFUs; (■) AI positive CFUs; (▲) total CFUs; (◆) total CFUs on YEPG containing tetracycline (14 µg/ml).

declined to $1.2\text{--}1.4 \times 10^7$ cfu/g over the following 10 days. The population of 5RLA as determined by selective plating on YEPG containing tetracycline followed the probing data and indicates that the population further declined to 2.4×10^6 cfu/g after another 9 days. The total population remained constant over 27 days at $5.7\text{--}7.3 \times 10^7$ cfu/g except for a increase to 1.8×10^8 cfu/g at 7 days.

P. fluorescens 5RLA was also able to persist in sterile MGP soil (Figure 4-2B). The initial concentration was 8.5×10^5 cfu/g as determined by *lux* and AI probing of colony blots taken from non-selective media. The concentration increased to 1.5×10^7 cfu/g after 7 days and declined to 2.5×10^6 cfu/g after 17 days. The population of 5RLA as determined by selective plating on YEPG containing tetracycline showed the concentration to remain at 2×10^6 . The total population was slightly higher than the 5RLA population. There were no CFUs when the sterile soil was initially moistened but at the time the experiment was begun there were 5×10^3 cfu/g.

The 5RLA population in MGP soil when amended with naphthalene impacted soil is shown in Figure 4-2C. The population of 5RLA did not persist at as high a level as in sterile or unamended MGP soil. The initial concentration of 5RLA was 2.3×10^5 cfu/g as determined by *lux* and AI probing of colony blots taken from non-selective media. The population increased to 3.3×10^6 cfu/ml after 7 days and declined below 10^4 cfu/ml after 17 days.

Co-introduction of *P. fluorescens* 5RLA and *P. putida* PB2440

P. fluorescens 5RLA was also able to persist under several different conditions in an MPG soil co-inoculated with *P. putida* PB2440. Figure 4-3A shows strain persistence for both strains in unamended MGP soil. The initial concentration of *P. fluorescens* 5RLA was 1.5×10^7 cfu/g of soil as determined by AI probing of colony blots from non-selective media. The population of 5RLA increased to 4.4×10^7 cfu/g after 2 days and declined to 4.5×10^5 cfu/g after 9 days followed by an increase to 2.1×10^6 cfu/g after 16 days of incubation. The 5RLA population declined to 1.2×10^3 after 69 days. The initial population of *P. putida* PB2440 was 1×10^7 cfu/g of soil as determined by selective plating on YEPG containing nalidixic acid (50 µg/ml). The population of PB2440 declined to 2.7×10^6 cfu/g after 2 days and further declined to 7.8×10^5 cfu/g after 9 days. The concentration increased to 1.9×10^6 cfu/g after 16 days and 2.1×10^6 cfu/g at the conclusion of the experiment. The population of 5RLA as determined by selective plating on YEPG containing tetracycline followed the AI probing data.

P. fluorescens 5RLA was also able to persist in sterile MGP soil that was co-inoculated with *P. putida* PB2440 (Figure 4-3B). The initial concentration of 5RLA was 6.7×10^6 cfu/g and increased to 5.1×10^7 cfu/g over 2 days then declined to 7.9×10^6 cfu/g over 69 days as determined by AI probing of colony blots from non-selective media. The 5RLA concentration followed the total population closely although there was a background of colony forming bacteria. The population of PB2440 was initially 5.5×10^6 cfu/g and increased slightly over 2 days. The concentration of PB2440 declined to 3.2×10^5 cfu/g over 8 days and declined to below 10 cfu/g over 16 days. The population of

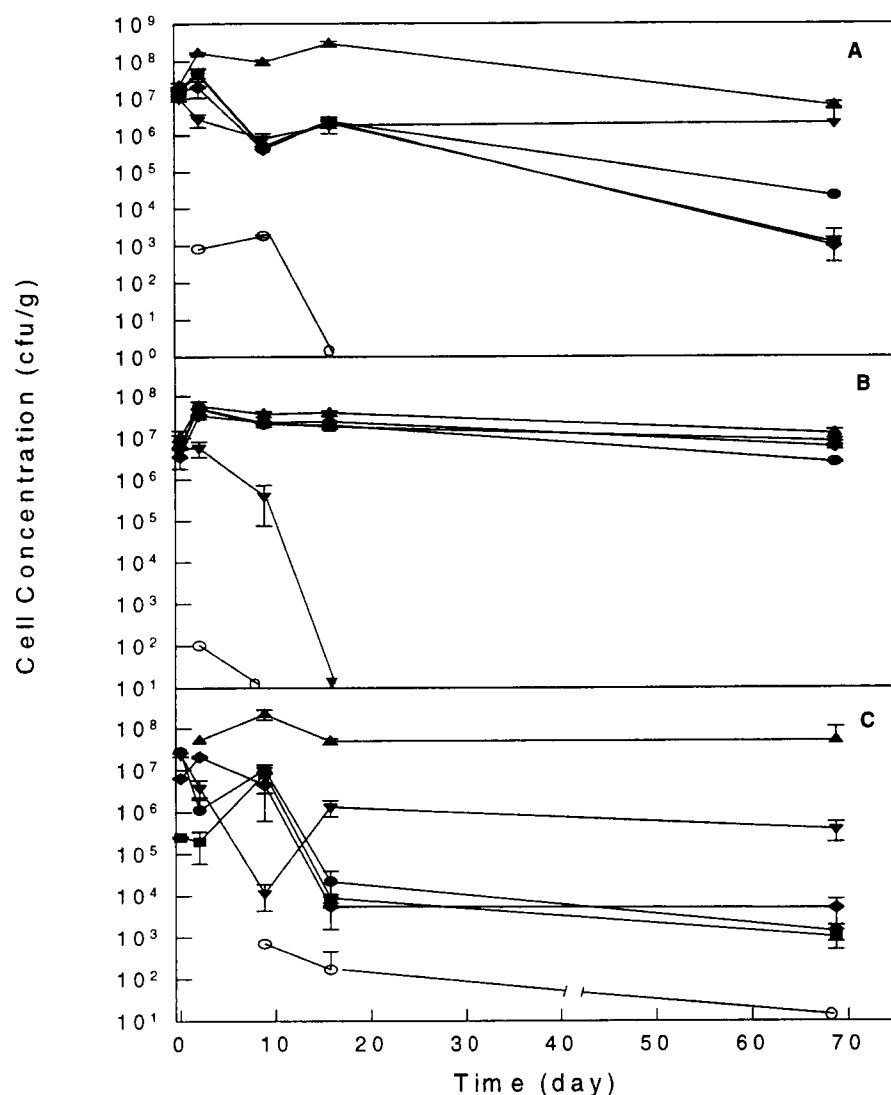


Figure 4-3. Persistence of *P. fluorescens* 5RLA and pUTK21 in an MGP soil co-inoculated with *P. putida* PB2440. Conditions: A. unamended soil; B. sterile soil; C. MGP soil amended with naphthalene impacted MGP soil. Symbols: (●) *lux* positive CFUs; (■) AI positive CFUs; (▲) total CFUs; (◆) total CFUs on YEPG containing tetracycline (14 μ g/ml); (▼) total CFUs on YEPG containing nalidixic acid (50 μ g/ml) and (○) transconjugants containing pUTK21.

5RLA as determined by selective plating on YEPG containing tetracycline followed the AI probing data.

The 5RLA population in MGP soil when amended with naphthalene impacted soil is shown in Figure 4-2C. The population of 5RLA did not persist at as high a level as in sterile or unamended MGP soil. The initial concentration of 5RLA was 2.5×10^5 cfu/g which decreased slightly over 2 days then increased to 8.1×10^6 cfu/g after 9 days as determined by AI probing of colony blots taken from non-selective plates. The 5RLA population declined to 8.8×10^3 cfu/g over 16 days and further declined to 1×10^3 cfu/g over 69 days. The population of PB2440 was initially 2.3×10^7 cfu/g as determined by selective plating on YEPG containing nalidixic acid. The PB2440 population declined to 3.8×10^6 cfu/g over 2 days and further declined to 1.1×10^4 cfu/g over 9 days. The level of PB2440 did increase to 1.3×10^6 cfu/g after 16 days followed by a decrease to 3.8×10^5 cfu/g. The population of 5RLA as determined by selective plating on YEPG containing tetracycline followed the AI probing data.

Transfer of pUTK21

Transfer of pUTK21 to indigenous bacteria

The *lux* and AI probing data in Figure 4-2A-C showed no evidence of transfer of pUTK21 to indigenous soil bacteria. Colony blots from non-selective media were overlain and colonies probing *lux* positive and AI negative were screened. None were detected.

Transfer of pUTK21 to *P. putida* PB2440

Transfer of pUTK21 was detected in microcosms where *P. putida* PB2440 was co-inoculated as a recipient. Transconjugants were detected in the unamended MGP soil in the first 2 days at a level of 8×10^2 cfu/g as determined by selective plating on YEPG plus tetracycline and nalidixic acid (Figure 4-3A). This gives a transfer frequency of 1.8×10^{-5} transconjugants per donor bacterium. The transconjugant level increased to 1.8×10^3 cfu/g in 9 days and declined to less than 10 cfu/g after 16 days. There was a significant difference in CFUs determined by AI and *lux* probing after 69 hours. There may have been a population of transconjugants not detected by selective media.

Transconjugants were also detected in microcosms containing sterile MGP soil at 1×10^2 cfu/g after 2 days when 5.1×10^7 5RLA CFUs were present giving a transfer frequency of 1.9×10^{-6} (Figure 3B). No transconjugants were detected at subsequent time points. This observation may be seen to correlate with the decline of PB2440 to below 10 cfu/g after 8 days.

In microcosms amended with naphthalene impacted MGP soil, transconjugants were detected at a level of 7×10^2 cfu/g after 9 days when 8.1×10^6 5RLA CFUs were present giving a transfer frequency of 8.6×10^{-5} transconjugants per donor bacterium. The transconjugant level declined to 1.7×10^2 cfu/g after 16 days. The level of PB2440 remained at 3.8×10^5 to 1.3×10^6 cfu/g during this period of time.

Discussion

Survival and persistence are important factors in determining the usefulness of GMOs in environmental applications as well as addressing risk assessment issues. *P. fluorescens* 5RLA, a genetically modified bacterium that serves as a cellular reporter for naphthalene and salicylate biodegradation, was inoculated into an MGP soil typical of a PAH contaminated site. *P. fluorescens* 5RLA persisted in a culturable state in unamended MGP soil at 10^6 cfu/g for 26 days. This is apparent in Figure 4-2A and 4-3A. The co-introduced strain *P. putida* PB2440 persisted at a higher level (10^6 cfu/g) than 5RLA (10^3 cfu/g) in unamended microcosms after 69 days. This is contrasted by the results from sterile MGP soil microcosms. *P. fluorescens* 5RLA persisted at 3×10^6 cfu/g after 27 days (Figure 4-2B) and 7.9×10^6 cfu/g over 69 days (Figure 4-3A) in sterile MGP soil. *P. putida* 2440 was not detected after 16 days. These data would indicate that *P. putida* PB2440 was less competitive than *P. fluorescens* 5RLA in sterile as compared to unamended MGP soil. *P. fluorescens* 5RLA increased in CFUs in both sterile and non-sterile MGP soil whereas PB2440 increased in non-sterile MGP soil but did not in sterile MGP soil.

In naphthalene amended MGP soil, *P. fluorescens* 5RLA increased from 2×10^5 cfu/g to 3×10^6 cfu/g over 7 days but declined below 10^4 cfu/g after 7 days (Figure 4-2C). This decline of *P. fluorescens* 5RLA was more pronounced than in unamended MGP soil or sterile MGP soil. The total CFUs indicate that the indigenous bacteria were more competitive when there was an addition of naphthalene to the system. The addition of

naphthalene had no effect on the total CFUs of PB2440 with the population being 2×10^6 cfu/g after 16 days and remaining at that level for 69 days.

P. putida PB2440 does not utilize naphthalene as a carbon source but must obtain carbon for growth and energy from other components of the MGP soil. Without the addition of naphthalene, PB2440 is more persistent than *P. fluorescens* 5RLA. In the unamended soil, *P. fluorescens* 5RLA persisted at levels useful as a biosensor for 17 days after which the level declined to 10^3 cfu/g. In naphthalene amended microcosms, *P. fluorescens* 5RLA persisted at useful levels for 9 days after which the population declined to 10^4 cfu/g. The decline over time relative to PB2440 may be due to a reduction in fitness due to the expression of the *lux* genes or since the microcosms were air tight, the available oxygen may have been consumed and the microcosms become anoxic. A persistence in population was observed for *P. fluorescens* 10585s/FAC510 in an uncontaminated soil. This strain carries a plasmid based bioluminescence system (*luxAB*) under the control of the constitutive tetracycline resistance promoter (Meikle et al, 1992). Of the initial inoculation of 10^9 cfu/g soil, the population decreased to 4×10^8 cfu/g over 92 days in sterile microcosms. This and subsequent studies (Amin-Hanjani et al., 1993) were aimed at examining bioluminescence as a quantitative tool for monitoring bacterial populations in sterile soil. No system similar to 5RLA has been examined in soil.

Studies examining the survival and persistence of *Pseudomonas fluorescens* strains in the rhizosphere have been reported (Kluepfel et al., 1991; Yeung et al., 1989). These studies demonstrated culturable populations that persisted for around 30 days and

populations were co-inoculated (Ramos-Gonzalez et al., 1991). It was shown that TOL transferred to intergeneric recipients at a frequency of 10^{-2} transconjugants per recipient but the intergeneric transfer rate was 10^{-3} to 10^{-7} transconjugants per recipient. The transfer of the 2,4-dichlorophenoxyacetic acid degradative plasmid pJP4 was studied in soil microcosms where a recipient population was added (Neilson et al., 1994). The frequency of transfer was 10^{-5} in sterile soil and 10^{-6} in 2,4-D amended soil. This observation contrasts with the results presented for transfer of pUTK21. There have been few reports of transfer of plasmids to indigenous soil bacteria. The transfer of a marked RP4 (60 kb) plasmid harbored in *P. fluorescens* was studied in the rhizosphere (Smit et al., 1991). They observed a transfer frequency of 10^{-4} when using 10^7 donor cells. Transfer of a 3-chlorobenzoate degradative plasmid was observed in sediment mesocosms (Wyndham et al., 1988; Fulthorpe & Wyndham, 1991). Transconjugants were observed at the conclusion of their experiment that were not the original host but were able to express the 3-chlorobenzoate degradative genes.

P. fluorescens 5RLA survived and remained culturable for 30 days at a level of 10^6 cfu/g of soil. These results agree with observations of other researchers using *Pseudomonas* sp. in bioaugmentation studies. While persistence for 30 days may not be long enough for bioremediation, it is promising for using the bioluminescence reporter strain as a short term bioavailability tool. In terms of risk assessment, the strain may persist for a period of time suitable for its purpose and then die off. No transfer of the reporter plasmid to indigenous bacteria was noted. Transfer was observed only when a

then declined. Other studies were conducted with *Pseudomonas putida* bearing a recombinant TOL plasmid (pWWO-EB62) for phosphinothricin biodegradation (Ramos et al., 1991). It was found that depending on the soil, the strain was culturable at $> 10^6$ cfu/g for 2-3 weeks. *Pseudomonas putida* harboring a *xyIE* marked plasmid was found to persist at 10^5 cfu/g for 2 weeks then rapidly decline with best survival on sandy and high clay soils (Macnaughton et al, 1992).

The transfer of pUTK21 was not observed in non-sterile MGP soil (Figure 4-2A-C). When a plasmidless recipient strain, *P. putida* PB2440, was added to microcosms, transfer was observed in the three treatments (Figure 4-3A-C). In non-sterile microcosms, transconjugants were observed at a frequency of 1.8×10^{-5} transconjugants per donor bacterium. The number of transconjugants increased over 9 days but this may have been due to growth of the transconjugant population. In sterile microcosms, transconjugants were observed after two days at a frequency of 1.9×10^{-6} transconjugants per donor bacterium. No transconjugants were detected after 2 days. In a sterile soil with little naphthalene for use as a growth substrate, these transconjugants may have died. In naphthalene amended MGP soil, transconjugants were detected at frequency of 8.6×10^{-5} transconjugants per donor bacterium. This level decreased over 9 days as opposed to the unamended MGP soil and none were detected after 69 days. This may have been due to competition for naphthalene.

This work represents the only report of transfer of a naphthalene degradative plasmid in soil. There have been few reports of the transfer of catabolic plasmids in soil. The transfer of the TOL plasmid pWWO-EB62 was examined in soil where recipient

plasmidless strain was added, which would be the optimal recipient. If transfer did occur, it occurred at very low frequencies (Frost, 1992).

PART 5

SUMMARY AND CONCLUSIONS

The specific conclusions of this work can be summarized as follows:

- The wild type naphthalene biodegradative plasmid, pKA1, and the *nah-lux* reporter plasmid, pUTK21, were maintained in 100% of the *P. fluorescens* 5R and *P. fluorescens* 5RL populations growing in glucose limited continuous culture.
- The reporter plasmid, pUTK21, was molecularly but segregationally unstable in *P. fluorescens* HK44 when growing in glucose limited continuous culture.
- The reporter plasmid pUTK9 was segregationally unstable in *P. putida* RB1351 but only a small fraction of the total culture existed without pUTK9.
- The bioluminescence reporter strains *P. fluorescens* 5RL was washed out of continuous cultures when competing with *P. fluorescens* 5R for naphthalene.
- The bioluminescence reporter strains *P. fluorescens* HK44 and *P. putida* RB1351 were maintained in mixed cultures when competing for naphthalene at low dilution rates. *P. fluorescens* HK44 was washed out at higher dilution rates.

- The bioluminescence reporter strains were able maintain detectable bioluminescence output under competitive continuous culture conditions.
- The Tn5Hg-AI insertion in *P. fluorescens* 5RL was confirmed as a chromosomal insertion and remained stable for 160 generations in continuous culture.
- The AI insertion was not found to be deleterious to *P. fluorescens* 5RL in any overt way since the strain could grow on minimal medium with either succinate or glucose as a sole source of carbon and energy.
- *P. flourescens* 5RLA was able to persist in unamended manufactured gas plant (MGP) soil for at ca. 10^6 cfu/g for 25 days.
- *P. flourescens* 5RLA was not able to persist in naphthalene amended MGP soil after 7 days ($<10^4$ cfu/g).
- No transfer of the reporter plasmid pUTK21 was noted except under optimal conditions such as the addition of a plasmidless recipient strain.

LITERATURE CITED

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1. **Amici, A., Bazzicalupo, M., Gallori, E., and others.** 1991. Monitoring a genetically engineered bacterium in a freshwater environment by rapid enzymatic amplification of a synthetic DNA "number-plate": *Appl. Microbiol. Biotechnol.* **36**:222-227.
2. **Amin-Hanjani, S., Meikle, A., Glover, L.A., and others.** 1993. Plasmid and chromosomally encoded luminescence marker systems for detection of *Pseudomonas fluorescens* in soil: *Mol. Ecol.* **2**:47-54.
3. **Austin, S.J.** 1988. Plasmid partition: *Plasmid* **20**:1-9.
4. **Bagash, J. M. A.** 1989, The actin genes of *Candida albicans* and *Candida tropicalis*, Knoxville, TN, The University of Tennessee.
5. **Baldwin, T.O., Devine, J.H., Heckel, R.C., and others.** 1989. The complete nucleotide sequence of the lux regulon of *Vibrio fischeri* and the lux ABN region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence: *J. Biol. Chem.* **4**:326-341.
6. **Balkwill, D.L., Rucinsky, T.E., and Casida, L.E.** 1977. Release of microorganisms from soil with respect to transmission electron microscopy viewing and plate counts: *Antonie Van Leeuwenhoek* **43**:73-87.
7. **Barkay, T., and Olson, B.H.** 1986. Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress: *Appl. Environ. Microbiol.* **52**:403-406.
8. **Barkay, T., Tripp, S.C., and Olson, B.H.** 1985. Effect of metal-rich sewage sludge applications on the bacterial communities of grasslands: *Appl. Environ. Microbiol.* **49**:333-337.
9. **Barkay, T.** 1987. Adaptation of aquatic microbial communities to Hg²⁺ stress: *Appl. Environ. Microbiol.* **53**:2725-2732.
10. **Barnsley, E.A.** 1975. The induction of the enzymes of naphthalene metabolism in *Pseudomonads* by salicylate and 2-aminobenzoate: *J. Gen. Microbiol.* **88**:193-196.
11. **Barnsley, E. A.** 1976. Role and regulation of the *ortho* and *meta* pathways of catechol metabolism in *Pseudomonads* metabolizing naphthalene and salicylate: *J. Bact.* **125**:404-408.

12. **Bentley, W.E., Mirjalili, N., Anderson, D.C., and others.** 1990. Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria: *Biotech. Bioeng.* **35**:668-681.
13. **Boronin, A.M., Filonov, A.E., Balakshina, V.V., and others.** 1985. Stability of naphthalene biodegradation plasmids NPL-1 and NPL-41 in populations of *Pseudomonas putida* under conditions of continuous culture: *Mikrobiologiya* **54**:489-494.
14. **Boye, M., Ahl, T., and Molin, S.** 1995. Application of a strain-specific rRNA oligonucleotide probe targeting *Pseudomonas fluorescens* Ag1 in a mesocosm study of bacterial release into the environment: *Appl. Environ. Microbiol.* **61**:1384-1390.
15. **Bradley, D.E., and Williams, P.A.** 1982. The TOL plasmid is naturally derepressed for transfer: *J. Gen. Microbiol.* **128**:3019-3024.
16. **Brenner, V., Hernandez, B.S., and Focht, D.D.** 1993. Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations: *Appl. Environ. Microbiol.* **59**:2790-2794.
17. **Bungay, H.R.I., and Bungay, M.L.** 1968. Microbial interactions in continuous culture: *Adv. Appl. Microbiol.* **10**:269-290.
18. **Burlage, R.S., Sayler, G.S., and Larimer, F.** 1990. Monitoring of naphthalene catabolism by bioluminescence with *nah-lux* transcriptional fusions: *J. Bact.* **172**:4749-4757.
19. **Burton, N.F., Day, M.J., and Bull, A.T.** 1982. Distribution of bacterial plasmids in clean and polluted sites in a south Wales river: *Appl. Environ. Microbiol.* **44**:1026-1029.
20. **Cane, P.A., and Williams, P.A.** 1982. The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1: *J. Gen. Microbiol.* **128**:2281-2290.
21. **Carmi, O.A., Stewart, G.S.A.B., Ulitzur, S., and others.** 1987. Use of bacterial luciferase to establish a promoter probe vehicle, capable of nondestructive real-time analysis of gene expression in *Bacillus* spp.: *J. Bact.* **169**:
22. **Catterall, F.A., Murray, K., and Williams, P.A.** 1971. *Biochim. Biophys. Acta* **237**:361.

24. **Church, G.M., and Gilbert, W.** 1984. Genomic Sequencing: Proc. Natl. Acad. Sci. USA **81**:1991-1995.
25. **Close, T.J., Zaitlin, D., and Kado, C.I.** 1984. Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTiC58: Plasmid **12**:111-118.
26. **Connors, M.A., and Barnsley, E.A.** 1982. Naphthalene plasmids in Pseudomonads: J. Bact. **149**:1096-1101.
27. **Davey, R. B., and Reanney, D. C.** 1981 Extrachromosomal genetic elements and the adaptative evolution of bacteria Hecht, M. K., Steere, W. C., and Wallace, B., Editores, Evolution Biology: New York, Plenum Publishing Corp., p. 113-147.
28. **Davies, J.I., and Evans, W.C.** 1964. Oxidative metabolism of naphthalene by soil Pseudomonads: Biochem. J. **91**:251-261.
29. **DiGrazia, P.M., King, J.M.H., Blackburn, J.W., and others.** 1991. Dynamic response of naphthalene biodegradation in a continuous flow soil slurry reactor: Biodegradation **2**:81-91.
30. **Dixon, B.** 1991. Commentary - Revelations Recurring: Bio/Technology **9**:403.
31. **Drahos, D.J., Hamming, B.C., and Macpherson, S.** 1986. Tracking recombinant organisms in the environment: β -galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads: Biotechnology **4**:439-443.
32. **Duetz, W.A., and van Andel, J.G.** 1991. Stability of TOL plasmid pWWO in *Pseudomonas putida* mt-2 under non-selective conditions in continuous culture: J. Gen. Microbiol. **137**: 1369-1374.
33. **Dunn, N.W., and Gunsalus, I.C.** 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*: J. Bact. **114**:974-979.
34. **Dykhuizen, D.E., and Hartl, D.L.** 1983. Selection in chemostats: Microbial Rev. **47**:150-168.
35. **Eaton, R.W., and Chapman, P.J.** 1992. Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions: J. Bact. **174**:7542-7554.
36. **Engbrecht, J., Simon, M., and Silverman, M.** 1985. Measuring gene expression with light: Science **227**:1345-1347.
37. **Ensley, B.D., and Gibson, D.T.** 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component: J. Bact. **155**:505.

36. **Engebrecht, J., Simon, M., and Silverman, M.** 1985. Measuring gene expression with light: *Science* **227**:1345-1347.
37. **Ensley, B.D., and Gibson, D.T.** 1983. Naphthalene dioxygenase: purification and properties of a terminal oxygenase component: *J. Bact.* **155**:505.
38. **Ensley, B.D., Gibson, D.T., and Laborde, A.L.** 1982. Oxidation of naphthalene by a multicomponent enzyme system from *Pseudomonas* sp. strain NCIB9816: *J. Bact.* **149**:948-954.
39. **Ensley, B.D., Ratzkin, B.J., Osslund, T.D., and others.** 1983. Expression of naphthalene oxidation genes in *Escherichia coli* in the biosynthesis of indigo: *Science* **222**:167-169.
40. **Farrar, W. E. Jr.** 1981, Evolution among antibiotic resistance plasmids in the hospital environment, Levy, S. B., Clowes, R. C., and Koenig, E. L. Eds., *In: Molecular biology, pathogenicity and ecology of bacterial plasmids*: New York, Plenum Publishing Corp., p. 1-10.
41. **Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., and others.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta-cleavage pathway: *Proc. Natl. Acad. Sci. USA* **78**:7458-7462.
42. **Fredrickson, A.G., and Stephanopoulos, G.** 1981. Microbial competition: *Science* **213**:972-979.
43. **Fulthorpe, R.R., and Wyndham, R.C.** 1992. Involvement of a chlorobenzoate-catabolic transposon, Tn5271, in a community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem: *Appl. Environ. Microbiol.* **58**:314-325.
44. **Genthner, F.J., Upadhyay, J., Campbell, R.P., and others.** 1990. Anomalies in the enumeration of starved bacteria on culture media containing nalidixic acid and tetracycline: *Microb. Ecol.* **20**:283-288.
45. **Glassman, D.L., and McNicol, L.A.** 1981. Plasmid frequency in natural populations of estuarine microorganisms: *Plasmid* **5**:231.
46. **Gottschal, J.C., and Thingstad, T.F.** 1982. Mathematical description of competition between two and three bacterial species under dual substrate limitation in the chemostat: a comparison with experimental data: *Biotech. Bioeng.* **24**:1403-1418.

47. **Hada, H.S., and Sizemore, R.K.** 1981. Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico: Appl. Environ. Microbiol. **41**:199-202.
48. **Haigler, B.E., and Gibson, D.T.** 1990. Purification and properties of ferredoxin_{nap}, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB9816: J. Bact. **172**:465-468.
49. **Hastings, J.W., Potrikus, C.J., Gupta, S.C., and others.** 1985. Biochemistry and physiology of bioluminescent bacteria: Adv. Microbial Physiol. **26**:235-291.
50. **Heitzer, A., Malachowsky, K., Thonnard, J.E., and others.** 1994. Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium: Appl. Environ. Microbiol. **60**:1487-1494.
51. **Heitzer, A., Webb, O.F., Thonnard, J.E., and others.** 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability using a bioluminescent catabolic reporter bacterium: Appl. Environ. Microbiol. **58**:1839-1846.
52. **Herrero, M., deLorenzo, V., and Timmis, K.N.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria: J. Bact. **172**:6557-6567.
53. **Hofte, M., Mergeay, M., and Verstraete, W.** 1990. Marking the *Rhizopseudomonas* strain 7NSK₂ with a Mu d(*lac*) element for ecological studies: Appl. Environ. Microbiol. **56**:1046-1052.
54. **Huang, J., and Schell, M.A.** 1991. In vivo interactions of the nahR transcriptional activator with its target sequences: J. Biol. Chem. **266**:10830-10838.
55. **Hwang, I., and Farrand, S.K.** 1994. A novel gene tag for identifying microorganisms released into the environment: Appl. Environ. Microbiol. **60**:913-920.
56. **Jain, R.K., Burlage, R.S., and Sayler, G.S.** 1988. Methods for detecting recombinant DNA in the environment: CRC Crit. Rev. Biotechnol. **8**:33-84.
57. **Jefferson, R.A.** 1989. The GUS reporter system: Nature **342**:837-838.
58. **Jerina, D.M., Daly, J.W., Jeffrey, A.M., and others.** 1971. Arch. Biochem. Biophys. **142**:394.

59. **Keshavarz, T., Lilly, M.D., and Clarke, P.H.** 1985. Stability of a catabolic plasmid in continuous culture: *J. Gen. Microbiol.* **131**:1193-1203.
60. **King, J.M.H., Digrazia, P.M., Applegate, B.M., and others.** 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation: *Science* **249**:778-781.
61. **King, R.J., Short, K.A., and Seidler, R.J.** 1991. Assay for detection and enumeration of genetically engineered microorganism which is based on the activity of a deregulated 2,4-dichlorophenoxyacetate monooxygenase: *Appl. Environ. Microbiol.* **57**:1790-1792.
62. **Kleupfel, D.A., Kline, E.L., Skipper, H.D., and others.** 1991. The release and tracking of genetically engineered bacteria in the environment: *Phytopathology* **81**:348-352.
63. **Leblond, J.D., Applegate, B.M., Menn, F.M., and others.** 1995. NAH plasmid-mediated metabolism of methyl-substituted naphthalenes: General Meeting of the American Society for Microbiology Q412.
64. **Lee, S.W., and Edlin, G.** 1985. Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*: *Gene* **39**:173-180.
65. **Levine, M.M., Kaper, J.B., Lockman, H., and others.** 1983. Recombinant DNA risk assessment studies in humans: efficacy of poorly mobilizable plasmids in biological containment: *J. Infect. Dis.* **148**:699-709.
66. **Levy, S. B., and Miller, R. V.,** 1989, *Gene Transfer in the Environment*: New York, McGraw-Hill, Inc.
67. **Macnaughton, S.J., Rose, D.A., and O'Donnell, A.G.** 1992. Persistence of a xylE marker gene in *Pseudomonas putida* introduced into soils of differing texture: *J. Gen. Microbiol.* **138**:667-673.
68. **Makemson, J.C., and Gordon, A.S.** 1989. Total energy flux in a marine bioluminescent bacterium: *FEMS Microbiol. Lett.* **57**:161-166.
69. **Molecular cloning:** a laboratory manual. 1990. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.
70. **Meighen, E.A.** 1988. Enzymes and genes form the lux operons of bioluminescent bacteria: *Ann. Rev. Microbiol.* **42**:151-176.

71. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence: Microbiol. Rev. **55**:123-142.
72. **Meikle, A., Killham, K., Prosser, J.I., and others.** 1992. Luminometric measurement of population activity of genetically modified *Pseudomonas fluorescens* in the soil: FEMS Microbiol. Lett. **99**:217-220.
73. **Menn, F.M., Applegate, B.M., and Sayler, G.S.** 1993. NAH plasmid-mediated catabolism of anthracene and phenanthrene to naphthoic acids: Appl. Environ. Microbiol. **59**:1938-1942.
74. **Meynell, G. G., and Meynell, E.** 1970. Theory and Practice in Experimental Bacteriology: Cambridge, UK, Cambridge University Press.
75. **Modi, R.I., Wilke, C.M., Rosenzweig, R.F., and others.** 1991. Plasmid macro-evolution of deletions during adaptation in a nutrient-limited environment: Genetica **84**:195-202.
76. **Monticello, D.J., Bakker, D., Schell, M., and others.** 1985. Plasmid-borne Tn5 insertion mutation resulting in accumulation of gentisate from salicylate: Appl. Environ. Microbiol. **49**:761-764.
77. **National Research Council (US) Committee on Environmental Epidemiology.** 1991. Environmental Epidemiology: Washington, DC, National Academy of Sciences.
78. **Neilson, J.W., Josephson, K.L., Pepper, I.L., and others.** 1994. Frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in soil: Appl. Environ. Microbiol. **60**:4053-4058.
79. **Patel, T.J., and Gibson, D.T.** 1974. Purification and properties of (+)-*cis*-naphthalene dihydrodiol dehydrogenase of *Pseudomonas putida*: J. Bact. **119**:879-888.
80. **Pavlou, S., Kevrekidis, I.G., and Lyberatos, G.** 1990. On the coexistence of competing microbial species in a chemostat under cycling: Biotech. Bioeng. **35**:224-232.
81. **Proctor, G.N.** 1994. Mathematics of microbial plasmid instability and subsequent differential growth of plasmid-free and plasmid-containing cells, relevant to the analysis of experimental colony number data: Plasmid **32**:101-130.
82. **Prosser, J.I.** 1994. Molecular marker systems for detection of genetically engineered micro-organisms in the environment: Microbiology **140**:5-17.

83. **Ramos-González, M.-I., Ruiz-Cabdlo, F., Bretlar, I., and others.** 1992. Tracking genetically engineered bacteria: monoclonal antibodies against surface determinants of the soil bacterium *Pseudomonas putida* 2440: J. Bact. **174**:2978-2985.
84. **Ramos-Gonzalez, M.-I., Duque, E., and Ramos, J.L.** 1991. Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids: Appl. Environ. Microbiol. **57**:3020-3027.
85. **Rogowsky, P.M., Close, T.J., Chimera, J.A., and others.** 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58: J. Bact. **169**:5101-5112.
86. **Sanseverino, J., Applegate, B.M., King, J.M.H., and others.** 1993. Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene: Appl. Environ. Microbiol. **59**:1931-1937.
87. **Sayler, G.S., Hooper, S.W., Layton, A.C., and others.** 1990. Catabolic plasmids of environmental and ecological significance: Microb. Ecol. **19**:1-20.
88. **Sayler, G.S., Shields, M.S., Tedford, E.T., and others.** 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples: Appl. Environ. Microbiol. **49**:1295-1303.
89. **Schaur, A.T., Ranes, M., Santamaria, R., and others.** 1989. Visualizing gene expression in time and space in the filamentous bacterium *Streptomyces coelicolor*: Science **240**:768-772.
90. **Schell, M.A.** 1986. Homology between nucleotide sequences of promoter regions of *nah* and *sal* operons of NAH7 plasmid of *Pseudomonas putida*: Proc. Natl. Acad. Sci. USA **83**:369-373.
91. **Schell, M.A., and Poser, E.** 1989. Demonstration, characterization, and mutational analysis of NahR protein binding to *nah* and *sal* promoters: J. Bact. **171**:837-846.
92. **Schell, Mark A.** 1990. Regulation of the naphthalene degradation genes of plasmid NAH7: Example of a generalized positive control system in *Pseudomonas* and related bacteria, S. Silver/A.M. Chakrabarty/B. Iglewski/S. Kaplan, *Pseudomonas - Biotransformations, Pathogenesis, and Evolving Biotechnology*, Chicago, Il, July 1989, p. 165-176.
93. **Schell, M.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product: Gene **36**:301-309.

94. **Schmidt, T.M., Kopecky, K., and Nealson, K.H.** 1989. Bioluminescence of the insect pathogen *Xenorhabdus luminescens*: Appl. Environ. Microbiol. **55**:2607-2612.
95. **Selifonova, O., Burlage, R., and Barkay, T.** 1993. Bioluminescent sensors for detection of bioavailable Hg(II) in the environment: Appl. Environ. Microbiol. **59**:3083-3090.
96. **Shaw, J.J., and Kado, C.I.** 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner: Biotechnology **4**:560-564.
97. **Shaw, J.J., Rogowsky, P.M., and Kado, C.I.** 1987. Working with bacterial bioluminescence: J. Mol. Plant-Microbe Interact. **1**:39.
98. **Shaw, J.J., Rogowsky, P., Close, T.J., and others.** 1987. Working with bioluminescence: Plant Mol. Biol. Rep. **5**:225-236.
99. **Shaw, J.J., Dane, F., Geiger, D., and others.** 1992. Use of bioluminescence for detection of genetically engineered microorganisms released into the environment: Appl. Environ. Microbiol. **58**:267-273.
100. **Shaw, J.J., Settles, L.G., and Kado, C.I.** 1988. Transposon Tn4431 mutagenesis of *Zanthomonas campestris* pv. *campestris*: characterization of a nonpathogenic mutant and cloning of a locus for pathogenicity: Mol. Plant-Microbe Int. **1**:39-45.
101. **Simon, M.J., Osslund, T.D., Saunders, R., and others.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4: Gene **127**:31-37.
102. **Smit, E., Venne, D., and Vanelas, J.D.** 1993. Mobilization of a Recombinant IncQ Plasmid Between Bacteria on Agar and in Soil via Cotransfer or Retrotransfer: Applied and Environmental Microbiology, v. 59, no. 7, p. 2257-2263.
103. **Spain, J.C., Pritchard, P.H., and Bourquin, A.W.** 1980. Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments: Appl. Environ. Microbiol. **40**:726-734.
104. **Starovoitov, I.I., Nefedova, M.Y., Yakovlev, G.I., and others.** 1975. Gentisic acid as a microbial oxidation product of naphthalene: Izv. Akad. Nauk USSR Ser. Khim. **9**:2091.

105. **Stephanopoulos, G., and Lapidus, G.R.** 1988. Chemostat dynamics of plasmid-bearing, plasmid-free mixed recombinant cultures: *Chem. Eng. Sci.* **43**:49-57.
106. **Stotzky, G., and Babich, H.** 1986. Survival of, and genetic transfer by, genetically engineered bacteria in natural environments: *Adv. Appl. Microbiol.* **31**:93-132.
107. **Summers, D.K.** 1991. The kinetics of plasmid loss: *Trends Biotech.* **9**:273-278.
108. **Top, E.** 1993. Horizontal gene transfer in the environment and the significance of broad host range plasmids, Gent, Belgium, University of Gent.
109. **Trevors, J.T.** 1988. Use of microcosms to study genetic interactions between microorganisms: *Microbiol. Sci.* **5**:132-136.
110. **Trevors, J.T., Barkay, T., and Bourquin, W.** 1987. Gene transfer among bacteria in soil and aquatic environments: a review: *Can. J. Microbiol.* **33**:191-198.
111. **Tsuda, M., and Iino, T.** 1990. Naphthalene degrading genes on plasmid NAH7 are on a defective transposon: *Mol. Gen. Genet.* **223**:33-39.
112. **Ulitzur, S., and Hastings, J.W.** 1979. Evidence for tetradecanal as the natural aldehyde in bacterial bioluminescence: *Proc. Natl. Acad. Sci. USA* **76**:265-267.
113. **Valentine, C. R. I., and Kado, C. I.** 1989. *Molecular Genetics of IncW Plasmids* Thomas, C., Editor, *Promiscuous Plasmids of Gram-Negative Bacteria*: New York, Academic Press, p. 125-163.
114. **Walter, M.V., Porteous, L.A., Fieland, V.P., and others.** 1991. Formation of transconjugants on plating media following in situ conjugation experiments: *Can. J. Microbiol.* **37**:703-707.
115. **Warnes, A., and Stephenson, J.R.** 1986. The insertion of large pieces of foreign genetic material reduces the stability of bacterial plasmids: *Plasmid* **16**:116-123.
116. **Wassel, R.A., and Mills, A.L.** 1983. Changes in water and sediment bacterial community structure in a lake receiving acid mine drainage: *Microb. Ecol.* **9**:155-169.
117. **Williams, D.R., and Thomas, C.M.** 1992. Active partitioning of bacterial plasmids: *J. Gen. Microbiol.* **138**:1-16.

118. **Williams, P.A., Taylor, S.D., and Gibb, L.E.** 1988. Loss of toluene-xylene catabolic genes of TOL plasmid pWVO during growth of *Pseudomonas putida* on benzoate is due to a selective growth advantage of 'cured' segregants: J. Gen. Microbiol. **134**:2039-2048.
119. **Williamson, W.** 1992. Environmental risks from the release of genetically modified organisms (GMOs) - the need for molecular ecology: Mol. Ecol. **1**:3-8.
120. **Wilson, Kate.** 1990. Chromosomal DNA Purification Ausubel, F. M., Editor, Current Protocols in Molecular Biology: New York, Greene Pub. Associates & Wiley-Interscience, p. 2.4.
121. **Winstanley, C., Morgan, J.A.W., Pickup, R.W., and others.** 1989. Differential regulation of lambda *pL* and *pR* promoters by a *cI* repressor in a broad-range thermoregulated plasmid marker system: Appl. Environ. Microbiol. **55**:770-777.
122. **Wolk, P.C., Cai, Y., and Panoff, J.-M.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium: Proc. Natl. Acad. Sci. USA **88**:5355-5359.
123. **Wyndham, R.C., Singh, R.K., and Straus, N.A.** 1988. Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes* sp. BR60: Arch. Microbiol. **150**:237-243.
124. **Yen, K.M., and Gunsalus, I.C.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation: Proc. Natl. Acad. Sci. USA **79**:874-.
125. **Yen, K.M., and Serdar, C.M.** 1988. Genetics of naphthalene catabolism in *Pseudomonas*: CRC Crit. Rev. Microbiol. **15**:247-267.
126. **Yeung, K.-H., Schell, M.A., and Hartel, P.G.** 1989. Growth of genetically engineered *Pseudomonas aeruginosa* and *Pseudomonas putida* in soil and rhizosphere: Appl. Environ. Microbiol. **55**:3243-3246.
127. **You, I.S., Ghosal, D., and Gunsalus, I.C.** 1988. Nucleotide sequence of plasmid NAH7 gene product *nahR* and DNA binding of the *nahR* gene product: J. Bact. **170**:5409-5415.
128. **Zünd, P., and Lebek, G.** 1980. Generation time-prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size: Plasmid **3**:65-69.
129. **Zaat, S.A.J., Slegtenhorst-eegdeman, K., Tommassen, J., and others.** 1994. Construction of *phoE-*caa**, a novel PCR- and immunologically detectable marker gene for *Pseudomonas putida*: Appl. Environ. Microbiol. **60**:3965-3973.

130. **Zuniga, M.C., Durham, D.R., and Welch, R.A.** 1981. Plasmid- and chromosome-mediated dissimilation of naphthalene and salicylate in *Pseudomonas putida* PMD-1: J. Bact. **147**: 836-843.

VITA

Wade Hampton Johnston was born in Summerville, South Carolina on November 17, 1963. He was raised in Harleyville, South Carolina and received primary and secondary education at Mims Academy and graduated high school in 1986. He graduated with a Bachelor of Science degree in microbiology from Clemson University in June 1986. He entered graduate school at Clemson University the following fall to study environmental microbiology. His research advisors were Dr. Malcolm Paynter and Dr. N. D. Camper. His research project involved the anaerobic biodegradation of Diquat in lake sediments and the aerobic biodegradation of aniline based pesticides in soil. He graduated with a Master of Science degree in microbiology from Clemson University in 1989. He entered the Ph.D. program in microbiology at the University of Tennessee in the Spring of 1989 with Dr. Gary S. Sayler as his advisor and major professor.

The author is married to Pamela Fox Johnston and they have one child, Andrew Wade Johnston. The Johnston family currently resides in Corvallis Oregon where the author is a post-doctoral research associate in environmental microbiology with the College of Veterinary Medicine at Oregon State University.