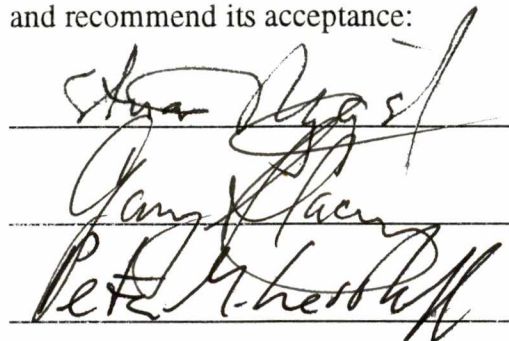


To the Graduate Council:

I am submitting herewith a dissertation written by Bruce Michael Applegate entitled "Construction of Recombinant Bacteria to Elucidate Catabolic Regulation and Critical Catabolic Reactions of Phenanthrene Metabolism by the NAH System." 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 Sayler, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate School



CONSTRUCTION OF RECOMBINANT BACTERIA TO ELUCIDATE CATABOLIC
REGULATION AND CRITICAL CATABOLIC REACTIONS OF PHENANTHRENE
METABOLISM BY THE NADH SYSTEM.

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

Bruce Michael Applegate
August 1997

Dedication

This dissertation is dedicated to

my wife

Pamela M. Applegate

my children

Michael and Sarah

and my parents

Henry P. and Betty R. Applegate

whose love, support and understanding made attaining this degree possible.

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Abstract

Pseudomonas putida strain PpG7 harbors the catabolic plasmid NAH7 which mediates the biotransformation of naphthalene. The plasmid contains the NAH system consisting of two distinct operons coding for two sets of enzymes: The first operon, *nah*, codes for the enzymes that metabolize naphthalene to salicylate (upper pathway) which is initiated by a dioxygenase attack on one ring followed by a dehydrogenation forming 1,2 dihydroxynaphthlene; The second operon, *sal*, codes for a set of enzymes (lower pathway) responsible for subsequent conversion of salicylate to TCA cycle intermediates. Both sets of enzymes are controlled by a LysR regulatory protein which induces both operons in the presence of salicylate. NAH7 and other NAH-type plasmids have also been shown to mediate mineralization of 9-¹⁴C labeled phenanthrene producing ¹⁴CO₂. However, this study shows strains harboring these plasmids can not utilize phenanthrene as a sole carbon and energy source. A transposon mutagenized NAH-type plasmid pUTK21 which can no longer mediate the transformation of salicylate to TCA cycle intermediates has been shown to mediate the biotransformation of phenanthrene to 1-hydroxy-2-naphthoic acid, a compound with a structure similar to salicylate. The strain harboring the plasmid pUTK21 designated 5RL will convert phenanthrene to 1-hydroxy-2-naphthoic acid but fails to produce ¹⁴CO₂. This failure to produce ¹⁴CO₂ is consistent with the production of the dead end metabolite 1-hydroxy-2-naphthoic acid. The absence of ¹⁴CO₂ directly links the enzymes responsible for salicylate metabolism to the further metabolism of 1-hydroxy-2-naphthoic acid. Hybridization results using a *nahG* (the first enzyme in the lower pathway) gene probe showed a direct connection of a negative result with lack of mineralization. The further metabolism of 1-hydroxy-2-naphthoic acid was thought to proceed by the conversion of 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene

by a simultaneous hydroxylation/decarboxylation mediated by the NahG. If this is correct 1,2-dihydroxynaphthalene would be further metabolized through the upper pathway to salicylate and ultimately TCA cycle intermediates. *nah-lux* bioluminescent reporter bacteria showed that inducing intermediates were not produced to allow induction of the enzymes. Since the hybridization results indicated that the *nahG* gene product was correlated with the ability to mineralize this gene was cloned and expressed in *Eschericia coli*. When cells expressing the *nahG* gene product were incubated with 1-hydroxy-2-naphthoic acid a characteristic orange color indicative of a ring cleavage product was produced. Results from this study show that the further metabolism of 1-hydroxy-2-naphthoic acid appears to consist of a ring cleavage of the 1-hydroxy-2-naphthoic acid and a subsequent aldolase reaction to form phthallic acid. These results show that the NAH system can only partially metabolize phenanthrene.

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Chapter I

Introduction

Over the past three decades there have been numerous investigations into the use of microorganisms to degrade xenobiotics as an approach to clean up the chemical aftermath of the industrial revolution. One of the primary groups of xenobiotics being examined for potential microbial degradation is polycyclic aromatic hydrocarbons (PAHs). PAHs are compounds with two or more fused benzene rings which also can contain various side group substitutions (e.g. methyl naphthalene). Research into the role microbes play in the degradation of various PAHs was begun in the first half of this century. Researchers studied the microbial degradation of a variety of PAHs with no forethought of the environmental problems ahead in the second half of the century. The following introduction is a series of synopses of early reports on microbial PAH degradation (primarily naphthalene and phenanthrene, the subject of this study) and attempts to elucidate their degradative pathways.

One of the first reports of the microbial degradation of PAHs was observed in 1927 by F. Tattersfield. Tattersfield examined the simplest of PAHs, naphthalene. His work consisted of determining the fate of naphthalene being used as an insecticide in garden soils. He reported that naphthalene degradation could be attributed to microbial decomposition, as sterile soil did not decompose naphthalene, while non-sterile soil did decompose naphthalene. He also observed that previous exposure to naphthalene accelerated the decomposition rate of naphthalene in non-sterile soil and that the overall heterotrophic population of bacteria increased after exposure to naphthalene. Another interesting observation made by Tattersfield concerned the fate of α -chloronaphthalene (1-chloronaphthalene) in soil. He found that α -chloronaphthalene persisted for a

longer time in the soils than did naphthalene. However, if naphthalene was added concurrently with α -chloronaphthalene, the α -chloronaphthalene disappeared at an accelerated rate. These results were the first report of co-oxidation, with naphthalene being the primary substrate and the α -chloronaphthalene being the fortuitously degraded substrate.

In the same year W.O. Tausson (1927) published a report on the ability of microorganisms to utilize naphthalene as a sole carbon source. Pure cultures of bacteria were isolated on mineral agar plates supplemented with naphthalene. Once obtained, the isolates were analyzed in liquid medium under a variety of growth conditions as well as with other sources of carbon. During the oxidation of naphthalene he observed that at first the medium is acidified, followed by a slow shift back through neutral pH toward alkalinity. This observation suggested that accumulation of free acids as terminal products was not plausible. As Tausson was also interested in the mechanism by which naphthalene was degraded, he examined the media for the presence of di- and triphenols as well as phthallic acid and its esters. None of these compounds was present and the strains were also unable to utilize these compounds or α - and β -naphthol to sustain growth. Therefore he deduced from his findings that naphthalene was not metabolized by cleavage of one of the rings but by cleaving both rings at the same time.

Using this approach Tausson (1928) also examined the microbial degradation of phenanthrene, a three-ringed PAH (Fig. 1). Tausson isolated three strains from oil soaked soils from the Baku, Gurien, and Maikop oil fields in Russia that could utilize phenanthrene as a sole carbon source. Two of the bacteria were designated at the time to be *Bacillus phenantheicus bakiensis* and *Bacillus phenantheicus guricus*; the third was an unknown isolate designated *Bacterium phenanthrecium*. Growth experiments using *o*-hydroxybenzyl alcohol as well as *o*-hydroxybenzyl aldehyde demonstrated

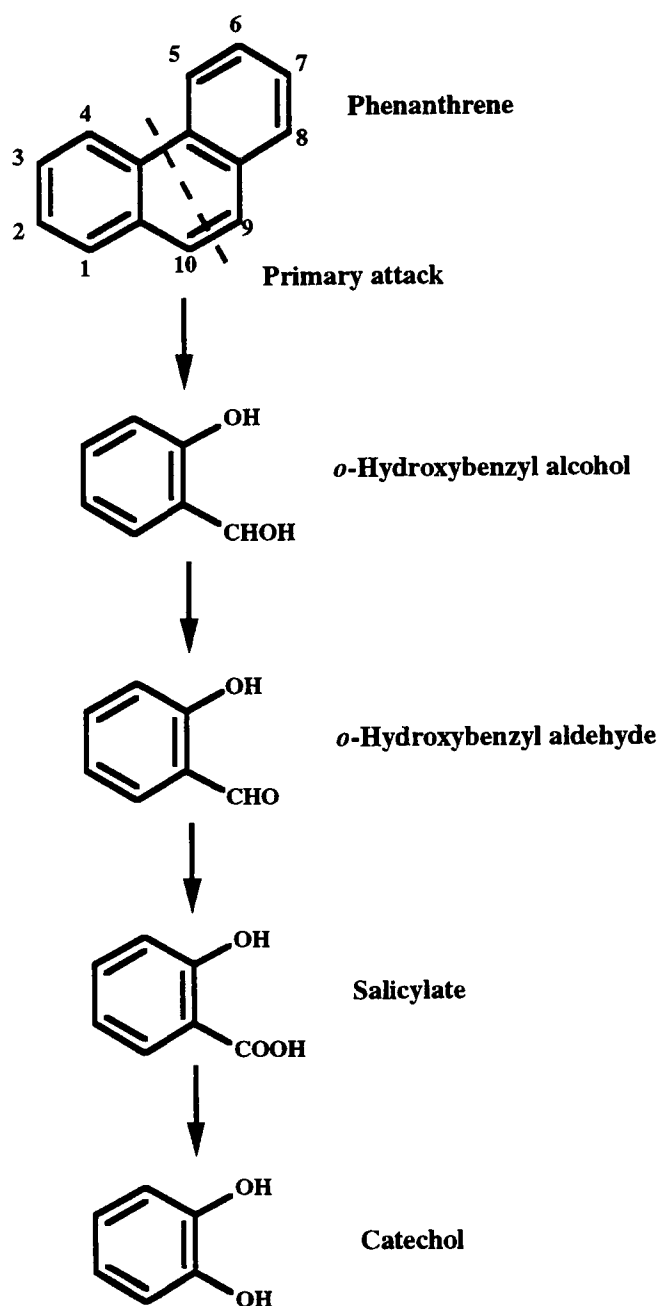


Figure 1. Phenanthrene degradative pathway as proposed by Tausson (1928). Phenanthrene is first cleaved into two molecules of *o*-hydroxybenzyl alcohol which are subsequently converted into *o*-hydroxybenzyl aldehyde and further transformed into salicylate then catechol.

that the strains capable of oxidizing phenanthrene also assimilated these compounds. However, the strains were not able to grow on phthalic acid. These observations caused Tausson to conclude that these particular bacterial strains degraded phenanthrene by an enzymatic attack at the 9,10 position cleaving the molecule in half forming the prior mentioned growth substrates. He then postulated the degradative pathway as seen in Figure 1. One other important observation made by Tausson was that the three strains could not grow using naphthalene as sole carbon source. This suggested that the degradation of naphthalene and phenanthrene must use two different pathways.

The ability of microbes to decompose higher molecular weight PAHs was ascertained in 1947 by Sisler and ZoBell. They examined naphthalene, anthracene, phenanthrene, 1,2-benzanthracene and 1,2,5,6-dibenzanthracene; the latter two had previously been determined to be carcinogenic (Hartwell, 1941). Their assay involved the application of 25 mg of a particular PAH to sand with subsequent inoculation with marine microorganisms and sea water (without a utilizable carbon source). Oxidation was measured by the evolution of CO₂ over time. Since the molecular weight of the PAHs were known, percent oxidations were determined empirically. The oxidation ranged from 13% for the 1,2,5,6-dibenzanthracene to 68% for the phenanthrene. Although this research was more directed toward finding potential therapeutic agents for cancer it is probably more significant for its report of the biological metabolism of PAHs with a higher molecular weight than that of naphthalene and phenanthrene.

The aforementioned research was basically concerned with the microbial degradation of PAHs with limited focus on the particular metabolic steps of degradation. Six months before the publication of Sisler and ZoBell's manuscript, R.Y. Stanier published a paper entitled *Simultaneous Adaptation: A New Technique For The Study of Metabolic Pathways* which outlined a procedure for the

determination of metabolic pathways (Stanier, 1947). The basic theory behind this method is the Kluver Axiom (Kluver, 1931) which states that "every dissimilation is the result of a series of simple, chemically intelligible step-reactions". Stanier took the axiom a step farther by also concluding that "complete oxidation of even a relatively small organic molecule will involve the formation of a large number of intermediate compounds". This was the basis for Stanier's technique. If a microorganism was grown on a particular substrate it would subsequently rapidly oxidize all intermediate substrates. Therefore, to determine if a compound was an intermediate it was simply added to the cells pregrown on the alleged parent substrate (to allow the enzymes to adapt for metabolism of that substrate) and oxygen uptake was followed manometrically. If rapid oxidation was observed, that particular compound was a possible intermediate of the parent compound's degradative pathway. Major drawbacks to this technique are: 1) the intermediate must be available, 2) it must be stable, and 3) the cell must be able to take it up. This approach was extensively used in the early years of pathway elucidation.

In 1957, Rogoff and Wender used the Simultaneous Adaptation approach to examine the dissimilation of phenanthrene. (However it is of interest to note that Stanier was not referenced in this report.) An organism which was tentatively identified as *Pseudomonas aeruginosa* was isolated from garden soil which could utilize phenanthrene as a sole carbon source. During growth on phenanthrene it was noted that the culture became "a deep buff color" and gave a positive ferric chloride test for phenolic compounds indicating a possible naphthoic acid. Cells were incubated with phenanthrene for 64 hours and subsequently pooled and extracted. A white precipitate was obtained after organic extraction and determined to be 1-hydroxy-2-naphthoic acid by comparing its infrared and ultraviolet spectra to that of an authentic compound. This was the first report of an intermediate detected in the oxidation of phenanthrene.

Phenanthrene grown cells were then used to determine other possible intermediates by adding them to adapted cell suspensions. Salicylate, catechol and protocatechuic acid were tested and the resultant oxygen uptake measured. Both catechol and salicylate produced a significant uptake of oxygen while protocatechuic acid produced marginal uptake. These results showed that the phenanthrene grown cells contain the enzymes to oxidize salicylate and catechol. These results allowed Rogoff and Wender to hypothesize a new pathway for the dissimilation of phenanthrene shown in Figure 2. Their pathway differs from that of Tausson as it indicates that the bacteria did not attack the molecule in the center at the 9,10-bond but at either end forming 1-hydroxy-2-naphthoic acid as an intermediate followed by salicylic acid and catechol.

The last of these reports to be presented is that of Evans et al. (1965) in which the phenanthrene degradative pathway was further elucidated using *Pseudomonas* strains P_F and P_G which had previously been isolated from garden soil. Since it had been reported by Colla et al. (1959) that 3,4-dihydro-3,4-dihydroxyphenanthrene was an intermediate, Evans hypothesized that the next intermediate was 3,4-dihydroxyphenanthrene. Since this compound was not commercially available at the time, it had to be synthesized. *Pseudomonas* strains P_F and P_G were grown on phenanthrene and stored at -15°C and subsequently used for simultaneous adaptation assays. Both whole cell assays and cell extracts showed the cleavage of 3,4-dihydroxyphenanthrene to the compound *cis*-o-hydroxynaphthylpyruvic acid. These results suggested that the ring cleavage occurred between the angular C and C-4 and was analogous to that of the 1,2-dihydroxynaphthalene previously described (Davies and Evans, 1964). The latter compound when further reacted with cell extracts was oxidized to 1-hydroxy 2-naphthaldehyde by an aldolase type attack on the side chain. The fate of the C₃ fragment was not determined. 1-Hydroxy-2-naphthaldehyde, which

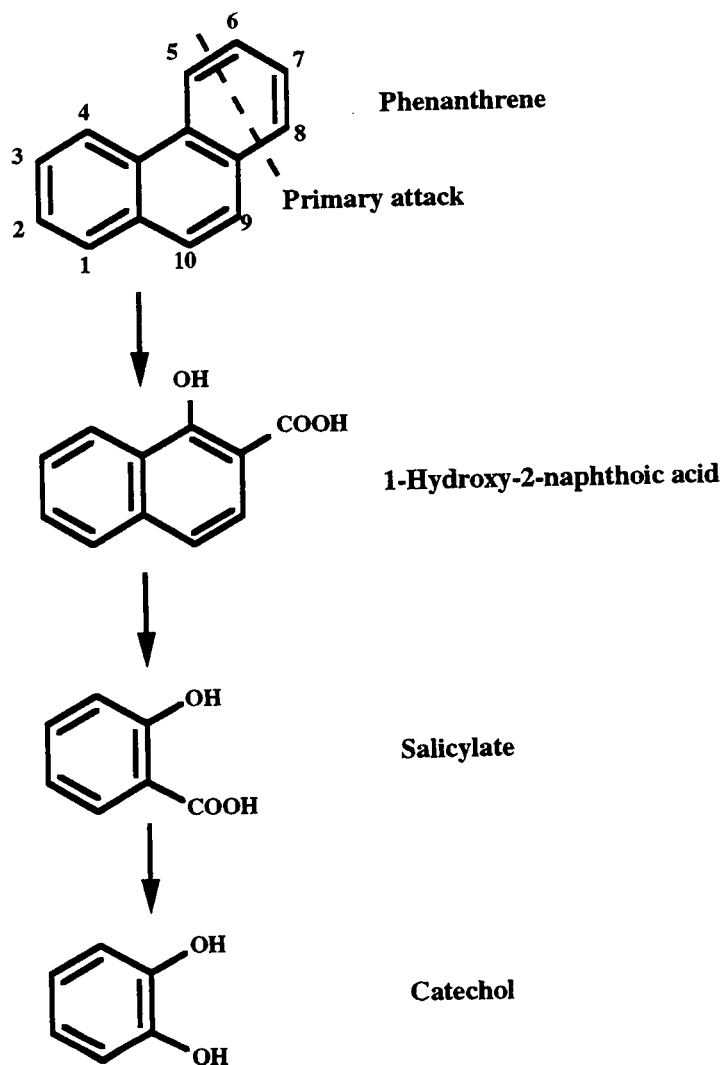


Figure 2. Phenanthrene degradative pathway as proposed by Rogoff and Wender (1957). Phenanthrene is cleaved at the end of the molecule resulting in the formation of 1-hydroxy-2-naphthoic acid and subsequently further metabolized to salicylate and then to catechol.

also had to be synthesized, was found to be further metabolized by cell extracts to 1-hydroxy-2-naphthoic acid by an NAD-dependent dehydrogenase. Since both *Pseudomonas* strains P_F and P_G pregrown on phenanthrene oxidized salicylate and catechol, it was hypothesized that 1-hydroxy-2-naphthoic acid was decarboxylated to 1,2-dihydroxy naphthalene and further metabolized via the naphthalene pathway (Davies and Evans, 1964). However, 1,2-dihydroxy naphthalene was never isolated from either cell extracts or cell suspensions when 1-hydroxy-2-naphthoic acid was assayed. This pathway is the primary pathway used by bacteria to metabolize phenanthrene to 1-hydroxy-2-naphthoic acid and has not been modified since it was proposed.

Since these early works on biodegradation were first reported, knowledge of the intricacies of microorganisms has grown considerably. In the years spanning the selected reports presented, DNA was discovered to be the genetic material (Avery et al., 1944), the structure of DNA was elucidated (Watson and Crick, 1953), and Jacob and Monod (1961) published "Genetic Regulator Mechanisms in the Synthesis of a Protein" in which the foundations for negative gene regulation were laid. These early microbial physiologists did not even consider regulation when they were performing their work as it did not affect the basic strategy of simultaneous adaptation. However, regulation would effect Tausson's approach in growing cells on prospective intermediates. This will be addressed in the discussion.

The last two decades have seen dramatic developments in analytical methods and instrumentation and molecular biological information applied to biotechnology. Simultaneous adaptation has given way to the use of recombinant DNA to construct clones of DNA coding for specific enzymes allowing transformation of compounds sequentially bypassing unstable intermediates. Reporter constructs allow screening of metabolites for their involvement in the regulation of these degradative pathways.

Analytical techniques, such as mass spectrometry, allow the identification of metabolites without intensive purification measures. However, unanswered questions remain concerning the molecular basis of phenanthrene metabolism and the role of the well characterized NAH System, and its contributions to phenanthrene biodegradation. The NAH system consists of two distinct operons coding for two sets of enzymes: The first operon, *nah*, codes for the enzymes that metabolize naphthalene to salicylate (upper pathway) which is initiated by a dioxygenase attack on one ring forming 1,2 dihydroxynaphthlene; The second operon, *sal*, codes for a set of enzymes (lower pathway) responsible for subsequent conversion of salicylate to TCA cycle intermediates. Both sets of enzymes are controlled by a LysR regulatory protein, which is divergently transcribed from the *sal* operon, induces both operons in the presence of salicylate. This study will employ an array of modern techniques and methods to ascertain the NAH involvement in metabolism of phenanthrene. However, this researcher looks back with admiration to these early scientists who used logical reasoning and imagination to ascertain the essential elements and mechanisms of PAH metabolism.

Chapter II

Literature Review

Physical Properties of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are composed of carbon and hydrogen and have a structure consisting of two or more fused benzene rings. The simplest of these PAHs is the bicyclic compound naphthalene. The higher molecular weight PAHs (three or more rings) can consist of a variety of geometric structures. They can be linear or have rings fused to linear configurations at angles of 120° . They also can be clustered with rings being surrounded on all sides. Figure 3 shows a variety of PAHs ranging in molecular weight and structure.

The physical and chemical properties of PAHs are directly related to their molecular weight and structure (Zander, 1983). Aqueous solubility of PAHs decreases as molecular weight increases. An illustration of this would be the difference in solubility of naphthalene (MW 128.16; solubility 33 mg/L) compared to that of phenanthrene (MW 178.22; solubility 1.29 mg/L). However, if you compare the two compounds phenanthrene and anthracene they have the same molecular weight (178.22) but their solubilities are markedly different, 1.29 mg/L versus 0.075 mg/L. The reason for the difference is that these two compounds are isomers (Fig. 3). Anthracene is a linear molecule of three fused rings while phenanthrene is linear with the third ring fused at an angle of 120° . The angular structure causes phenanthrene to be more polar than anthracene. Another example of structure affecting solubility are four isomers of a four ringed PAH: chrysene, benzanthracene, pyrene and triphenylene. These compounds have the same molecular weight (228.30) but differ in

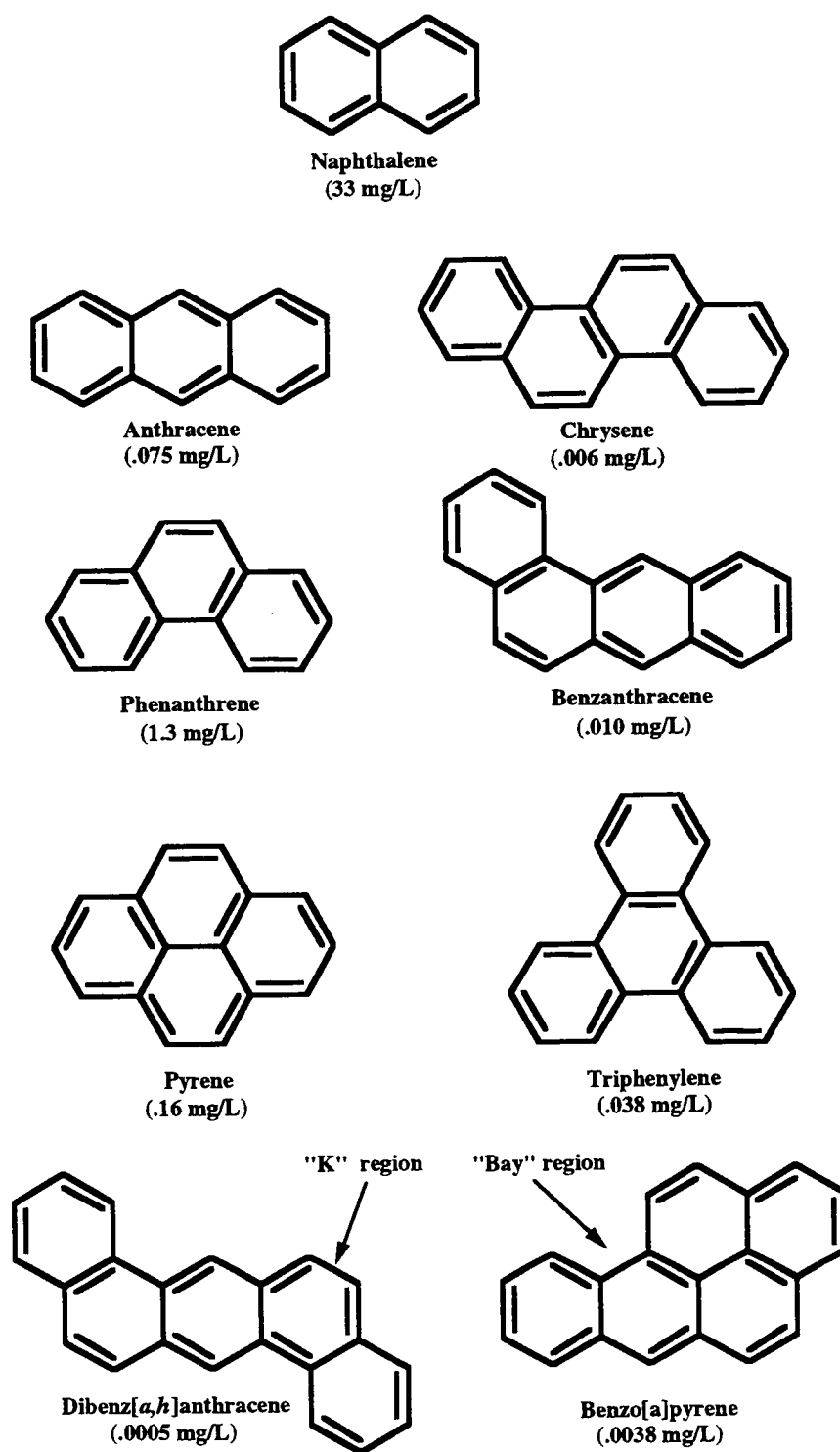


Figure 3. Various polycyclic aromatic hydrocarbons (PAHs). PAHs ranging from the lowest molecular weight PAH naphthalene to isomers consisting of four fused rings. Solubilities are enclosed in parenthesis.

structure (Fig. 3). Solubilities range from the most soluble, pyrene (0.16 mg/L) to the least soluble chrysene (.006 mg/L).

Toxicology of PAHs

PAHs are extremely nonpolar and lipophilic in nature which correlates with their low water solubility. PAHs are of environmental concern because they are: 1) persistent in the environment, 2) can be both carcinogenic and mutagenic, and 3) pose the potential to bioaccumulate. Some PAHs are carcinogenic and were first determined to be chemical carcinogens by their link to snuff (John Hill 1761), chimney soot (Pott, 1775), and coal tar (Yamagiwa and Ichikawa, 1915). PAHs greater than three rings with the characteristic angular region (Bay-Region) are termed DNA-reactive carcinogens because they require metabolic activation of the initial compound to its resultant carcinogenic metabolite (Williams and Weisburger, 1986). One of the most widely studied PAHs for its carcinogenic effect is benzo(a)pyrene. This particular PAH undergoes metabolic activation in the mammalian liver to form a bay-region diol-epoxide which reacts with DNA resulting in mutagenicity and subsequent carcinogenicity (Boyland and Sims, 1965a, 1965b; Phillips, 1985). Phenanthrene, the subject of this study, is not considered to be mutagenic or carcinogenic. However, it has been reported that the K-region metabolites 9,10-oxide, 9-phenanthrol and phenanthrene *trans*- 9,10-dihydrodiol are weakly mutagenic (Bücker et al., 1979).

In general, phenanthrene itself is considered nontoxic to mammals (Yoshikawa et al., 1985). However it is toxic to green algae as it significantly reduces photosynthesis (Kusk, 1981) and also causes digestive cell death in marine mussels (Pipe and Moore, 1986). Phenanthrene is also considerably toxic to young

rainbow trout and decreases reproduction and increases mortality in crustaceans (Savino and Tanabe, 1989). Passino-Reader et al. (1995) also found phenanthrene to be toxic to rainbow trout fry with an LC50 of 0.2 mg/L in a large constant flow water system.

Sources and Distribution of PAHs in the Environment

PAHs are ubiquitous environmental pollutants and are primarily produced from the incomplete combustion of organic material (Harvey, 1991; Edwards, 1983 ; Blumer, 1976). PAHs can come from a variety of pyrolytic processes both natural and anthropogenic (Edwards, 1983; Sims and Overcash, 1983). Some of the natural origins of PAHs are from forest fires and volcanoes, while the anthropogenic sources of PAHs include burning of fossil fuels, cigarette smoke, and the coking of coal to produce charcoal and combustible gas. The latter of these processes was a major source of PAH production for nearly a century as coal was gasified and the resultant gas was used as fuel for heating, lighting and cooking (Rhodes, 1979). The residue from this process, primarily coal tar PAHs, was released into the environment due to poor byproduct recovery and inefficient material handling. The burning of fossil fuels and subsequent release of PAHs into the air results in atmospheric deposition. Runoff from roadways is also a significant source of PAHs in the environment (Malthy et al., 1995). This results in these compounds being ubiquitous environmental pollutants. The concentration of PAHs in soils vary but shows an overall trend that relates concentrations from high to low as one moves from an industrial area to a rural area (Jones et al., 1989).

Biological PAH Degradation

PAHs are degraded biologically by two predominant oxidative enzyme attacks divided basically along prokaryotic and eukaryotic lines (Fig. 4) (Cerniglia, 1993; Cerniglia and Heitkamp, 1989; Ribbons and Eaton, 1982). The objective of eukaryotic metabolism of PAHs is predominantly conversion of the PAH into a more hydrophilic molecule which can be excreted. Prokaryotes generally metabolize the larger PAH into smaller constituents which may allow assimilation.

Eukaryotes predominantly degrade PAHs via a monooxygenase attack catalyzed by cytochrome P450 resulting in the formation of a ring epoxide. The resultant arene oxide is then transformed by an epoxide hydrolase to a *trans*-dihydrodiol or it can undergo a nonenzymatic rearrangement to form phenols. The resultant compound is more hydrophilic than the parent compound and thus more readily excreted. The P450 system in the fungus *Cunninghamella elegans* has been examined for its ability to metabolize a variety of PAHs ranging from the simplest naphthalene to the most potent carcinogen benzo(a)pyrene to the corresponding dihydrodiols or phenolic compounds (Cerniglia et al., 1980; Pothuluri et al., 1990; Pothuluri et al., 1992; Pothuluri et al., 1993). However, there are numerous reports showing the lignolytic fungus *Phanerochaete chrysosporium* is capable of metabolizing a variety of compounds including various PAHs (Bumpus et al, 1985; Sanglard, 1986; Bumpus 1987; Bumpus, 1989; Hammel, 1989; Hammel, 1991; Morgan, 1991) using enzymes other than the P450 monooxygenase. Lignolytic breakdown of PAHs is nonspecific and is the result of extracellular lignolytic peroxidases (Kirk and Ferrell, 1987; Tien, 1987; Hammel and Moen, 1991; Moen and Hammel, 1994). These peroxidases react with their substrate to form cation radicals which result in a chain reaction of decomposition. Recently the white rot fungus *Pleurotus ostreatus* has been

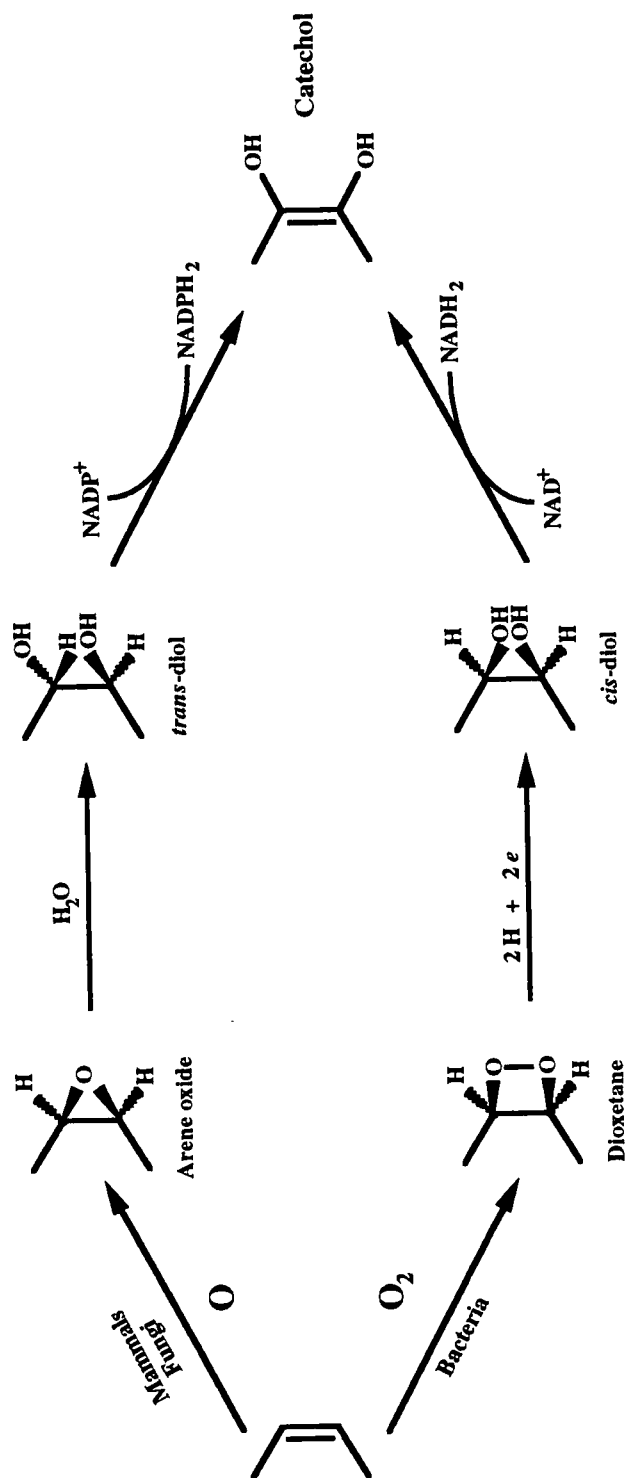


Figure 4. Differences in the eukaryotic and prokaryotic initial metabolism of PAHs to catechols. (Adapted from Ribbons and Eaton, 1982)

shown to mineralize various PAHs via a route other than extracellular peroxidases (Belzal et al., 1996b). Although extensive work has been done on fungal metabolism of PAHs, the predominant work is prokaryotic.

The bacterial metabolism of PAHs is predominantly done by aerobic microorganisms and has been extensively reviewed (Cerniglia 1984, 1992, 1993; Gibson and Subramanian, 1984; Sutherland et al., 1995). The primary initial oxidation of PAHs by aerobic bacteria is the addition of molecular oxygen across the carbon-carbon double bond of the ring forming a *cis*-dihydrodiol (Fig. 4) (Cerniglia, 1992). Reports of bacterial oxidation of PAHs are inversely proportional to their molecular weight. This observation is due to decreasing solubilities and the structure of the molecule as clustered PAHs tend to be more recalcitrant in the environment (Bossert, et al., 1984). One of the most studied PAHs in terms of its degradation is naphthalene. There are numerous bacteria that are capable of metabolizing naphthalene; *Pseudomonas putida*, (Davies and Evans, 1964, Eaton and Chapman, 1992), *Pseudomonas fluorescens* (Sanseverino et al., 1993), *Mycobacterium* (Heitkamp et al., 1988), *Rhodococcus* (Grund et al., 1992). The aforementioned examples all contain a common link; they biochemically mediate naphthalene degradation through salicylate as a metabolic intermediate. The further degradation of salicylate is a point of divergence in the bacterial metabolism of naphthalene as there are two distinct pathways known. The primary route of salicylate metabolism is by a simultaneous decarboxylation/hydroxylation forming catechol which is subsequently degraded through an *ortho* or *meta* cleavage pathway. The second pathway consists of the oxidation of salicylate to gentisate and the subsequent ring cleavage product maleylpyruvate (Lack, 1959). Both pathways further degrade their particular ring cleavage products to TCA cycle intermediates (Stanier, 1966).

There are few reports of bacteria able to utilize the more complex PAHs of four or more rings as a sole carbon source. It is more likely that these compounds are fortuitously degraded when cultures are provided with an alternative carbon source (Cerniglia, 1984). However, Boldren et al. (1993) have isolated a *Mycobacterium* sp. that is capable of using the four ringed PAHs pyrene and fluoranthene as sole carbon and energy sources.

Fungal Phenanthrene Degradation

Phenanthrene is readily metabolized by the non-lignolytic fungus *Cunninghamella elegans* to the corresponding *trans* 1,2-, *trans* 3,4- and *trans* 9,10-dihydrodiols (Fig. 5A) (Cerniglia et al., 1989), while *Synecephalustrum racemosum* metabolizes phenanthrene to *trans* 3,4- and *trans* 9,10-dihydrodiols but not the 1,2-*trans* dihydrodiol (Sutherland et al., 1993). It is important to note that in both the aforementioned cases no mineralization occurs. In contrast to the previously mentioned examples, lignolytic fungi possess the ability to cleave aromatic rings and mineralize PAHs (Barclay et al., 1995; Bezalel et al., 1996a; Bumpus, 1989). Due to its ionization potential of 8.19 eV, phenanthrene is not considered to be a lignin peroxidase substrate (Hammel et al., 1992). However, under lignolytic conditions *Phanerochaete chrysosporium* is able to mineralize phenanthrene by the conversion of phenanthrene to phenanthrene-9,10-quinone followed by ring cleavage to 2,2'-diphenic acid and subsequent release of CO₂ (Fig. 5B) (Hammell et al., 1992; Heitkamp et al., 1988). It is of interest to note that under nonlignolytic conditions *Phanerochaete chrysosporium* metabolizes phenanthrene to phenanthrene *trans* 1,2-, *trans* 3,4- and *trans* 9,10-dihydrodiols and 9-phenanthrol (Sutherland et al., 1991). Recently Bezalel et al. (1996b) working with the white rot fungus *Pleurotus ostreatus* elucidated a pathway

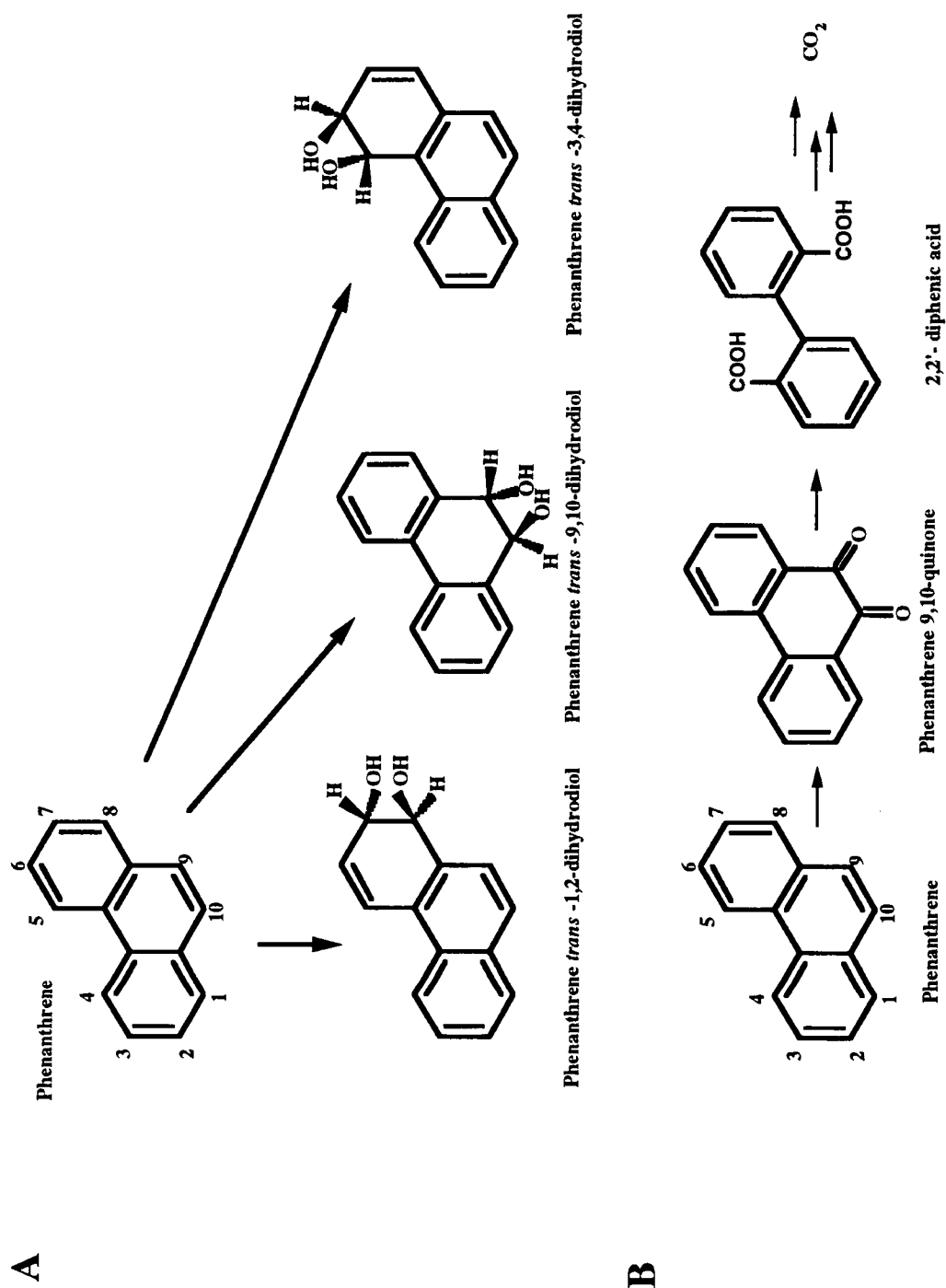


Figure 5. Fungal metabolism of phenanthrene. A.) Oxidation of phenanthrene to dihydrodiols by *Cunninghamella elegans*. B.) Proposed phenanthrene degradative pathway utilized by *Phanerochaete chrysosporium*. (Adapted from Sutherland et al., 1995)

for phenanthrene mineralization which is initiated by a stereoselective oxidation of phenanthrene to a *trans* 9,10-dihydrodiol followed by the formation of 9,10-phenanthrenequinone with resultant cleavage to 2,2'-diphenic acid and subsequent release of CO₂. However, it is important to note that although 94% of the phenanthrene was metabolized by *Pleurotus ostreatus* only 3% was mineralized to ¹⁴CO₂.

Bacterial Metabolism of Phenanthrene

Microorganisms which are capable of metabolizing phenanthrene as their sole carbon and energy source are easily isolated from soil. The initial bacterial oxidation of phenanthrene occurs by the addition of a molecule of oxygen (O₂) across the double bond at the 3,4-position to form a 3,4-dihydroxy-3,4-dihydrodiol. This intermediate was first isolated from a *Flavobacterium sp.* which was grown on phenanthrene. The stereochemistry of the phenanthrene dihydrodiols was confirmed to be the phenanthrene-*cis*-3,4-dihydrodiol by two mutant strains *Beijenickia* B-836 and *Pseudomonas putida* 119 which were deficient in the dihydrodiol dehydrogenase activity (Jernia et al., 1976). With the dihydrodiol dehydrogenase activity present, the phenanthrene-*cis*-3,4-dihydrodiol is converted to 3,4-dihydroxyphenanthrene. Evans et al. (1965) showed that cell free extracts of three *Pseudomonas* strains contained enzymes which could cleave 3,4-dihydroxyphenanthrene on the Bay side of the 4 position to form *cis-o*-hydroxynaphthylpyruvic acid. This ring cleavage product is further oxidized by a hydratase-aldolase cleavage of the isomerized side chain to form 1-hydroxy-2-naphthaldehyde and pyruvate. The 1-hydroxy-2-naphthaldehyde is subsequently converted to 1-hydroxy-2-naphthoic acid by oxidation of the keto group to a carboxyl group by a dehydrogenase. A summary of the stepwise degradation of

phenanthrene to 1-hydroxy-2-naphthoic acid can be seen in Figure 6. At this point in the metabolic pathway of phenanthrene degradation there is a divergence as 1-hydroxy-2-naphthoic acid can be further metabolized via two distinctly different pathways.

1-hydroxy-2-naphthoic acid can be decarboxylated to form 1,2-dihydroxynaphthalene where it is then degraded via the naphthalene pathway as proposed by Evans et al. (1965). Kiyohara and Nagao (1978) isolated numerous strains of bacteria that were capable of phenanthrene utilization. In particular, they isolated an *Aeromonas* sp. that metabolized phenanthrene to 1-hydroxy-2-naphthoic acid by the same pathway Evans et al. (1965) hypothesized (Fig. 6). However, the further degradation of 1-hydroxy-2-naphthoic acid did not involve the decarboxylation to 1,2-dihydroxynaphthalene but an intradiol cleavage to form 2'-carboxybenzylpyruvate which is then oxidized to *o*-phthalate and degraded through protocatechuic acid. The *Aeromonas* strain also was able to grow on naphthalene and salicylate. However, there was minimum O₂ uptake when these compounds were used in simultaneous adaptation assays with cells grown on phenanthrene, suggesting that there were two distinct pathways present in the cell. Barnsley (1983a) isolated a Gram-negative coccus which was able to grow on phenanthrene and phthalic acid as sole carbon source. This seems to suggest a similar catabolic pathway as demonstrated by Kiyohara and Nagao (1978). Barnsley (1983b) further analyzed the metabolism of phenanthrene by this strain and found both the formation of 1-hydroxy-2-naphthoic acid and intradiol cleavage to form 2'-carboxybenzylpyruvate. He also showed the aldolase cleavage of 2'-carboxybenzylpyruvate to phthallic acid and pyruvate using cell extracts. Figure 7 shows the divergence of the further metabolism of 1-hydroxy-2-naphthoic acid. It has been shown that the NAH system for naphthalene degradation can also metabolize phenanthrene. [This is the subject of this study and will be addressed in the section following a thorough description of the NAH system.]

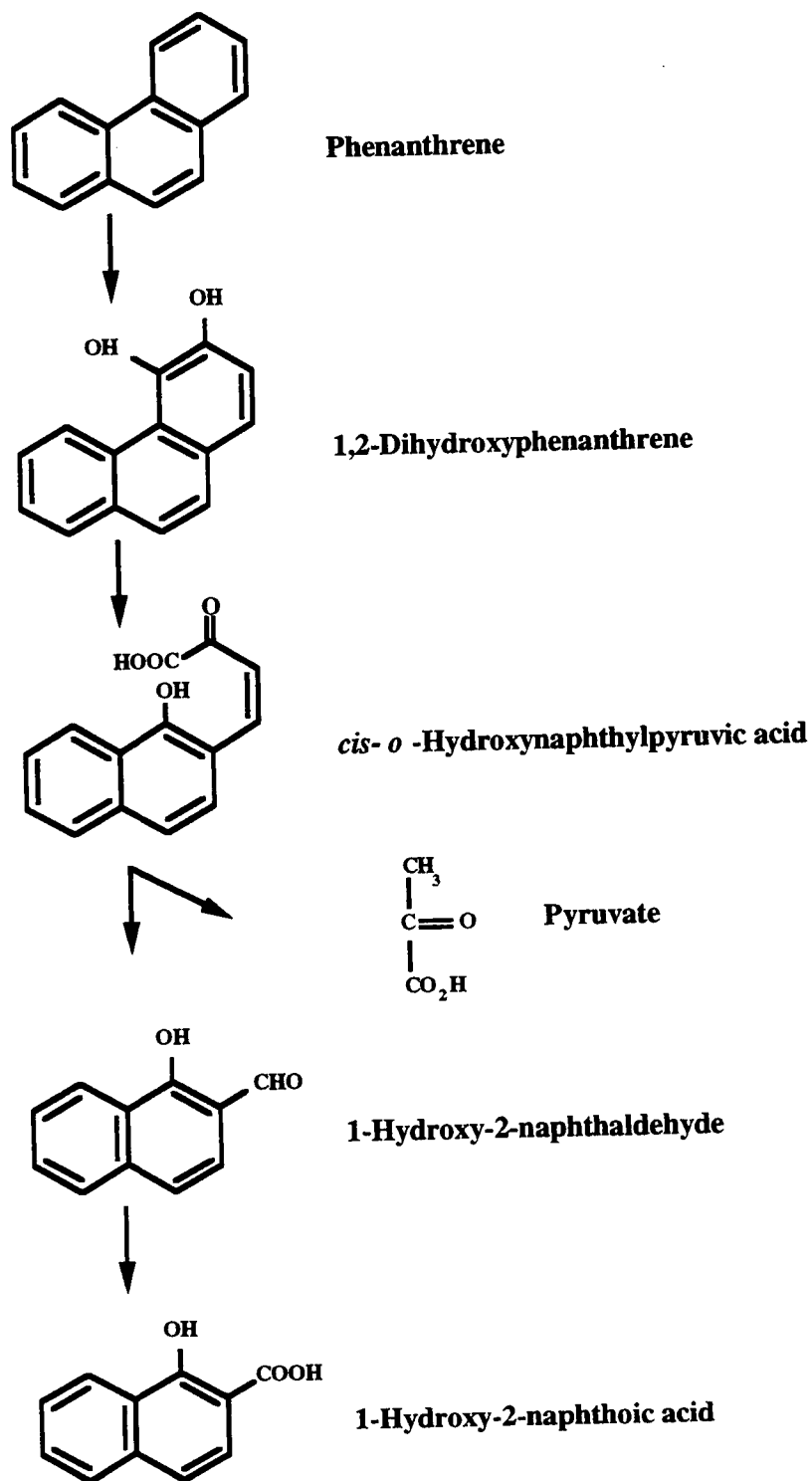


Figure 6. Metabolic pathway for the degradation of phenanthrene to 1-hydroxy-2-naphthoic acid as outlined in the text.

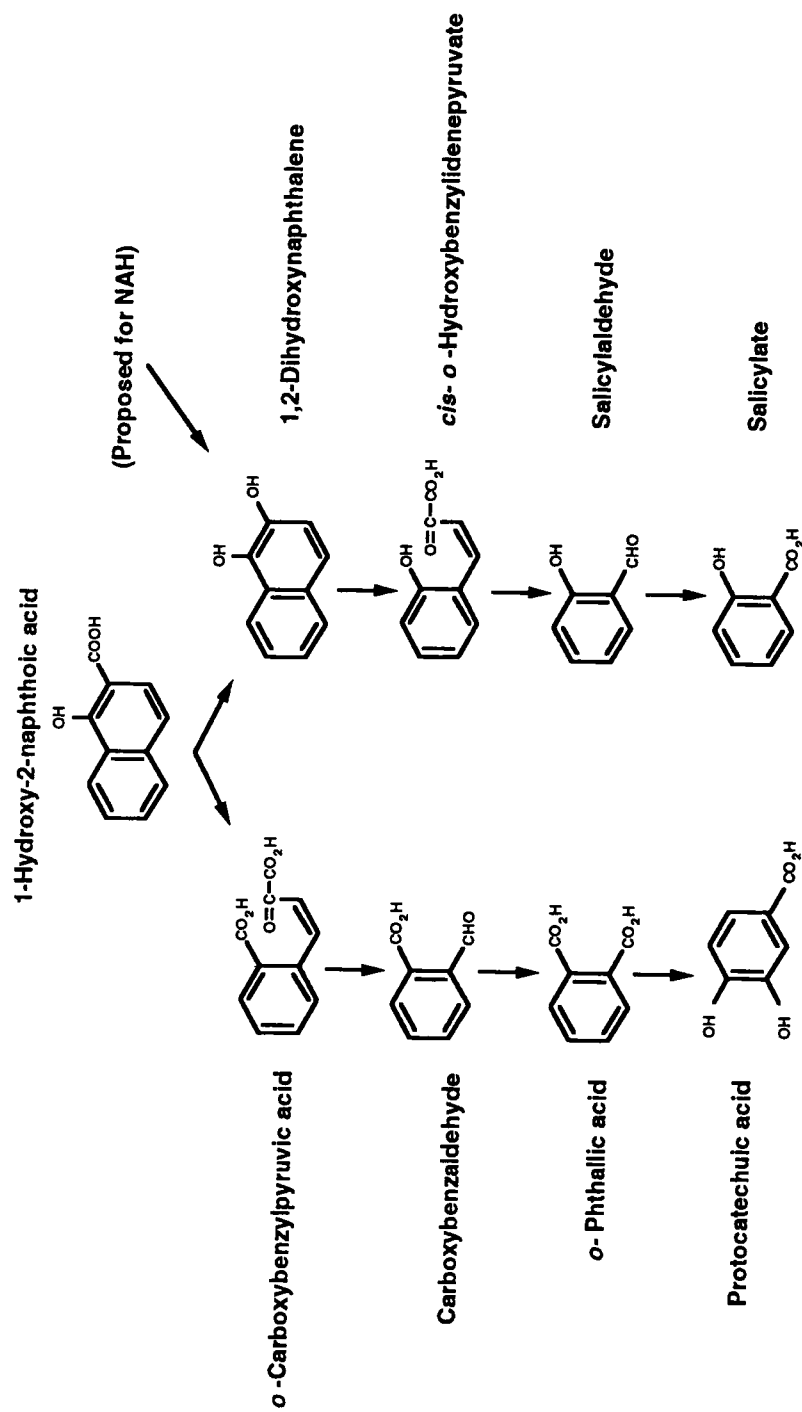


Figure 7. Divergent pathways for the further bacterial metabolism of 1-hydroxy-2-naphthoic acid.

NAH7 and Archetypal NAH Plasmids

NAH7 is a large catabolic self transmissible plasmid harbored in the *Pseudomonas putida* PpG7 (Dunn and Gunsalus, 1973). This plasmid contains the genes which code for the metabolism of naphthalene to TCA cycle intermediates. The biochemistry and genetics of this degradative pathway have been extensively studied and reviewed (Yen and Serdar, 1988; Eaton and Chapman, 1992; Eaton, 1994). Plasmids conferring the ability to metabolize naphthalene have been isolated. These include the plasmid pDTG1 harbored in NCIB 9816-1, and its derivatives in NCIB9816-2, NCIB 9816-3 and NCIB 9816-4 (Connors and Barnsley, 1982; Yen and Serdar, 1988). The degradative pathway for naphthalene coded for on both the NAH7 plasmid and that of the variant of pDTG1 harbored in NCIB 9816 are very similar (Cane and Williams, 1986). Denome et al. (1993) isolated a 75 kb plasmid that contains an identical *nah* operon as determined by sequence analysis. Other archetypal NAH plasmids have been isolated from strains from Manufactured Gas Plant (MGP) sites including plasmids pKA1, pKA2, and pKA3 from the *Pseudomonas fluorescens* strains 5R, DFC 49, and DFC 50 (Sanseverino et al., 1993). These plasmids were approximately 100 kb in size, self transmissible and hybridized to NAH derived gene probes. An earlier report examining both the presence and the restriction fragment length polymorphism (RFLP) of plasmids harbored in strains from MGP sites from around the United States showed a high prevalence of plasmid incidence for naphthalene degrading strains and a variable diversity of RFLP patterns depending on the soil (Sanseverino et al., 1991). These data suggest an almost ubiquitous nature for plasmid encoded naphthalene degradation as there have been few reports of genes for NAH degradative capacity on the chromosome (Callicotte, 1995).

The NAH catabolic system conferred by the NAH7 plasmid is composed of two genetically distinct operons separated by a regulatory gene and is shown in Figure 8 (Yen and Serdar, 1982). The first of these operons is referred to as *nah* and codes for the upper biochemical pathway responsible for the metabolism of naphthalene to salicylate. The second operon referred to as *sal* codes for the further biochemical transformation of salicylate to TCA cycle intermediates. The lower pathway (*sal*) consists of two distinct genetic elements, *nahR-nahG* and the catechol *meta* cleavage operon from pWWO (plasmid which mediates toluene metabolism) which have been combined. Yen and Serdar (1988) propose that *nahR-nahG* evolved independently of the rest of the lower pathway. The evidence for this is two-fold. First there are 831 bp between the end of *nahG* and the beginning of *nahH* the first enzyme in the catechol *meta*-cleavage operon. This region also contains a 327 bp ORF with apparently no known function (You et al., 1991). This region could be left from a possible recombination event between the *nahR-nahG* operon and that of the catechol *meta* cleavage operon from pWWO. The second piece of data to suggest this proposal is that when one compares the sequence similarity of *nahH* and *xylE* from pWWO, there is an 81% sequence identity between the two genes (Ghosal et al., 1987). These two pieces of data tend to support the hypothesis that the lower operon is composed of two distinct genetic elements.

***nah* Operon**

The *nah* operon codes for the genes responsible for the enzymes that metabolize naphthalene to the intermediate salicylate (upper NAH pathway) as shown in Figure 9. The first step of the biodegradation of naphthalene consists of the conversion of naphthalene to *cis*-naphthalene dihydrodiol (Catteral et al., 1971 and Jernia et al., 1971).

NAH Catabolic Gene Organization and Regulation

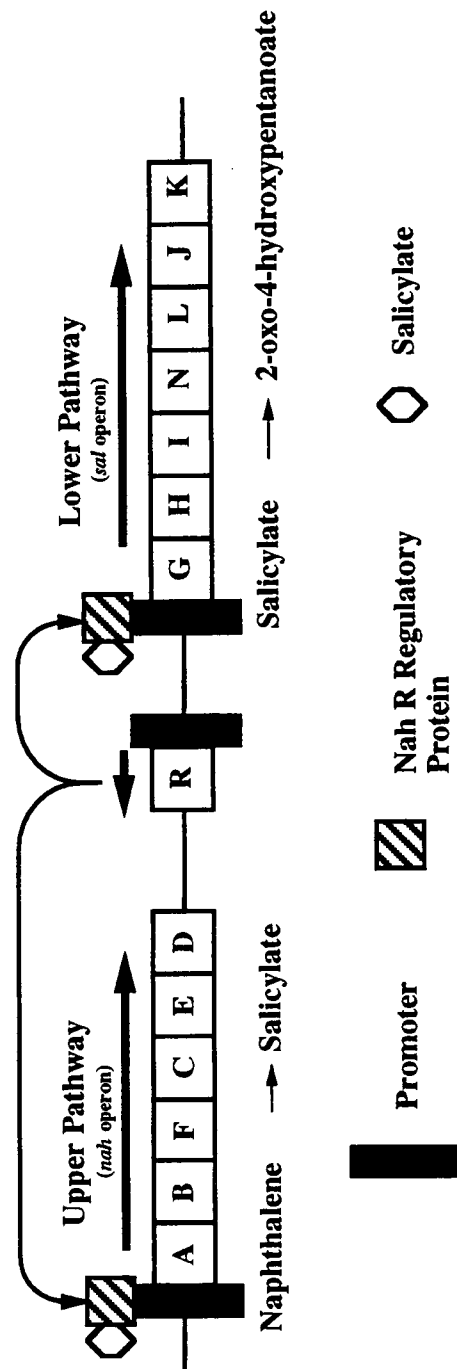


Figure 8. Schematic representation of the NAH system for the degradation of naphthalene. The upper pathway consists of the *nah* operon which codes for the enzymes to metabolize naphthalene to salicylate. The lower pathway consists of the *sal* operon which further metabolizes salicylate to TCA cycle intermediates. The regulatory protein is divergently transcribed from the *sal* operon and interacts with salicylate to induce both the *nah* and *sal* operons.

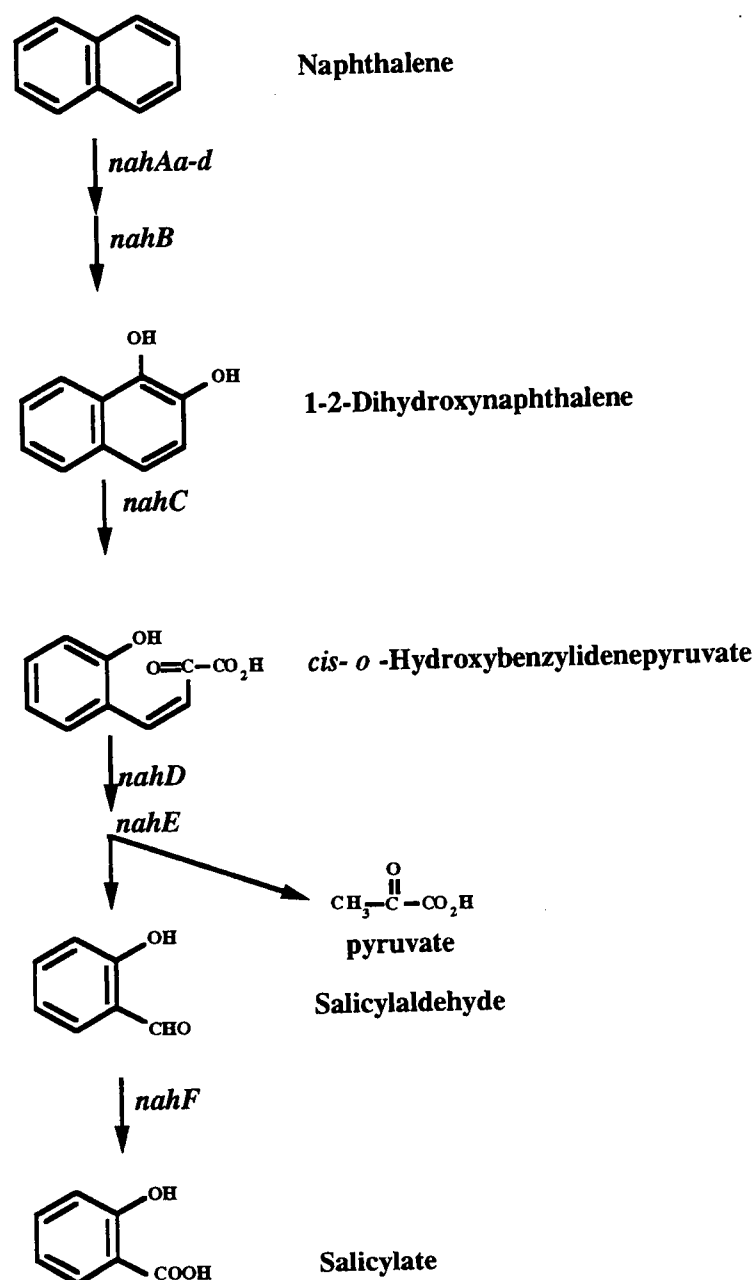


Figure 9. Stepwise degradative pathway of naphthalene to salicylate by the enzymes encoded by the *nah* operon (upper pathway). Enzymes: *nahAa-d* naphthalene dioxygenase; *nahB* *cis*-naphthalene dihydrodiol dehydrogenase; *nahC* 1,2-dihydroxynaphthalene dioxygenase; *nahD* 2-hydroxychromene-2-carboxylase isomerase; *nahE* *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase; *nahF* salicylaldehyde dehydrogenase.

The reaction consists of adding molecular oxygen across the double bond at the 1,2 position of naphthalene by the naphthalene dioxygenase coded for by the *nahA* gene cluster *nahA_aAbA_cAd*. The naphthalene dioxygenase is a multicomponent enzyme consisting of beta2 quaternary structure consisting of two subunits with molecular masses of 55 kdal and 22 kdal and a total molecular mass of 158 kdal (Ensley and Gibson, 1983).

In the second step of the NAH degradative pathway *cis*-naphthalene dihydrodiol is subsequently dehydrogenated by an NAD dependent oxidation to form 1,2-dihydroxynaphthalene by the *cis*-naphthalene dihydrodiol dehydrogenase (Patel and Gibson, 1974). 1,2-Dihydroxynaphthalene was first hypothesized to be an intermediate in naphthalene degradation by simultaneous adaptation studies performed by Fernly and Griffith (1958). The *cis*-naphthalene dihydrodiol dehydrogenase coded for by the *nahB* was also purified and characterized by Patel and Gibson (1974).

The third step in the oxidation of naphthalene is the first of two ring cleavage reactions. The reaction is catalyzed by the ring cleavage enzyme 1,2-dihydroxynaphthalene dioxygenase coded for by *nahC* (Patel and Barnsley, 1980 and Harayama and Rekik, 1989). The ring cleavage reaction involves the addition of molecular oxygen across the double bond at the 1,2 position. Early work performed by Davies and Evans (1964) suggested that the initial ring cleavage product of 1,2-dihydroxynaphthalene was 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate spontaneously isomerizing to form *cis*-2-hydroxybenzylidenepyruvate which is then degraded to salicylate and pyruvate. However, Barnsley (1976) proposed that the ring cleavage product was 2-hydroxychromene-2-carboxylate and that it was converted by an enzyme, which had been partially purified, to *cis*-2-hydroxybenzylidenepyruvate. Sixteen years later Eaton and Chapman (1992) solved the discourse in the earlier work by using a *nahABC* clone that converted naphthalene

to a mixture of 2-hydroxychromene-2-carboxylate and *trans*-2-hydroxybenzylidenepyruvate. These results suggested that Barnsley had incorrectly identified *trans*-2-hydroxybenzylidenepyruvate as its *cis* isomer. However, Eaton and Chapman proposed that 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate is the actual ring cleavage product which is spontaneously isomerized to 2-hydroxychromene-2-carboxylate via a *cis*-2-hydroxybenzylidenepyruvate intermediate. The 2-hydroxychromene-2-carboxylate subsequently can undergo spontaneous isomerization to *trans*-2-hydroxybenzylidenepyruvate. Eaton and Chapman also demonstrated the gene product from *nahD* was the isomerase 2-hydroxychromene-2-carboxylate isomerase which increases the rate of isomerization to *trans*-2-hydroxybenzylidenepyruvate. After isomerization by either spontaneous or enzymatic action the resultant *trans*-2-hydroxybenzylidenepyruvate is further metabolized to pyruvate and salicylaldehyde by a hydratase-aldolase catalyzed reaction encoded by a single enzyme coded for by *nahE*.

The final enzymatic step in the upper pathway is the conversion of salicylaldehyde to salicylate by the gene product of *nahF*. This NAD⁺ requiring dehydrogenase oxidizes the keto group to a carboxyl group forming salicylate. The salicylate is then involved in the overall regulation of the NAH system and further metabolized by the *sal* operon.

***sal* Operon**

The *sal* operon encodes the lower NAH pathway consisting of the enzymes necessary to degrade salicylate to TCA cycle intermediates pyruvate and acetylaldehyde. The first enzyme in the lower pathway is the salicylate hydroxylase encoded by the gene *nahG*. This enzyme catalyzes the simultaneous hydroxylation/decarboxylation of

salicylate to catechol as seen in Figure 10. Salicylate hydroxylase is a flavoprotein and has a molecular mass of approximately 45 kdal. It has been purified and the amino terminus sequenced (You et al., 1990). The gene coding for this enzyme has also been cloned and expressed in *Escherichia coli* (You et al., 1991). The obtained sequence information is in agreement with the overall molecular weight and amino terminus previously ascertained.

Catechol is further metabolized through a meta cleavage pathway as seen in Figure 11. The first step in the *meta* cleavage pathway is the initial ring cleavage of the catechol by catechol 2,3-dioxygenase which is encoded by the *nahH* which has been cloned and sequenced (Ghousal et al., 1987). The resultant cleavage product, 2-hydroxymuconic semialdehyde, is bright yellow and has a specific absorbance at 375 nmol. The resultant 2-hydroxymuconic semialdehyde undergoes a series of enzymatic steps outlined in Figure 12 to form 2-oxo-4-hydroxypentanoate followed by an aldolase cleavage to form pyruvate and acetaldehyde (Yen and Serdar, 1988).

NAH Regulation

The NAH system for the degradation of naphthalene is positively regulated by the NahR protein. Salicylate, a metabolite produced by the degradation of naphthalene by the upper NAH pathway, binds to the NahR promoter complexes (*sal* and *nah*) causing the formation of an operational RNA transcriptional complex and initiation of transcription (Schell, 1985). NahR is a 36 kdal protein encoded by *nahR* which has been sequenced (Schell and Wender, 1986; You et al., 1988). Analysis of the amino acid sequence composing the NahR protein showed that it was a member of the LysR family of regulatory proteins. LysR proteins are a group of positive regulatory

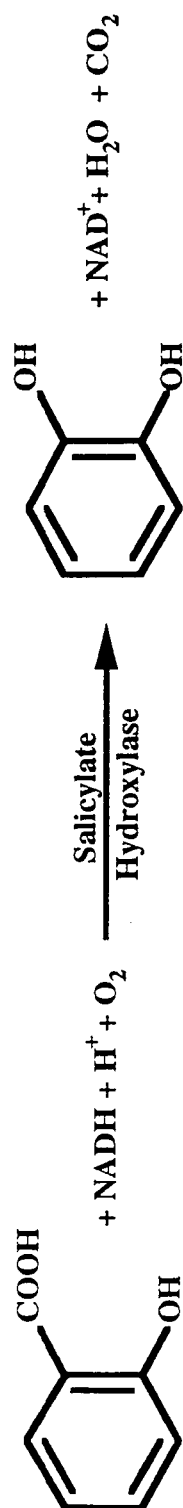


Figure 10. The NADH dependant simultaneous hydroxylation/decarboxylation of salicylate to catechol by the *nahG* encoded salicylate hydroxylase. (Adapted from You et al., 1991)

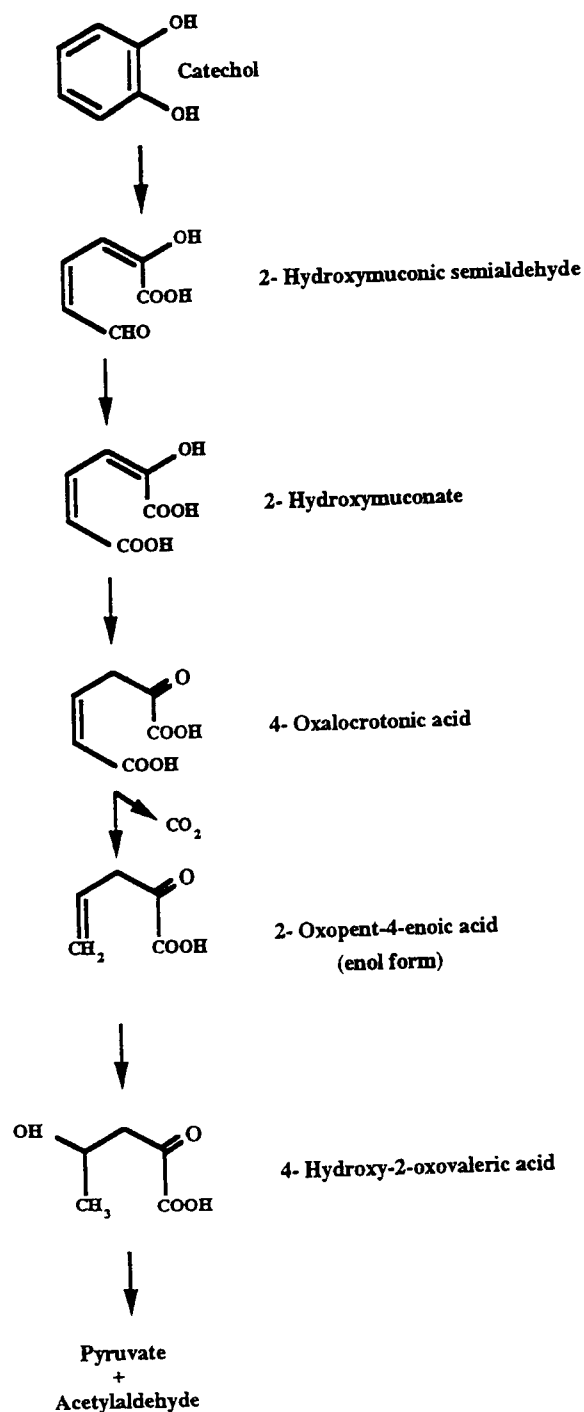


Figure 11. Metabolic pathway of catechol degradation to TCA cycle intermediates through the *meta* cleavage enzymes encoded by the *sal* operon.

proteins which have similar characteristics in their structure and require an effector molecule to initiate transcription. These proteins contain three major domains which are conserved among members. The first of these is the DNA binding domain which contains a helix-turn-helix DNA binding motif and is found at the amino terminus and usually contained in the first 66 amino acids; the second domain consists of the coinducer recognition/response domain which binds the effector molecule; the third conserved domain is that found at the carboxy terminus between amino acids 227-253. The function of this region varies with different members of the LysR family (Schell, 1993). As well as protein homology, these regulatory proteins share other characteristics. They will bind to the promoter sequence whether or not the coinducer is present. They are divergently transcribed and usually the promoter overlaps the promoter for the gene which is being regulated. In the case of the NAH system *nahR* is divergently transcribed from *nahG*, the first gene in the lower pathway as seen in Figure 8. The latter of these characteristics as well as the proteins binding the promoter of the regulated gene in the absence of coinducer allows self-regulation which is thought to be a 2-to 3-fold reduction in the case of *nahR* (Schell and Faris, 1987).

Early work involving the regulation of the NAH system demonstrated that salicylate was the inducer of both the *nah* and *sal* operons and resulted in a 30-fold increase in their transcript level (Schell, 1985). Early work also showed that 2-aminobenzoate also induced the transcription of the *nah* and *sal* operons (Barnsley, 1975). It is interesting to note that the inducer in this system is not naphthalene itself but a metabolite of naphthalene degradation. This shows that there must be a basal level of transcription of the genes of the *nah* operon to maintain enzyme levels to metabolize naphthalene to salicylate. Until recently only the two aforementioned compounds were known inducers of the NAH system. Using a *nah-lux* bioluminescent reporter strain 5RL, constructed by Tn4431 transposon mutagenesis of

the wild type NAH plasmid pKA1, LeBlond et al., (1995) investigated various substituted methylsalicylates for their ability to induce the NAH system. They found that, of the methyl substituted salicylates, only 4-methyl salicylate induced the NAH system as measured by increased bioluminescence as well as the corresponding methyl substituted naphthalenes (parent compounds). In another study examining amino-, chloro-, and hydroxy- substituted salicylates as well as amino substituted benzoates it was found that only the 4-chlorosalicylate induced transcription as measured by bioluminescence (Applegate et al., 1996). These results seem to conflict with the earlier work done with 2-aminobenzoate.

NAH-Mediated Metabolism of Phenanthrene

Direct evidence of the NAH-mediated metabolism of phenanthrene to 1-hydroxy-2-naphthoic acid has been reported by several groups of researchers. Sanseverino et al. (1993) reported that archetypal NAH7 plasmids mediated the mineralization of phenanthrene. In this report it was shown that the strain 5RL harboring the bioluminescent reporter plasmid pUTK21 (King et al., 1990), which has an inactivated lower pathway, due to a Tn4431 insertion, was unable to mineralize phenanthrene and accumulated a metabolite during phenanthrene degradation. Menn et al. (1993) further examined the NAH metabolism of phenanthrene using strain 5RL and ascertained that the accumulated metabolite was 1-hydroxy-2-naphthoic acid. Denome et al. (1993) cloned a 9.8 kb chromosomal DNA fragment from a *Pseudomonas* strain designated C18 that encoded the genes for enzymes that would degrade phenanthrene to 1-hydroxy-2-naphthoic acid. The fragment was sequenced and determined to have almost 100% identity to the upper pathway of NAH7 and the gene order is the same. Since these genes were initially isolated for their mediation of dibenzothiophene they

were labeled *dox*. Kiyohara et al. (1994) cloned a 25 kb fragment from *Pseudomonas putida* OUS82 which encoded the genes for the biotransformation of phenanthrene to 1-hydroxy-2-naphthoic acid. These genes were subsequently designated *pah* for polycyclic aromatic hydrocarbons. Using RFLP and subsequent hybridization with NAH gene probes it was determined that the *pah* genes were similar to the *nah* genes. Yang et al. (1994) cloned an 8.5 kb DNA fragment from the archetypal NAH plasmid pDTG1 which coded for the enzymes to metabolize phenanthrene to 1-hydroxy-2-naphthoic acid. This fragment contains the upper pathway *nah* genes. All of these reports share one common element; all of these gene clusters share a high sequence similarity and gene order. Therefore, one can correlate the individual enzymatic steps of naphthalene degradation to that of phenanthrene degradation as shown in Figure 12. However, the further metabolism of 1-hydroxy-2-naphthoic acid by the NAH system, primarily by the *sal* operon (lower pathway), has yet to be elucidated.

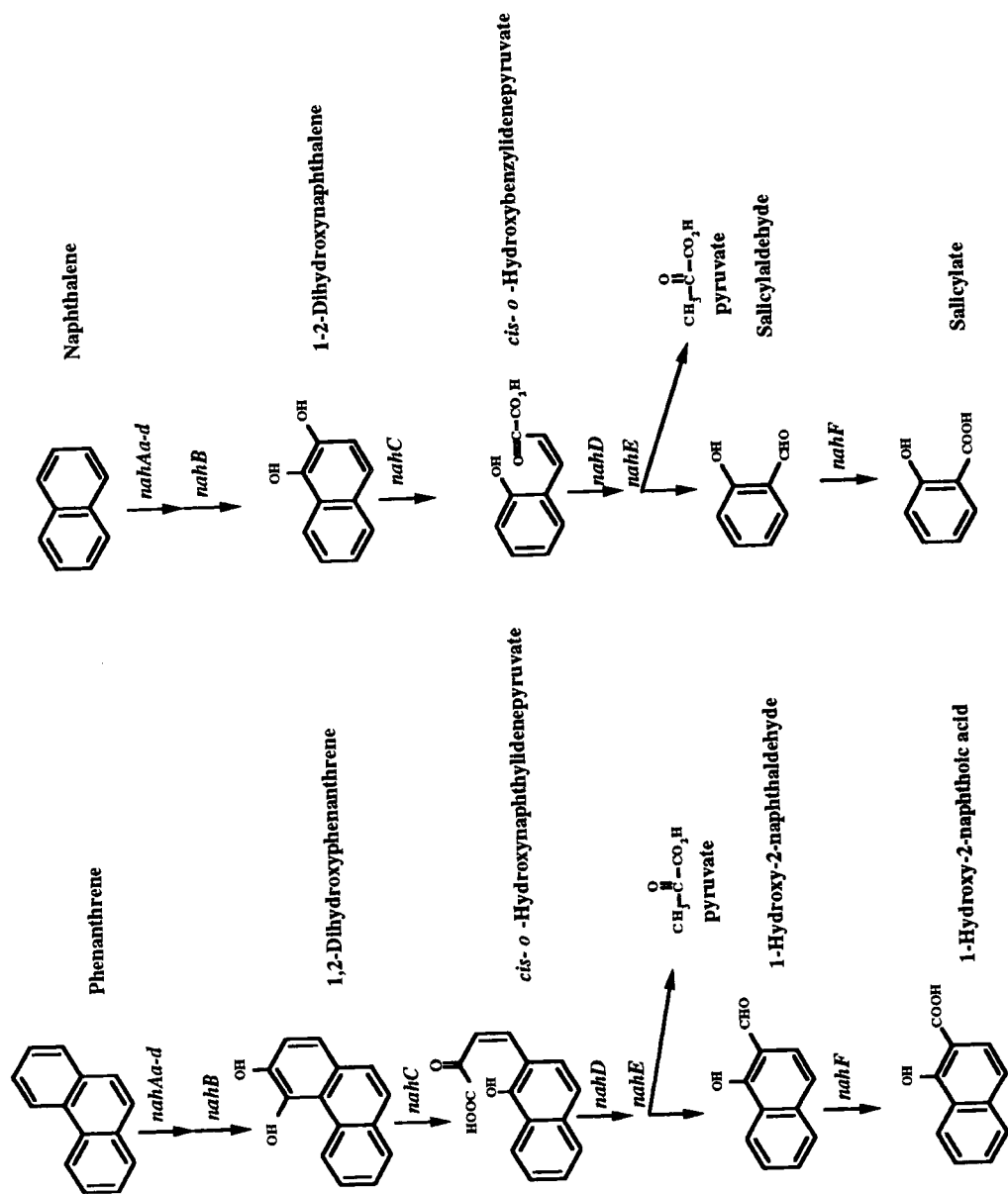


Figure 12. Naphthalene metabolism by the enzymes of the *nah* operon and the analogous steps in phenanthrene degradation. Enzymes: *nahAa-d* naphthalene dioxygenase; *nahB* *cis*-naphthalene dihydrodiol dehydrogenase; *nahC* 1,2-dihydroxynaphthalene dioxygenase; *nahD* 2-hydroxynaphthalene-2-carboxylase isomerase; *nahE* *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase; *nahF* salicylaldehyde dehydrogenase.

Chapter III

Objectives

The overall objective of this research is to test the following hypothesis:

Phenanthrene is metabolized by the NAH system by the conversion of phenanthrene to 1-hydroxy-2-naphthoic acid by the enzymes of the *nah* operon, subsequent conversion of 1-hydroxy-2-naphthoic acid to 1,2 dihydroxynaphthalene by salicylate hydroxylase, the further degradation of this compound by the upper pathway to salicylate (the inducing intermediate), and its subsequent conversion to acetaldehyde and pyruvate by the enzymes of the *sal* operon. This hypothesis is presented in the form of a pathway in Figure 13.

Specific Objectives

1. Determine if microorganisms with the NAH system genotype can grow on phenanthrene as a sole carbon and energy source.
2. Confirm the role of the NAH system in the biotransformation of phenanthrene to 1-hydroxy-2-naphthoic acid.
3. Construction of a mini-Tn5 transposon vector system that will allow stable insertion of genetic constructs to accomplish the last two objectives.
4. Determine the involvement of the NAH system in the further biotransformation of 1-hydroxy-2-naphthoic acid.
5. Determine if any metabolites produced by the biotransformation of phenanthrene are capable of inducing the NAH system.

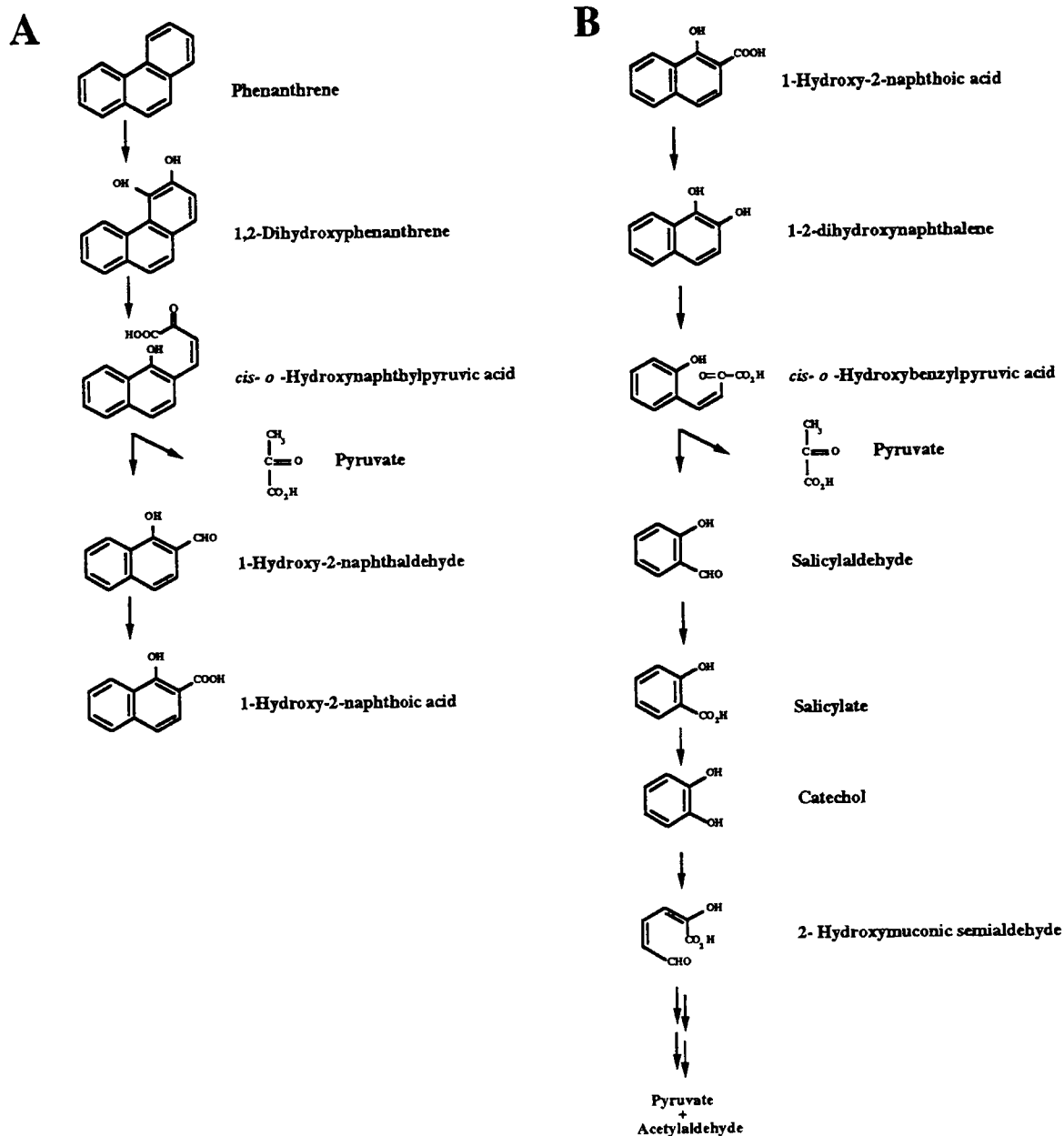


Figure 13. Hypothesized degradative pathway for the metabolism of phenanthrene to TCA cycle intermediates by the NAH system. A). Metabolism of phenanthrene to 1-hydroxy- 2-naphthoic acid by the enzymes of the *nah* operon. B). Further metabolism of 1-hydroxy- 2-naphthoic acid to 1,2-dihydroxynaphthalene by salicylate hydroxylase, followed by metabolism through the *nah* and *sal* pathways.

Chapter IV

Experimental Design

To meet the objectives, research was carried out in six areas of investigation:

1.) NAH positive strains were subjected to physiological screening to ascertain the extent of phenanthrene metabolism by the NAH system. Phenanthrene growth assays were performed to ascertain whether or not strains containing the NAH system could utilize this PAH as a sole carbon and energy source. Growth of the strains on phenanthrene would suggest extensive metabolism.

2.) To further examine the extent of phenanthrene degradation by the NAH system various strains were further analyzed using phenanthrene spray plate assays. Strains were also subjected to ^{14}C mineralization assays. These data were then compared to correlate a link between mineralization and the capacity to clear a phenanthrene spray plate.

3.) To verify the involvement of the NAH system in the degradation of phenanthrene to 1-hydroxy-2-naphthoic acid and the necessary enzymes to generate a clear zone in phenanthrene spray plate assays recombinant strains harboring a *lac-nahA* and a *lac-nahABFCE* fusion were constructed. These strains allow the isolation of the genes encoding the enzymes responsible for the aforementioned transformation. They also determine whether the initial dioxygenase attack by the naphthalene dioxygenase is sufficient to produce clear zones on phenanthrene spray plates or if other enzymes are necessary.

4.) DNA:DNA hybridizations using various NAH system gene probes were performed as well as characterizing the Tn4431 mutagenized strain 5RL. This was

done to determine if there was a link between positive and negative hybridization results, and the location of the Tn4431 insertion and the previous physiological data.

5.) Genes implicated in the mediation of the further transformation of 1-hydroxy-2-naphthoic acid were cloned and expressed in *E. coli*. This allowed the determination of both the genes involved and the metabolites produced in the further transformation of 1-hydroxy-2-naphthoic acid.

6.) A mini-Tn5 transposon vector system was constructed to generate a *nah-lux* reporter transposon as well as an expression vector for gene complementation. The *nah-lux* reporter transposon was used to generate a bioluminescent reporter strain to ascertain whether or not metabolites produced in the metabolism of phenanthrene induced the NAH system. The expression transposon was used to complement the mutation in the disrupted *nahG* of *P. fluorescens* 5RL.

Chapter V

Materials and Methods

Bacterial Strains and Plasmids

All bacterial strains used in this study are described in Table 1. Plasmids used in this study are found in Table 2. Strains were routinely stored by mixing an equal amount of sterile glycerol (50% glycerol) with overnight cultures grown with appropriate antibiotic selection then aliquoted into 2 ml microfuge tubes and frozen in a dry ice ethanol bath and stored at -70°C until needed.

Chemicals

All chemicals used in this study were purchased from Malinkrodt (Paris, KY) unless otherwise specified. Phenanthrene, 1-hydroxy-2-naphthoic acid, and phthallic acid were obtained from Aldrich Chemical (Milwaukee, WI). The various substituted salicylates were obtained from Sigma Chemical (St. Louis, MO). Media constituents were obtained from Difco BRL (Gaithersburg, MD). All chemicals used in this study were of the highest purity available.

Media and Growth Conditions

All *E. coli* strains were grown at 37°C unless otherwise noted in Luria -Bertani (LB) broth (10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl per liter, pH 7.5) with

Table 1. Bacterial strains used in this study.

Strains	Relevant genotype/characteristics	References
<i>E. coli</i> DH5	F- 80 <i>lac</i> ZM15 (<i>lac</i> ZYA- <i>arg</i> F)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR</i> 17(rK-,mK+) <i>phoA</i> <i>supE</i> 44 - <i>thi</i> -1 <i>gyrA</i> 96 <i>relA</i> 1	Gibco BRL
<i>E. coli</i> S17-l(pir)	<i>pir</i> , <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> - M+, RP4:2-Tc:Mu:Km Tn7TpRSmR; mobilizing strain for pUT mini-Tn5 derivatives	Herrero, et al., 1990
<i>E. coli</i> INVF'	Strain used with TA cloning vector, pCRII: F' 80 <i>lac</i> ZM15 (<i>lac</i> ZYA- <i>arg</i> F)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR</i> 17(rK-,mK+) <i>phoA</i> <i>supE</i> 44 - <i>thi</i> -1 <i>gyrA</i> 96 <i>relA</i> 1	Invitrogen
<i>P. putida</i> PpG7	Contains an 83 kb plasmid designated NAH7	Dunn and Gunsalus, 1973
<i>P. fluorescens</i> 5R	Contains archetypal NAH plasmid pKA1	Sanseverino et al., 1993
<i>P. fluorescens</i> 5R rif	Rif mutant of 5R	King et al., 1990
<i>P. fluorescens</i> DFC49	Contains archetypal NAH plasmid pKA2	Sanseverino et al., 1993
<i>P. fluorescens</i> DFC50	Contains archetypal NAH plasmid pKA3	Sanseverino et al., 1993
<i>P. fluorescens</i> 5RL	Contains Tn4431 mutagenized plasmid pKA1 resulting in the <i>nah-lux</i> bioluminescent reporter plasmid pUTK21	King et al., 1990
<i>P. fluorescens</i> 18H	Strain has a Sal ⁺ phenotype utilizing an ortho cleavage pathway	King et al., 1990, Callicotte, 1995
<i>P. fluorescens</i> HK44	Strain 18H containing bioluminescent reporter plasmid pUTK21, Tc ^R	King et al., 1993
<i>Acidovorax</i> <i>delafielii</i>	Strain is able to utilize phenanthrene as a sole carbon and energy source	Robinson et al., 1994
<i>Burkholderia</i> <i>cepacia</i> JS150	Contains chromosomally encoded upper NAH operon and a gentisate <i>ortho</i> cleavage operon	Spain and Nishino, 1987, Callicotte, 1995
<i>Pseudomonas</i> <i>putida</i> RB1351	Bioluminescent reporter strain containing plasmid NAH7 as well as a <i>nah-lux</i> reporter plasmid pUTK9	Burlage et al., 1990.

Table 2. Plasmids used in this study.

Plasmid designations	Relevant genotype/characteristics	Reference
pUCD615	Promoterless <i>luxCDABE</i> cassette, <i>ori</i> pSa, <i>ori</i> pBR322, Ap ^R , Km ^R	Rogowsky et al., 1987
pKK223-3	Expression vector containing the 5S ribosomal terminator <i>rrnB</i> T1T2, Ap ^R	Pharmacia
pBSKS	pBluescript II KS+ with multicloning site (MCS) <i>Kpn</i> I- <i>Sac</i> I, Ap ^R	Stratagene
pUT	5.2 kb cloning vector containing <i>mob</i> RP4, <i>ori</i> R6K and Tn5 <i>tnp</i> lacking <i>Not</i> I sites, Ap ^R	Herrero et al., 1990
pCRII	3.9 kb cloning vector for PCR products with 3' A overhangs, Ap ^R , Km ^R	Invitrogen
pCRII <i>nahRG_p</i>	pCRII containing a previously amplified 1.3 kb <i>nahRG_p</i> fragment from pKA1	Sanseverino, 1996
NAH7	83 kb self transmissible catabolic plasmid containing the NAH system for the metabolism of naphthalene.	Dunn and Gunsalus, 1973
pKA1	101 kb self transmissible catabolic plasmid containing the NAH degradative operons.	Sanseverino et al., 1993
pKA2	101 kb self transmissible catabolic plasmid containing the NAH degradative operons.	Sanseverino et al., 1993
pKA3	101 kb self transmissible catabolic plasmid containing the NAH degradative operons.	Sanseverino et al., 1993
pUTK21	<i>nah-lux</i> reporter plasmid, generated by a Tn4431 insertion into the <i>sal</i> operon	King et al., 1990

appropriate antibiotic selection. *Acidovorax delafieldiis* and *Pseudomonas* strains were grown at 28°C unless otherwise noted on either YEPG (0.2 g yeast extract, 0.2 g NH_4NO_3 , 2.0 g polypeptone, 1.0 g dextrose per liter, pH 7.5.) (Sayler et. al, 1979) or YEPSS (0.2 g yeast extract, 0.2 g NH_4NO_3 , 2.0 g polypeptone, 2.7 g sodium succinate, 0.5 g sodium salicylate per liter, pH 7.2) (Sanseverino et al., 1993b). Solid medium was prepared by adding agar to 1.7%. Minimal salts medium (MSM) was composed of 0.68 g KH_2PO_4 , 1.73 g K_2HPO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.0 g NH_4NO_3 per liter of deionized water (Davis and Mingioli, 1950). After sterilization by autoclaving the medium was supplemented with one-tenth volume of a trace elements solution consisting of: 10.0 g MgO , 2.94 g CaCl_2 , 5.4 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.44 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25g CuSO_4 , 0.062 g H_3BO_4 , and 0.49 g $\text{Na}_2\text{Mo}_4 \cdot \text{H}_2\text{O}$ per liter of deionized water (Meynell and Meynell, 1965).

Phenanthrene Growth Assays

Phenanthrene liquid growth assays were performed by adding 5 ml of a 4% solution of phenanthrene dissolved in acetone to previously sterilized 250 ml Erlenmeyer flasks (Hunt, 1993). The flasks were then placed into a laminar flow hood uncapped to allow the acetone to evaporate. After the acetone had evaporated leaving a thin layer of phenanthrene on the bottoms of the flasks they were capped and removed from the hood. One hundred ml of MSM containing trace elements was added to the flasks. *Pseudomonas* strains 5R, DFC49, DFC50, and PpG7 were grown to an OD_{546} of 1.0 in YEPG broth. One ml aliquots were centrifuged in 1.5 ml eppendorf tubes and washed twice with 50 mM KH_2PO_4 . Cell pellets were suspended in 100 ml of MSM and added to the flasks and incubated in a rotary incubator at 28°C. The positive control *Acidovorax delafieldii* was inoculated by scrapping it from a phenanthrene

minimal medium agar plate (Shiaris and Cooney, 1983). The bacteria were then suspended in 100 μ l 50 mM KH_2PO_4 and inoculated into the phenanthrene flasks. Positive controls for the *Pseudomonas sp.* consisted of MSM with 500 ppm sodium salicylate. Negative controls consisted of flasks in which 5 ml acetone alone was added. Phthallic acid and 1-hydroxy-2-naphthoic acid growth assays were as above except MSM was amended with either substrate at 200 ppm.

Molecular Biology Techniques

Unless otherwise specified all molecular biology techniques were performed as outlined in Sambrook et al. (1989). All enzymatic reactions such as restriction endonuclease digestions, ligations, and labeling reactions were performed as indicated by the manufacturer's protocol. All DNA modifying enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Plasmid Isolation

Plasmid DNA was isolated from both environmental isolates and *E. coli* using a modified alkaline lysis protocol described in Promega technical bulletin (1992). Plasmid DNA was further purified by cesium chloride:ethidium bromide ($\text{CsCl}:\text{EtBr}$) bouyant density ultracentrifugation (Sambrook, 1989). Samples were extracted with water saturated butanol to remove the EtBr. Plasmid DNA was further purified and concentrated by ethanol precipitation and suspended in TE (10mM Tris-HCl, 1mM EDTA). Samples were stored at -20°C .

Mini plasmid preparations for routine screening of clones were performed using the rapid boiling mini-prep technique of Holmes and Quigley (1981). This technique

provided enough DNA for restriction enzyme analysis of the clones. However due to the crudeness of the DNA prep, long incubation times are not recommended as significant degradation will occur from residual Dnase.

PCR Reactions

PCR amplifications were performed using the AmpliTaq kit and a DNA Thermal Cycler purchased from Perkin Elmer Cetus (Norwalk, CT). Primers were synthesized by Genosys Inc. (The Woodlands, TX) or synthesized using a Beckman Oligo 1000 DNA synthesizer (Palo Alto, CA) following the manufacturer's protocol. Primer sets and amplification parameters are described in Table 3. All PCR reactions consist of a 5 min denaturation step, 38 amplification cycles, and a 10 min final elongation step unless otherwise stated. Reaction products were routinely analyzed using 1% agarose gels electrophoresed at 70 volts for 2 hours in TBE buffer (Sambrook et al., 1989). Gels were stained in a 10 mg/l EtBr staining solution and examined on a UV transilluminator.

Probe Generation

DNA probes were generated using the PCR as described by Applegate et al. (1995). Probe templates were generated from the wild-type strain whenever possible. Templates for probe generation were made by reamplifying dilutions of previously amplified products. This was done to avoid any contamination from flanking genes. Probes were generated using the same conditions as previously outlined in the PCR section (Table 3) except with these modifications: only one primer was used,

Table 3. Genes, primer sets, and amplification conditions for PCR reactions and oligonucleotide probes.

Target Gene	characteristics/primers ^a	amplification conditions	Reference
<i>nahA</i>	1 kb fragment encoding the 5' region of <i>nahAc</i> amplified from NAH7 5' CCCTAGCGCGTAACTACCC 3' GGTCCAGACCTCGGYTG GTG	94°C (1 min) 55°C (1 min) 72°C (1 min)	Simon et al., 1993
<i>nahG</i>	1.3 kb DNA fragment encoding the entire <i>nahG</i> amplified from NAH7 and pKA1 5'GCCATCACGAGTACAGC 3'CGCGATCGGTGTCAACG	94°C (1 min) 55°C (1 min) 72°C (2 min)	You et al., 1991
<i>nahH</i>	1 kb DNA fragment encoding the entire <i>nahH</i> amplified from NAH7 and pKA1 5'GTATCGAGCGTTACTAGTAGCCG 3'CGTCCTAGGTCATAACGGTCATG	94°C (1 min) 55°C (1 min) 72°C (1 min)	Ghosal et al., 1987
<i>nahGH</i>	3.1 kb DNA fragment encoding <i>nahGH</i> amplified from NAH7 and pKA1 5'GCCATCACGAGTACAGC 3'CGTCCTAGGTCATAACGGTCATG	94°C (1 min) 55°C (1 min) 72°C (3 min)	You et al., 1991; Ghosal et al., 1987
16S	Universal 16S oligonucleotide probe 5'ACGGGCGGTGTGTRC	NA ^b	Lane et al., 1985

^a Primers are designated 5' or 3' in relevance to their annealing sites on the gene.

^b Probe was end labeled with ³²P.

radiolabeled dCTP was substituted into the reaction mix for unlabeled dCTP, 20 ng of template was used for amplification. Unincorporated radiolabel was removed by size exclusion chromatography using a NucTrap push column (Stratagene, LaJolla, CA). End labeling of the oligonucleotide probe was performed using a 5' end labeling kit according to manufacturer's protocol (Bethesda Research Laboratories, Gaithersburg, MD).

Strain Construction for Biotransformations

Appropriate strains for biotransformations were constructed using recombinant DNA techniques. Restriction enzymes and DNA ligase were obtained from Gibco/Bethesda Research Laboratories (Gaithersburg, MD). All restriction digests and ligations were performed according to manufacturer's protocol.

Construction of an *E. coli* strain containing the genes from the upper pathway of NAH7 was accomplished by cloning an 11 kb *EcoRI-HindIII* containing *nah ABFCE* into pBluescript II S.K. (Stratagene, LaJolla, CA). These two cleavage sites allow the directional cloning of this fragment downstream of the *lac* promoter to form a *lac-nah ABFCE* transcriptional fusion. NAH7 DNA and pBluescript were cleaved in double digests using *EcoRI-HindIII* and ethanol precipitated. The DNAs were then mixed together in a ligation reaction. The ligation reaction was then used in a transformation of subcloning efficiency DH5a competent cells purchased from Gibco/BRL according to manufacturer's protocol. Cells were plated onto LB agar plates with 50 µg ampicillin/ml. Clones were screened for the appearance of blue colonies. Blue colonies result from the naphthalene dioxygenase reacting with indole (produced from the catabolism of tryptophan) converting it into indigo (Ensley, 1983).

Plasmid DNA isolated from positive colonies was cleaved with the appropriate restriction endonucleases to verify the insert.

After the aforementioned construction was completed the resultant plasmid was cleaved with *XhoI* and the reaction ethanol precipitated, diluted, and religated. DNA was transformed as above and plasmid DNA from blue colonies was screened by *EcoRI* restriction digests to check colonies for the removal of the *nahBFCE*. The plasmid was designated pUTK202.

nahG, *nahH*, and *nahGH* clones were generated by amplifying these particular DNAs from pKA1 as previously described (Table 3). PCR products were directly cloned into the TA cloning vector pCRII (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Transformants were plated on LB agar plates with 50 µg ampicillin/ml and 40 µl of an Xgal dimethyl formamide solution (1mg/ml) spread per plate. White colonies indicating the presence of inserts were screened for orientation by adding catechol to the plates for the *nahGH* and *nahH* clones. Positives are detected by the yellow ring cleavage product produced from catechol. Positive *nahGH* clones were screened with salicylate, yellow colonies indicate a functional NahG and NahH. *nahG* clones were screened for functionality by adding salicylate to 2 ml broth cultures of white colonies and incubating overnight. The supernatants from these tubes were then mixed with a NahH positive strain. If the characteristic yellow ring cleavage product was produced, the clone was judged positive for the production of NahG. Plasmid DNA from positive clones was also screened by cleaving with appropriate restriction endonucleases.

Spray Plate Assays

Spray plate assays were performed by spotting 20 µl samples of cells from an overnight culture onto YEPSS agar plates supplemented with blue food coloring for contrast (Kroger Food Corp., Cincinnati, OH). The *nahA-D* clone and the *nahA* clone were streaked across LB agar plates. After the colonies or streaks obtained sufficient biomass, a 1% solution of phenanthrene dissolved in acetone was sprayed on the plates (Kiyohara et al., 1982). After the evaporation of the acetone, a thin opaque layer of phenanthrene was left on the plate. Plates were incubated at 28° C and monitored periodically for zones of clearing around the colonies and streaks. Positives were scored if they produced a clear zone around the colony.

Spray plates using a 1% solution of 1-hydroxy-2-naphthoic acid dissolved in acetone were performed as above.

Colony Hybridizations

Colony blots were prepared as above by spotting test cultures onto YEPG agar plates and incubated at 28°C until a significant biomass was obtained. Once the bacteria had grown, they were subjected to colony hybridization according to the manufacture's protocol (ICN Biomedicals, Cincinnati, OH). Biotrans™ colony lift membranes were placed onto the plates for 1 min to allow the bacterial colonies to adhere to the membrane. The colonies adhered to the membranes and were placed colony side up for 5 min on 2 ml of 0.5 N NaOH which had previously been spotted onto saran wrap. The NaOH lyses the cells and affixes the DNA to the membrane. The membranes were then blotted on filter paper and allowed to dry. The dry membranes were then baked at

80°C for 1 h to fix the DNA to the membrane. Filters were stored at room temperature until needed.

Membranes were hybridized using a modified method of Sayler et al. (1985) (Applegate et al., 1995). Membranes were prehybridized in 0.5 M Na₂HPO₄, 1 mM EDTA, 7% SDS, pH 7.2 for one hour at 65°C before the addition of the appropriate gene probe (Church and Gilbert, 1984). After the addition of probe the membranes were hybridized overnight (>12h) at 65°C. After hybridization the membranes were washed 4 times at 15 min intervals with a high stringency wash buffer (0.59 g NaCL, 2.42 g Tris base, 0.37 g EDTA, and 5.0 g SDS per liter at pH 7.5) (Sayler, 1985). The membranes were then dried and subjected to autofluorography using X-ray film (Kodak XRP-5, Eastman Kodak, Rochester, NY) at -70°C (Sambrook, 1989). Film was developed after 24 h.

Mapping Tn4431 Insertion Site

The insertion site of Tn4431 was mapped using the following primers and PCR conditions. Primer 1 (GCGCATCGGTATCGTCGGCGGCGG) was chosen at the 5' end of the *nahG* gene sequence (You et al., 1991) while primer 2 (CGTGGCCGGGGCGCATTACACC) was chosen from the 5' of *nahH*. The sequence of primer 3 (ACGATTTTTTCCGAATTCTGCGG) was determined from the insertion sequence of Tn1721 (Schmitt et al., 1985), the parental transposon of Tn4431. This primer can be used for amplification in both directions since the insertion sites of a transposon are inverted repeats.

The template DNA was initially denatured at 94°C for 5 min. A total of 25 cycles were run during which DNAs were denatured at 94°C for 1 min, primers were annealed at 55°C for 1 min, and the strands extended at 72°C for 2 min. The positive

control consisted of using plasmid NAH7 and pKA1 with primers 1 and 2 while the negative control consisted of pKA1 with primer combinations of 1 and 3, as well as 2 and 3.

Construction of Cloning Vector pLJS

The cloning vector, pLJS, was constructed from pBluescript II (KS) (Stratagene, LaJolla, CA) by cleaving with *Bss*H II and religating to remove the multicloning site (MCS). Ligated DNA was transformed into DH5a subcloning efficiency competent cells using manufacturer's protocol and spread on LB plates supplemented with ampicillin (50 mg/ml) and X-gal (40 mg/ml). Transformants without the MCS were white since they were incapable of α -complementation. The resultant plasmid was named pBSMCS(-). Two oligonucleotides (a 47-mer and a 44-mer) with base substitutions were synthesized as previously described to regenerate the multicloning site and add the following restriction sites, *Xba*I, *Nhe*I, *Spe*I, and *Avr*II. The sequences and orientation of the added sites can be seen in Figure 14. The new multicloning site was amplified from pBluescript II (KS) using the manufacturer's protocol with the following thermocycler conditions: 94°C initial denaturation for 5 min; 38 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 1 min, extension at 72°C for 30 s; final extension at 72°C for 15 min. The amplified fragment was cleaved with *Bss* II, ligated into pBSMCS(-) and transformed into DH5a. Transformants were screened on LB agar with ampicillin (50 mg/l) and X-gal (40 mg/ml). Blue colonies were selected since they indicated restored α -complementation. The construct was sequenced to confirm the base substitutions and integrity of the MCS.

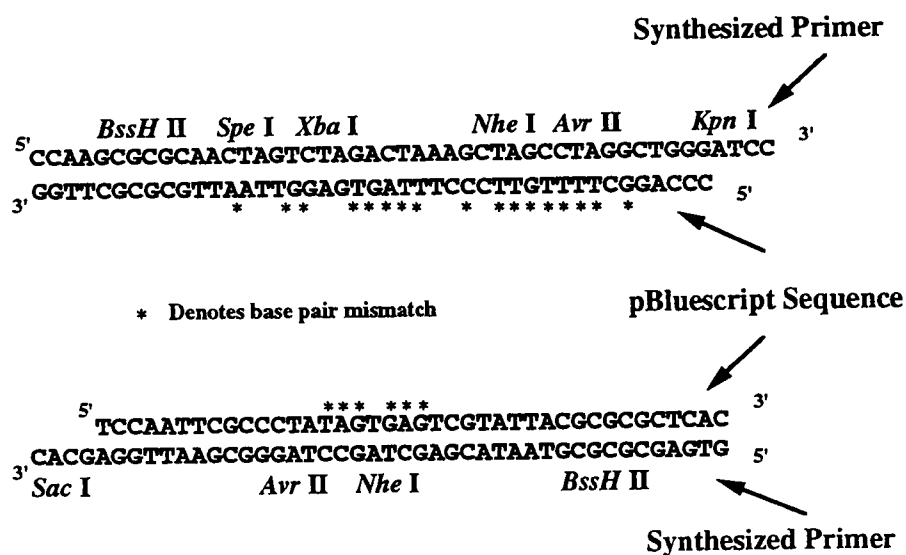


Figure 14. Sequence of synthesized primers and base substitutions for site directed mutagenesis of the multicloning site of pBluescript SK.

Construction of Various Clones in pLJS

Plasmid pLJST2 was generated by directionally-cloning the 0.77 kb *Hind* III-*Hinc*II fragment containing the 5S ribosomal *rrnB* T₁T₂ transcription terminator from pKK223-3 (Pharmacia, Piscataway, NJ) into pLJS cleaved with *Hind*III and *Sma*I. Transformation was performed as previously described. Transformants were screened on LB agar with ampicillin (50 mg/l) and X-gal (40 mg/ml). White colonies were verified by restriction endonuclease analysis.

Plasmid pLJS*lux* was generated by directionally cloning an 8.35 kb *Kpn*I-*Pst*I fragment from pUCD615 (Rogowsky et al., 1987) into pLJS. Resultant transformants were screened for bioluminescence as proper insertion would generate a *lac-lux* transcriptional fusion. Bioluminescent colonies were further verified by cleavage with restriction endonucleases.

Plasmid pLJS*nahRG_p*- was generated by heterologous directional cloning of a 1.2 kb *Kpn*I-*Eco*R V fragment containing the complete *nahR* and promoter region from pCRII (Gift from John Sanseverino) into the *Kpn*I-*Sma*I sites of pLJS. Transformants were screened as above. White colonies were verified to contain proper inserts by restriction endonuclease analysis.

Electroporation

Electrocompetent cells were prepared as outlined by the manufacturer (BTX, San Diego, CA). Electroporations were performed using a BTX Electroporator 600 with the following conditions: 40 ml cells, 1 ml ligation mixture, a 2.5 kV pulse for about 4.7 ms using a 2 mm gap cuvette. After the pulse, cells were immediately

resuspended in LB (to 1 ml) and allowed to recover for 1 h at 37°C shaken at 200 rpm before plating on LB plates with the appropriate antibiotic selection.

***nahRG_p-lux* Reporter Transposon Construction**

The transposon mini-Tn5Km*NX* was constructed using site-directed mutagenesis and the polymerase chain reaction. Two 58 base oligonucleotides, 5' and 3' with respect to the kanamycin resistance gene (Km^R) in pCRII (Invitrogen, San Diego, CA) were synthesized using a Beckman Oligo 1000 DNA synthesizer (Palo Alto, CA) following the manufacturer's protocol. Base substitutions were made to generate both I and O insertion sequences as well as unique *NotI* and *XbaI* sites inside the transposon for cloning. An *EcoRI* site and a *NheI* site were added to the end of each oligonucleotide, respectively, to allow cloning of mini-Tn5Km*NX* into the delivery vector pUT (Herrero et al., 1990, DeLorenzo et al., 1990). The sequence and base changes can be seen in Figure 15. The primers were used to amplify the kanamycin resistance gene from pCR II using Touchdown PCR (Don et al., 1991) with the following thermocycler conditions: 94°C initial denaturation, 5 min; 5 cycles at 94°C for 1 min, 72°C annealing for 1 min, 72°C extension for 2 min; the annealing temperature was lowered 5°C every 5 cycles until 42°C at which 8 cycles were run, followed by a final extension of 15 min at 72°C. The 1.3 kb product was cloned into pCRII using a TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. The resultant plasmid pUTK209 containing the mini-Tn5Km*NX* was sequenced to verify the incorporation of both the I and O insertion sequences. After confirmation, pUTK209 was cleaved with *NheI* and *EcoRI* and gel-purified using agarose gel electrophoresis (Sambrook et al., 1989). The purified mini-Tn5Km*NX* fragment was ligated into the mini-Tn5 delivery vector, pUT. One

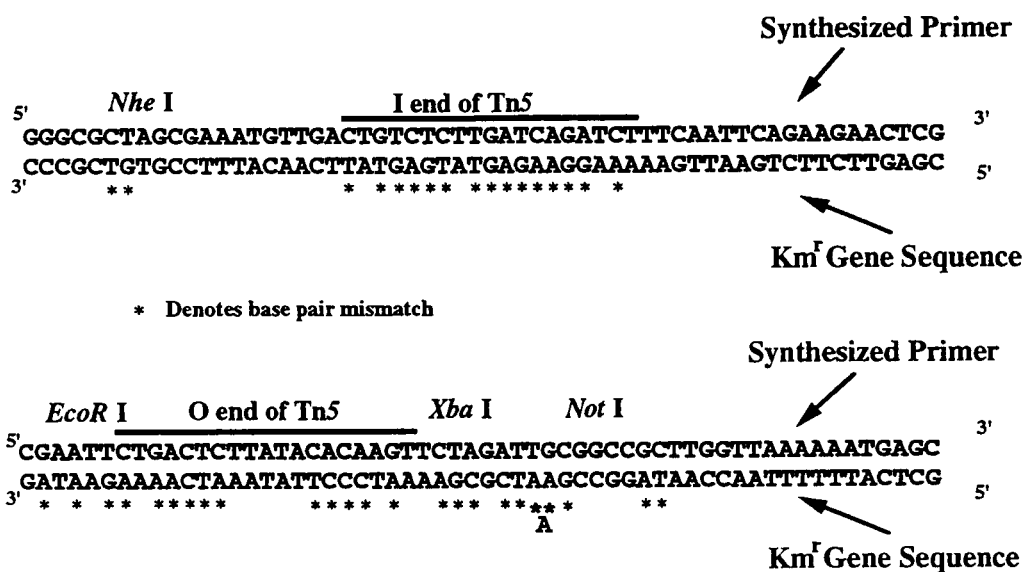


Figure 15. Sequence of synthesized primers for the construction of mini-Tn5kmNX.

microliter of the ligation reaction was electroporated into *E. coli* S17-1(λ pir).

Electroporants with the proper inserts were selected on LB plates with 50 mg/ml kanamycin. DNA minipreps (Holmes et al., 1981) were obtained and inserts were verified by cleavage with restriction endonucleases. The pUT-mini-Tn5KmNX was designated pUTK210. The further assembly of the reporter transposon is outlined below. All ligation mixtures were electroporated and electroporants were screened as above. All resultant constructs were verified by cleavage with appropriate restriction endonucleases.

The terminator was subsequently cloned into the *NotI*-*XbaI* site of mini-Tn5KmNX by cleaving pLJST2 with *NotI* and *AvrII*, allowing the subsequent destruction of the mini-Tn5KmNX *XbaI* site by heterologous ligation and the regeneration of the *NotI* and *XbaI* unique sites in mini-Tn5KmNX downstream of the terminator. The resultant construct was designated pUTK211 with the transposon Tn5KmNX T2. Subsequently the 1.2 kb *NotI*-*AvrII* *nahRG_p* fragment from pLJS*nahRG_p* was directionally cloned into the *NotI* *XbaI* site of pUTK 211. Similar to above the mini-Tn5KmNX T2 *XbaI* site was destroyed and *NotI* *XbaI* sites regenerated downstream of the *nahG_p*. The resultant construct was designated pUTK212 containing mini-Tn5Kmn*nahRG_p*XN. This construct allows the construction of *nahG* transcriptional fusions. To generate the final construct an 8.35 kb *AvrII* -*NotI* *lux* fragment from pLJS*lux* was cloned into the *XbaI* *NotI* sites of Tn5Kmn*nahRG_p*XN generating a *nahG-lux* transcriptional fusion. The resultant construct containing mini-Tn5Kmn*nahRG_p*-*lux* was designated pUTK213.

Construction of a mini-Tn5Km*nahRG_p*-*nahGH*

A salicylate inducible *nahGH* mobile genetic element was generated by cloning the 3.1 kb *SpeI-NotI nahGH* fragment from pUTK200 into the *XbaI NotI* sites of mini-Tn5Km*nahRG_p*XN in pUTK215. Electroporants were screened by restriction endonuclease analysis with the appropriate enzymes.

DNA Sequencing

Manual sequencing of pLJS was performed by the dideoxy chain termination method of Sanger et al. (1977). Sequencing reactions were performed using a Δ Taq Version 2.0 sequencing kit (United States Biochemical, Cleavland, OH). Double stranded sequencing templates were prepared according to manufacture's protocol from plasmid DNA isolated as previously described. The modified multicloning site was sequenced in both directions using both forward and reverse sequencing primers obtained from Stratagene. Primers were labeled at the 5' end with ^{32}P using the forward reaction with polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ ^{32}P] ATP. Sequencing reactions were analyzed by polyacrylimide gel electrophoresis using 6% (wt/vol) acrylimide-8M urea gels. Gels were subjected to autofluorography and the subsequent autoradiogram was analyzed for sequence determination.

The mini-Tn5Km/VX in pCRII was sequenced to confirm that the site-directed mutagenesis was successful using both the forward and reverse sequencing primers for pCRII. Sequencing was performed using an Applied Biosystems Model 373A (Foster City, CA) at the Molecular Biology Resource Facility, University of Tennessee.

Transposon Mutagenesis

E. coli S17-1(λ pir) containing mini-Tn5KmnahRG_p-lux was mated into *P. fluorescens* 5R rif (King et al., 1990) by plate mating. Donor and recipient cells were mixed in a ratio of approximately 5 to 1 and spotted onto LB plates which were incubated at 25°C for 24 hr. Mutants were selected on LB agar supplemented with 50 mg/ml kanamycin and 50 mg/ml rifampicin. Colonies were subsequently sub-cultured to grid plates and exposed to naphthalene vapor (Kehrmeyer, 1997). Bioluminescent strains were further evaluated for fitness by the ability to grow on naphthalene MSM. A strain designated 135R75 was chosen for further study.

E. coli S17-1(λ pir) containing mini-Tn5KmnahRG_p-nahGH was mated into *Pseudomonas putida* 5RL by mixing liquid cultures of recipient and donor at a ratio of 10 to 1 in a microfuge tube. The tube was then centrifuged at 10,000 x g and the supernatant removed. The cell pellets were then incubated for 24 h at room temperature. After 24 h the cells were plated on LB agar containing Tc at 14 µg/ml and kanamycin at 50 µg/ml. Plates were subsequently exposed to naphthalene vapor.

Bioluminescent Assays

Bioluminescent assays were conducted as described by Heitzer et al. (1992) with slight modifications. An overnight culture from a frozen stock of the specific bioluminescence reporter was prepared in 250 ml erlenmeyer flasks containing 100 ml YEPG media with appropriate antibiotic selection. A sub-culture was prepared in YEPG, grown to an OD₅₄₆ of 0.35-0.45. Two ml of this culture was added to 25 ml mineralization vials containing 2 ml of minimal salts buffer with test compounds. All test compounds were added in amounts that would produce equal molar amounts of the

potential inducer. Positive controls consisted of salicylate and naphthalene and the negative control consisted of cells with minimal salts buffer alone. Light measurements were taken every 30 min for two hours. The test vials were inserted into a light tight measurement cell and the light output was measured using a liquid light cable connected to a photomultiplier (model 77340, Oriel, Stratford, CT) and a digital display as seen in Figure 16 (model 7070 Oriel, Stratford, CT). Bioluminescence measurements were recorded every 30 min and plotted over a 3 h period. After the final time point of the experiment the OD₅₄₆ was measured. All samples were performed in triplicate and reported as averages.

Assays conducted using saturated solutions of phenanthrene were conducted with the following modification. Cells were concentrated 10-fold by centrifugation and 200 µl of cell suspension was added to the test vials which contained 4 ml consisting of 2 ml YEPG and 2 ml test solution.

Test solutions were prepared by dilutions of saturated solutions of phenanthrene and naphthalene in MSM. Stock 100 ppm solutions of salicylate, 1-hydroxy-2-naphthoic acid, and phthalic acid were diluted in MSM as previously described.

Mineralizations

To ascertain whether or not strains could mineralize phenanthrene, they were incubated with radiolabeled [9-¹⁴C]phenanthrene (10.4 mCi/mmol: purity 99%, Sigma Chemical Company, St. Louis, MO.) and monitored for radiolabeled ¹⁴CO₂ evolution (Fig. 17A). Test vials were prepared as follows: 4 ml of YEPSS medium was added to 40 ml EPA vials (Pierce Chemical Company, Rockford, IL). An 8 ml vial containing 0.5N NaOH was inserted into the EPA vial containing the medium to be used as a CO₂ trap (Fig. 17B). Approximately 100 µl of an overnight culture were

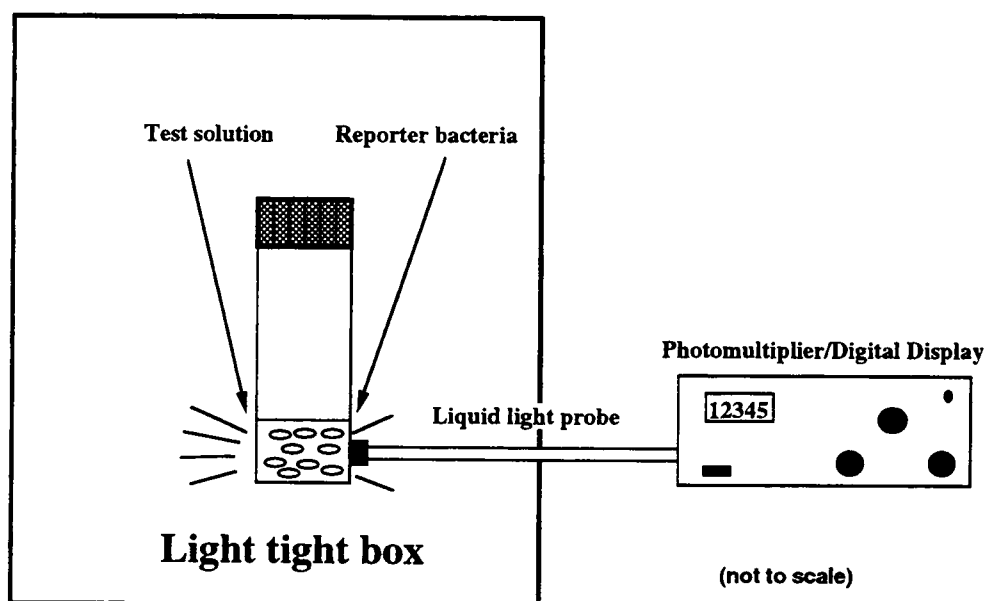


Figure 16. Light measuring apparatus for bioluminescent assays consisting of: light tight measurement cell, liquid light cable connected to a photomultiplier, (model 77340, Oriel, Stratford, CT), and digital display (model 7070 Oriel, Stratford, CT).

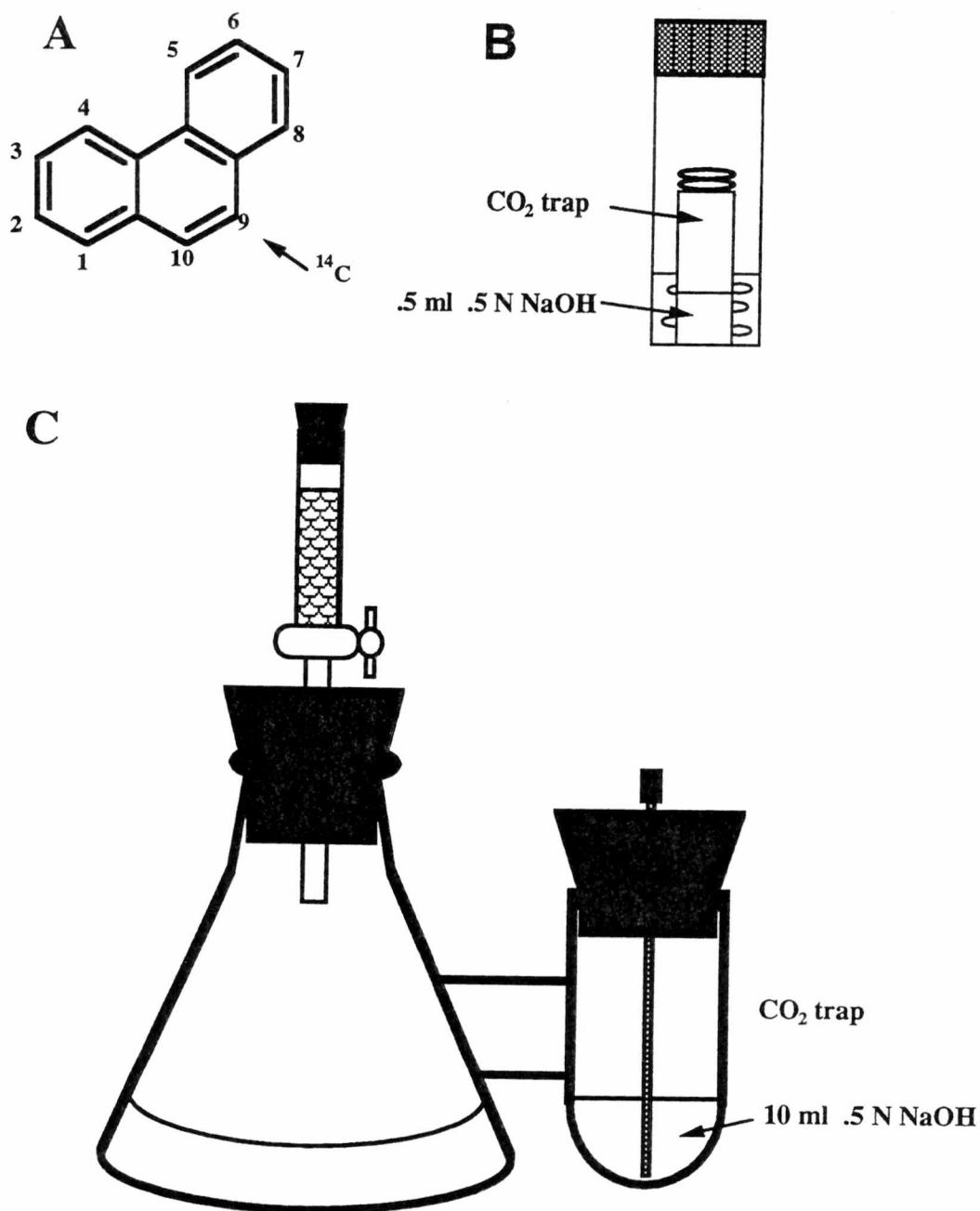


Figure 17. A. Position of radiolabeled carbon in Phenanthrene. B. Mineralization vial set up. C. Biometer Flask

added to the vials along with 100,000 dpm of [9- ^{14}C]phenanthrene dissolved in 10 ml acetone. Cultures were incubated for 72 hrs at room temperature. Negative controls consisted of: biological *P. fluorescens* 18H (Sanseverino et al., 1993), and an abiotic control was a killed biological sample for each strain.

Assays were terminated by the addition of 0.5 ml 4N H_2SO_4 by injection through the septa of the vial. Vials were allowed to shake for an additional hour after termination. For mass balance purposes, 2 ml of a 4:1 hexane isopropanol solution was also injected into the vials and allowed to shake for another 1 hr. The contents of the traps were removed and added to 1 ml of deionized water and 10 ml of Beckman Readysafe scintillation fluor. Aqueous phase (0.5 ml) from the vial was also added to 1 ml of deionized water and 10 ml of Beckman Readysafe scintillation fluor. The organic phase (2 ml) was added to 10 ml of DuPonts Econofluor. Scintillation vials were stored in the dark for 24 h before being counted (Beckman Liquid Scintillation Counter Model LS5000). Conversion of counts per min to disintegrations per min were calculated using standard quench curves. Assays were performed in triplicate.

Mineralization assays were also performed using biometer flasks to allow the monitoring of radiolabeled CO_2 evolution over time (Bartha, 1965). A diagram of this flask can be seen in Figure 17C. Cells were grown in 2 liter Erlenmeyer flasks with 1 liter of YEPSS broth to an OD_{546} of 1 and washed with 50 mM K_2HPO_4 buffer (pH 7.5) and subsequently resuspended in 25% of the initial volume (4-fold concentration increase), 50 ml of this suspension was added to the biometer flasks with varying amounts of radiolabeled phenanthrene. Ten ml of 0.5 N NaOH was added to the trap. At defined intervals, 0.5 ml aliquots were removed from the trap and counted as above. All samples were performed in triplicate and cumulative dpms ($^{14}\text{CO}_2$) evolved were plotted versus time.

Biotransformation Assays

Phenanthrene biotransformation experiments were performed by growing cells to an OD₅₄₆ of 1.0 in 1 L of YEPSS in a 2 L Erlenmeyer flask. The cells were subsequently washed 3 times with equal volumes of 50 mM KH₂PO₄ (pH 7.0)(Menn et al., 1993). The cells were then suspended in 100 ml of 50 mM KH₂PO₄ buffer and added to 500 ml Erlenmeyer flasks. Substrates were added by dissolving 10 mg in 250 µl of *N,N*-dimethylformamide and dispensing into the flask. Cultures were incubated overnight at 28°C. Controls consisted of cultures without the addition of substrate and substrate alone in the 50 mM NaH₂PO₄.

After incubation, cultures were centrifuged at 10,000 rpm to remove the cells. The supernatants were extracted three times with 100 ml volumes of ethyl acetate (neutral extract) and pooled. The supernatant was acidified with 2 N H₂SO₄ to a pH below 2.0 and extracted three more times (polar extract). Both ethyl acetate extracts were subsequently dried over anhydrous sodium sulfate and the solvent was removed using a Roto vap at 30°C under a vacuum. The yellow residues were suspended in 2 ml of acetone and concentrated under a nitrogen stream. Samples were stored at -20°C until needed.

Biotransformation assays using 1-hydroxy-2-naphthoic acid and the recombinant *E.coli* strains were performed as above except that LB broth was used instead of YEPSS. Cultures were 100 ml as opposed to 1 L and were incubated at 37°C rather than 28°C.

Meta Cleavage Assays

To ascertain the substrate range of the salicylate hydroxylase encoded by *nahG*, *E. coli* harboring pUT200 was used in a series of biotransformation assays. Plasmid

pUTK200 contains both *nahG* and *nahH* and they are expressed as a *lac Z* transcriptional fusion by the *lac* promoter. Salicylate hydroxylase activity was determined by the meta cleavage of the resultant substituted catechol. Cells were grown at 28° C in 100 ml LB broth to an OD₅₄₆ of 1 and subsequently washed 3 times with equal volumes of 50 mM KH₂PO₄ (pH 7.0) and suspended in 250 ml of the same buffer. Fifty ml of the cell suspension was added to 250 ml Erlenmeyer flasks and incubated at 28°C and shaken at 200 rpm. Negative control was *E. coli* INVF' harboring pCRII without an insert. One ml samples were taken every 30 min, placed into an Eppendorf tube and centrifuged at 14,000 rpm for 5 min in Brinkman microfuge. The supernatant was analyzed by scanning it through the ultraviolet and visible wavelengths (200 nmol to 600 nmol) using a Beckman DU-70 spectrophotometer (Palo Alto, CA). The characteristic yellow meta cleavage product absorbs between 375 and 385 nmol.

Thin Layer Chromotography

Ethyl acetate extracts from biotransformation assays were suspended in acetone and separated on thin layer chromatography (TLC) plates. TLC analysis were performed with Silica Gel 60 (F254) on a precoated plastic sheet (EM Separations, Gibbstown, NJ) or preparative C18-Silica on glass (Sigma Aldrich, St. Louis, MO) Phenanthrene biotransformations were run with a solvent system of 90% chloroform and 10% methanol. All plates were run with solvent prior to use with samples. Equal amounts of metabolites and standards (determined empirically) were spotted onto plates and then developed with the aforementioned solvent system. Metabolites were visualized under UV light. Separated spots were subsequently scrapped from the TLC plate and placed into a pasteur pipette with a siliconized glass wool plug. Metabolites

were then eluted from the plate material by passing methanol through the column. The sample was then concentrated by evaporating the methanol under a nitrogen stream. Samples were suspended in hexane for gas chromatography and mass spectrometry (GC-MS) analysis.

Gas Chromatography Mass Spectrometry

Metabolites purified from TLC plates were analyzed by GC-MS using a Hewlett Packard 5995A with a GC-MS capillary interface model 18964A and a flame ionization detector model 18965A (Menn et al., 1993). Mass spectral analyses was carried out using the electron impact mode at an ionization voltage of 70 eV. A BP5 capillary column (0.22 mm x 12 m) (SGE, Inc, Austin, TX) was used for gas chromatography. Samples were injected at 250°C and held isothermally for 2 min, the oven temperature was programmed to increase to 250°C at a rate of 10°C per min. Spectra of metabolites were compared to authentic compounds for identification.

Chapter VI

Results

Growth Assays

To determine whether or not bacterial strains with the NAH system could grow on phenanthrene as a sole carbon source *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, and *P. fluorescens* DFC50 were all subjected to growth assays with phenanthrene and possible metabolic intermediates. Results of these assays are seen in Table 4. None of the NAH strains showed growth on phthallic acid or 1-hydroxy-2-naphthoic acid. Only the positive control *Acidovorax delafieldii* was capable of growing on phenanthrene and phthallic acid as a sole carbon and energy source attaining an OD₅₄₆ of 0.26 and 0.31 after 14 days, respectively. Only the NAH strains were capable of growth on salicylate.

Phenanthrene Spray Plate Assays

The strains, *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 were examined for their ability to degrade phenanthrene by the spray plate assay. YEPSS medium was used as it would induce the NAH system and allow more rapid clearing (Yang et al., 1994). All of the strains except for *P. fluorescens* 18H were able to produce zones of clearing as shown in Figure 18. Since *P. fluorescens* 5RL was able to produce a clear zone and it was sal⁻ (Sanseverino et al., 1993), it was determined whether the naphthalene dioxygenase converting the

Table 4. Growth of NAH strains on phenanthrene and putative metabolites.

Strain	Growth/Carbon Source				
	Negative Control	Salicylate (200 ppm)	Phthallic acid (200 ppm)	1-Hydroxy-2-naphthoic acid (200 ppm)	Phenanthrene (1.3 ppm)
<i>P. putida</i> PpG7	-	+	-	-	-
<i>P. fluorescens</i> 5R	-	+	-	-	-
<i>P. fluorescens</i> DFC49	-	+	-	-	-
<i>P. fluorescens</i> DFC50	-	+	-	-	-
<i>Acidovorax delafieldii</i>	-	-	+	-	+

† = cell density of OD₅₄₆ 0.1 or greater.

- = no measurable increase in OD.

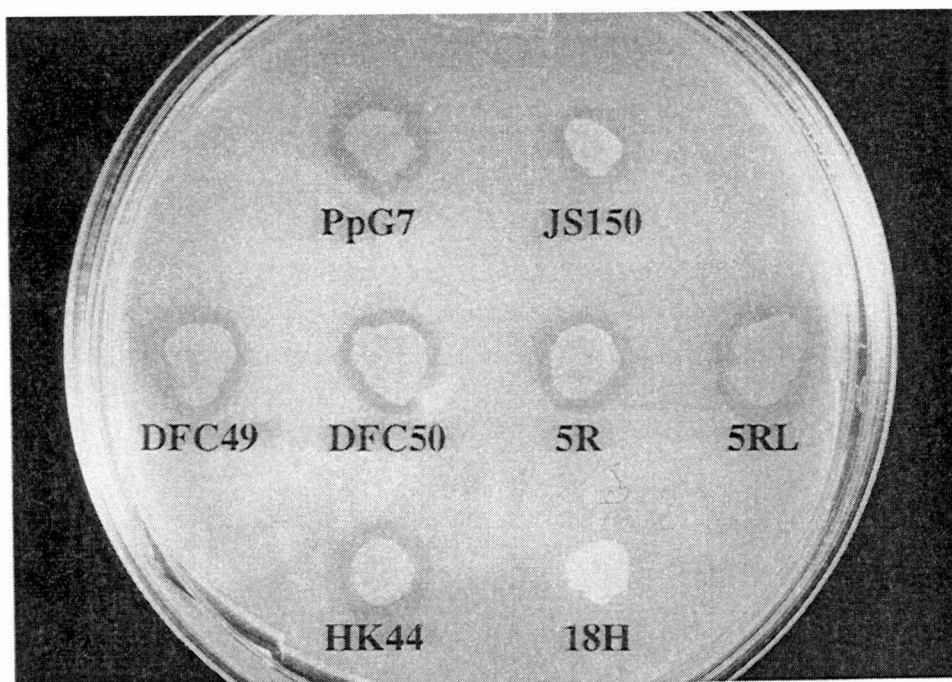


Figure 18. Photograph of *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 phenanthrene spray plate. Strains are identified by their designation.

phenanthrene to phenanthrene-3,4-dihydroxy-3,4-dihydrodiol was enough to produce clear zones. Two strains were constructed: *E. coli* DH5 α harboring either a *lac-nahA* fusion (pUTK201) or a *lac-nahABCFE* fusion (pUTK 202). Both strains were streaked onto LB plates with ampicillin (50 mg/ml) and incubated at 28°C. Once sufficient biomass had been obtained the plates were sprayed with phenanthrene and continued incubating at 28°C. The strain harboring the *lac-nahA* fusion was unable to produce a clear zone while the *lac-nahABCFE* strain cleared phenanthrene (Fig. 19). These results indicate that the phenanthrene-3,4-dihydroxy-3,4-dihydrodiol must be further metabolized to produce zones of clearing.

Mineralization Assays

Since it was previously shown that zones of clearing could be produced with the gene products from *nahABCFE*, it was necessary to examine the extent of phenanthrene metabolism using ^{14}C mineralization assays. Since [9- ^{14}C]phenanthrene is labeled at the 9 position, in the center ring, the evolution of $^{14}\text{CO}_2$ shows metabolism past the intermediate 1-hydroxy-2-naphthoic acid. Mineralization assays were performed using *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, *B. cepacia* JS150, and *E. coli* DH5 α (*lac-nahABCFE*). The results are shown in Table 5. *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, and *P. fluorescens* DFC50 all mineralized [9- ^{14}C]Phenanthrene while *E. coli* DH5 α (*lac-nahABCFE*),

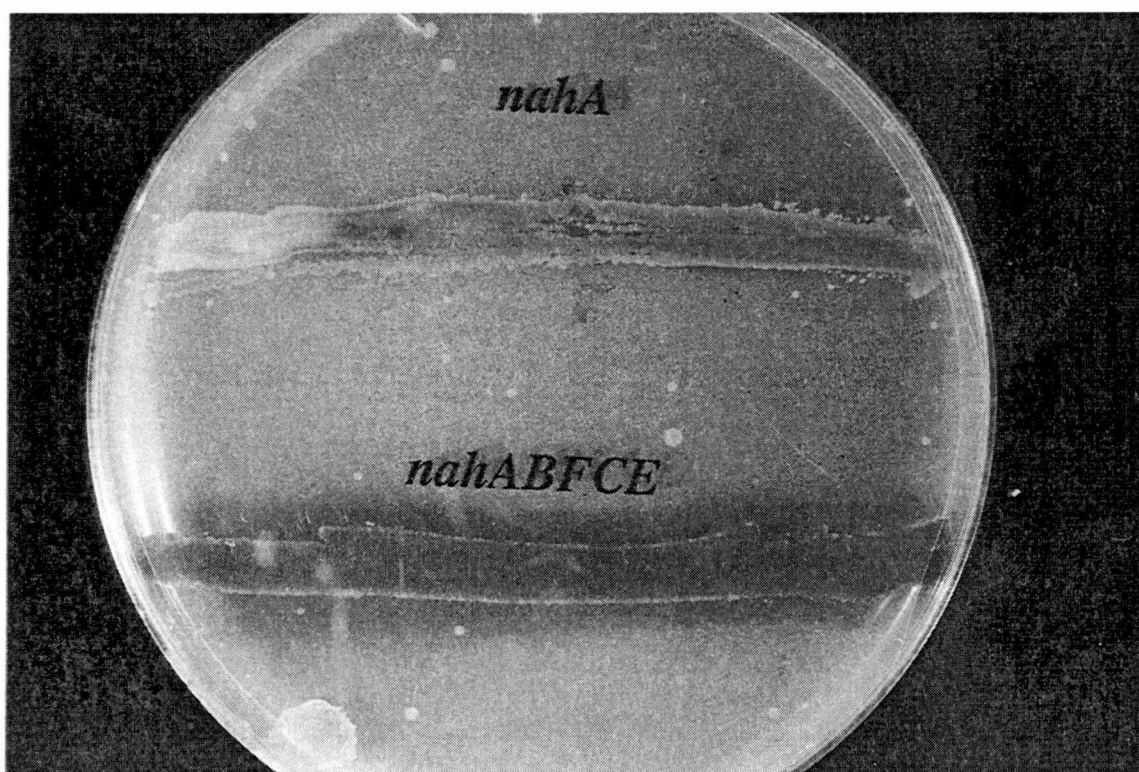


Figure 19. Phenanthrene spray plate of *E. coli* DH5 α (pUTK201) and *E. coli* DH5 α (pUTK202).

Table 5. [9-¹⁴C]Phenanthrene mineralization assays

Strain	[9- ¹⁴ C]Phenanthrene Mineralization	
	% ¹⁴ C as CO ₂	Total ¹⁴ C Recovered
<i>P. putida</i> PpG7	14.3 ± 3.5	92 ± 10.2
<i>P. fluorescens</i> 5R	23.3 ± 2.2	103 ± 13
<i>P. fluorescens</i> DFC49	20.1 ± 4.2	89 ± 9.3
<i>P. fluorescens</i> DFC50	31.1 ± 5.6	78 ± 9.1
<i>P. fluorescens</i> 5RL	nd	103 ± 5.3
<i>P. fluorescens</i> 18H	nd	102 ± 10
<i>P. fluorescens</i> HK44	nd	88 ± 8.7
<i>Burkholderia</i> <i>cepacia</i> JS150	nd	94 ± 12
<i>E. coli</i> DH5α (<i>lac</i> - <i>nahABC</i> FE)	nd	97 ± 15

Values are the averages of three replicates ± one std deviation.
nd = not detected.

P. fluorescens 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 did not.

Biotransformation Assays

Since *P. fluorescens* 5RL, *P. fluorescens* HK44, *B. cepacia* JS150, and *E. coli* DH5 α (pUTK202) did not mineralize phenanthrene but did clear spray plates, it was of interest to examine the extent of their metabolism of phenanthrene. Using these strains resting cell biotransformation assays were performed with phenanthrene. The extracts were analyzed by TLC and visualized using UV light. The results of the TLC plate can be seen in Figure 20. All four strains produced a single polar metabolite which matched the retention factor of 1-hydroxy-2-naphthoic acid. For further confirmation, each spot was scraped from the TLC plate and subjected to GCMS as described in chapter 4. Retention factors (RF) and mass spectral identification are summarized in Table 6. All of the strains produced 1-hydroxy-2-naphthoic acid as confirmed by MS data major metabolite. A representative mass spectrogram showing 1-hydroxy-2-naphthoic acid from the polar extract from 5RL can be seen in Figure 21.

DNA:DNA Hybridization Analysis

The strains: *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 were subjected to DNA:DNA hybridization. Probes for *nahA*, *nahG*, and *nahH* were used to ascertain the relevant genotype of the strains. The 16S oligonucleotide probe for all eubacteria was used to validate the technique for the strains

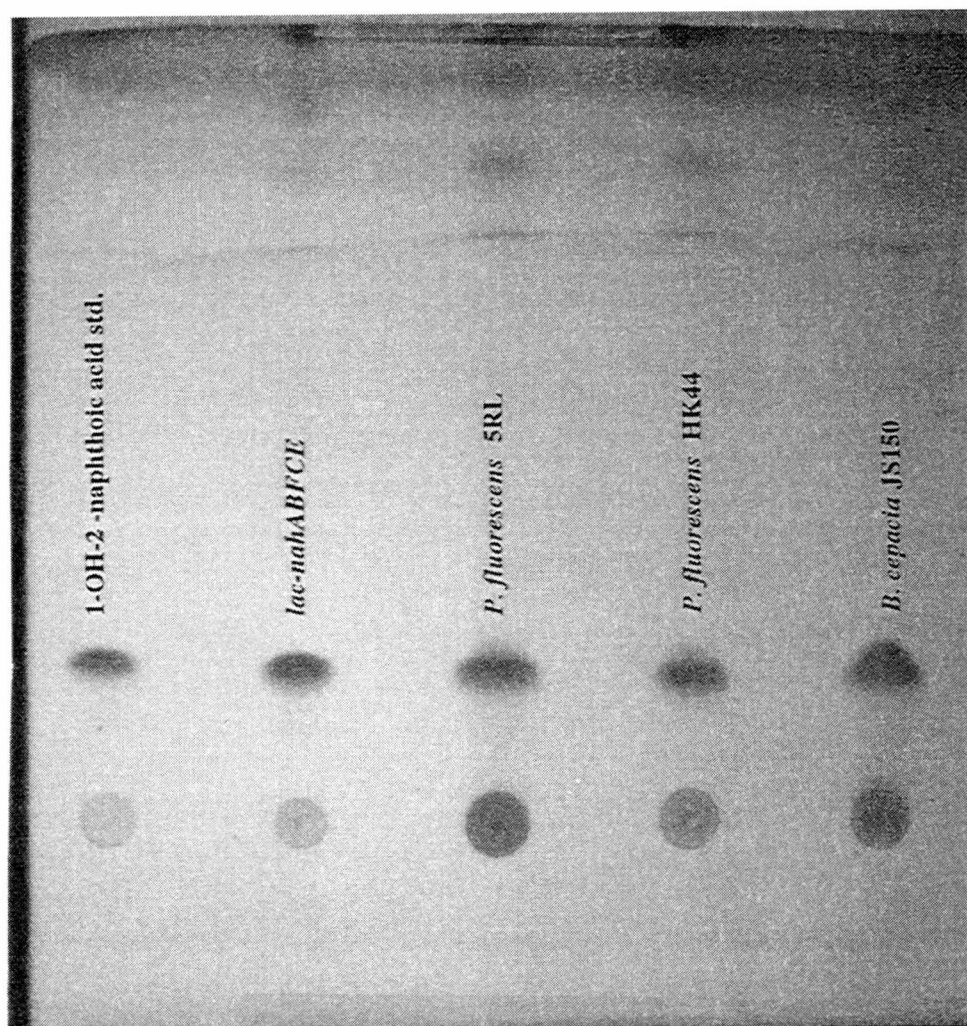


Figure 20. TLC analysis of non-mineralizing strains. UV illuminated TLC plate showing the major polar metabolite 1-hydroxy-2-naphthoic acid from acid extracts of tested strains *P. fluorescens* 5RL, *P. fluorescens* HK44, *B. cepacia* JS150, and *E. coli* DH5 α (pUTK202).

Table 6. Mass spectra data of putative 1-hydroxy-2-naphthoic acid eluted from TLC plates.

Strain	RF value	Mass Spectral Identification
Authentic 1-hydroxy-2- naphthoic acid	.25	1-hydroxy-2-naphthoic acid
DH5 α	.24	1-hydroxy-2-naphthoic acid
<i>P. fluorescens</i> SRL	.24	1-hydroxy-2-naphthoic acid
<i>P. fluorescens</i> HK44	.24	1-hydroxy-2-naphthoic acid
<i>Burkholderia</i> <i>cepacia</i> JS150	.24	1-hydroxy-2-naphthoic acid

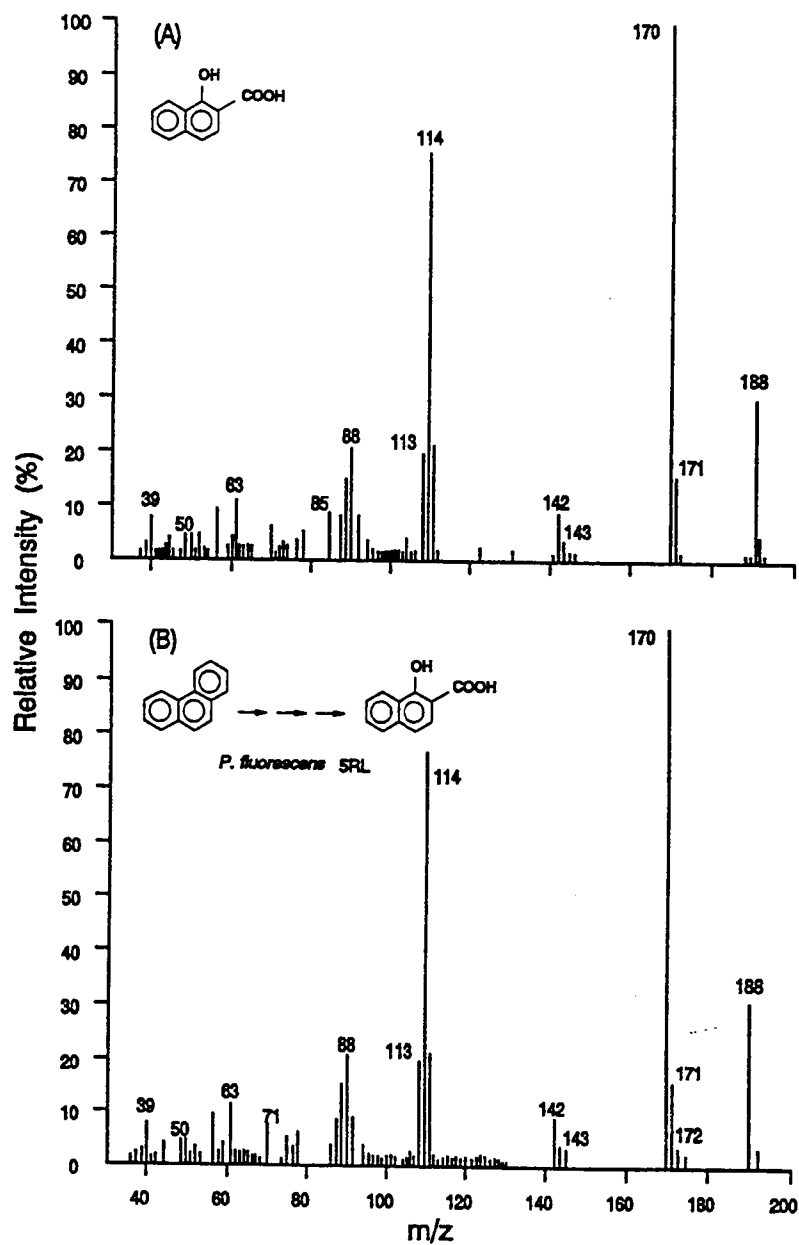
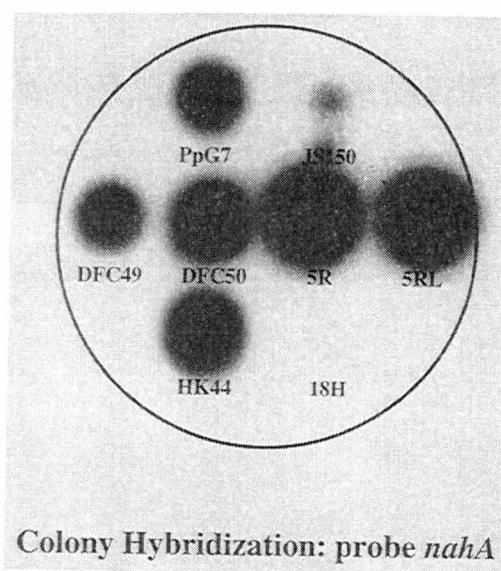


Figure 21. Representative mass spectrum of major polar metabolite from TLC plate. A. Mass spectrum of polar metabolite from 5RL. B. Mass spectrum of authentic compound 1-hydroxy-2-naphthoic acid.

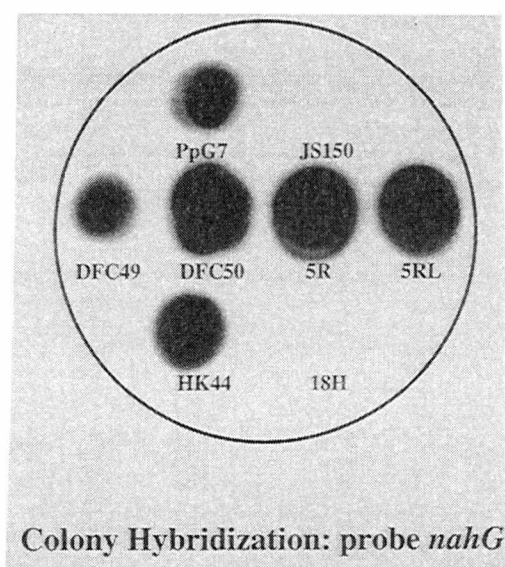
tested (i.e., there was DNA from the cells on the filter). The 16S oligonucleotide probe was also used to probe filters with negative results to eliminate the possibility the negative colonies simply did not lyse. Colony blots are shown in Figure 22. The results are summarized in Table 7.

Mapping Tn4431 Insertion Site in 5RL

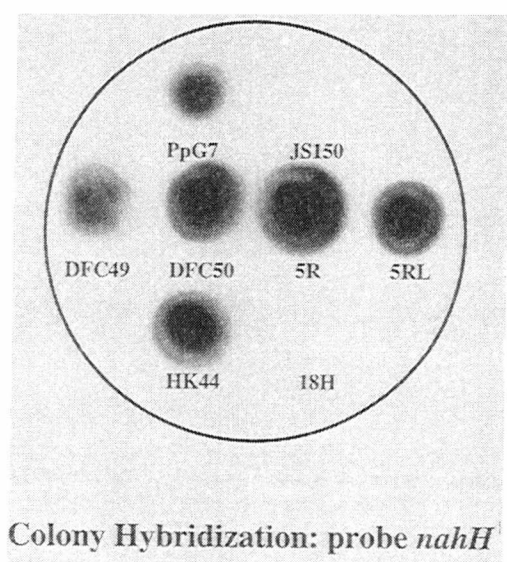
Since strain 5RL was used to analyze phenanthrene degradation, it was necessary to determine the location of the Tn4431 insertion. The insertion site was proposed within the salicylate hydroxylase gene (*nahG*) by analyzing metabolites of naphthalene degradation which revealed the accumulation of salicylate (King et al., 1990). Southern hybridization using *xylE*, which shows high sequence similarity (81%) to *nahH*, (Ghosal et al., 1987) localized the transposon to a 1.6 kb *Pst* I fragment adjacent to *nahH*. These data, assuming a NAH7 gene organization, indicated the transposon inserted into the *nahG*. Therefore it was determined that using the polymerase chain reaction and three primers could verify the location of insertion. The parent plasmid pKA1 (King et al., 1990) and the highly characterized plasmid NAH7 were subjected to PCR using primers 1 and 2 (described in Chapter 4), thus generating a 2100 bp fragment (Fig. 23, lane 4; NAH7 data not shown) in both reactions. Using primer 1 and primer 2, no amplified fragment was detected after PCR with pUTK21 as the template DNA. The 2100 bp fragment seen with the parent plasmid pKA1 was absent because the insertion of the 15 kb Tn4431 exceeded the approximately 3000 bp limit for PCR amplification under these amplification conditions. Amplification with PCR using primer 1 and primer 3, and primer 2 and primer 3, generated DNA fragments of 300 bp and 1800 bp, respectively (Fig. 23, lanes 2 and 3).



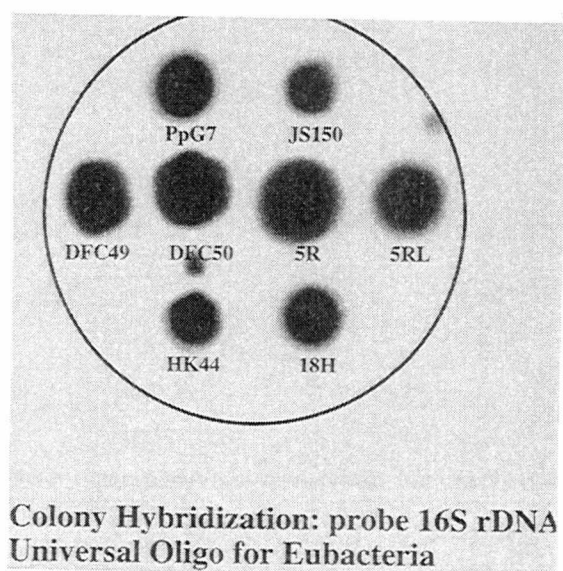
A



B



C



D

Figure 22. Autoradiograms of colony hybridizations. Blots were hybridized using ^{32}P labeled *nahA* (A), *nahG* (B), *nahH* (C), and 16S (D) universal oligo DNA probes

Table 7. Colony Hybridization Results.

Strain	DNA Probe/Hybridization $\pm$			
	<i>nahA</i>	<i>nahG</i>	<i>nahH</i>	16S Universal oligo ^a
<i>P. putida</i> PpG7	+	+	+	+
<i>P. fluorescens</i> 5R	+	+	+	+
<i>P. fluorescens</i> DFC49	+	+	+	+
<i>P. fluorescens</i> DFC50	+	+	+	+
<i>P. fluorescens</i> 5RL	+	+	+	+
<i>P. fluorescens</i> 18H	-	-	-	+
<i>P. fluorescens</i> HK44	+	+	+	+
<i>Burkholderia</i> <i>cepacia</i> JS150	+	-	-	+

^a All colony blots with negative results were verified by hybridization with 16S universal oligo.

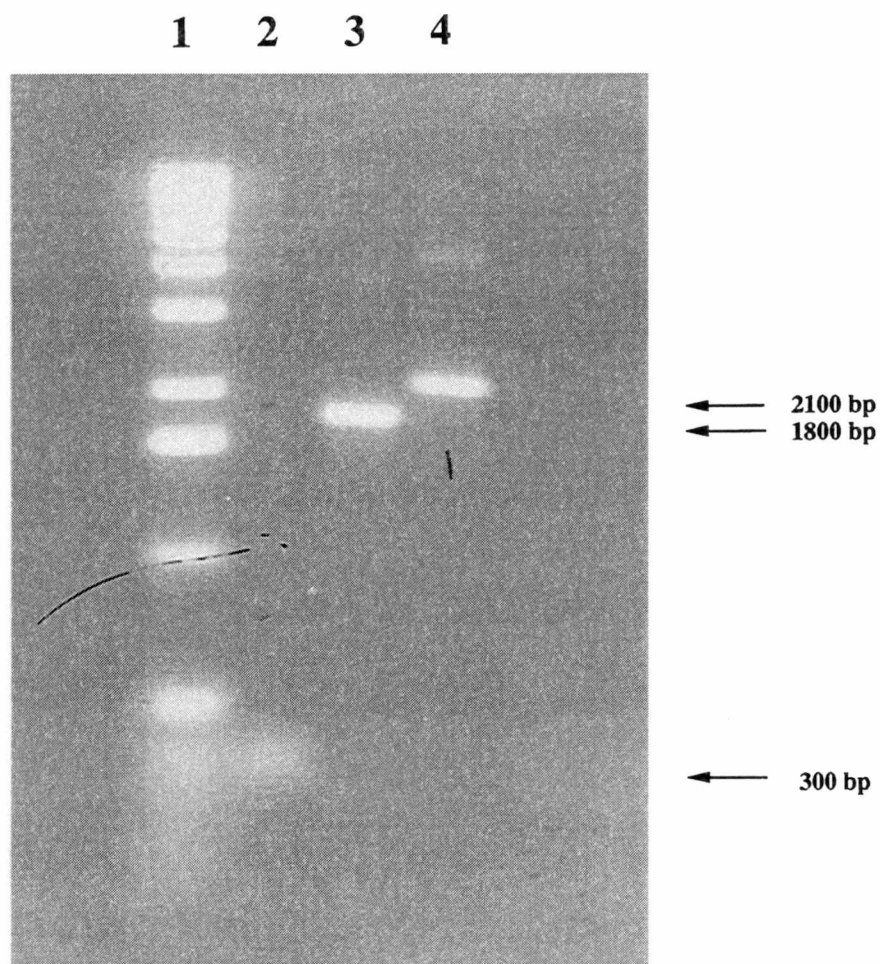


Figure 23. Mapping of Tn4431 in pUTK21 using PCR. 1% agarose gel of PCR products from amplifications using various primer combinations and plasmid pUTK21 as template DNA. Lane 1, 1 kb ladder; lane 2, *nahG*-IS of Tn 4431 of pUTK 21 using primers 1 and 3; lane 3 IS of Tn 4431-*nahG* of pUTK 21 using primers 3 and 2; lane 4, *nahG-nahH* of pKA 1 using primers 1 and 2.

Figure 24 is a schematic representation of the electrophoresis results in Figure 23. Using the same primer pairs on the parent plasmid pKA1 did not produce any amplified products (data not shown). These data show there was no sequence complementary to primer 3 on pKA1 that would generate the results with 5RL. Using the polymerase chain reaction and the appropriate primers obtained from previously published sequence data, confirmation of NAH7 gene order of the lower pathway of pKA1 and mapping of the insertion site of TN4431 300 bp from the start of *nahG* was accomplished.

Construction of pLJS

To facilitate the construction of the reporter transposon, pBlueScript II KS was modified to provide a MCS flanked by *Xba*I, *Nhe*I, *Spe*I, and *Avr*II restriction sites (as described in Chapter 4). The construct was sequenced to determine the integrity of the MCS and confirmation of the added sites (Fig. 25). The plasmid retained the ability to provide α -complementation despite the base substitutions. A diagram of the resultant plasmid pLJS can be seen in Figure 26. Since all of these enzymes generate the same cohesive end (CTAG) they can be heterologously cloned. This heterologous cloning destroys the recognition sites. However it is very advantageous, as it allows assemblage of DNA fragments cloned into the MCS of pLJS by the destruction and recreation of unique sites.

Resultant pLJS and pCRII Clones

The various pLJS and pCRII clones are listed in Table 8. All pLJS clones are harbored in *E. coli* DH5 α while all pCRII amplification product clones are in *E. coli* INVF'.

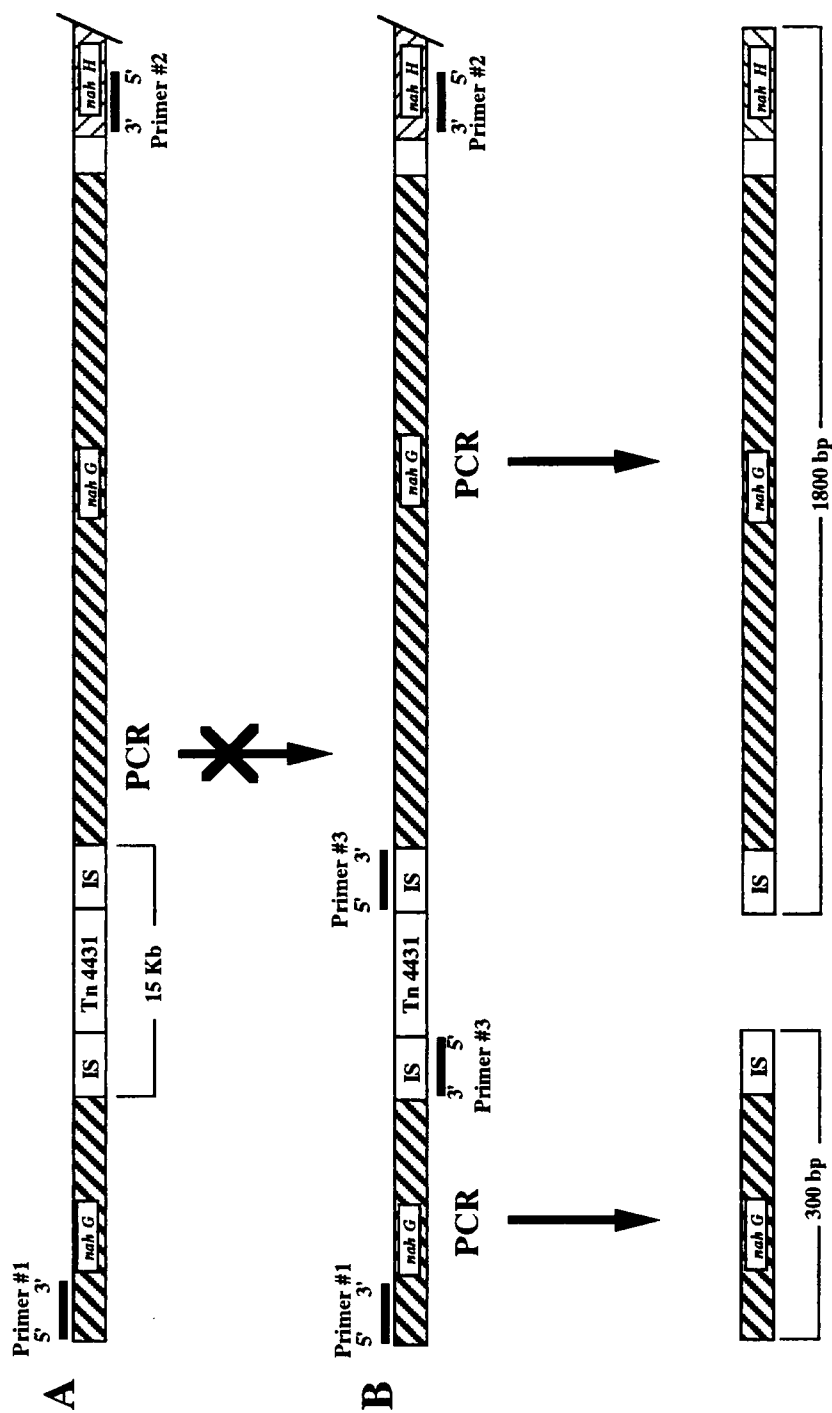


Figure 24. Schematic representation of the location of the amplification products. A. No amplification occurs due to the insertion of Tn 4431. B. Products mapping the insertion site of Tn 4431.

5'

A GTACCGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATT
 |||||
 B GTACCGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATT

A CCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCCGGTGGGA
 |||||
 B CCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCCGGTGGGA

A GCTCCAATTCGCCCTATAGTGAGTCGTATTACG^{3'}
 |||||
 B GCTCCAATTCGCCCTATAGTGAGTCGTATTACG

Figure 25. Comparison of analyzed sequence to that of the theoretical sequence of pLJS. Strand A is theoretical sequence. Strand B is actual sequence.

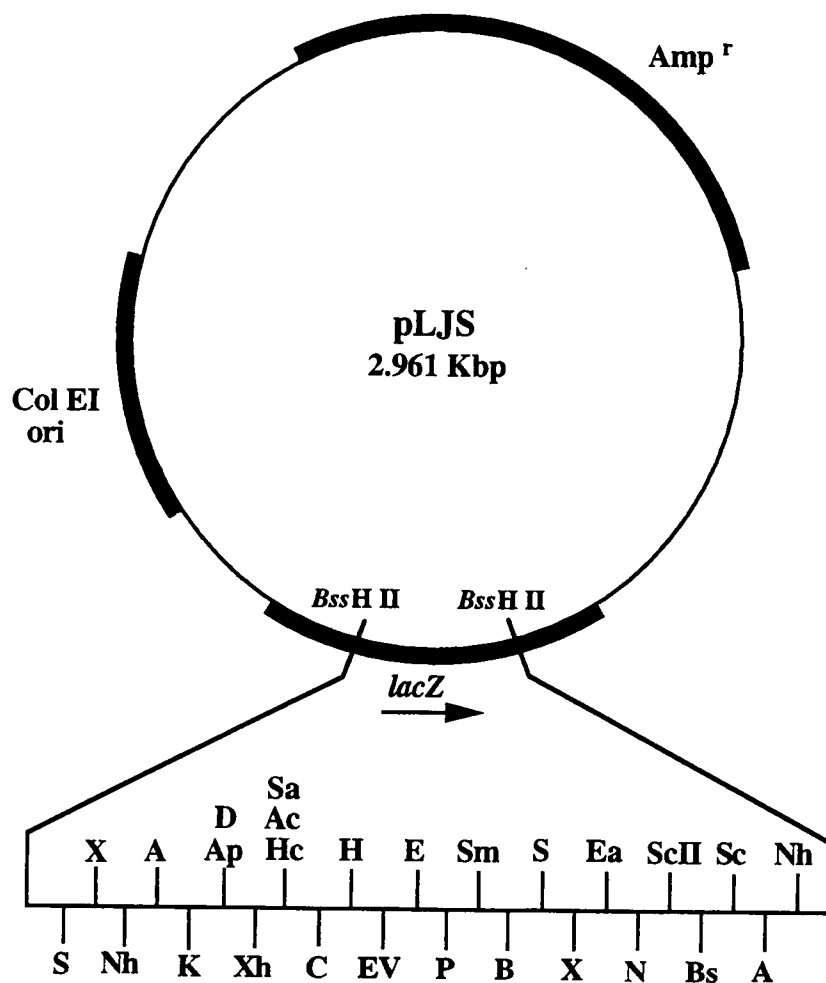


Figure 26. Cloning plasmid pLJS with unique restriction sites. Abbreviations: A, *AvrII*; Ac, *AccI*; Ap, *Apal*; B, *BamH I*; Bs, *BstX I*; C, *Clal*; D, *DraII*; E, *EcoR I*; Ea, *EagI*; EV, *EcoR V*; H, *HindIII*; Hc, *HincII*; K, *KpnI*; N, *NotI*; Nh, *NheI*; P, *PstI*; S, *SpeI*; Sa, *Sall*; Sc, *SacI*; ScII, *SacII*; Sm, *SmaI*; X, *XbaI*; Xh, *XhoI*.

Table 8. pLJS and pCRII (pUTK) constructs generated in this study.

Plasmids	Relevant Characteristics
pLJS- <i>lux</i>	pLJS containing the 8.35 kb <i>Kpn</i> I- <i>Pst</i> I <i>lux</i> CDABE cassette from pUCD615, Ap ^R
pLJST2	pLJS containing the 0.77 kb <i>Hind</i> III- <i>Hinc</i> II fragment from pKK223-3 cloned into <i>Hind</i> III- <i>Sma</i> I site, Ap ^R
pLJS <i>nahRG_p</i>	pLJS containing the 1.3 kb <i>Eco</i> RV- <i>Kpn</i> I <i>nahRG_p</i> fragment from pCRII <i>nahRG_p</i>
pUTK198	pCRII containing 1 kb <i>nahH</i> DNA fragment amplified from pKA1, codes for complete catechol 2,3 dioxygenase
pUTK199	pCRII containing 1.3 kb <i>nahG</i> DNA fragment amplified from pKA1 codes for complete salicylate hydroxylase
pUTK200	pCRII containing 3.1 kb <i>nahGH</i> DNA fragment amplified from pKA1 codes complete salicylate hydroxylase and catechol 2,3 dioxygenase

Construction of Bioluminescent Reporter Transposon mini-Tn5KmnahRG_p-lux and Resultant Construction of Bioluminescent Reporter *P. fluorescens* 135R75

Tn5KmnahRG_p-lux was constructed by the following steps: The first step was to construct a core transposon, mini-Tn5KmNX with unique *Not*I and *Xba*I sites. This core unit allowed a stepwise assembly of directionally cloned inserts from pLJS (*Not*I-*Avr*II fragments) as shown in Figure 27.

E. coli S17-1(λ pir) containing mini-Tn5KmnahRG_p-lux was successfully mated into *P. fluorescens* 5R rif (King et al., 1990) by plate mating. The resultant construct was designated 135R75 and evaluated for fitness and performance (Kehrmeyer et al., 1997). The strain was used in subsequent *nah*-lux induction assays.

Bioluminescence Induction Assays

Bioluminescence induction assays were performed as outlined in Chapter 4. The results are presented in Figs. 28, 29 and 30. This procedure allows maximum time for the parent substrate to be converted to the inducer and subsequent bioluminescence. All three *nah*-lux bioluminescence reporters gave negative induction results for both 0.65 ppm and 1.3 ppm (saturation) phenanthrene. Equimolar amounts of naphthalene were used for positive controls and showed a significant response for all three strains. The modification to the growing cell assay (adding concentrated cells) appeared to cause a lag in induction as seen in the positive control except for 135R75. However, the overall performance was similar. Naphthalene caused a significant bioluminescent response in all three of the modified assays.

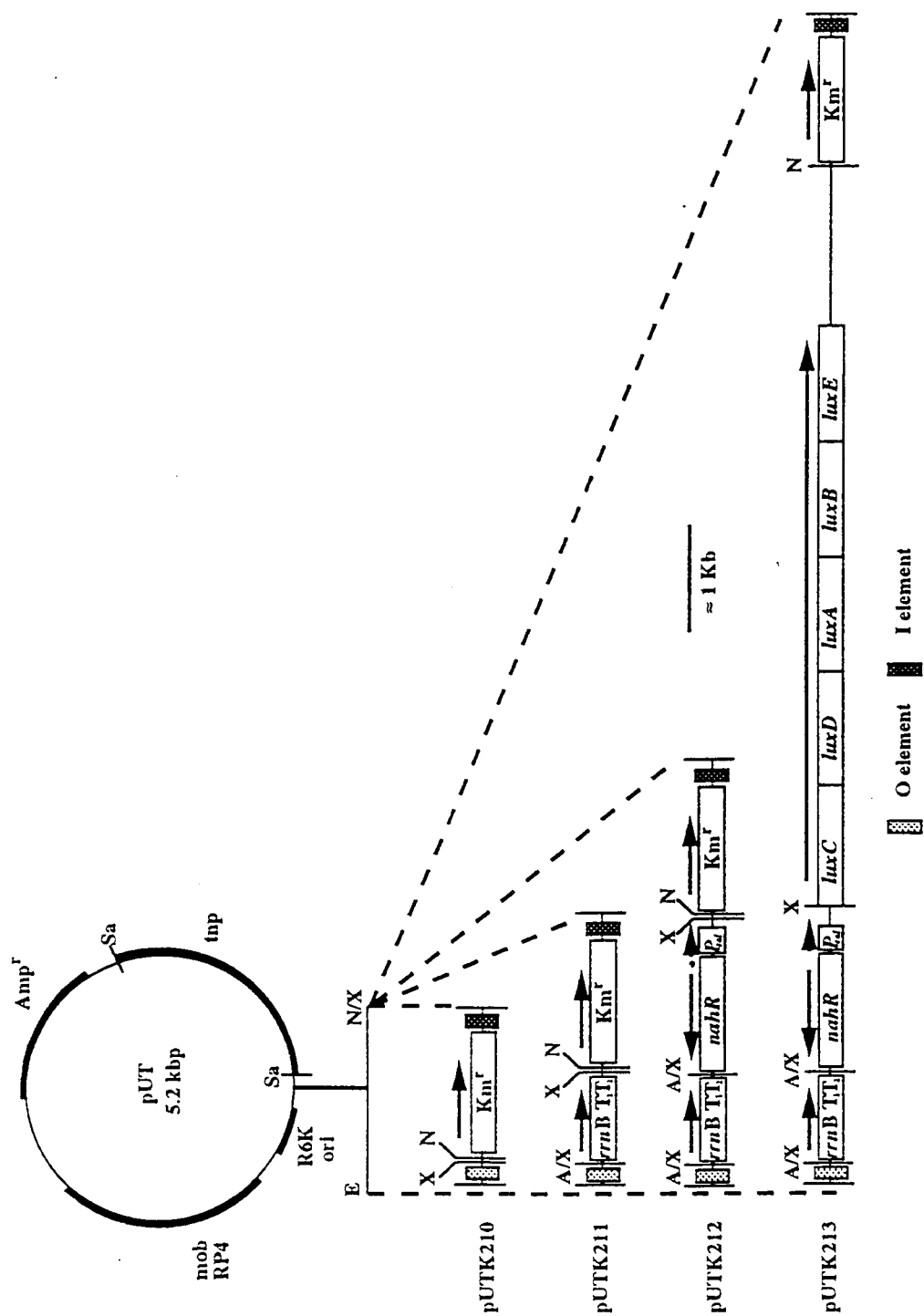


Figure 27. Schematic representation of the assembly of mini-Tn5KmNX by heterologous cloning. Restriction sites are: N, *NotI*; X, *XbaI*; Sa, *SacI*; E, *EcoRI*; and A/X represents the heterologous cloning of *AvrII* and *XbaI*.

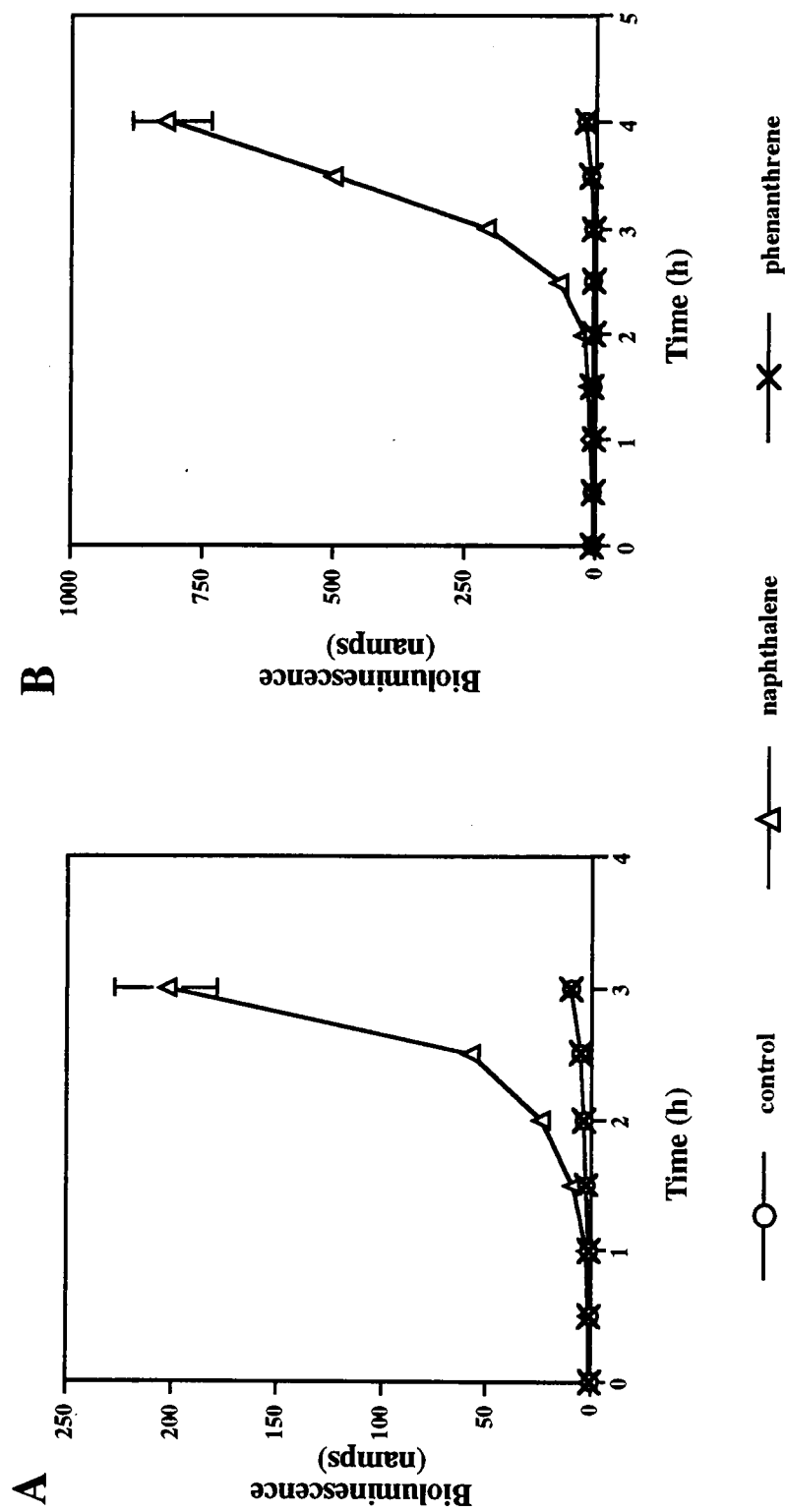


Figure 28. Induction assays of the *nah-lux* reporter strain 5RL. A.) Standard growing cell assay with 0.65 ppm phenanthrene, 0.50 ppm naphthalene (approximately equal molar values). B.) Bioluminescence response to 1.3 ppm phenanthrene and equal molar concentration of naphthalene at approximately 1.0 ppm.

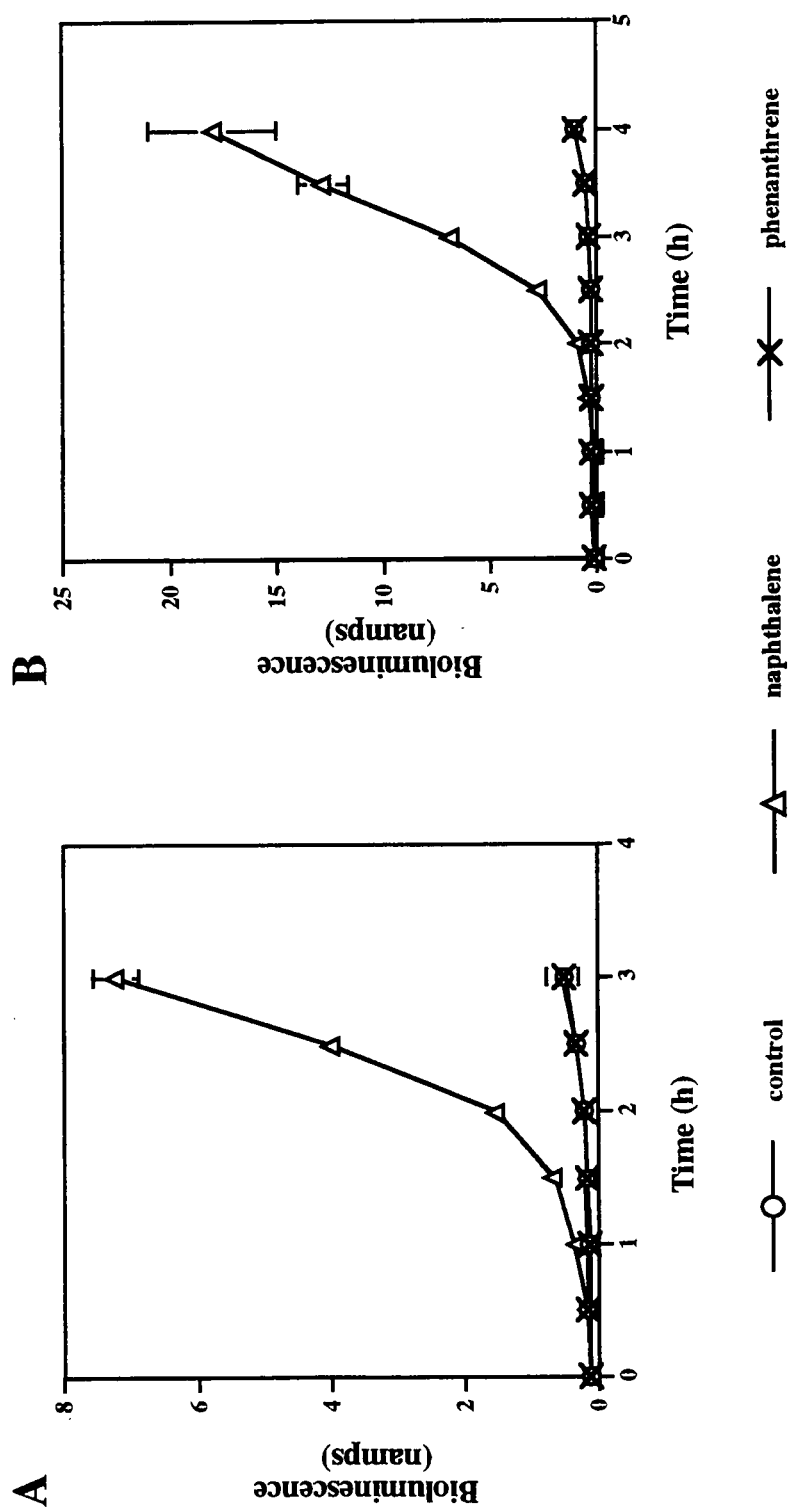


Figure 29. Induction assays of the *nah-lux* reporter strain RB1351. A.) Standard growing cell assay with 0.65 ppm phenanthrene, 0.50 ppm naphthalene (approximately equal molar values). B.) Bioluminescence response to 1.3 ppm phenanthrene and equal molar concentration of naphthalene at approximately 1.0 ppm.

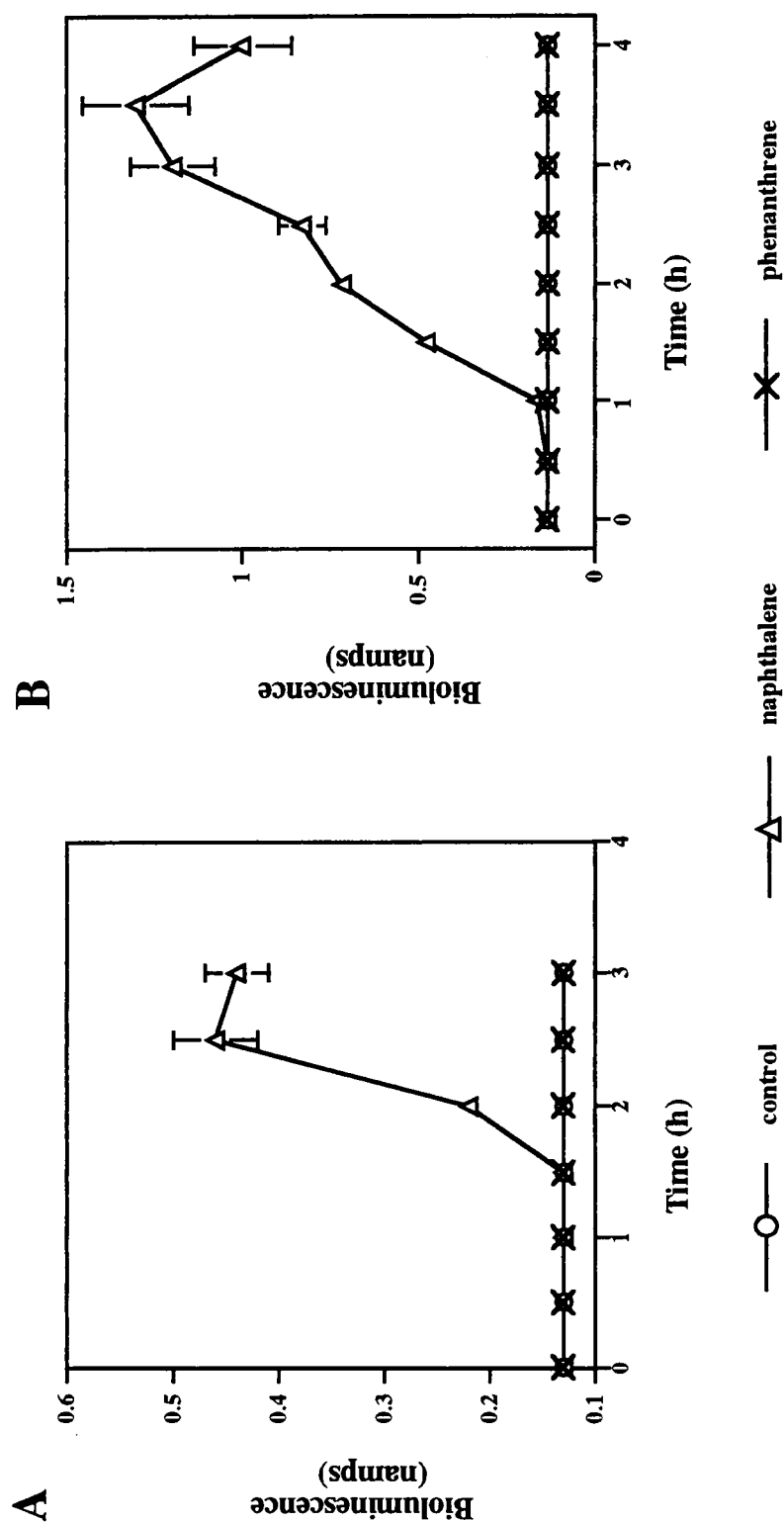


Figure 30. Induction assays of the *nah-lux* reporter strain 135R75. A.) Standard growing cell assay with 0.65 ppm phenanthrene, naphthalene 0.50 ppm (approximately equal molar values). B.) Bioluminescence response to 1.3 ppm phenanthrene and equal molar concentration of naphthalene at approximately 1.0 ppm.

Putative metabolites 1-hydroxy-2-naphthoic acid and phthallic acid were also examined at molar equivalents to phenanthrene at both 0.65 ppm and 1.3 ppm with negative results i.e. no bioluminescence increase (data not shown). Therefore, these data show that phenanthrene is not metabolized to intermediates capable of interacting with NahR and inducing transcription at relevant physiological concentrations.

Mineralization Assays using *P. fluorescens* 5R

Resting cell mineralizations using *P. fluorescens* 5R were performed to ascertain the maximum amount (%) of ^{14}C that could be recovered as $^{14}\text{CO}_2$ from [9- C^{14}]phenanthrene. Biometer flasks containing resting cells were amended with: 1×10^5 dpm (4.3 nmol), 5×10^5 dpm (21.5 nmol), and 1×10^6 dpm (46.5 nmol). Percent $^{14}\text{CO}_2$ was plotted versus time and is shown in Figure 31.

All three concentrations of phenanthrene were rapidly mineralized in 24 h to a value that approached 50% $^{14}\text{CO}_2$ evolution. To determine whether or not this plateau was an artifact of the assay a subsequent experiment was performed in which 1.65×10^6 dpm (70.95 nmol) of phenanthrene were added to biometer flasks. Evolution of $^{14}\text{CO}_2$ was monitored over time as above (Fig. 32). After 9 h, the recovered dpm as $^{14}\text{CO}_2$ began to plateau (similar to previous experiments) at which time another 1.65×10^6 dpm (70.95 nmol) of [9- C^{14}]phenanthrene was added. $^{14}\text{CO}_2$ evolution immediately began to increase and plateaued at approximately 50% of the additional substrate as $^{14}\text{CO}_2$. The rates of $^{14}\text{CO}_2$ evolution over time were determined from the linear part of the curve and were similar at an initial rate of $1.27 \times 10^5 \pm 1.12 \times 10^4$ $^{14}\text{CO}_2$ dpm/hr followed by a rate of $1.35 \times 10^5 \pm 2.9 \times 10^4$ $^{14}\text{CO}_2$ dpm/hr. These data suggest that the reason for the approximate 50% plateau is substrate limitation since readdition of the substrate resulted in the same amount of mineralization eliminating other potential limiting

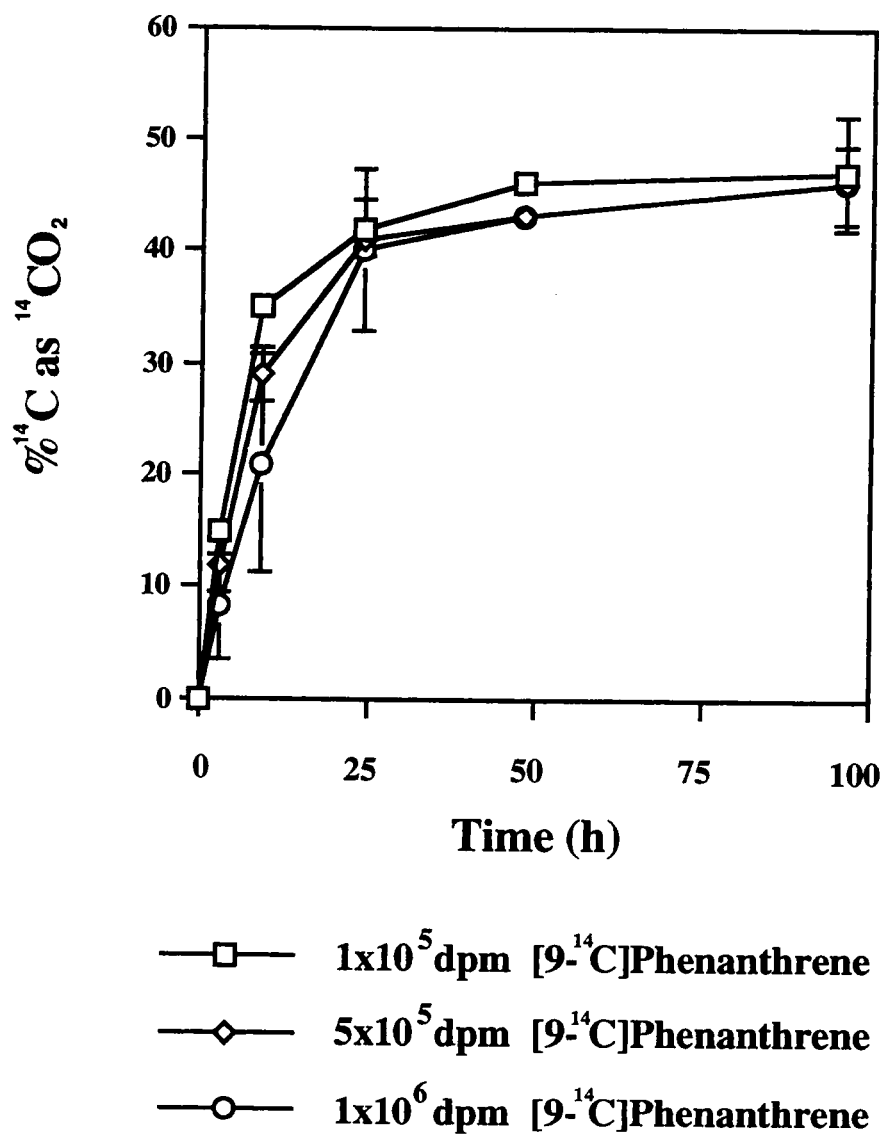


Figure 31. Mineralization experiment using different amounts of $[9\text{-C}^{14}]$ phenanthrene.

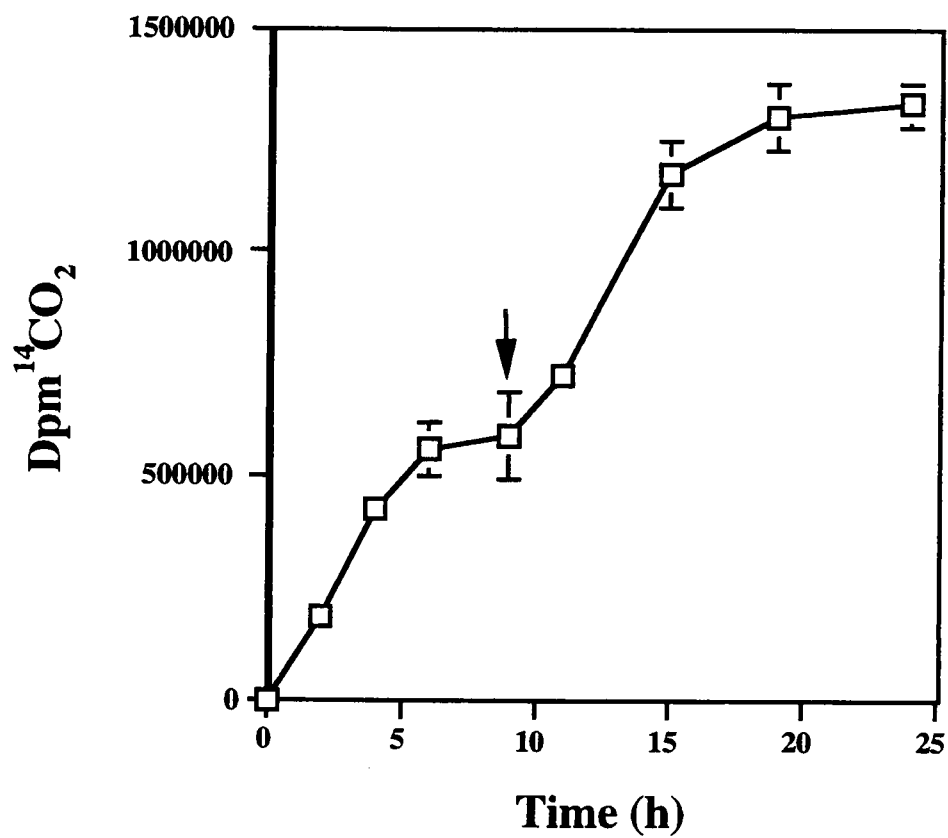


Figure 32. Mineralization experiment with readdition of $[9-^{14}\text{C}]$ phenanthrene substrate. Arrow indicates point of addition of another 1.65×10^6 dpm $[9-^{14}\text{C}]$ phenanthrene.

factorse.g. oxygen, loss of enzyme activity etc. Supernatants from these experiments were extracted as biotransformation assays at the termination of the experiment as previously described.

Phenanthrene Biotransformation Assays using *P. fluorescens* 5R

Biotransformation assays were performed using *P. fluorescens* 5R as previously described in Chapter 4. Extracts were suspended in acetone and analyzed by GCMS. Acid extracts of transformations revealed the presence of two major peaks: The first peak was identified as 1-hydroxy-2-naphthoic acid (9.49 rt, peak area 22,511), while the second major peak was identified as phthallic acid (16.44 rt, peak area 159,312). Mass spectrum is shown in Figure 33. Phthallic acid was not presence in the control extracts. However, since phthallates are plasticizers and are common GCMS contaminants (Ende et. al, 1982), it was neccessaryto validate this result with an alternative method.

Radiolabeled transformation products from the mineralization assays were analyzed using TLC. Approximately 100,000 dpm of putative phthallic acid was loaded on a TLC plate with a phthallic standard (subjected to extraction procedure) and developed on chloroform. Under these conditions neither of the two spots moved. The radiolabeled spot was subsequently scraped off the plate and eluted with methanol as previously described. Approximately 90% of the counts were recovered suggesting that the majority of the radiolabel was this polar metabolite. These data imply that the radiolabeled polar compound is phthallic acid or a similar compound.

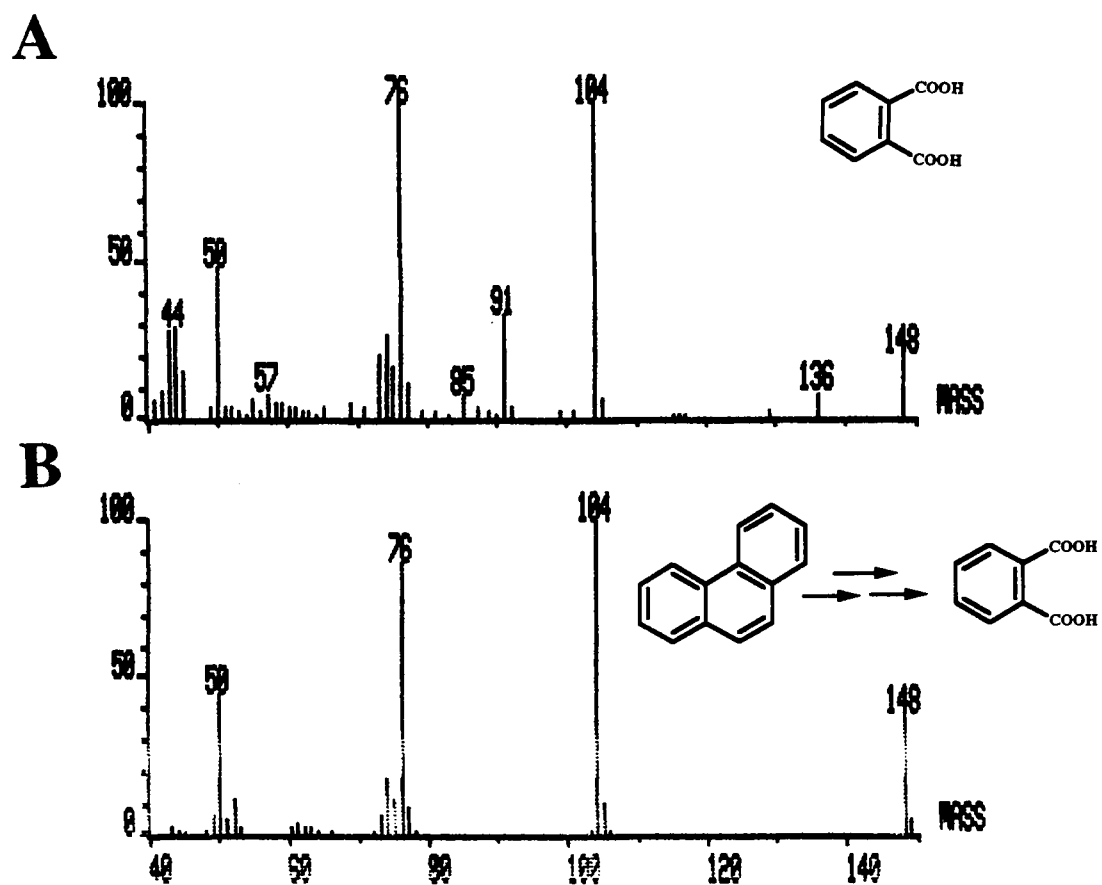


Figure 33. Mass spectrum of phthalic acid. A). Authentic phthalic acid. B). Metabolite extracted from phenanthrene biotransformation using *P. fluorescens* 5R.

Meta Cleavage Assays

Meta cleavage assays were performed to ascertain the substrate specificity of the salicylate hydroxylase. Using *E. coli* INVF' *lac-nahGH*, various substituted salicylates were assayed for the ability to be decarboxylated to the corresponding catechol and subsequent ring cleavage. The results are shown in Table 9.

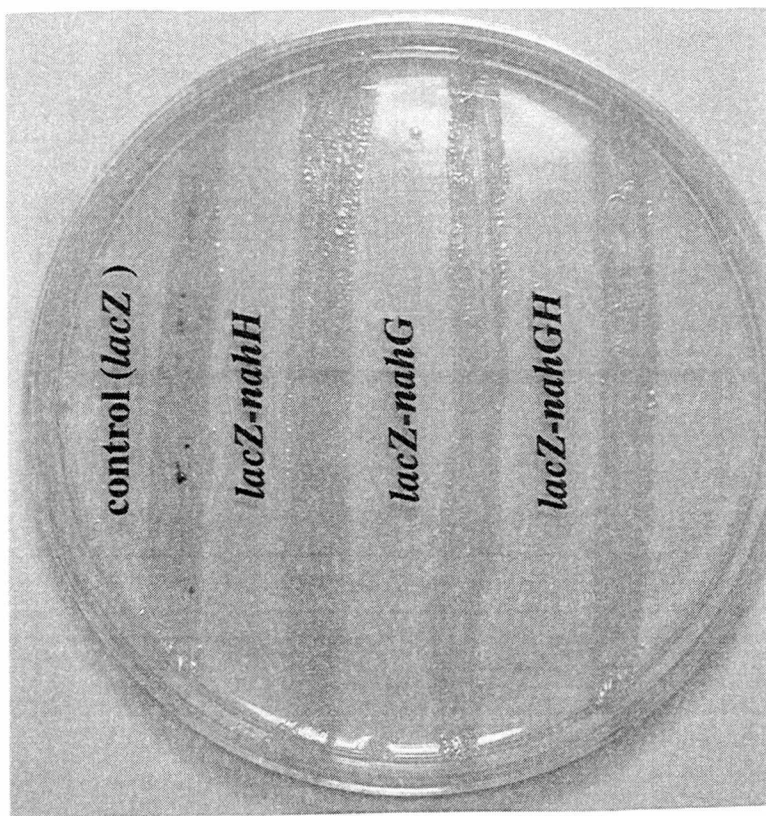
Salicylate Hydroxylase Transformation of 1-Hydroxy-2-naphthoic acid

Three strains of *E. coli* INVF' harboring a *lac-nahH*, *lac-nahG*, and *lac-nahGH* transcriptional fusion were streaked on LB agar plates with 50 µg/ml Km. Negative control consisted of pCRII in *E. coli* INVF'. *E. coli* INVF' has a deletion in the *lac* repressor and therefore, the strains do not require induction (IPTG etc.). The plates were then sprayed with 1-hydroxy-2-naphthoic acid and incubated at 28°C. After approximately 18 h the *E. coli* INVF' with the *lac-nahG* and *lac-nahGH* developed an orange color while the *E. coli* INVF' *lac-nahH* and pCRII did not (Fig. 34).

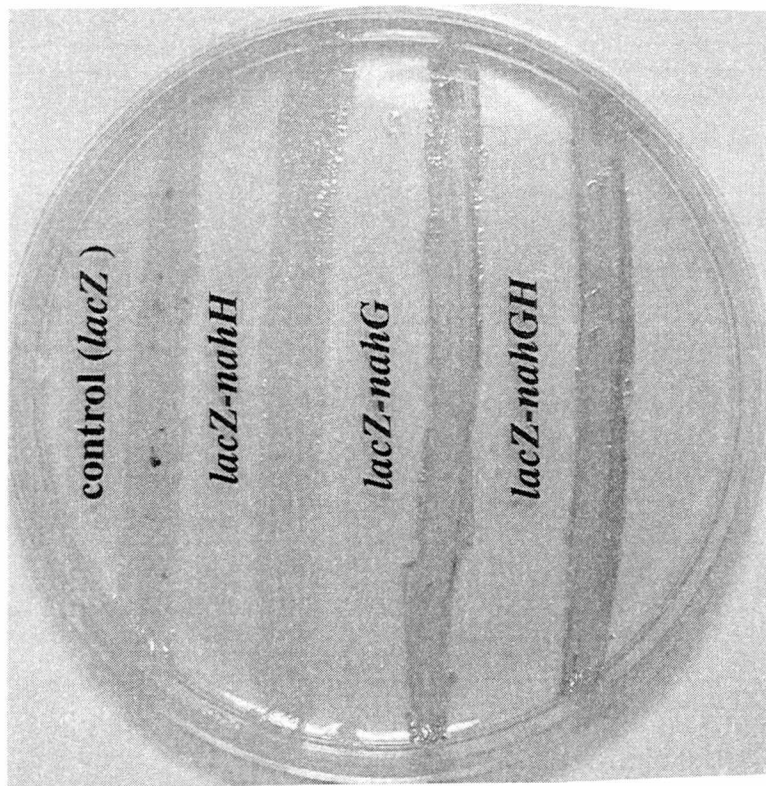
Resting cell biotransformations were also performed using these strains with the similar results. Supernatants were orange for both the *lac-nahGH* and *lac-nahG* and clear for the *lac-nahH* and pCRII strains. Supernatants were extracted and concentrated as previously described. The sample along with standards of 1-hydroxy-2-naphthoic acid as well as 1,2-dihydroxynaphthalene, were analyzed by TLC as previously described. The two fusions containing the salicylate hydroxylase contained an orange polar substrate which remained at the bottom of the plate as well as a large amount of 1-hydroxy-2-naphthoic acid. However, there were no spots from the sample which were similar to the 1,2-dihydroxynaphthalene.

Table 9. Metacleaveage product formation by salicylate hydroxylase and catechol dioxygenase of *E. coli* INV^F' *lac-nahGH*.

Test compounds	Absorbance maximum (nm) of Product
5-Methylsalicylate	380
4-Methylsalicylate	380
3-Methylsalicylate	385
5-Hydroxysalicylate	none
4-Hydroxysalicylate	none
3-Hydroxysalicylate	none
5-Chlorosalicylate	380
4-Chlorosalicylate	380
5-Aminosalicylate	none
4-Aminosalicylate	none
Salicylate	375
Negative control	none



A



B

Figure 34. Photograph of LB plates with various *lac* transcriptional fusions sprayed with 1% solution of 1-hydroxy-2-naphthoic acid in acetone. A) Plate initially after spraying. B) Plate after 24 h.

Complementation of Tn4431 Insertional Mutation in *P. fluorescens* 5RL

The 3.1 kb *nahGH* fragment was cloned into plasmid pUTK212 (previously constructed during the construction of the mini-Tn5 bioluminescent reporter transposon) containing Tn5Km*nahRG_p* to generate Tn5Km*nahRG_p*-*nahGH*. The construct was subsequently inserted into *P. fluorescens* 5RL to complement the disrupted *nahG*. The strains were selected for on naphthalene vapor to allow light induction and the generation of the yellow ring cleavage product from catechol.

The resultant strain 5RLTnGH3 was subjected to mineralization assays to ascertain the ability to mineralize phenanthrene (complementation of the mutation). Resting cell assays were performed as previously mentioned except they were performed in vials. The strain 5RLTnGH3 was able to mineralize phenanthrene to $^{14}\text{CO}_2$. However, only $6.6 \pm .8 \%$ (mass balance 89%) of the radiolabeled was returned as $^{14}\text{CO}_2$ while 5R produced $43 \pm 5.1\%$ (mass balance 91%).

Chapter VII

Discussion

There are numerous reports showing that organisms containing NAH type plasmids and naphthalene catabolic operons homologous to NAH are capable of degrading phenanthrene (Menn et al., 1993; Sanseverino et al., 1993; Denome et al., 1993; Yang et al., 1994; Kiyohara et al., 1994). In this study NAH plasmid containing environmental strains *P. fluorescens* DFC49, *P. fluorescens* DFC50, and *P. fluorescens* 5R, along with *P. putida* PpG7 and the putative chromosomal NAH pathway in *B. cepacia* JS150 (Callicotte, 1995) were extensively studied for their involvement in the degradation of phenanthrene.

P. fluorescens DFC49, DFC50, and 5R, along with *P. putida* PpG7 were not able to utilize phenanthrene as a sole carbon and energy source. One might attribute this to the low solubility of phenanthrene. However, the *nahA*⁻ isolate *A. defieldii* (Hunt, 1994) was capable of producing significant biomass in the assay suggesting that the layer of phenanthrene on the bottom of the flask allowed a constant concentration of phenanthrene for utilization. Barnsley (1983a) also reported a Gram-negative coccus that could utilize phenanthrene as evidenced by increased cell density as determined plate counts. However in the same report *Pseudomonas* P_G, which later was designated NCIB9816 (containing an archetypal NAH plasmid) was not capable of growing on phenanthrene and the actual number of cells decreased over time. This later report supports the observations in this dissertation.

Although unable to grow on phenanthrene as a sole carbon source, these strains as well as the mutant strain, *P. fluorescens* 5RL, were capable of clearing phenanthrene spray plates. However, when these strains were assayed for the ability to mineralize

[9-¹⁴C]phenanthrene, only *P. fluorescens* DFC49, DFC50, and 5R, along with *P. putida* PpG7 produced significant amounts of ¹⁴CO₂. *P. fluorescens* 5RL, *P. fluorescens* 18H, *P. fluorescens* HK44 and *B. cepacia* JS150 did not mineralize [9-¹⁴C]phenanthrene. These results show that the ability to produce a clear zone on a spray plate is not correlated with mineralization. Since the radiolabeled phenanthrene is not uniformly labeled but at the 9 position (on the interior ring), the position of the label is crucial as it provides information on the extent of degradation. Significant degradation must occur in order to allow the release of the labeled carbon as CO₂. The importance of using radiolabeled compounds is obvious, as it allows the distinction between disappearance and metabolism to CO₂. This can be seen in the previously mentioned example of *P. chrysosporium* rapidly converting phenanthrene to its quinone intermediate but only 3% CO₂ is evolved (Sutherland et al., 1995).

The genotypes of *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 18H, *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 were determined. All strains hybridized to the *nahA* gene probe except for *P. fluorescens* 18H. However, only *P. putida* PpG7, *P. fluorescens* 5R, *P. fluorescens* DFC49, *P. fluorescens* DFC50, *P. fluorescens* 5RL, and *P. fluorescens* HK44 hybridized with *nahH* and *nahG*.

When one examines the phenotypic and hybridization data summarized in Table 10 an important link emerges. Strains which are capable of producing clear zones on phenanthrene are *nahA*⁺. Strains able to mineralize phenanthrene are *nahA*⁺, *nahG*⁺, and *nahH*⁺. All of the strains which were *nahA*⁺ but *nahH*⁻ and *nahG*⁻ and the mutant strains which exhibited the same phenotype as the *nahH*⁻ and *nahG*⁻ were not capable of mineralizing phenanthrene. These data provide a direct link between the lower *nah* operon and the mineralization of phenanthrene.

Table 10. Genotypic and phenotypic characteristics of strains used in this study.

Strain	Classification	Phenotype			Hybridization Results		
		Growth	Spray Plates	Phenanthrene Mineralization	<i>nahA</i>	<i>nahG</i>	<i>nahH</i>
5R	<i>P. fluorescens</i>	-	+	+	+	+	+
pPG7	<i>P. putida</i>	-	+	+	+	+	+
5RL	<i>P. fluorescens</i>	-	+	-	+	- <i>nahG</i> :Tn4431	+
HK44	<i>P. fluorescens</i>	-	+	-	+	- <i>nahG</i> :Tn4431	+
JS150	<i>P. cepacia</i>	-	+	-	+	-	-
DFC49	<i>P. fluorescens</i>	-	+	+	+	+	+
DFC50	<i>P. fluorescens</i>	-	+	+	+	+	+
18H	<i>P. fluorescens</i>	-	-	-	-	-	-

The fact that *P. fluorescens* 5RL, *P. fluorescens* HK44, and *B. cepacia* JS150 did not mineralize phenanthrene, but cleared spray plates, suggested the probability of an accumulated metabolite. These strains were subjected to biotransformation assays and analysis of the extracts demonstrated an accumulation of 1-hydroxy-2-naphthoic acid. It is important to note presence of the upper *nah* operon appears to be sufficient to produce clear zones in phenanthrene spray plates as well as the metabolite 1-hydroxy-2-naphthoic acid. However, it was demonstrated that the naphthalene dioxygenase alone was not sufficient to cause clearing. The *lac-nahABFCE* construct and *P. fluorescens* 5RL both were able to convert phenanthrene to 1-hydroxy-2-naphthoic acid. In contrast, Yang (1994) reported that an *E. coli* strain harboring pE317 (Ensley and Gibson, 1983), which contains the same *nahABFCE* gene cassette from NAH7 cloned into pBR322, was unable to transform phenanthrene to 1-hydroxy-2-naphthoic acid. While this observation directly conflicts with the data presented here, there is a possible explanation that can be found in the literature. The original clone was found to express low levels of naphthalene dioxygenase activity in both orientations (Ensley and Gibson, 1983). Yang's strains also have a very low activity as very little salicylate is detected in his naphthalene transformation assays with this strain. The *nahABFCE* construct used in this study is a *lac* fusion which results in higher expression and subsequent higher levels of enzymes. Therefore, Yang's result was most likely due to low expression levels.

Gene expression is an important aspect of metabolism: if the enzymes are not produced then the substrate is not metabolized. One of the unique features of the NAH system is that a metabolite (salicylate) and not the substrate (naphthalene) causes induction. This characteristic requires the cells to maintain a low level of expression of the *nah* operon such that significant enzyme levels are produced to convert naphthalene to salicylate. In this study three bioluminescent reporter strains each with a specific

attribute were used to ascertain whether or not inducing metabolites are generated during the metabolism of phenanthrene. The first reporter, *P. fluorescens* 5RL, has a blocked lower pathway such that when exposed to naphthalene, it is metabolized to salicylate with the resulting increase in bioluminescence. This strain is not capable of further metabolizing salicylate, so a high intracellular salicylate concentration which results in high light output. Since 5RL accumulates 1-hydroxy-2-naphthoic acid, this allows the determination if 1-hydroxy-2-naphthoic acid induces the NAH system.

P. putida RB1351 was constructed by cloning the *nah_P* in front of the promoterless *lux* gene cassette of pUCD615 (Burlage et al., 1990). This strain can further metabolize salicylate. Therefore it elucidates whether metabolites of the further metabolism of 1-hydroxy-2-naphthoic acid by the lower pathway can induce the NAH system. The primary advantage of strain RB1351 is the multicopy nature of the plasmid. Numerous DNA/NahR complexes could compete with salicylate hydroxylase for the inducing intermediate (salicylate).

Strain 135R75 was constructed with the same strategy as above except the *nah-lux* was introduced into the chromosome. Since there is less competition for inducer by the DNA/NahR due to only one copy, this strain provides a more accurate observation of the metabolism by the cell. The previous two strains were either not able to degrade the inducer (5RL) or less efficient (RB1351). This chromosomally encoded *nah-lux* strain 135R75 should allow us to determine if the inducing substrate is metabolized by the basal level of enzymes at a rate to inhibit induction. All three strains were uninduced when exposed to phenanthrene, indicating either that a slow rate of metabolism by the basal level of enzymes or an inducing metabolite is not produced.

Although not the subject of this research, it is of interest to note that the naphthalene control data validates the theory behind the use of the three different strains. This is seen in a comparison of the light output of the three strains

(5RL>RB1351>135R75) and duration of response as the light level of 135R75 drops at the end of the assay.

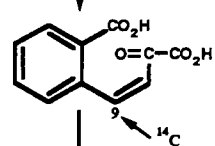
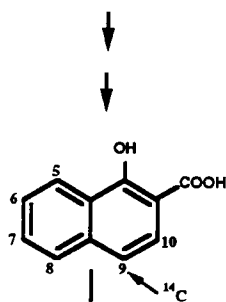
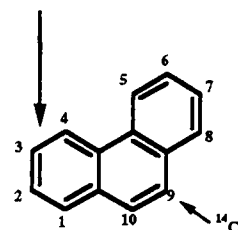
To ascertain whether or not an inducing intermediate is produced, biotransformation assays using strain 5R were conducted. The results of the GCMS showed a significant peak that was determined to be phthallic acid. This result is questionable as phthalates are common plasticizers and this could just be an artifact (these compounds are common GCMS contaminants) (Ende, 1982). However, phthallic acid was not detected in the controls. These data were substantiated by radiolabeled transformations demonstrating this was a dead end product.

Salicylate hydroxylase transformations of 1-hydroxy-2-naphthoic acid showed that this enzyme did not decarboxylate 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene. The biotransformation assays did not yield any detectable 1,2-dihydroxynaphthalene and generated an unknown orange polar metabolite. Salicylate hydroxylase was also shown to decarboxylate a variety of substituted salicylates by the *meta* cleavage assay. Although proposed by Evans (1964), he never isolated 1,2-dihydroxynaphthalene either. These data further suggest that phthalate is a metabolic end product of phenanthrene metabolism and is reinforced by the results below.

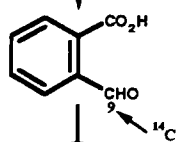
Phenanthrene mineralization experiments were performed with *P. fluorescens* 5RLTnGH3. Since the disrupted *nahG* was complemented in *trans* it was expected that wild-type mineralization capacity would be restored. However, 5RLTnGH3 was only capable of mineralizing 16% of that of the wild type strain 5R. These data also suggest that the salicylate carboxylase does not decarboxylate 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene which simply re-enters the *nah* upper pathway and is further metabolized. These data also suggest that other genes in the lower pathway are probably involved in the further metabolism of 1-hydroxy-2-naphthoic acid.

Phthallate as an end point is also indicated by the mineralization experiments. The first experiment used three different concentrations of phenanthrene to ascertain the extent of mineralization. Mineralization with all three concentrations approached 50%. The phthallate explanation is tied into the significance of the location of the labeled carbon in the phenanthrene molecule. Since phenanthrene is a symmetrical molecule, the initial dioxygenase attack is arbitrary. Therefore, it will hydroxylate either the number 4 and 5 carbons or the number 2 and 3 carbons. Since the labeled carbon is at the number 9 position it can be located at either of two different positions relative to the functional groups on 1-hydroxy-2-naphthoic acid depending on the initial dioxygenase attack (Fig. 35). Further metabolism of this compound, assuming it goes through *o*-phthalate and can not be further metabolized, explains the mineralization data when the position of the label is considered (Fig. 35). Only half of the label can be converted to $^{14}\text{CO}_2$.

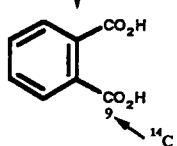
Initial dioxygenase attack



o-Carboxybenzylpyruvic acid



Carboxybenzaldehyde



o-Phthallic acid

Initial dioxygenase attack

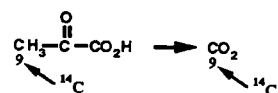
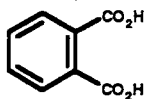
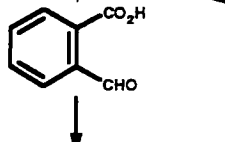
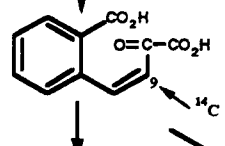
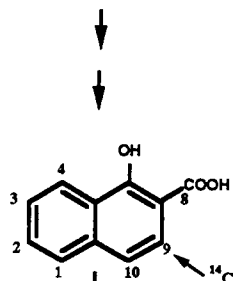
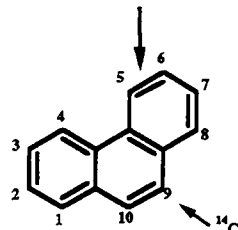


Figure 35. Pathway of 1-hydroxy-2-naphthoic acid degradation through phthallic acid with the different label position.

Chapter VIII

Summary

The primary purpose of this study was to test the following hypothesis:

Phenanthrene is metabolized by the NAH system by the conversion of phenanthrene to 1-hydroxy-2-naphthoic acid by the enzymes of the *nah* operon, subsequent conversion of 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene by salicylate hydroxylase, the further degradation of this compound by the upper pathway to salicylate (the inducing intermediate), and its subsequent conversion to acetylaldehyde and pyruvate by the enzymes of the *sal* operon.

The results of this investigation indicates that phenanthrene is degraded to 1-hydroxy-2-naphthoic acid by the enzymes coded by the *nah* operon. This result validates the first part of the hypothesis tested (Fig. 36). However, the latter part of the hypothesis is rejected (Fig. 36). It appears that a simultaneous hydroxylation/decarboxylation of 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene does not occur. Apparently 1-hydroxy-2-naphthoic acid is cleaved by the salicylate hydroxylase forming a polar metabolite compound X (Fig. 37). Compound X is subsequently converted to 2'-carboxybenzylpyruvate by the tautomerse (*nahJ*) and metabolized to phthalate and pyruvate by the aldolase-hydrotase (*nahE*). These results as well as the lack of induction reject the latter part of the hypothesis.

Therefore, these results suggest that phenanthrene is metabolized by the pathway in Figure 37 as opposed to the one hypothesized in Figure 36.

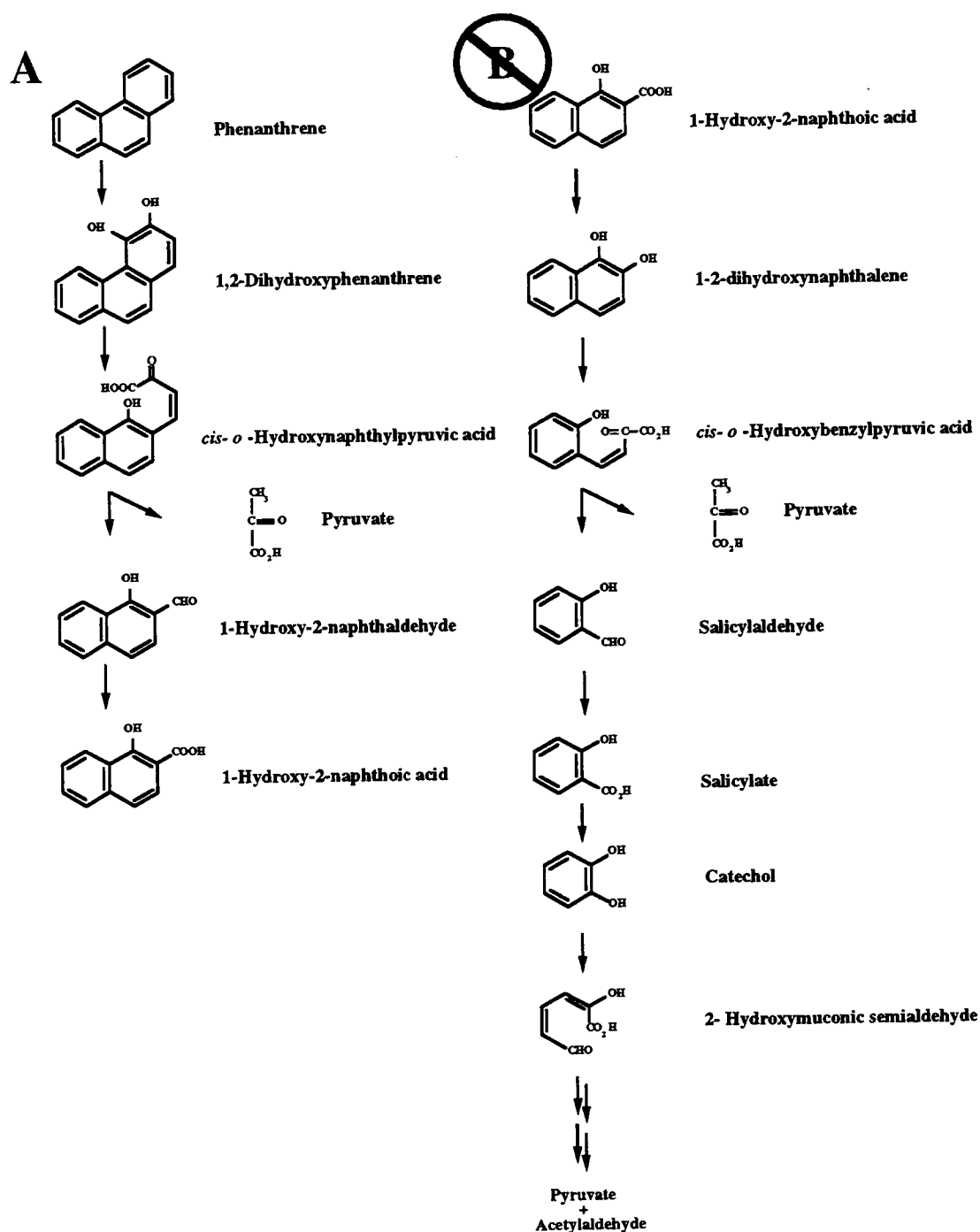


Figure 37: Hypothesized degradative pathway for the metabolism of phenanthrene to TCA cycle intermediates by the NAH system. A). Metabolism of phenanthrene to 1-hydroxy-2-naphthoic acid by the enzymes of the *nah* operon. B). Further metabolism of 1-hydroxy-2-naphthoic acid to 1,2-dihydroxynaphthalene by salicylate hydroxylase, followed by metabolism through the *nah* and *sal* pathways does not occur.

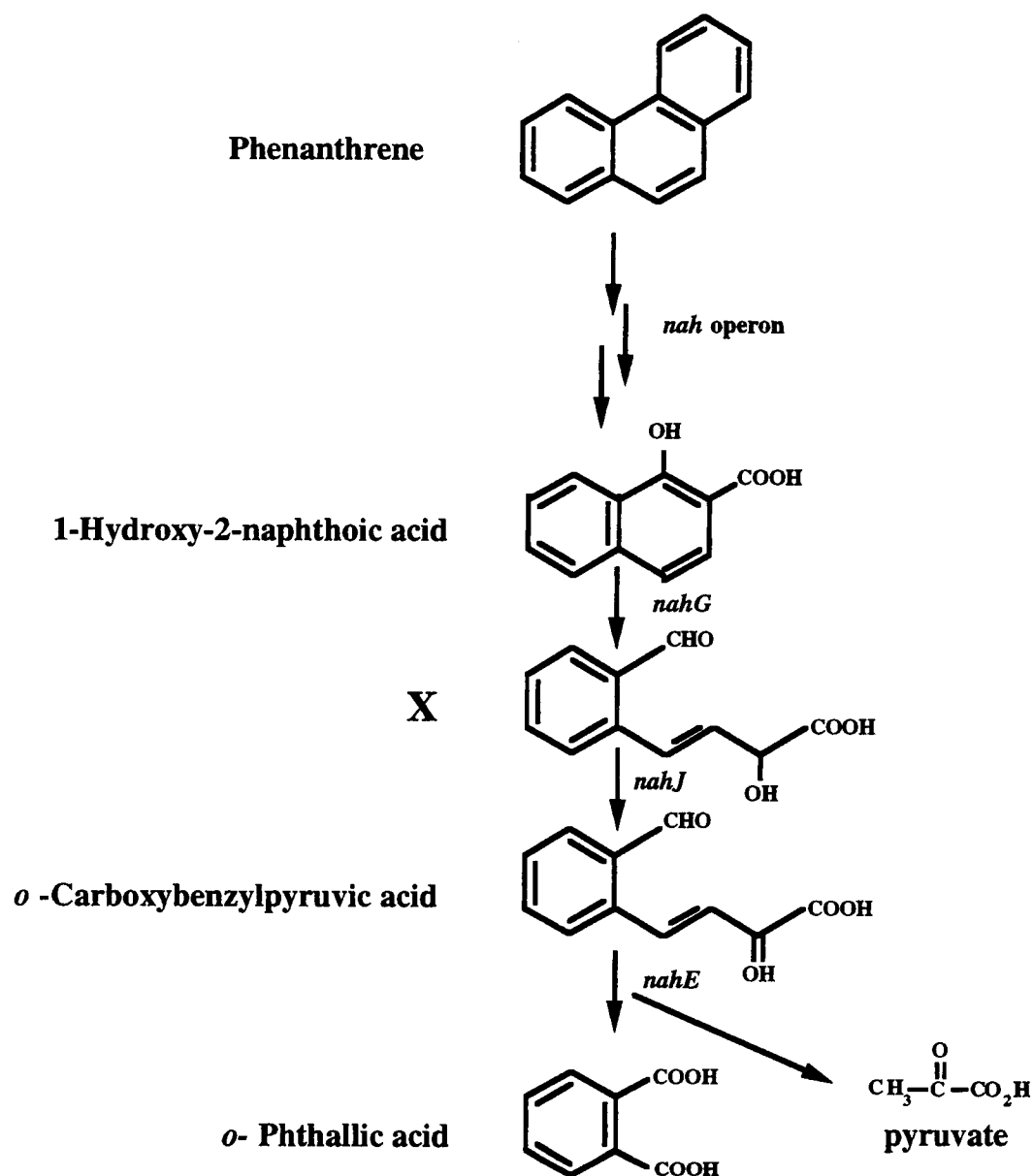


Figure 38: Theoretical Pathway for Phenanthrene Degradation by the NAH System. Phenanthrene is metabolized by the by the enzymes encoded by the *nah* operon to 1-hydroxy-2-naphthoic acid, which is subsequently converted to compound X (Fig 38) by the salicylate hydroxylase (*nahG*), tautomerized (*nahJ*) to 2'carboxybenzylpyruvate, which is cleaved by the aldolase-hydrotase (*nahE*) to phthalate (dead end) and pyruvate.

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Vita

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