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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

ANN M. MAUER

ENTITLED THE ISOLATION AND HYBRIDIZATION OF DNA FROM

PSEUDOMONAS AERUGINOSA PaG158

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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Stephen C. Selye
Instructor in Charge

APPROVED: *Concepcion*

HEAD OF DEPARTMENT OF Biochemistry

THE ISOLATION AND HYBRIDIZATION OF DNA

FROM *PSEUDOMONAS AERUGINOSA* PaG158

BY

ANN M. MAUER

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INTRODUCTION

Within the biosphere there are numerous oxygenation processes mediated by a group of enzymes referred to as oxygenases. One of the largest classes of enzymes which function as monooxygenases are categorized as the cytochrome P-450s. As monooxygenases, these enzymes cleave molecular oxygen by a two-electron reduction which produces one water molecule and one oxygenated substrate molecule. The cytochrome P-450 enzymes are identified by their unique spectral absorption at 450nm which is observed when the system is reduced and saturated with carbon monoxide. These P-450 heme proteins catalyze a broad spectrum of biological processes in prokaryotes and eukaryotes where they display an enormous diversity in their substrate affinity and specificity. For example, in mammalian systems cytochrome P-450s are involved in steroid hormone biosynthesis, carcinogen activation, and numerous catabolic pathways. In microorganisms, cytochrome P-450s are essential for the degradation of organic compounds from the environment including aliphatic, aromatic, and alicyclic hydrocarbons.

A *Pseudomonas putida* bacterial strain (ATCC 17453) which can use camphor as its sole carbon source was isolated in 1950 (1). Further work by Hildegaard and Gunsalus revealed a portion of the reaction pathway and the enzymes involved in the degradation of camphor by this soil pseudomonad (2). The first step of the camphor catabolic pathway, the stereospecific hydroxylation of camphor to 5-exo-hydroxycamphor, is catalyzed by the cytochrome P-450_{cam} (Figure 1) (2,3,4). The cytochrome P-450_{cam} system contains three protein

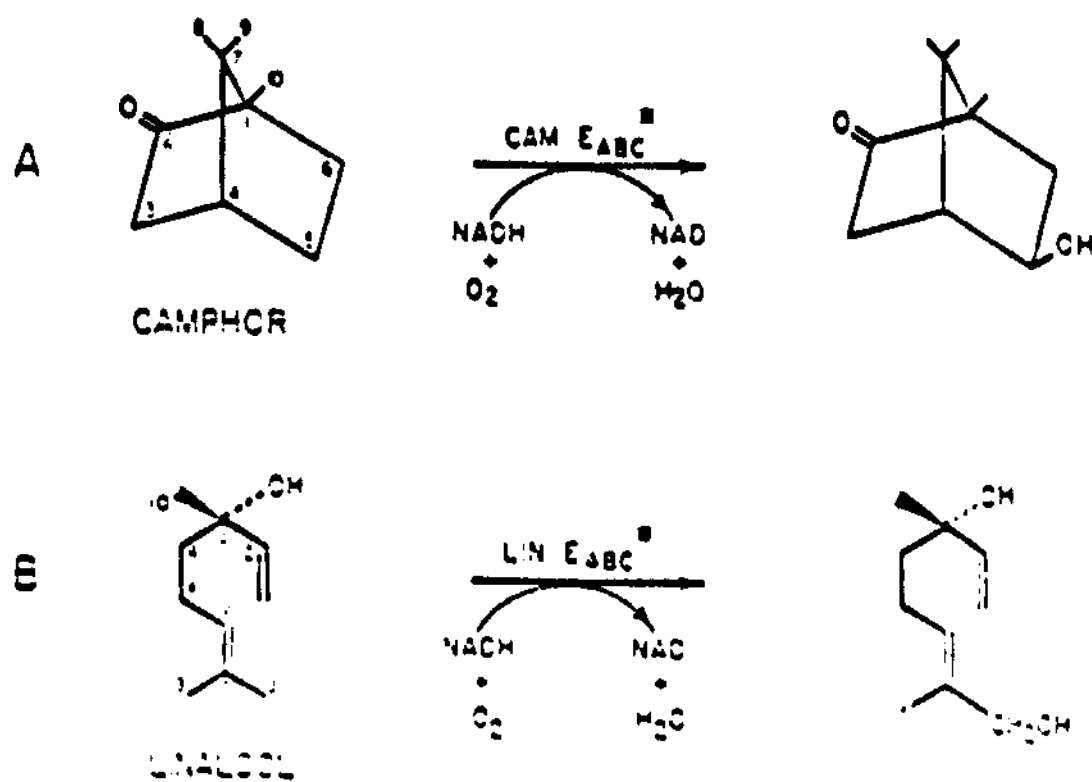


FIGURE 1. The hydroxylation of camphor to 5-*exo*-hydroxycamphor catalyzed by P-450_{cam} (A). The hydroxylation of linalool catalyzed by P-450_{lin} (B).

components: an NADH specific FAD-containing putidaredoxin reductase, an iron-sulfur protein putidaredoxin, and P-450_{cam} (5). These proteins are encoded by the *camA* gene, the *camB* gene, and the *camC* gene respectively.

Genetic studies by Rheinwald (6) and Chakrabarty (7) demonstrated that the genes encoding for cytochrome P-450_{cam} were present on a 230 kilobase plasmid, the CAM plasmid. Later Koga cloned the gene for cytochrome P-450_{cam} by mutant *trans* complementation in *P. putida* (8). To clone this gene, the CAM plasmid DNA was isolated then digested with the restriction enzyme *Pst*I. A ligation of this digested DNA into a *Pst*I site of the broad-host-range vector pKT240 resulted in the plasmid pKG201 (Figure 2). Characterization of this plasmid revealed that it carries a 2.2-kb insert of the CAM plasmid including the *camC* gene (8). The insert from pKG201 was ligated into the *Escherichia coli* vector pBR322 to yield pKG300. When this plasmid was transformed into *E. coli*, P-450_{cam} was produced at high concentrations indicating the initiation of transcription by vector promoters. The cytochrome P-450_{cam} produced by *E. coli* under the *lac* promoter was compared with the *P. putida* CAM plasmid protein in respect to its molecular weight, catalytic activity, optical spectrum and N-terminal amino acid sequence. The two proteins were indistinguishable in the above mentioned characteristics (10). The genes encoding for putidaredoxin reductase (*camA*) and putidaredoxin

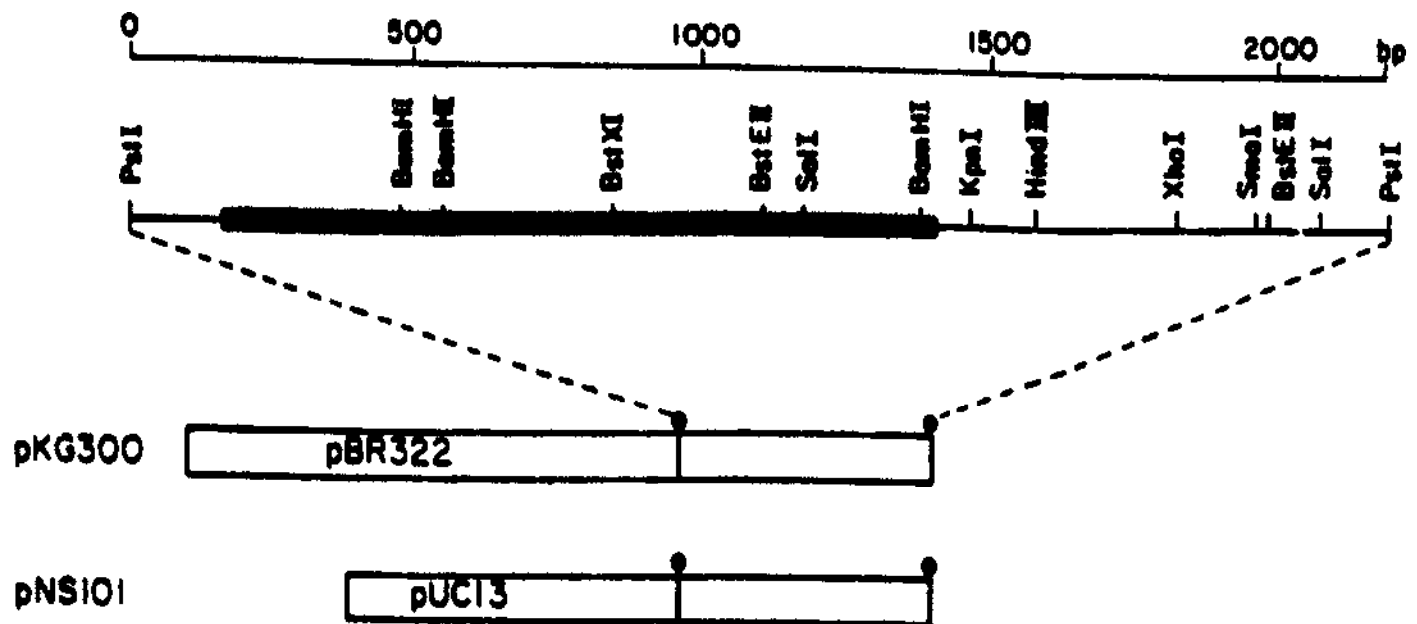


FIGURE 2. RESTRICTION MAP OF pKG300 and pNS101. The top panel is the restriction map of a 2.2-kbp fragment of the CAM plasmid where the heavy region represents the P-450_{cam} gene. The bottom two panels represent the cloned plasmids.

(camB) have also been cloned in the Koga laboratory (11,12). In addition to the genetic studies of the P-450_{cam} system, many of which have not been cited above, there has been much research on the P-450_{cam} system thus making it one of the best characterized P-450 systems.

A *Pseudomonas putida* strain which metabolizes linalool was isolated by Madhyastha (11). In this strain, a cytochrome P-450 monooxygenase is responsible for the 8-methyl and 10-methyl hydroxylation of linalool in the initial step of the catabolic pathway (12). A variant of this strain, *P. Putida* PpG777, also possesses a cytochrome P-450 monooxygenase (P-450_{lin}), but differs from the parental strain because it catalyzes only the 8-methyl hydroxylation of linalool (Figure 1) (12). The P-450_{lin} system in PpG777 includes a NADH-specific flavoprotein reductase and a 2Fe2S ferredoxin (12). All three components of the P-450_{lin} system have been isolated, purified, and studied. This P-450 system has exhibited similarities to the P-450_{cam} system in terms of its optical, chemical, and physical properties.

Several *Pseudomonas* strains which metabolize p-cymene and a variety of related structures have also been isolated. One of these strains is the PL strain also referred to as the PL-W or JT101 strain (13-16). The overall p-cymene metabolic pathway for this strain involves the oxidation of p-cymene to a carbonyl followed by a 2,3

ring hydroxylation and a meta fission (Figure 3). The initial steps of p-cymene metabolism in the PL strain include the hydroxylation of the p-cymene methyl group to p-cumic alcohol followed by an oxidation to p-cumate (14,15). It is expected that this metabolic pathway involves a cytochrome P-450 system, however this has not been documented in this strain.

Another bacterial strain which degrades p-cymene is *Pseudomonas aeruginosa* PaG158. This strain was also derived from the Madhyastha *P. putida* linalool strain in the Gunsalus laboratory. It is known that the initial steps in the degradation of p-cymene by PaG158 are identical to those of the PL strain, however this has not been confirmed for the later steps of the pathway (17,18). The PaG158 strain possesses a cytochrome P-450 monooxygenase (P-450_{cym}) which has been isolated (17). Cytochrome P-450_{cym} catalyzes the initial hydroxylation of p-cymene to p-cumic alcohol (17,18). The other protein components of the CYM P-450 system have not been isolated.

Several studies have been done to compare the CYM monooxygenase to the CAM and LIN monooxygenases (18). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins demonstrated that the sizes of the CAM, LIN, and CYM monooxygenases are very similar. One study showed that the P-450_{cam} and P-450_{lin} proteins elute from a DE-52 anion exchange column at the same salt concentrations while P-450_{cym} elutes at a lower salt concentration. Another study was done

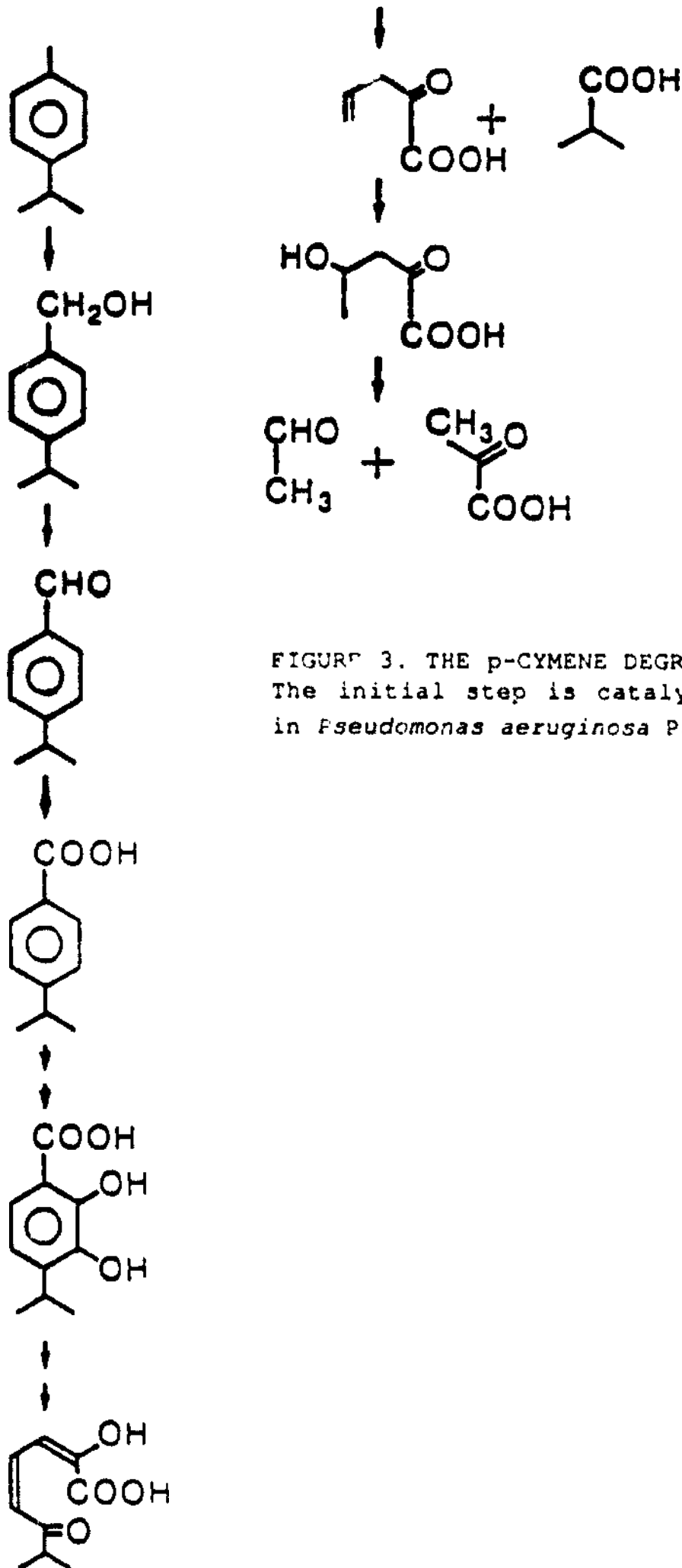


FIGURE 3. THE p-CYMENE DEGRADATIVE PATHWAY. The initial step is catalyzed by P-450_{cym} in *Pseudomonas aeruginosa* PaG158.

involving the heterologous reconstitution of hydroxylase activities. The P-450_{cym} was combined with the redox components of the CAM and LIN P-450 systems and the hydroxylase activity was measured. The CAM and LIN P-450s were also reconstituted with their homologous redox partners. This study (Table I) showed that the enzyme activities were much higher for the homologously reconstituted systems than the heterologous systems. This variation occurred due to the differences in the proteins. Based on the different hydroxylase activities for the CYM and LIN heterologous systems, Suhara concluded that the P-450_{cym} protein resembles the P-450_{cam} more than the P-450_{lin} (18).

In addition to the P-450 systems mentioned above, there have been numerous P-450 systems discovered. Many of these proteins have been isolated and characterized. Among these characterized P-450 proteins, the structure is retained in terms of size, physical and optical properties. Within all of the proteins there are two domains of amino acid sequences which are conserved (19). The amino acid regions which are not conserved include the domains of the substrate pockets which must be unique to achieve substrate specificity. Based on the demonstrated homology of P-450 systems, an attempt was made to clone the P-450_{cym} gene using the P-450_{cam} gene as a probe. It is expected that the P-450_{cym} and the P-450_{cam} gene have similar DNA sequences with many conserved regions because they are found in the same genus, *Pseudomonas*.

TABLE IP-450 HYDROXYLASE CROSS REACTIVITIES^a

E_C (P-450)	E_B (redoxin)	E (reductase)	Substrate	TON^b
CAM	CAM	CAM	Camphor	36
CAM	LIN	LIN	Camphor	0
LIN	LIN	LIN	Linalool	36
LIN	CAM	CAM	Linalool	12
CYM	CAM	CAM	p-Cymene	6.6
CYM	LIN	LIN	p-Cymene	2.4

^aP-450_{cam/lin/cym} = E_{A/B/C}

^bTON, Turn over number (umol/sec/umol enzyme).

MATERIALS AND METHODS

ISOLATION OF TOTAL DNA

The *Pseudomonas aeruginosa* bacterial strain PaG158 was obtained from I.C. Gunsalus at the University of Illinois and streaked on PAS plates (Appendix A) supplemented daily with 0.1g of p-cymene (Aldrich Chemical). From this plate, an isolated colony was used to inoculate a 50ml culture of PAS broth supplemented with 1.0g of p-cymene. The cells were grown for 48 hours at 30°C, then the entire culture was used to inoculate one liter of Luria-Bertani broth (20). After 16 hours the cells were harvested in late log phase by centrifugation for 10 minutes at 5000g and 4°C. The total DNA was isolated from these cells using a variation of the Marmur method (21). About 5g of cells were washed with 40ml of 0.9% NaCl and resuspended in 40ml of 0.9% NaCl, 1mM EDTA. Cell lysis was achieved by adding 10% sodium dodecyl sulfate to a final concentration of 2% and gently mixing the solution in a 60°C water bath. The suspension was cooled and 5M sodium perchlorate was added to a final concentration of 1M. An equal volume of a phenol-chloroform mixture (1:1) was mixed with the suspension to give an emulsion which was centrifuged at 8000g and 4°C for 10 minutes. The aqueous phase was collected in a beaker and the DNA was precipitated by overlaying 2 volumes of 100% ethanol and spooling with a glass rod. The precipitated DNA was dissolved in TE buffer (Appendix A) and treated with DNAase free RNAase at a concentration of 50mg/ml for 1 hour at 37°C. Protein was removed from the DNA solution using chloroform-isoamyl (24:1) extractions until no protein could be seen at the interface between the

two phases. After the salt concentration of the DNA solution was adjusted to 0.2M NaCl, the DNA was precipitated with 100% ethanol by spooling and washed with 70% ethanol. The DNA was dissolved in TE buffer and stored at 4°C.

PREPARATION OF THE HYBRIDIZATION PROBE

The hybridization probe was prepared from pNS101 (Figure 2) , a pUC13 derivative containing the CAM plasmid fragment from (ATCC 17453). This vector was constructed by H. Nikkila by subcloning the 2.2-kbp CAM plasmid fragment from pKG300 into the PstI site of pUC13 vector (9). A restriction digest of 100ug of pNS101 with PstI (Bethesda Research Laboratories) for 4 hours at 37°C generated the CAM plasmid fragment and the pUC13 vector. The resulting fragments were separated by electrophoresis on a 1% agarose gel (14.5x14.5cm) with ethidium bromide (0.5ug/ml). The gel was run at 20mA in a TBE buffer system (Appendix A) until the bands separated as evidenced by ethidium bromide staining. The CAM plasmid was eluted from the gel onto a DEAE membrane (Schleicher and Schuell) by placing the membrane directly in front of the corresponding DNA band and running the gel at 150V until binding to the membrane was complete. The DNA was recovered from the membrane by placing the membrane and 150-250ul of high salt buffer (Appendix A) in an eppendorf tube and incubating the tube at 60°C for 1 hour. The resulting DNA solution was desalted and reduced to a volume of 100ul using a Centricon concentrator (Amicon).

The isolated CAM DNA fragment was radiolabelled with alpha-³²P (Amersham) using a nick translation kit (Amersham). The nick translation was performed using a variation of the manufacturer's protocol. The following reaction mixture was used: 10ul of the kit nucleotide buffer (Appendix A), 1ug of DNA, 80uCi of alpha-³²P dCTP, 5ul of the kit enzyme solution (DNAase and DNA polymerase), and enough sterile water to bring the total volume to 50ul. The reaction was carried out at 15°C for 1.5 hours then a ten-fold excess of nick translation stop buffer (Appendix A) was added. The DNA was precipitated with 2 volumes of cold 100% ethanol overnight at -20°C. The resulting pellet was dried and dissolved in 100ul of TE buffer. Before the hybridization, the DNA was denatured by placing it in a 100°C bath for 10 minutes.

GENOMIC SOUTHERN BLOT

RESTRICTION DIGEST OF TOTAL DNA

The DNA used in the genomic Southern analysis were prepared by digesting the *Pseudomonas aeruginosa* chromosomal DNA with six cutter restriction enzymes (HindIII, PstI, SalI, EcoRI, and BamHI) according to the manufacturer's directions (Bethesda Research Laboratories). The following reaction mixture was used: 10ug of DNA, 100 units of restriction enzyme, 50ul of 10X reaction buffer, and sufficient sterile water to bring the reaction volume to 500ul. The DNA was digested at 37°C for 4 hours then the salt concentration was adjusted to 0.3M NaOAc with 3M NaOAc. Two volumes of 100% ethanol were added and the DNA was

precipitated using dry ice for 15 minutes. After centrifugation for 10 minutes and removal of the supernatant, the pellet was washed with 70% cold ethanol and recentrifuged. The resulting DNA pellet was dried and dissolved in TE buffer.

ELECTROPHORESIS AND TRANSFER OF DNA TO MEMBRANE

Eight micrograms of the DNA from the restriction digest plus an aliquot of bromophenol blue loading dye were mixed and loaded into a 1 cm long sample well of a 1% agarose gel (14.5x14.5cm). Two molecular weight markers, a HindIII digest of lambda DNA and a BstEII digest of lambda DNA, were also loaded on the gel. Next the gel was stained in ethidium bromide solution and photographed under UV light. The DNA was denatured by soaking the gel in 0.25N HCl for one 15 minute interval and in 0.2N NaOH, 0.6M NaCl for two 15 minute intervals. Next the gel was neutralized in 25mM K₂HPO₄/KH₂PO₄ buffer, pH 6.5 for 1 hour with several changes of solution. The GeneScreen™ hybridization membrane (New England Nuclear, Boston, MA) cut to the size of the gel was also soaked in 25mM K₂PO₄/KH₂PO₄ buffer for 20 minutes. The DNA fragments were transferred from the gel to the membrane using an electroblot procedure (22,23). The transblotter apparatus (Bio-Rad Laboratories) was assembled according to the manufacturer's instructions. Following the electrophoretic transfer, the DNA fragments were UV cross-linked to the wet membrane for 2 minutes (24).

HYBRIDIZATION OF DNA

Two separate DNA hybridization methods were utilized. Method I is an adaptation of the protocol provided by the GeneScreen™ Membrane manufacturer, New England Nuclear. The membrane was sealed in a plastic bag then a prehybridization solution with dextran sulfate (Appendix A) was added to the bag using a syringe and needle. The bag was incubated with constant agitation at a prehybridization temperature specific for that hybridization scheme. After 24 hours, the hybridization solution (Appendix A) and the denatured radioactive probe were added into the prehybridization bag with a syringe and needle. The bag was resealed and incubated with constant agitation at the prehybridization temperature. After 24 hours, a corner of the bag was cut off and the bag was drained of the hybridization solution. The bag was flushed with 6X SSC (Appendix A), then the membrane was placed in a shallow pan. The membrane was washed at room temperature with 400ml of 6X SSC for 2 twenty minute intervals then rinsed with cold H₂O. The wet membrane was placed on two pieces of filter paper; wrapped in saran wrap; and placed in an X-ray film holder with a sheet of X-ray film. The film was exposed for 24 hours then developed.

If non-specific signals were observed on the X-ray, further washes of the membrane were done to obtain a more specific signal. These washes were done by placing the membrane in 400ml of 0.1% SDS in 6X SSC for 15-20 minutes under constant agitation at 5°C greater than the temperature of the previous wash. Following the wash, the membrane was

rinsed with water and exposed as previously. Washes were continued until a specific signal could be observed on the membrane.

Method II was also a variation of the procedure suggested by New England Nuclear. The membrane was sealed in a plastic bag and the prehybridization buffer with formamide (Appendix A) was added to the bag. The prehybridization bag was incubated for 24 hours at 30°C under constant agitation. The prehybridization buffer was drained from the bag and the hybridization buffer including the denatured radioactive probe was added to the bag. The bag was resealed and incubated at 30°C under constant agitation for 24 hours. The membrane was removed from the bag and washed with 400ml of 0.5% SDS in 1X SSC at room temperature for two 20 minute intervals. The membrane was exposed to X-ray film as above to obtain an autoradiographic image. If necessary, further washes were carried out at 5°C above the previous wash temperature in 0.5% SDS in 1X SSC.

RESULTS AND DISCUSSION

As stated in the introduction, the purpose of this research project was to clone the cytochrome P-450_{cym} gene from of *P. aeruginosa* PaG158. Fundamentally the cloning of a gene involves inserting a specific gene from one organism into another organism. The desired gene fragment is inserted into a vector--either plasmid or phage DNA--which is introduced into a bacterial cell where it is copied. Gene cloning provides a way to obtain large amounts of the gene and the gene product. These large quantities are necessary to study the gene's structure, function, and regulation. Therefore through the cloning of the P-450_{cym} gene it will be possible to sequence the P-450_{cym} gene and express the protein in *E. coli*. Genetic control of the CYM gene would aid in studying its enzymology and provide a bacterial comparision to P-450_{cam}.

There are two basic techniques which may be utilized to clone a particular gene when genomic DNA is used as the starting material. One technique involves a preliminary purification of the DNA segments before insertion into the vector thereby reducing the number of clones which have to be screened. This method is advantageous when a specific gene product is desired, and thus this strategy was utilized to clone the P-450_{cym} gene. The second cloning technique, shotgun cloning, involves making a total gene library. Using this method, the entire genome is cloned with all DNA sequences present in some vector. This cloning strategy allows for greater flexibility, but it requires screening of a large number of clones.

The first step of a cloning strategy is the isolation of the DNA from the donor organism. The procedure used for DNA isolation depends on whether the DNA sought is chromosomal or extrachromosomal. For this project, total DNA was isolated according to the procedure outlined in the Materials and Methods section.

There were several technical problems in the isolation of the total DNA from PaG158. The bacteria cell walls did not lyse sufficiently under several conditions. It was determined that the degree of lysis was critically dependent of the amount of cells in the suspension. A dilute cell suspension lyses more efficiently than a dense suspension. It was also difficult to lyse cells grown in PAS media enriched with p-cymene; thus all DNA had to be isolated from cells grown in Luria broth. This difficulty may be explained by the possibility that the cells grown in PAS media reached stationary phase at a faster rate and consequently became more difficult to lyse. Another problem which occurred during the DNA isolation was the mechanical shearing of DNA. Long molecules of DNA are easily sheared randomly along the genome because of their large length-to-width ratio. This shearing of DNA is undesirable because it creates fragments which are too short to be spooled with a glass rod. Thus the best method to isolate total DNA requires a dilute suspension and great care be taken not to mechanically shear the DNA.

Following the DNA isolation, the concentration of DNA was measured using ultraviolet spectroscopy. This calculation was based on the

relationship that 1 A₂₆₀ unit equals 50ug of DNA per ml. In addition, an approximate measure of the DNA purity was determined by calculating the A₂₆₀/A₂₈₀ ratio. For pure *E. coli*, the A₂₆₀/A₂₈₀ ratio is 1.95. The DNA isolated from *P. aeruginosa* PaG158 had an average A₂₆₀/A₂₈₀ ratio of 2.

The next step toward isolating the gene from the entire genome is to break the genome into fragments of smaller size. These fragments can be yielded from the total DNA by mechanical shearing or by enzymatic cleavage. The random cleavage of DNA by shearing produces fragments which are undesirable for subsequent cloning procedures. The best technique for generating fragments is to digest the genomic DNA with restriction endonucleases. These enzymes catalyze specific, nonrandom breaks in the DNA by cleaving short specific DNA sequences of 4 to 6 base pairs. The frequency of the DNA cleavage is dependent on the length of the sequence recognized by the enzyme. The probability that a restriction cleavage site will occur is estimated using the following formula:

$$\text{Site frequency} = (1/4)^N$$

where N = the length of the restriction site sequence. The probability of a particular hexameric sequence is 1/4000. The distribution of specific restriction sequence site on the genome is random and does not occur every 4000 base pairs. When the genome is digested, a continuum of fragments is generated. Another advantage of using restriction

enzymes to cleave the genomic DNA is that each fragment generated ends in the same known sequence containing a sticky end and therefore can be easily ligated into a vector.

The ideal restriction digest generates one fragment containing the entire gene. This experiment was done with several different restriction enzymes having different recognition sequences in order to increase the chances of creating a fragment containing the entire gene. This experiment utilized the following restriction endonucleases: BamHI, Eco RI, HindIII, PstI, and SalI (see Appendix B for specific recognition sequences). These sequences were used because they recognize hexameric DNA sequences and generate longer fragments than enzymes which recognize fewer base pairs.

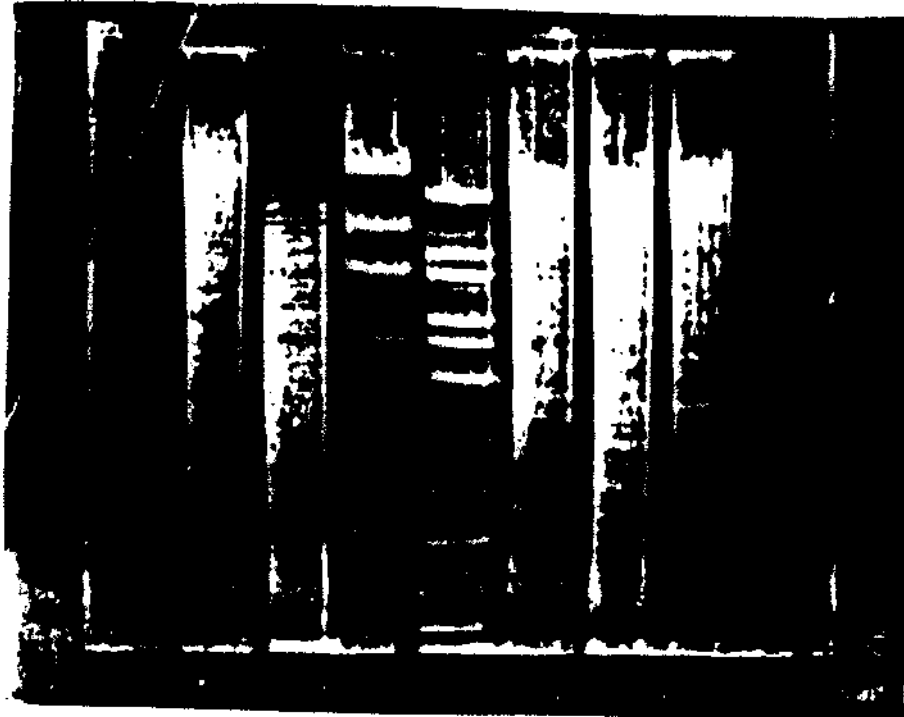
Following the restriction digests, the DNA fragments were electrophoresed on an agarose gel. This method was used to separate and identify DNA fragments based on their size; the shortest fragments migrate the farthest. The photograph in Figure 4 shows an electrophoretic gel used to separate the fragments produced by a restriction digest. Each vertical lane corresponds to a particular restriction digest or molecular weight marker. The molecular weight markers (lanes 5,6) are DNA fragments of known size which are used to estimate the size of other fragments based on a comparison of their positions. By looking closely at the lanes corresponding to the restriction digests (lanes 2,3,7,8), it is possible to see that each lane contains many separate fragments of DNA which results in a ladder-like banding pattern on the gel. It may also be noticed that at

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1. The first part of the document is a list of names of the members of the committee. The names are listed in alphabetical order. The names are: [illegible]



the top of lanes 2, 3, 7 and 8 the bands are very intense due to a higher concentration of DNA in these areas. The variations in intensity along these lanes are due to the incomplete cutting by the restriction enzymes. Lane 2 exhibits a uniform distribution of fragments throughout the gel and is the ideal pattern for use in later hybridization procedures. This pattern is observed only when the restriction digests are complete.

After the DNA fragments have been separated and isolated into distinct bands, the next step in the cloning procedure involves indentifying which DNA fragment contains the desired gene. In bacteria, the genome is about 5000-kbp in size. The P-450_{cym} gene is probably about 1.5-kbp and therefore represents only about 0.3% of the total DNA (8,18). One way to identify this small portion of the genome is to utilize a specific probe which reacts with the gene. A good probe is a fragment of DNA which is complementary to the DNA sequence of interest. The probe is radioactively labelled so that the amount of duplex formation can be measured using autoradiographic techniques.

The probe used in this study was a 2.2-kbp fragment of the CAM plasmid. This probe was chosen because it was believed that its DNA sequence displays homology to the DNA sequence of P-450_{cym}. Previous studies of the various isolated and purified cytochrome P-450s have demonstrated that certain regions of the cytochrome P-450 amino acid sequence are conserved among organisms. The probe was radioactively labelled using a nick translation procedure.

The DNA fragments in an electrophoresis gel cannot be conveniently identified using a hybridization procedure, so the fragments are immobilized on a filter or membrane by a process called Southern blotting (23). This solid support is placed in a hybridization solution which contains the radioactive probe. Only the DNA fragments which contain the desired gene hybridize with the probe. The positions of these fragments are identified by an image (a dark band) on the autoradiograph. If the gene is contained in two or more fragments, more than one fragment will be visualized on the autoradiograph.

The rate and extent of the hybridization reaction is dependent upon the hybridization conditions. Some parameters which may be varied include: the concentrations of the reactants; the ionic strength of the hybridization buffer; the temperature of hybridization; the concentration of organic solvents in the hybridization buffer; and the degree of homology between the two hybridizing sequences. The temperature at which one half of the DNA of the probe is double stranded with the DNA sequence is defined as the melting temperature, T_m . The maximum rate of DNA hybridization occurs at 25°C below the melting temperature, thus most hybridization reactions are carried out at $T_m-25^\circ\text{C}$. The hybridization temperature may be calculated using the following equation:

$$T_m = 16.6(\log[\text{Na}^+]) + 0.41(\text{G+C}\%) + 81.5$$

The first term accounts for the hybridization temperature dependence on the salt concentration (25). This dependence is due to the repulsive

forces between negatively charged phosphate groups on the DNA. A stabilization of the DNA structure is observed when salt is added to the hybridization reaction. Generally the hybridization is faster at higher salt concentrations, but concentrations greater than 6X SSC do not significantly raise the rate.

The second term of the equation accounts for the T_m dependence on the average base composition of the DNA. A GC base pair results in greater stability of the DNA duplex because it contains three H bonds compared to the two H bonds exhibited by an AT base pair. If the GC content of the probe is unknown, calculations are done assuming 50% base pairing.

Several organic solvents such as formamide, urea, and dimethyl sulfoxide depress the thermal stability of DNA duplexes and thereby decrease the rate of hybridization. By utilizing this property, hybridizations can be carried out at lower temperatures so there is less thermal scission of the strand (27). Formamide lowers the T_m 0.7°C for each percent of formamide used in the hybridization (29). The following equation summarizes this:

$$T_m(\text{formamide}) = T_m(\text{H}_2\text{O}) - (0.7^\circ\text{C})(\% \text{ formamide})$$

The equations above define T_m for homologous DNA duplexes where the DNA probe and DNA sequences are exactly matched. Most experimental DNA hybridizations involve heterologous DNA duplexes. In general, the rate

of hybridization for heterologous DNA sequences is about two times slower than for homologous sequences (29). This difference in hybridization rates occurs because there are fewer hydrogen bonds between the mispaired strands to stabilize the formation of the duplex. To account for this, the optimal T_m for heterologous duplexes is lowered 1° for each 1% increase in the number of mismatched pairs (29).

When hybridization occurs, it is possible that the probe will anneal with DNA sequences other than the specific gene. This non-specific binding produces a background on the autoradiograph and may make it difficult to detect the desired signal. Several techniques may be used to increase the stringency of the hybridizations and consequently decrease the non-specific binding. A higher degree of stringency is achieved at higher temperatures. Therefore after washing the membrane at a temperature greater than the hybridization temperature, the level of background may be reduced. Another technique to increase stringency utilizes surfactants such as bovine serum albumin (BSA), SDS, polymers (Ficoll, polyvinylpyrrolidone) or heterologous DNA (denatured salmon sperm DNA). These surfactants work by saturating some of the sites where non-specific binding could occur. Another additive, 10% dextran sulfate, acts by volume exclusion; it takes up water in the hybridization solution so the effective concentrations of the reactants are greater.

Several hybridization experiments were done in an attempt to identify the P-450_{cym} gene from the restriction digest. The hybridization

experiments were carried out at varying conditions and their autoradiographs are shown in figures 5,6, and 7. In experiment I, the hybridization was done at 25°C in the presence of dextran sulfate. The hybridization temperature and conditions were chosen based on previous hybridization experiments by Heli Nikkila in a similar system. Following the hybridization several washes of the membrane were done at 30°C, 36°C, 42°C, 47°C, 52°C, and 58°C to remove the non-specific signal. After all of these washes it was not possible to detect a specific signal on the autoradiograph. This hybridization experiment was ineffective because the DNA on the membrane was not sufficiently separated and isolated in the desired ladder-like banding pattern. This occurred because the membrane was prepared from incomplete restriction digests.

A new batch of DNA was isolated because it was suspected that the DNA from hybridization I contained enzyme inhibiting contaminants. Restriction digests were done on this new DNA and an aliquot of each digest was run on a gel. The HindIII, PstI, EcoRI, and BamHI digests were more complete than in experiment I, but not total digests. The SalI digest was completed and displays an ideal banding pattern on the gel. Before the next series of restriction digests, several DNA purification procedures were done to remove contaminants such as phenol, ethanol and salt. Despite these purifications, total restriction digests could not be achieved using HindIII, PstI, EcoRI, and BamHI. To confirm that the restriction enzymes and buffers were not contaminated, a set of restriction digests was done using new enzymes and buffers.

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FIGURE 6. AUTORADIOGRAPH FROM HYBRIDIZATION WITH DEXTRAN SULFATE METHOD.





Again the digests using HindIII, PstI, EcoRI, and BamHI were slightly incomplete. A membrane was made using these digests because they were much more complete than those used in experiment I. This membrane was also hybridized in the presence of dextran sulfate, but the hybridization temperature was increased to 30°C to increase the stringency of hybridization. Washes of membrane II were done at 35°C, 40°C, and 45°C, but it was impossible to detect a specific signal after each wash (Fig.6).

A third hybridization was done in an attempt to increase the stringency of the hybridization conditions. Hybridization III was done in the presence of formamide without dextran sulfate. The hybridization temperature, 30°C, was calculated based on the equations presented earlier in this discussion. After the initial wash, the membrane was exposed to X-ray film. The resulting autoradiograph displayed an enormous amount of background which masked any specific signals which may have been present (Fig.7). This background could not be removed with further washes. By comparing this hybridization experiment with the previous two hybridization experiments, it appears that the dextran sulfate plays an important role in reducing the non-specific binding.

Based on the hybridization schemes described above, it has not been possible to identify a DNA fragment which contains the P-450_{cym} gene. In order to identify this fragment using the hybridization method, it will be necessary to obtain a higher signal intensity and a lower signal background on the membrane. A lower signal background will be achieved by increasing the stringency of hybridization.

Due to time limitations, I was unable to investigate all variables which effect hybridization. Some possible experiments to be investigated are listed below. One such experiment would involve determining the optimal hybridization temperature for this system. The optimal temperature would represent the temperature at which the most non-specific binding could be removed without significantly decreasing the specific binding. In the future, it may also be useful to do a hybridization experiment in the presence of formamide and dextran sulfate. Under these conditions, the background would be minimized by the dextran sulfate and the stringency would be increased by the formamide. Another obvious suggestion for further work includes determining a method for obtaining complete restriction digests.

This research project was based on two assumptions. The first assumption was that the P-450_{cam} gene and the P-450_{cym} gene exhibit complementary DNA sequences. It can be assumed that the amino acid sequences of P-450_{cam} and P-450_{cym} exhibit homology based on the homologies present between other P-450 systems. However, it is less than certain that a homology between the DNA sequences exists. Due to the degenerate genetic code, homologous amino acid sequences are not always represented by homologous DNA sequences. If homology is displayed only in a small percentage of the total probe DNA sequence or the DNA homology does not exist, the P-450 gene will not serve as a good probe. A cDNA copy of mRNA which represents the P-450_{cym} protein would

be the best probe because the cDNA probe is exactly complementary to the gene of interest. In hybridization experiments using a cDNA probe, the hybridization is faster and stronger thus increasing the intensity of the signal.

The second assumption made when planning this experiment was that the P-450_{cym} gene could be cloned using this cloning strategy. Perhaps another cloning strategy such as cDNA cloning or shot gun cloning would have been more effective. In conclusion, there are several possible experiments and strategies which should be investigated in order to clone the P-450_{cym} gene.

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APPENDIX A

PAS Media

77.5 ml/l PA Concentrate
10 ml/l 100X salts
18 g/l agar for plates

100X Salts

10g/l Bacto-tryptone
5g/l Bacto-yeast extract
10g/l NaCl
Adjust pH to 7.5 with sodium hydroxide.

TE Buffer

10mM Tris.Cl (pH 7.4)
1mM EDTA (pH 8.0)
0.002M EDTA

High Salt Buffer

1M NaCl
20mM Tris, pH 8.0
0.1mM EDTA

Amerham Nick Translation Nucleotide Buffer Solution

100uM dATP, dGTP, dTTP in Tris/HCl pH 7.8
Magnesium chloride
2-mercaptoethanol

5X Hybridization P-Buffer

1.0% Ficoll, polyvinylpyrrolidone, BSA
250mM Tris.HCl
0.5% Sodium pyrophosphate

Prehybridization buffer for dextran sulfate method

2ml 10% SDS
4ml 5M NaCl
4ml 5X P-buffer
2ml H₂O
8ml 25% dextran sulfate

Hybridization buffer for dextran sulfate method

0.5ml 10% SDS
1.0ml H₂O
1.0ml 5X P-buffer
2.0ml 25% dextran sulfate

PA Concentrate

1.0M K₂HPO₄
1.0M KH₂PO₄
1.0M NH₄Cl

Luria-Bertani Media

5.0g MnSO₄·H₂O
19.0g Mn SO₄
5.0g FeSO₄·7H₂O
0.3g CaCl₂·2H₂O
1.0g Ascorbic acid

TBE Buffer

0.089M Tris-borate
0.089M boric acid

20X SSC

3M NaCl
0.3M sodium citrate
Bring to pH 7.4

APPENDIX A (CONTINUED)

Prehybridization and hybridization buffer formamide method

50% formamide

2X SSC

0.05M Na₂HPO₄ /NaH₂PO₄

0.02% polyvinylpyrrolidone, BSA, Ficoll

≥ 100ug/ml denatured salmon sperm DNA

APPENDIX B

Restriction Enzyme Recognition Sequences

EcoRI

G AATTC
CTTAA G

BamHI

G GATCC
CCTAG G

SalI

G TCGAC
CAGCT G

HindIII

A AGCTT
TTCGA A

PstI

CTCGA G
G AGCTC

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